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An application of the C^* concept in predicting the topical efficacy of finite dose acyclovir in the treatment of cutaneous HSV-1 infections in hairless mice

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Summary

This study has examined C^* (and its temporal pattern) as a parameter for predicting topical antiviral efficacy of acyclovir (ACV) formulations in a recently developed hairless mouse model for the treatment of HSV-1 cutaneous infections. C^* referred to here is the free drug concentration at the target site, which is believed to be the basal cell layer of the epidermis. Two different topical ACV formulations, one available commercially (Zovirax 5% ointment) and the other prepared in our laboratories (3% ACV suspension) were investigated in a finite dose multiple dosing regimen (twice a day application) to simulate the clinical situation. C^* was calculated from in vitro flux data and information obtained from previous baseline studies. In vitro ACV flux determinations involved an in vivo-in vitro experimental design that was believed to approximate closely the actual in vivo antiviral treatment protocol. For the in vivo antiviral efficacy studies, a 1-day delayed (after virus inoculation) 4-day treatment protocol was implemented and the animals wore a Velcro jacket to protect the formulations from being removed during the drug application period. With Zovirax 5% ointment given 3 mg every 12 h, our results showed a 0% topical efficacy which was consistent with the C^* predictions. In the case of the laboratory prepared 3% ACV suspension, a dose of 2 μl was given every 12 h and our results showed a modest level (33–44%) of topical effectiveness, which was somewhat higher than predicted (0–25%) from C^* considerations. Also, in the efficacy studies (but not in the in vitro flux studies) with the laboratory prepared formulation, there were instances (20–33%) of severe skin irritation/damage noted at the end (day 5) of the experiments. Taking this into consideration, it is suggested that these studies represent a good correlation between predictions (C^*) based on the in vitro data and the in vivo antiviral efficacy and validate the predictive value of this approach.

Introduction

In a recent review (Su et al., 1991), the concept of C^* (i.e., the free drug concentration at a skin

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target site) was introduced as a true measure of topical bioavailability and discussed as to how it may be used to predict the effectiveness of dermatological formulations. As an example, data (Gonsho et al., 1990; Lee et al., 1992) on the topical and the systemic efficacy of acyclovir (ACV) in the treatment of cutaneous herpes simplex virus type 1 (HSV-1) infections in hairless mice were discussed, and it was shown how C^* may be related to the ACV skin flux through an appropriate set of in vivo data. Briefly, C^* may be calculated from the equation,

$$C^* = \frac{J}{P_D} \tag{1}$$

where J is the ACV skin flux and P_D is the in vivo dermis permeability coefficient. P_D was accessed from the equation,

$$P_D = \frac{J_{50}}{C^*_{50}} \tag{2}$$

where J_{50} is the ACV flux for which lesion inhibition was found in 50% of the mice *treated topically* at this flux value and C^*_{50} was estimated from (by equating it to) plasma levels of ACV that inhibited cutaneous lesion formation in 50% of the animals *treated systemically*. It was implicit in this approach, therefore, that C^* would have the same effect on in vivo cutaneous HSV-1 virus replication whether it came about from systemic ACV treatment or from local (topical) ACV delivery.

The above analysis gave a P_D value of 1.4×10^{-3} cm/s and a C^*_{50} value of 2.5×10^{-7} g/ml. This in vivo P_D value is noted as being about 20-times larger than the P_D value (6.8×10^{-5} cm/s) for dermis obtained in in vitro experiments: this is, however, consistent with expectations based on blood flow effects upon the diffusional behavior of permeants in the dermis (Song, 1989; Su et al., 1991). Also, it is important to note that the in vivo C^*_{50} value of 2.5×10^{-7} g/ml is generally in good agreement with literature values of ID_{50} for ACV obtained in vitro from Vero cell cultures (McLaren et al., 1982, 1983; Parris and Harrington, 1982; Smee et al., 1985; Al-Hasani et al., 1986; Pulliam et al., 1986; Mansuri et al., 1987). Here, ID_{50} is the ACV concentration required in vitro to inhibit HSV-1 induced cytopathogenicity or viral plaques by 50% in these cell cultures.

All previous data referred to in the above analysis were obtained under (relatively) constant flux conditions using (trans)dermal patches with rate-controlling membranes, and an ultimate goal in our research has been to develop a method for predicting the effectiveness of actual dermatological formulations, such as creams and ointments. Accordingly, this report describes the results of our first attempts to utilize Eqns 1 and 2 in 'predicting' the effectiveness of topical formulations of ACV in a moderately realistic finite dose, dosing regimen (twice a day treatment). The present study has examined the approach using two different topical ACV formulations, one available commercially (Zovirax 5% ointment) and the other prepared in our laboratories.

Materials and Methods

Drugs, enhancers, and formulation bases

Acyclovir (ACV) was obtained as its sodium salt (Zovirax) from Burroughs Wellcome Co., Research Triangle Park, NC and was converted to its free acid form before use as previously described (Gonsho et al., 1990). The commercially available topical formulation of ACV (Zovirax 5% ointment) was also purchased from Burroughs Wellcome Co.

The skin permeation enhancer *N*-dodecyl-2-pyrrolidone was obtained from GAF Chemicals Corp., Wayne, NJ and Azone (1-dodecylazacycloheptan-2-one) was a gift from Nelson Research Corp., Irvine, CA. Both were used as received.

Lanolin anhydrous, USP (Mallinckrodt Chemical Works, St. Louis, MO) and isopropyl myristate (Sigma Chemical Co., St. Louis, MO) were used as received in the preparation of topical formulations.

Virus and animals

Samples of the same batch of herpes simplex virus type 1, strain E-377, with a titer of 1.35×10^8 plaque-forming units (PFU)/ml were used throughout this study. They were stored in aliquots at −70°C until used. The preparation and assay of the virus have been previously described (Kern et al., 1973).

Female hairless mice (strain SKH/HR1), 5–6 weeks old with body weight 23.8 ± 2.0 g, were purchased from Charles River, Bloomington, MA and used in this study.

Preparation of formulations

The preparation of topical formulations was carried out with a mortar and pestle. Anhydrous lanolin (200 mg) was added in small portions to 570 μl of isopropyl myristate and then well mixed by levigation. To this mixture were added 200 μl of *N*-dodecyl-2-pyrrolidone. Finally, 30 mg of ACV were added to the mixture by trituration. The formulations (3% ACV suspensions) were prepared 1 day before use and the same batch was used in both in vitro and in vivo studies.

Fabrication of (trans)dermal patches

As in the previous study (Gonsho et al., 1990), polymeric hydrogel membranes were used for controlling ACV fluxes through the Azone-pretreated hairless mouse skin. The preparation and characterization of these hydrogel membranes have been previously described in detail (Miyajima et al., 1987; Gonsho et al., 1990). The fabrication of the (trans)dermal patch has also been described in the previous paper (Gonsho et al., 1990). The drug reservoir in each (trans)dermal patch was loaded with a suspension of ACV sufficient for constant rate release over the experiment period.

Strategy of studies

As stated above, the purpose of the present study was to assess C^* from in vitro flux data (with the hairless mouse model) and to predict the topical efficacy of ACV in the treatment of cutaneous HSV-1 infections with two different topical formulations, one available commercially (Zovirax 5% ointment) and the other prepared in our laboratories, applied to the skin at defined 'finite dose' levels and in multiple dosing regimens to simulate the actual clinical situations. This study was therefore to entail, in the first part, the determination of in vitro fluxes and the assessment of C^* from these flux results (using Eqn 1) and then in the second part, an evaluation of topical efficacy from in vivo antiviral studies. From these results, it was to be determined whether there would (or would not) be a correlation between the C^* predictions and the in vivo efficacy.

During the early phase of experimental design, two major practical problems asserted themselves. A discussion of these problems and how they were addressed follows.

The first problem encountered was how to conduct a meaningful in vitro 3-day or a 4-day flux study when it is well-documented (Bond and Barry, 1988; Lambert et al., 1989) that hairless mouse skin is not stable, in vitro, beyond around 24 h in a diffusion cell experiment. Faced with this situation, it was felt that the best solution would be to try to obtain the flux data for the C^* calculations from in vivo experiments with the same protocol as in the in vivo antiviral efficacy studies; this way, any flux changes with time during the treatment period would be expected to be nearly the same for the two sets of experiments. However, there are no known in vivo methods (e.g., plasma level determinations or a 'top-wash' procedure) for skin flux determinations that would provide data of sufficient precision. Accordingly, an experimental design was conceived and it was believed to be the next best solution to the problem: it was planned to implement a combined in vivo-in vitro experiment to obtain relevant in vitro fluxes. Specifically, topical ACV formulations are applied onto live animals following the same protocol as in the in vivo efficacy studies, then at the predetermined point in time, the animals are killed and the ACV-treated skin excised, and the Franz cell in vitro flux experiments run for 24 h. For example, to obtain the first day flux results, skin samples are excised from freshly killed animal to determine the in vitro fluxes for 24 h; to obtain the day 2 flux, the in vivo experiment is run for 1 day

before skin samples are excised to determine the in vitro fluxes on day 2; to obtain the day 3 flux, the in vivo experiment is conducted over the first 2 days and the in vitro fluxes determined on day 3; and so on. The complete experimental design is presented in Table 1.

The second major problem encountered was how to protect the topically applied formulation from being licked off, scratched off, and/or rubbed off once applied onto the hairless mouse skin. It was thought that Velcro (Velcro USA Inc., Manchester, NH), a light-weight material, which is a hook-and-loop type fastener with a sticky back may work. A Velcro jacket was made which covered the entire trunk of the hairless mouse except for the part (1 inch × 1 inch in dimension) at the left back area, which was designated for the application of formulations. A Velcro cover for protecting the drug treated part of the animal but allowing for good ventilation was also made. The details of this Velcro protection jacket are illustrated in Fig. 1. It has turned out

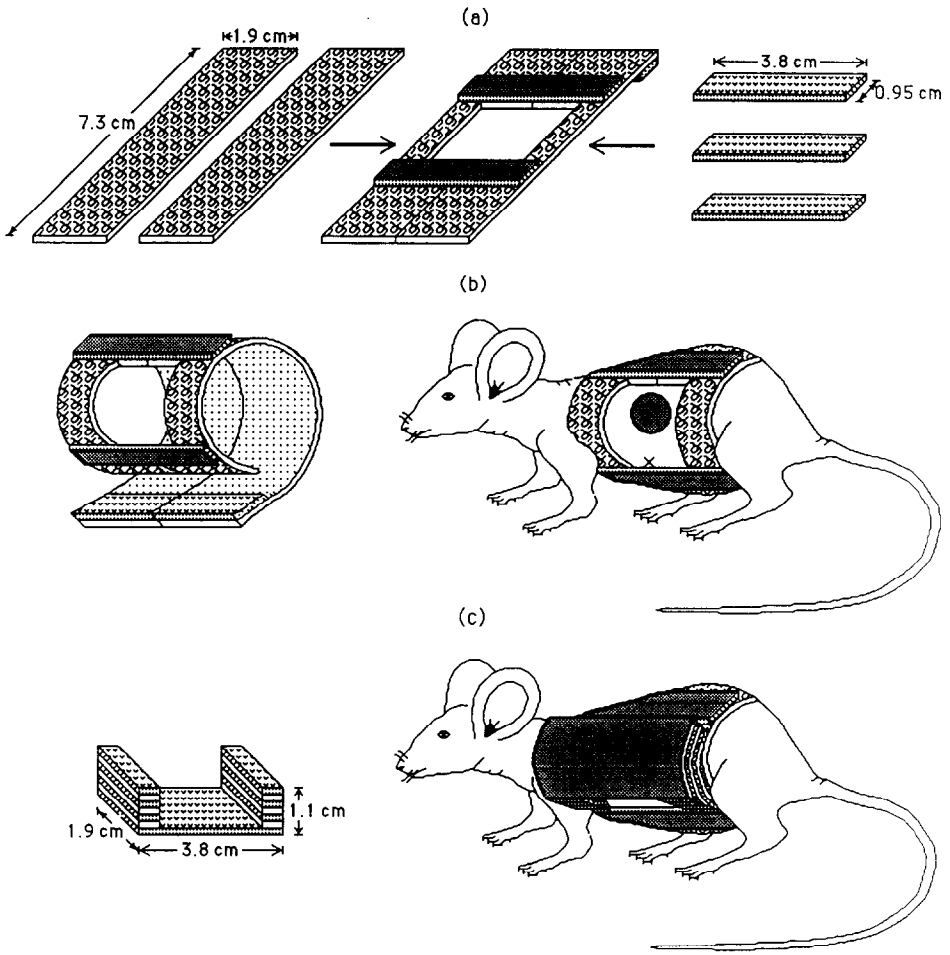

Fig. 1. A schematic illustration of a Velcro protection jacket which was fabricated by fastening two pieces of the hook-and-loop Velcro together with a 1 inch × 1 inch opening in the center to expose the virus inoculation site and the skin area (1 cm^2) for application of the finite dose formulation. The finished jacket was wrapped around the trunk of a hairless mouse. Once the formulation was applied, another piece of Velcro cover was mounted over the opening for protection but allowing for good ventilation.

TABLE 1

The combined in vivo-in vitro experimental design for determining the in vitro ACV flux across the hairless mouse skin using finite dose formulations [a]

Results obtained	Day				
	Day 1	Day 2	Day 3	Day 4	Day 5
First day flux	– [b]				
Second day flux	** [c]	– [b]			
Third day flux	** [c]	** [c]	– [b]		
Fourth day flux	** [c]	** [c]	** [c]	– [b]	
Fifth day flux	** [c]	** [c]	** [c]	** [c]	– [b]

[a] Applicable to both Zovirax 5% ointment and the self-prepared 3% ACV suspension.

[b] In vitro flux determinations conducted with freshly prepared skin samples using a Franz cell at 37°C for 24 h.

[c] The in vivo part of a combined in vivo-in vitro experiment. Every 12 h, the residual ACV formulation on the skin was removed and a fresh dose reapplied. Each animal wore a Velcro protection jacket during this period and was individually housed.

that this Velcro protection jacket serves its purpose quite well and the mouse is able to lead a normal life pattern wearing this jacket.

Although the commercially available Zovirax 5% ointment provided us with a convenient sample to test with our hairless mouse model, it was considered desirable to examine one or two more topical formulations so that our claim on the applicability of this model approach would be much more firm. By trial and error, a topical formulation (3% ACV suspension) was found suitable for the present work.

In vitro ACV flux determinations and in vivo C assessments*

As mentioned above, a combined in vivo-in vitro experimental design was employed to determine the in vitro ACV fluxes across the hairless mouse skin. The same procedure was used for both Zovirax 5% ointment and the self-prepared formulation and the experiment was run in triplicate. During the in vivo part of the combined in vivo-in vitro experiment, a circular skin site with an area equivalent to the diffusional area (1 cm^2) of the Franz cell on the left back area of a hairless mouse was treated with finite dose topical formulations every 12 h. For Zovirax 5% ointment, the dose given was 3 mg and was applied onto the skin with inunction using a small glass rod, while for the self-prepared 3% ACV suspension, the dose given was 2 μl and was slowly added onto the skin using a micropipette; this maximized the chance for an even distribution of the drug suspension over the skin surface. Each hairless mouse wore a Velcro protection jacket during the entire in vivo experimental period and was individually housed. At the end of each dosing interval, the small piece of Velcro cover over the formulation application site was temporarily detached and the residual formulation on the skin was wiped clean with a cotton-tipped applicator prewetted with saline prior to the application of the next dose.

To begin the in vitro part of the combined in vivo-in vitro experiment, the Velcro protection jacket was removed from each hairless mouse and the remaining drug formulation on the skin was wiped clean. The hairless mouse was then killed by cervical dislocation and the formulation-pretreated skin site with its surrounding tissue was excised. The freshly prepared skin sample was sandwiched between the donor and the receiver chamber of a Franz cell with the formulation-pretreated skin site centered. The receiver chamber (with a small magnetic stirring bar) was filled with saline containing 0.02% sodium azide and a finite dose of the topical formulation was added to the skin membrane surface in the same manner as described above. The sampling port of the receiver chamber was covered except for the times of sample withdrawal, while the donor chamber was left open to the atmosphere to mimic the in vivo experimental conditions. The experiment was initiated by turning on the magnetic stirrer and carried out at 37°C for 24 h. At predetermined time intervals, 1 ml of sample was taken from the receiver chamber and was immediately replaced with the same volume of fresh saline. The collected samples were analyzed for their ACV contents using HPLC as previously described (Gonsho et al., 1990). The amount of ACV permeated through the hairless mouse skin was plotted as a function of time and the instantaneous ACV flux was estimated from the slope of the line connecting the two consecutive time points.

C^* was calculated using Eqn 1 with $P_D = 1.4 \times 10^{-3}$ cm/s.

In vivo antiviral efficacy studies

The in vivo antiviral efficacy studies with finite dose topical ACV formulations were conducted using 10 animals in each group. The hairless mice were inoculated with HSV-1 following the procedures previously described (Lee et al., 1992). For the treatment, the finite dose formulations were applied onto a circular skin site (1 cm^2 in area) on the left back area about 1 cm distance dorsal to the virus inoculation site and in the predicted path of lesion development and a 1-day delayed 4-day treatment protocol (Lee et al., 1992) was used (i.e., the treatment would start 1 day after the virus inoculation and continue for 4 consecutive days). The dosing interval, the doses given, and the ways to apply the doses and to remove the residual formulations were exactly the same as those described above in the in vivo part of the combined in vivo-in vitro experiment. Each hairless mouse wore a Velcro protection jacket during the treatment period and was individually housed. At 5 days postinoculation, the Velcro protection jacket was removed and the lesion development was scored for each mouse and used to calculate the antiviral efficacy of ACV as previously described (Lee et al., 1992).

For control, a placebo group was always run along with each experimental group. For the present study, the placebo group was treated with a (trans)dermal patch containing aqueous Carbopol (B.F. Goodrich Co., Cleveland, OH) gel but without ACV as in our earlier studies (Gonsho et al., 1990; Lee et al., 1992).

Also run for control purpose in each experiment was the group(s) treated with a (trans)dermal patch containing an ACV suspension to ensure that the results were reproducible under different circumstances. This experiment also permitted a direct comparison of a current study with previous baseline efficacy studies using the (trans)dermal patch delivery system. The treatment with (trans)dermal patches used a 2-day delayed 3-day treatment protocol (Lee et al., 1992).

Table 2 summarizes the treatment protocols for the different groups used in the in vivo antiviral efficacy studies.

TABLE 2

Treatment protocols for the different groups used in the in vivo antiviral efficacy studies

Group	Day					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Placebo group treated with (trans)dermal patches [a]	inoculation of virus	pretreatment with 25 mg Azone for 24 h	application of (trans)dermal patches			removal of (trans)dermal patches and observation of results
Control group treated with (trans)dermal patches [a]	inoculation of virus	pretreatment with 25 mg Azone for 24 h	application of (trans)dermal patches			removal of (trans)dermal patches and observation of results
Experimental group treated with finite dose formulations [b]	inoculation of virus	application of finite dose formulation and removal of residual formulation every 12 h with the hairless mouse wearing a Velcro protection jacket during this period				removal of Velcro protection jacket and observation of results

[a] Run along with the experimental group in each study.

[b] Applicable to the experimental group treated with Zovirax 5% ointment or with the self-prepared formulation.

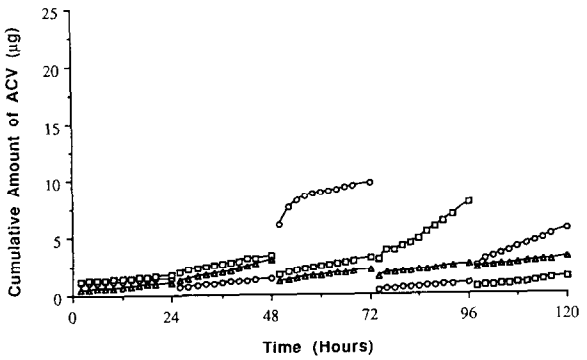

Fig 2. The accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using a 3 mg finite dose Zovirax 5% ointment in a combined in vivo-in vitro experiment, The experiment was run in triplicate.

Results and Discussion

In vitro ACV flux determinations and in vivo C assessments using finite dose Zovirax 5% ointment*

Fig. 2 shows the accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using finite dose (3 mg) Zovirax 5% ointment in a combined in vivo-in vitro experiment. Unlike the in vivo part of the combined in vivo-in vitro experiment where the finite doses were given every 12 h, the in vitro flux determinations were run for 24 h. This is because (a) it was found difficult to remove the residual formulation and to apply a new dose onto the isolated skin samples which became very delicate through hydration for 12 h; and (b) our preliminary studies had shown that the ACV fluxes (the slopes) usually remained relatively constant for 24 h during the in vitro Franz cell experiments.

It is clear from Fig. 2 that except for one skin sample from each of the third, the fourth and the fifth day, the in vitro ACV flux results (the slopes) were quite reproducible and remained relatively similar over the 5-day experimental period. This outcome showed that the variations of C^* during the treatment period were relatively small and therefore allowed for a more exact interpreting of the results.

Fig. 3 shows the temporal pattern of in vivo (predicted) C^* assessed according to Eqn 1 (with $P_D = 1.4 \times 10^{-3}$ cm/s) using the instantaneous

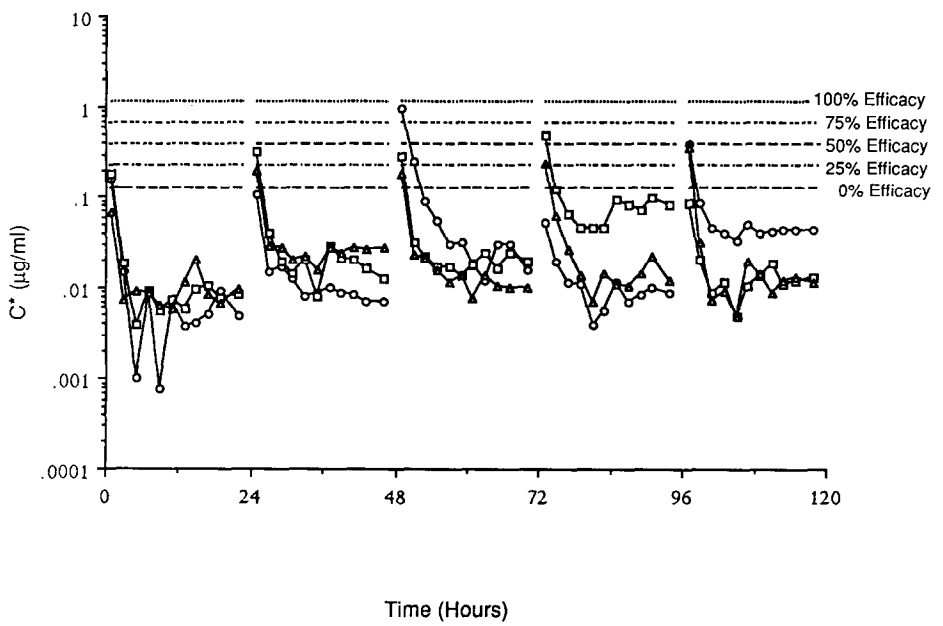

Fig 3. The temporal pattern of C^* assessed for Zovirax 5% ointment according to Eqn 1 using the instant ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Fig. 2. The broken lines represent the C^* values corresponding to the different antiviral efficacy calculated based on our previous results (Lee et al., 1992).

ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Fig. 2. As can be inferred from the in vitro ACV fluxes (the slopes), the in vivo C^* also remained relatively constant throughout the 5-day period. The efficacy levels (dotted lines) shown in Fig. 3 were calculated from the constant flux vs efficacy data of Lee et al. (1992) using Eqn 1 and a P_D value of 1.4×10^{-3} cm/s. It is important to note here that the C^* values stayed far below that corresponding to a 0% topical efficacy; consequently, these results predict poor efficacy for Zovirax 5% ointment in this model.

In vivo antiviral efficacy study using finite dose Zovirax 5% ointment and correlation between the in vitro flux and the in vivo efficacy results

The results of the in vivo antiviral efficacy study obtained from the experimental group treated with finite dose (3 mg) Zovirax 5% ointment and from the placebo and the two control groups treated with (trans)dermal patches are presented in Table 3. The five lesion categories used here were exactly the same as those previously described (Gonsho et al., 1990; Lee et al., 1992), namely, the lesion development went through (Th), stopped (St) at the edge of, jumped (J) over, did not reach (NR), and totally missed (M) the designated skin site for treatment with the (trans)dermal patch or with the finite dose formulation.

The placebo group treated with a (trans)dermal patch containing no ACV showed a 100% infection rate, i.e., all 10 animals in this group displayed a 'Th' lesion category, indicating that the batch of virus used was viable and there should be no doubt about our technique for virus inoculation.

The two control groups were treated with (trans)dermal patches containing ACV employing a 2-day delayed 3-day treatment protocol. For control group I, the (trans)dermal patch used a rate-controlling membrane made up of 100% 2-hydroxyethylmethacrylate (HEMA) with 1 mm thickness. The ACV flux for this (trans)dermal patch was 416 $\mu g/cm^2$ per day determined in vitro using the method described previously (Gonsho et al., 1990) and was 325 $\mu g/cm^2$ per day estimated in vivo using the method of extraction (Lee et al., 1992). The possible reasons for the in vivo flux being smaller than the in vitro prediction have been discussed in detail in our last paper (Lee et al., 1992). The control group I demonstrated a 100% topical efficacy which was consistent with our previous results (Lee et al., 1992) and a 10% systemic efficacy, which was a little lower than our previous results (Lee et al., 1992). For control group II, a rate-controlling

TABLE 3

Results of the in vivo antiviral efficacy study using the finite dose Zovirax 5% ointment

Group	Lesion category for mouse no.										Antiviral efficacy (%)	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	Th	Th	Th	Th	0	0
Control I [b]	St	St	St	St	St	NR	St	St	M	St	100	10
Control II [c]	St	Th	Th	NR	St	Th	J	Th	Th	Th	40	10
Experimental [d]	Th	Th	Th	Th	Th	Th	Th	Th	Th	– [e]	0	0

[a] Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a 2-day delayed 3-day treatment protocol.

[b] Treated with a (trans)dermal patch containing an ACV suspension employing a 2-day delayed 3-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 100% HEMA with 1 mm thickness.

[c] Same as [b] except that the (trans)dermal patch used a rate-controlling membrane made up of 90% HEMA and 10% styrene with a thickness of 0.5 mm.

[d] Treated with a finite dose (3 mg) Zovirax 5% ointment every 12 h employing a 1-day delayed 4-day treatment protocol.

[e] The mouse died prior to the lesion scoring.

membrane made up of 90% HEMA and 10% styrene with a thickness of 0.5 mm was used in the preparation of (trans)dermal patches. The results in Table 3 show that this second control group had a topical efficacy of 40% and a systemic efficacy of 10% and both were in general agreement with our previous results (Lee et al., 1992).

For the experimental group, the animals were treated with finite dose (3 mg) Zovirax 5% ointment every 12 h using a 1-day delayed 4-day treatment protocol. This treatment protocol was considered a reasonable choice; previously, it had been shown with the (trans)dermal patch that 0-day, 1-day, and 2-day delayed treatment protocols gave essentially the same results (Lee et al., 1992). As can be seen from the bottom row of Table 3, Zovirax 5% ointment gave a 0% efficacy which was in good agreement with the theoretical C^* prediction. This lack of antiviral efficacy demonstrated by Zovirax 5% ointment is thus likely due to poor percutaneous ACV delivery from its polyethylene glycol vehicle (Spruance and Crumpacker, 1982; Freeman and Spruance, 1986). The present results differ somewhat from those of Shannon et al. (1985), who reported a significant reduction in the mean peak lesion score when the virus-infected hairless mice were topically treated with Zovirax 5% ointment. However, a detailed experimental procedure was not revealed by these authors and, therefore, a comparison between their results and those of the present study is impossible.

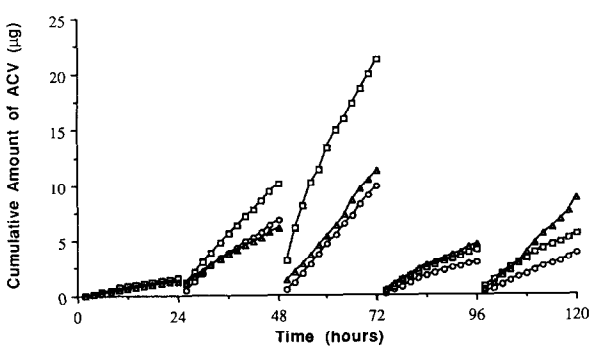

Fig 4. The accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using a 2 μl finite dose 3% ACV suspension in a combined in vivo-in vitro experiment. The experiment was run in triplicate.

The skin samples treated with finite dose Zovirax 5% ointment gave the same general appearance as those untreated and it can be stated with confidence that the results presented in Table 3 were not complicated by the problem of skin irritation/damage.

In vitro ACV flux determinations and in vivo C^* assessment using the finite dose 3% ACV suspension

The procedures involved here were the same as those described above for Zovirax 5% ointment, except that a smaller (2 μl) finite dose was used for the self-prepared 3% ACV suspension. A finite dose of 3 μl or larger in the form of a suspension was more than what could be maintained on the skin surface of 1 cm^2. These higher doses were found to drain/drip from the application site; it was felt that if this were to happen in vivo, the antiviral efficacy results might be compromised by contact of the drug formulation with the inoculation site.

Fig. 4 shows the accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using finite dose (2 μl) 3% ACV suspension in a combined in vivo-in vitro experiment. Comparing these data to the Zovirax 5% ointment results, it is seen that the 3% ACV suspension demonstrated a somewhat larger variability over the 5 day experimental period, with the flux being the lowest on the first day, the highest on the third day, and about the same for the other three days. This same pattern was retained in Fig. 5 where the C^* values calculated from the in vitro fluxes are presented. Excluding the first day results, the C^* values for the remaining 4 days were found to be in the range from somewhere below that corresponding to a 0% topical efficacy to that for around a 25% topical efficacy.

In vivo antiviral efficacy studies using the finite dose 3% ACV suspension and correlation between the in vitro flux and the in vivo efficacy results

Table 4 summarizes the results of the first in vivo antiviral efficacy study obtained from the

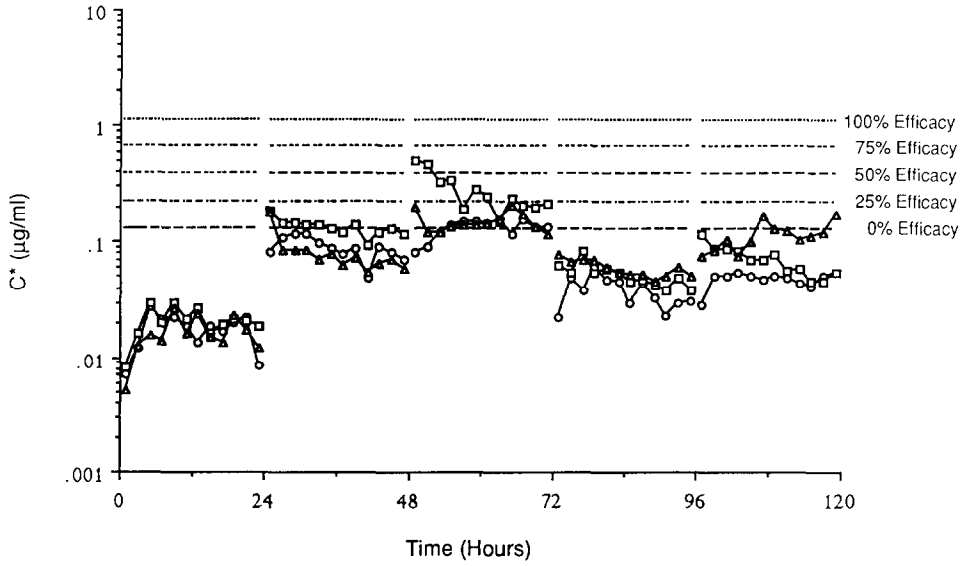

Fig 5. The temporal pattern of C^* assessed for 3% ACV suspension according to Eqn 1 using the instantaneous ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Fig. 4. The broken lines represent the C^* values corresponding to the different antiviral efficacy calculated based on our previous results (Lee et al., 1992).

experimental group treated with the finite dose (2 μl) 3% ACV suspension and from the accompanying placebo and the control groups. The placebo group was treated with a (trans)dermal patch containing the aqueous Carbopol gel but without ACV. It is important to note that all the mice in the placebo group developed a 'Th' lesion category and this validates our inoculation technique. The control group was treated with a (trans)dermal patch which used a 100% HEMA hydrogel membrane with 1 mm thickness to control the delivery of ACV. The ACV flux for this (trans)dermal patch was 422 $\mu g/cm^2$ per day determined in vitro (Gonsho et al., 1990) and was 399 $\mu g/cm^2$ per day estimated in vivo using the method of extraction (Lee et al., 1992). The con-

TABLE 4

Results of the first in vivo antiviral efficacy study using the finite dose 3% ACV suspension

Group	Lesion category for mouse no.										Antiviral efficacy (%)	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	- [d]	Th	Th	Th	0	0
Control [b]	NR	St	NR	NR	NR	St	NR	St	St	NR	100	60
Experimental [c]	NR	NR	NR	Th	Th	Th	Th	NR	Th	- [d]	44	44
	(1) [e]	(4)	(2)	(1)	(1)	(1)	(1)	(4)	(1)			

[a] Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a 2-day delayed 3-day treatment protocol.

[b] Treated with a (trans)dermal patch containing an ACV suspension employing a 2-day delayed 3-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 100% HEMA with 1 mm thickness.

[c] Treated with a finite dose (2 μl) 3% ACV suspension every 12 h employing a 1-day delayed 4-day treatment protocol.

[d] The mouse died prior to the lesion scoring.

[e] The number in the parenthesis under each lesion category in the experimental group was the corresponding skin irritation/damage score with 0 representing a nonirritative case and 4 being the most severe case.

trol group showed a 100% topical efficacy and a 60% systemic efficacy and both results are consistent with our previous findings (Lee et al., 1992).

For the experimental group treated with the finite dose (2 μl) 3% ACV suspension in this first study, a 44% topical efficacy and a 44% systemic efficacy were obtained. These efficacy results were somewhat higher than those predicted. Based upon the flux data (Fig. 4) and the C^* results (Fig. 5), a topical efficacy of somewhere between 0 and 25% and a systemic efficacy of 0% (Lee et al., 1992) would have been expected. The somewhat higher than predicted topical efficacy results may be still considered to be in satisfactory agreement with the C^* prediction in view of statistics of small sample numbers; the rather higher degree of systemic efficacy (4 NRs) is however, more difficult to explain.

It was observed that the animals in the experimental group of the antiviral efficacy study (but not in the in vivo-in vitro flux study) exhibited a high frequency and a high degree of skin irritation and skin damage. This may be characterized by an erythema over the formulation application site in the early stage of the treatment and by dryness of the application site and, in the most severe cases, scabbing of the application site during the later stages. The cause(s) of this skin irritation was not investigated in the present study; however, the problem was believed to have been related to the presence in the formulation of 20% *N*-dodecyl-2-pyrrolidone which has been reported (Ansell and Fowler, 1988) to cause varying degrees of dermal irritation in rabbits depending on the concentration used. For the purpose of providing some measure of skin irritation/ damage in the experiments, an arbitrary scoring system was adopted which assigns a number from 0 to 4 to the various degrees of skin irritation/damage with 0 being the nonirritative case and 4 representing the most severe case. For the animals in the in vivo-in vitro experiment, skin irritation was barely perceptible in the majority of the cases; most would score a 0 and some might score a 1 using this scoring system. For the animals in the in vivo efficacy study, the scores were generally higher and these are presented in Table 4 (within the parentheses). It can be seen in Table 4 that only mouse no. 2 and mouse no. 8 showed severe (score 4) skin irritation; the other mice demonstrated slight (score 1) to mild (score 2) skin irritation. If the two mice with the severe skin irritation score were excluded from the calculation of antiviral efficacy, the adjusted topical and systemic efficacy would both be 29% which would provide better agreement with the in vitro flux and C^* predictions.

For the following reasons, it was decided to carry out a second antiviral efficacy study with the 3% ACV suspension. First, alerted to the

TABLE 5

Results of the second in vivo antiviral efficacy study using the finite dose 3% ACV suspension

Group	Lesion category for mouse no.										Antiviral efficacy (%)	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	Th	Th	Th	Th	0	0
Control [b]	J	St	Th	J	NR	St	St	NR	St	St	90	20
Experimental [c]	Th	Th	Th	J	NR	Th	Th	NR	– [d]	Th	33	22
	(1) [e]	(1)	(1)	(2)	(4)	(3)	(4)	(4)		(1)		

[a] Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a 2-day delayed 3-day treatment protocol.

[b] Treated with a (trans)dermal patch containing an ACV suspension employing a 2-day delayed 3-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 95% HEMA and 5% styrene with a thickness of 0.5 mm.

[c] Treated with a finite dose (2 μl) 3% ACV suspension every 12 h employing a 1-day delayed 4-day treatment protocol.

[d] The mouse died prior to the lesion scoring.

[e] The number in the parentheses under each lesion category in the experimental group was the corresponding skin irritation/ damage score with 0 representing a nonirritative case and 4 being the most severe case.

problem of skin irritation/damage, this aspect could be more closely observed in a second study. Also, it was thought prudent to run a repeat as the predictions vs actual results were less than ideal in the first study.

Table 5 presents the results of the second in vivo antiviral efficacy study with the self-prepared 3% ACV suspension. Firstly, the placebo group showed zero efficacy: this again was assurance that the inoculation technique was reliable in this set of experiments. Secondly, the results for the control group (topical efficacy = 90%; systemic efficacy = 20%) were again in general agreement with those reported (Lee et al., 1992) in our earlier studies for the same rate-limiting membrane system (95% HEMA, 5% styrene; thickness = 0.5 mm).

For the experimental group treated with finite dose (2 μl) 3% ACV suspension, a 33% topical efficacy and a 22% systemic efficacy were observed. These values are not far from those predicted (predictions on the basis of in vitro flux and C^*: topical efficacy = 0 to 25%; systemic efficacy 0%). Again, skin irritation of varying degrees were observed in this experimental group. It is interesting to note that if mice with an irritation score of 4 are excluded, the adjusted topical efficacy would be 17% and the adjusted systemic efficacy would be 0% for the other mice; these results would then be in very good agreement with predictions.

Assessment of the C^* Approach Based on the Mechanism of Action of ACV

ACV exerts its antiviral effect on HSV-1 by interfering with viral DNA synthesis and thereby inhibiting viral replication (Elion, 1982, 1983). In in vitro cell culture studies, ACV is selectively phosphorylated by cells infected with HSV-1 to ACV monophosphate via virus-coded thymidine kinase; the monophosphate is subsequently converted to the diphosphate and the triphosphate via other cellular enzymes (Elion et al., 1977). In vitro antiviral studies (Furman et al., 1979; St. Clair et al., 1980) have indicated that ACV triphosphate is the pharmacologically active form of the drug, the non-phosphorylated ACV, ACV monophosphate and ACV diphosphate are found to have minimal or no antiviral activity.

In the present study, the concentration of the active drug species, ACV triphosphate, at the skin target site (the epidermal basal layer) was not determined. Instead, the ACV concentration (C^*) in the aqueous compartment bathing the epidermal basal cells was estimated from the in vitro flux (using Eqn 1) and this was used for making predictions of the in vivo antiviral efficacy of ACV. This is believed to be very reasonable and is based on the following arguments: (a) the uptake of ACV into human erythrocytes from the surrounding medium has been found (Mahony et al., 1988) to take place very fast (equilibration time of the order of minutes) and there is no reason to believe that the in vivo transport of ACV into the epidermal basal cells of a hairless mouse would take place at greatly slower rates; (b) the extent of formation of ACV triphosphate in virus-infected cells is directly related to the concentration of ACV in the surrounding medium (Elion et al., 1977); and (c) it has been shown (Furman et al., 1981) that once the cells are deprived of their ACV source, the ACV triphosphate contents in the cells quickly decline with a half-life of 1–2 h depending on the remaining concentration of ACV in the medium. The above arguments support the view that intracellular ACV triphosphate should likely be a single-valued function of C^* (for ACV) during a 3- or 4-day treatment period; consequently, the present approach of employing C^* as a predictor of in vivo efficacy appears to possess a sound foundation.

Conclusion

Despite the problem of skin irritation/damage which somewhat compromised the interpretation of our results, especially for the self-prepared formulation, a relatively good correlation was observed between the in vitro ACV flux (and the C^* predictions) and the in vivo antiviral efficacy in the present study. It is suggested that this good correlation supports the validity of this hairless

mouse model approach involving the use of in vitro ACV flux and C^* to predict in vivo antiviral efficacy. More studies along these lines are needed to establish the generalizability of this approach.

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References

Al-Hasani, A.M., Barton, I.G., Al-Omer, L.S., Kinghorn, G.R. and Potter, C.W., Susceptibility of HSV strains from patients with genital herpes treated with various formulations of acyclovir. *J. Antimicrob. Chemother.*, 18 (Suppl. B) (1986) 113–119.

Ansell, J.M. and Fowler, J.A., The acute oral toxicity and primary ocular and dermal irritation of selected *N*-alkyl-2-pyrrolidones. *Food Chem. Toxicol.*, 26 (1988) 475–479.

Bond, J.R. and Barry, B.W., Limitations of hairless mouse skin as a model for in vitro permeation studies through human skin: hydration damage. *J. Invest. Dermatol.*, 90 (1988) 486–489.

Elion, G.B., Mechanism of action and selectivity of acyclovir. Acyclovir Symposium. *Am. J. Med.*, 73 (1982) 7–13.

Elion, G.B., The biochemistry and mechanism of action of acyclovir. *J. Antimicrob. Chemother.*, 12 (Suppl. B) (1983) 9–17.

Elion, G.B., Furman, P.A., Fyfe, J.A., De Miranda, P., Beauchamp, L. and Schaeffer, H. J., Selectivity of action of antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. USA*, 74 (1977) 5716–5720.

Freeman, D.J. and Spruance, S.L., Efficacy of topical treatment for herpes simplex virus infections: predictions from an index of drug characteristics in vitro. *J. Infect. Dis.*, 153 (1986) 64–70.

Furman, P.A., St. Clair, M.H., Fyfe, J.A., Rideout, J.L., Keller, P.M. and Elion, G.B., Inhibition of herpes simplex virus induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. *J. Virol.*, 32 (1979) 72–77.

Furman, P.A., De Miranda, P., St. Clair, M.H. and Elion, G.B., Metabolism of acyclovir in virus-infected and uninfected cells. *Antimicrob. Agents Chemother.*, 20 (1981) 518–524.

Gonsho, A., Imanidis, G., Vogt, P., Kern, E.R., Tsuge, H., Su, M.-H., Choi, S.-H. and Higuchi, W.I., Controlled (trans)dermal delivery of an antiviral agent (acyclovir) I: An in vivo animal model for efficacy evaluation in cutaneous HSV-1 infections. *Int. J Pharm.*, 65 (1990) 183–194.

Kern, E.R., Overall, J.C. and Glasgow, L.A., *Herpesvirus hominis* infection of newborn mice I: An experimental model and therapy with iodoxyuridine. *J. Infect. Dis.*, 128 (1973) 290–299.

Lambert, W.J., Higuchi, W.I., Knutson, K. and Krill, S.L., Effects of long-term hydration leading to the development of polar channels in hairless mouse stratum corneum. *J. Pharm. Sci.*, 78 (1989) 925–928.

Lee, P.H., Su, M.-H., Kern, E.R. and Higuchi, W.I., Novel animal model for evaluating topical efficacy of antiviral agents: flux versus efficacy correlations in the acyclovir treatment of cutaneous HSV-1 infections in hairless mice. *Pharm. Res.*, 9 (1992) 979–989.

Mahony, W.B., Domin, B.A., McConnell, R.T. and Zimmerman, T.P., Acyclovir transport into human erythrocytes. *J. Biol. Chem.*, 263 (1988) 9285–9291.

Mansuri, M.M., Ghazzouli, I., Chen, M.S., Howell, H.G., Brodfuehrer, P.R., Benigni, D.A. and Martin, J.C., 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil. A highly selective antiherpes simplex agent. *J. Med. Chem.*, 30 (1987) 867–871.

McLaren, C., Corey, L., Dekket, C. and Barry, D.W., In vitro sensitivity to acyclovir in genital herpes simplex viruses from acyclovir-treated patients. *J. Infect. Dis.*, 148 (1983) 868–875.

McLaren, C., Sibrack, C.D. and Barry, D.W., Spectrum of sensitivity to acyclovir of herpes simplex virus clinical isolates. Acyclovir Symposium. *Am. J. Med.*, 73 (1982) 376–379.

Miyajima, M., Okano, T., Kim, S.W. and Higuchi, W.I., Preformulation of an Ara-A transdermal delivery system: membrane fabrication and characterization. *J. Controlled Release*, 5 (1987) 179–186.

Parris, D.S. and Harrington, J.E., Herpes simplex virus variants resistant to high concentrations of acyclovir exist in clinical isolates. *Antimicrob. Agents. Chemother.*, 22 (1982) 71–77.

Pulliam, L., Panitch, H.S., Baringer, J.R. and Dix, R.D., Effect of antiviral agents on replication of herpes simplex virus type 1 in brain cultures. *Antimicrob. Agents Chemother.*, 30 (1986) 840–846.

Shannon, W.M., Westbrook, L., Higuchi, W.I., Sugibayashi, K., Baker, D.C., Kumar, S.D., Fox, J.L., Flynn, G.L., Ho, N.F.H. and Vaidyanathan, R., Influence of 1-dodecylazacyclohepton-2-one (Azone) on the topical therapy of cutaneous herpes simplex virus type 1 infection in hairless mice with 2′,3′-di-*O*-acetyl-9-β-D-arabinofuranosyladenine and 5′-*O*-valeryl-9-β-D-arabinofuranosyladenine. *J. Pharm. Sci.*, 74 (1985) 1157–1161.

Smee, D.F., Campbell, N.L. and Matthews, T.R., Comparative anti-herpesvirus activities of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, acyclovir, and two 2′-fluoro-pyrimidine nucleosides. *Antiviral Res.*, 5 (1985) 259–267.

Song, W.-Q., Estimation of the acyclovir concentration in the epidermis following transdermal drug delivery and the correlation with its antiviral efficacy. M.S. Thesis, University of Utah, Salt Lake City, UT (1989).

Spruance, S.L. and Crumpacker, C.S., Topical 5 percent acyclovir in polyethylene glycol for herpes simplex labialis: antiviral effect without clinical benefit. Acyclovir Symposium. *Am. J Med.*, 73 (1982) 315–319.

St. Clair, M.H., Furman, P.A., Lubbers, C.M. and Elion, G.B., Inhibition of cellular and virally induced deoxyribonucleic acid polymerases by the triphosphate of acyclovir. *Antimicrob. Agents Chemother.*, 18 (1980) 741–745.

Su, M.-H., Lee, P.H., Ghanem, A.H. and Higuchi, W.I., A novel method to assess bioavailability and to predict efficacy for dermatological formulations. *Chin. Pharm. J.*, 43 (1991) 265–274.