Influence of the Treatment Protocol upon the in Vivo Efficacy of Cidofovir (HPMPC) and of Acyclovir (ACV) Formulations in Topical Treatment of Cutaneous HSV-1 Infection in Hairless Mice

MOHSEN I. AFOUNA, SAMIR C. MEHTA,[†] ABDEL-HALIM GHANEM, WILLIAM I. HIGUCHI,* EARL R. KERN,[‡] ERIK DECLERCQ,[§] AND HAMED H. EL-SHATTAWY[||]

Contribution from *Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 30 So. 2000 E, Salt Lake City, Utah 84112; GlaxoWellcome Inc., 5 Moore Dr., Research Triangle Park, North Carolina 27709; The Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama 35294; Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium; and the Department of Pharmaceutics, Al-Azhar University, Nasr City, Cairo, Egypt.*

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Abstract □ In recent studies we found that the topical effectiveness of acyclovir (ACV) formulations was a single-valued function of *C**—the target site free drug concentration. The topical efficacy was the same when the therapy was initiated 0, 1, or 2 days after intracutaneous herpes simplex virus type-1 (HSV-1) inoculation in hairless mice. The purpose of the present study was to examine the hypothesis that the topical effectiveness of cidofovir (HPMPC) would not be a single valued function of *C** and that it would be dependent upon when the therapy was initiated relative to the time of viral infection. Formulations of HPMPC and ACV in 95% DMSO as a vehicle were used. Hairless mice intracutaneously infected with HSV-1 were used, and 20 μL of the test formulation was topically applied twice a day. In protocol A, the treatment was continued until the fourth day after virus inoculation, whereas in protocol B the treatment was terminated on the day of virus inoculation. Treatment was initiated on various days ranging from day −6 to day 4, and the lesions were scored on day 5. Treatment of ACV according to protocol A proved efficacious whether started as early as 6 days before virus inoculation or later, whereas the efficacy of ACV was annihilated if applied following protocol B. For HPMPC, on the other hand, the in vivo efficacies were found to be strongly dependent on how early the therapy was initiated, and significant efficacy was observed even when the treatment was terminated on the day of virus inoculation. This difference was attributed to the virus-independent intracellular phosphorylation of HPMPC and slow clearance of its metabolites from the cell. It was also noted that, similar to ACV, for HPMPC the topical efficacy is likely to be a function of *C** for a fixed protocol. However, unlike for ACV, for HPMPC the efficacy was not a single-valued function of *C**.

Introduction

In a previous study,[1] we found that the topical effectiveness of acyclovir (ACV) formulations was essentially the same when the therapy was initiated 0, 1, or 2 days after intracutaneous herpes simplex virus type-1 (HSV-1) inoculation in hairless mice. Also, a good relationship was found between the free drug concentration at the skin target site (*C**) calculated from in vitro flux data and the in vivo antiviral efficacy for a variety of ACV formulations (i.e., topical efficacy was found to be a single-valued function of *C**).[2] We have hypothesized that this relatively straightforward behavior exhibited by ACV is the consequence of the relatively rapid local pharmacokinetics that ACV may exhibit at the cellular level. A long-term objective of this research has been to investigate the *C** concept using other anti-herpes model drugs that are similar to, as well as different from, ACV. A recent report assessed the relationship of *C** with the in vivo efficacy of (*E*)-5-(2-bromovinyl)-2-deoxyuridine (BVDU), an antiherpetic drug with a mechanism of action similar to that of ACV.[3] Cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine or HPMPC) represents a new class of broad-spectrum antiviral agents that are active against a broad range of herpes viruses and exhibit rather slow cellular kinetics; particularly noteworthy is that the active metabolites are retained intracellularly for a very long time.[4] The purpose of the present study was to examine the hypothesis that because of the slow cellular pharmacokinetics, the topical effectiveness of HPMPC formulations would be strongly dependent upon when therapy is initiated relative to the time of virus infection and that, accordingly, the final outcome may not be a single-valued function of *C**.

Materials and Methods

Animals—Female hairless mice strain SKH/HR1 (Charles River, Bloomington, MA), 6−8 weeks old with average body weight of 22−27 g, were used throughout this study.

Virus—Samples from the same batch of herpes simplex virus type-1, strain E-377, with a final titer of 4×10^7 PFU/mL, were used for inoculation. They were stored at −70 °C in aliquots until used. The preparation and assay methods of the virus have been previously reported.[5]

Drug Formulations—HPMPC was generously provided by Gilead Sciences, Inc. (Foster City, CA). ACV was obtained from Thera Tech, Inc. (Salt Lake City, UT). DMSO was purchased from Baker Chemical Company (Phillipsburg, NJ). The formulations were made with 0.5 and 1% HPMPC and 0.1% ACV in 95% DMSO as a vehicle and 1% hydroxypropyl cellulose (Klucel, Hercules, Wilmington, DE) was added as a thickening agent.

In Vitro Flux Measurement and *C Predictions**—For the in vitro determination of HPMPC and ACV fluxes and *C** predictions, a combined in vivo−in vitro experimental procedure reported earlier[1,6] was adopted and performed in triplicate for each formulation. Briefly, a finite dose of 20 μL of the test formulation was applied over a rectangular skin area of 2 cm^2 on the animal

* To whom correspondence should be addressed.
[†] GlaxoWellcome Inc.
[‡] University of Alabama.
[§] Rega Institute for Medical Research.
[||] Al-Azhar University.

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Table 1—Treatment Protocols Followed for the in Vivo Efficacy Experiments. X Indicates Two Applications on that Day

	protocol A							protocol B			
	–6A	–4A	–3A	–2A	–1A	0A	1A	–6B	–4B	–2B	0B
day –6	X							X			
day –5	X							X			
day –4	X	X						X	X		
day –3	X	X	X					X	X		
day –2	X	X	X	X				X	X	X	
day –1	X	X	X	X	X			X	X	X	
day 0 (virus inoculation)	X	X	X	X	X	X					
day 1	X	X	X	X	X	X	X				
day 2	X	X	X	X	X	X	X				
day 3	X	X	X	X	X	X	X				
day 4	X	X	X	X	X	X	X				
day 5	scoring	scoring	scoring	scoring	scoring	scoring	scoring	scoring	scoring	scoring	scoring

dressed in a Velcro jacket twice a day for 2 days. The in vivo pretreatment was followed by the in vitro flux experiment in which the pretreated skin was excised from the animal and mounted on a Franz diffusion cell. Flux measurements were carried out as reported earlier[1] after applying a 10 μL/cm^2 dose of the test formulation. ACV and HPMPC concentrations were analyzed using the previously reported reversed phase HPLC methods.[2,7]

The cumulative amount of the test drug transported into the receiver chamber was plotted as a function of time, and the instantaneous flux J was estimated from the slope of the line connecting the two consecutive points. The instantaneous C^* estimates were then calculated using the following equation.

$$C^* = J/P_D \quad (1)$$

where J is the skin flux and P_D is the in vivo dermis permeability coefficient of the drug. The details of the derivation of this equation have been reported in our previous study.[8] A P_D value of 1.4×10^{-3} cm/s was obtained for ACV. Since the molecular size of HPMPC is sufficiently close (for the present purpose) to that of ACV, it was deemed reasonable to use the in vivo P_D value of ACV for HPMPC as a good approximation.

In Vivo Antiviral Efficacy Studies—The in vivo antiviral efficacy was evaluated using a group of 8–10 hairless mice per experiment, and an average of two to six experiments were performed for each drug formulation. In each experiment a group of animals treated with a placebo formulation similar to the test formulation without the drug was always included as a negative control. For the purpose of these experiments, the animals were dressed in a Velcro jacket. The details of dressing the animals in the Velcro jacket and of virus inoculation have been described earlier.[2] Two general treatment protocols were followed. For both protocols, 20 μL of the test formulation was topically applied twice a day. In protocol A, the treatment was continued until the fourth day after virus inoculation, whereas in protocol B the treatment was terminated on the day of virus inoculation. The day of virus inoculation was always considered as day 0. Treatment was initiated on various days ranging from day −6 to day 4, as outlined in Table 1. The lesions were scored on day 5 as previously described,[9] and the antiviral efficacies were calculated using the following equations:[1]

$$\text{topical efficacy (\%)} = \frac{N_{St} + N_J + N_{NR}}{N_{Th} + N_{St} + N_J + N_{NR}} \times 100 \quad (2)$$

$$\text{systemic efficacy (\%)} = \frac{N_{NR}}{N_{Th} + N_{St} + N_J + N_{NR} + N_M} \times 100 \quad (3)$$

where N_{Th}, N_{St}, N_J, N_{NR}, and N_M are the number of animals corresponding to each of the five lesion categories and represent the cases where the lesion passes through (Th), stops (St) at the edge of, jumps (J) over, does not reach (NR), or misses (M) the treatment area, respectively.

During the treatment protocol coded −6A, the dosing was performed over 11 days, and hence the animal was dressed with the Velcro jacket for that period of time. This raised the question of the possible effect of stress (induced due to prolonged dressing in Velcro jackets) on the efficacy results. To assess this, an experiment was performed in which the animals were dressed in Velcro jackets on day −6 and the treatment with 0.5% HPMPC was initiated on day 1. The results of this experiment were compared with the results obtained with protocol coded 1A (where the dressing in the jacket and the treatment were both initiated on day 1). The results of these two experiments were comparable indicating that prolonged dressing in the Velcro jacket did not compromise the efficacy results (data not shown). The same conclusion was drawn from separate experiments with 0.1% ACV formulation.

Table 2—Mean C^* Estimates ($n = 3$) for 0.5% and 1% HPMPC and 0.1% ACV Formulations

formulation	C^* (μg/mL)	SD
0.5% HPMPC	1.2	0.14
1% HPMPC	2.13	0.38
0.1% ACV	0.12	0.02

Results

Table 2 shows the mean C^* estimates obtained from the combined in vivo–in vitro experiments for the three topical formulations. For HPMPC, an increase in the applied concentration resulted in an increase in the predicted C^* levels. The saturation solubility of HPMPC in DMSO was >10%, and hence an increase in the applied concentration resulted in essentially a proportional increase in the flux, which in turn resulted in proportionally higher C^* levels. These results are consistent with those previously observed with other anti-herpes agents such as ACV and BVDU.[2,3]

Figure 1 shows the results (mean ± SD) of our preliminary experiments with 1% HPMPC in 95% DMSO as vehicle. For this formulation, when the therapy was initiated 1 day after virus inoculation (and continued for 4 days, protocol 1A), around 45% topical efficacy and 0% systemic efficacy was observed. However, when the treatment was initiated sooner, high efficacies were obtained, rising to ~100% topical efficacy and ~45% systemic efficacy when the treatment was started 2 days before virus inoculation (and continued for 4 days after virus inoculation, protocol coded −2A).

To further extend these findings, we investigated the effect of starting the treatment even sooner (up to 6 days before virus inoculation). Also, a lower HPMPC level (of 0.5%) was employed to provide a greater dosage range, and therefore greater differentiation in the effects. Figure 2A presents the results of these experiments. As can be seen here, in general the earlier the treatment was initiated, the higher were the topical and systemic efficacies with both reaching a maximum of 100% with protocol coded −6A (treatment initiated 6 days prior to virus inoculation).

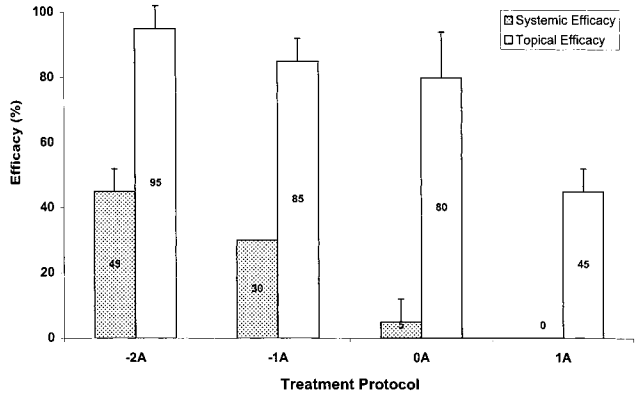

Figure 1—The topical and systemic efficacy of 1% HPMPC in 95% DMSO as a function of the treatment protocol ($n = 3$).

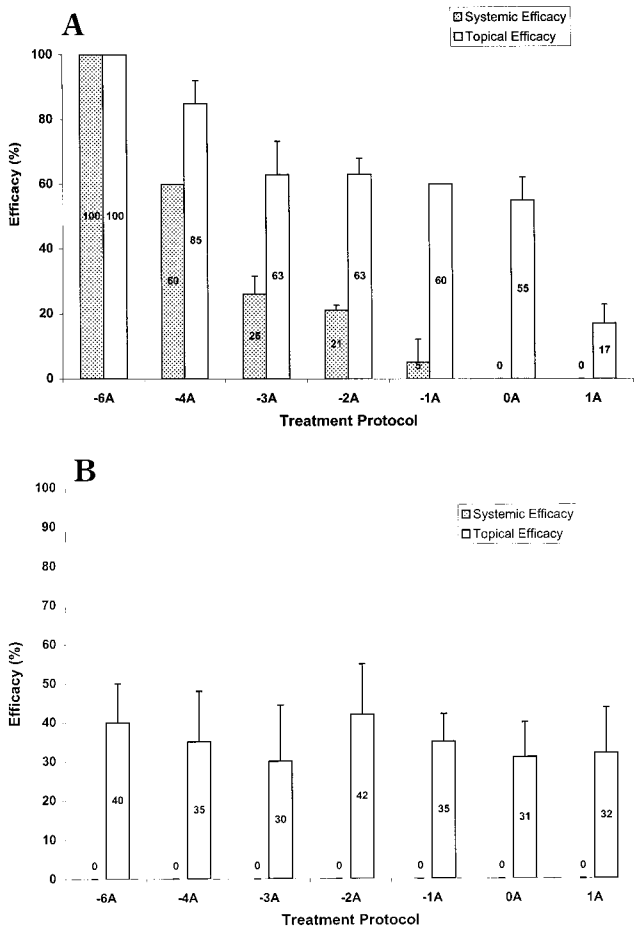

Figure 2—The topical and systemic efficacy of (A) 0.5% HPMPC and (B) 0.1% ACV in 95% DMSO as a function of the treatment protocol ($n = 3$–6).

A remarkable contrast is seen when the HPMPC results are compared to the ACV results. Figure 2B presents the results of similar experiments with 0.1% ACV. The figure clearly shows that treatment with ACV beginning as early as 6 days before virus inoculation (protocol coded −6A) had no effect on the outcomes when compared to the standard experiment of beginning the treatment 1 day after virus inoculation (protocol coded 1A). Clearly, the strong time dependency seen with HPMPC is absent with ACV.

In another series of experiments, the antiviral efficacies of 0.5% HPMPC and 0.1% ACV were tested as per protocol B (Figures 3A and 3B, respectively). In these experiments, the treatment was stopped on the day of virus inoculation. There was consequently no application of the drug formulation during the period of virus replication. Once again, there was a sharp difference in the outcomes with ACV and HPMPC. For ACV, terminating the treatment on the day of virus inoculation essentially rendered the treatment completely ineffective (Figure 3B), indicating that the application of ACV was required during the time of virus replication. However, for HPMPC, this was not the case. Topical efficacies ranging from 25 to 60% were found when the treatment was initiated 2−6 days prior to virus inoculation and stopped on the day of virus inoculation (Figure 3A). HPMPC applied prior to the infection acted prophylactically and continued to act against viral replication even though no HPMPC was applied during the time when the infection was progressing. The efficacy seen in this case seemed to be related to the duration of the exposure prior to the treatment, a longer duration of exposure with a given HPMPC formulation resulting in higher efficacy. For example, the topical efficacy obtained with 2 days (−2B) and 6 days (−6B) of exposure were 25 and 61%, respectively.

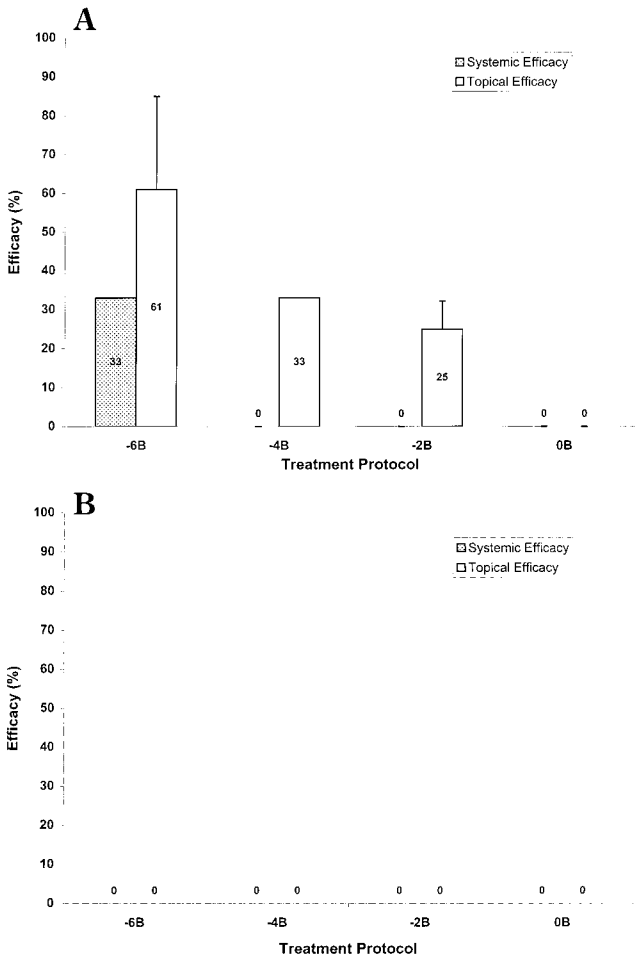

Figure 3—The topical and systemic efficacy of (A) 0.5% HPMPC and (B) 0.1% ACV in 95% DMSO as a function of the treatment protocol ($n = 2$).

Discussion

For ACV as the anti-herpes agent, starting treatment early did not alter its efficacy against cutaneous HSV-1 infections. These results extend the earlier work of Lee et al.,[1] who, employing controlled delivery transdermal systems, found that topical efficacy was independent of whether ACV treatment was started on day 0, 1, or 2 after

virus inoculation. The present data show that initiating treatment with ACV several days prior to virus inoculation essentially has the same effect as initiating treatment 1 day after virus inoculation. These results are consistent with our earlier findings[2] that topical efficacy of ACV formulations is a single valued function of *C** and can be explained by the relatively rapid pharmacokinetics of ACV at the cellular level.

Cell culture studies have shown that ACV is converted to its mono-, di-, and triphosphorylated forms (ACVp, ACVpp, and ACVppp, respectively) in cells infected with HSV-1.[10] The initial phosphorylation of ACV to ACVp is catalyzed by the HSV-1-induced thymidine kinase. Subsequent phosphorylation to ACVpp and ACVppp is carried out by cellular enzymes. In the uninfected cells, phosphorylation of ACV occurs to a very limited degree. The end product of phosphorylation, ACVppp, is a selective inhibitor of viral DNA polymerase and is the active metabolite responsible for efficacy.

The intracellular concentration of the active metabolite (species) can be regarded as a function of (a) *C** (extracellular drug concentration), (b) the rate of cellular uptake of the drug, (c) the rate of conversion of the drug to the active species, and (d) the rate of elimination of the active species. When the treatment with ACV is started prior to virus inoculation, step c is very slow. The uninfected cells lack the virally induced thymidine kinase which is responsible for conversion of ACV to ACVp (a required precursor for the active species). The rate of formation of ACVppp in the uninfected cells is approximately several orders of magnitude smaller than that in the infected cells.[11] On the other hand, step d, the rate of elimination of active species, is fairly rapid for ACV. In cell culture studies, the levels of ACVppp declined rapidly after the removal of the drug from the medium. The initial half-life of the triphosphate was 1.2 h in the absence of ACV in the medium with the levels reaching a plateau after 6 h.[11] The combined effect of the above would mean that the intracellular levels of the active species of ACV are negligible until the infection sets in. This can explain the results obtained with protocol B, where no protective effect was seen when ACV was applied as pretreatment only. With protocol A there was no additional effect afforded by ACV pretreatment. It should also be noted that in the infected cells, steps b−d are relatively rapid for ACV, and a steady state is reached quickly. Due to this rapid uptake and elimination, the intracellular level of the active ACV species and its efficacy seems to be a single-valued function of *C**, the extracellular drug concentration.

HPMPC is somewhat different from ACV in its mechanism of action. HPMPC is a nucleoside phosphonate (thus a nucleotide) analogue, in a way similar to ACVp. In cells, HPMPC is phosphorylated by host enzymes to its mono- and diphosphates (HPMPCp and HPMPCpp, respectively). HPMPCpp (similar to ACVppp) is a selective inhibitor of viral DNA polymerase and is the active species responsible for efficacy.[12] The HPMPCp−choline adduct is another intracellular moiety identified in cell culture studies. A significant difference from ACV is that the phosphorylation is carried out by the host cell enzymes and is therefore not dependent on virus infection. It has been shown that following treatment with a fixed extracellular HPMPC concentration, levels of HPMPCp, HPMPCpp, and HPMPCp−choline adduct in the cells infected with HSV-1 are the same as those in the uninfected cells.[12] This allows the opportunity for building up metabolites prior to virus infection and priming the cells to resist viral replication. It would also explain, at least in part, the results of Figure 3A where pretreatment with HPMPC acted prophylactically to resist a subsequent virus infection.

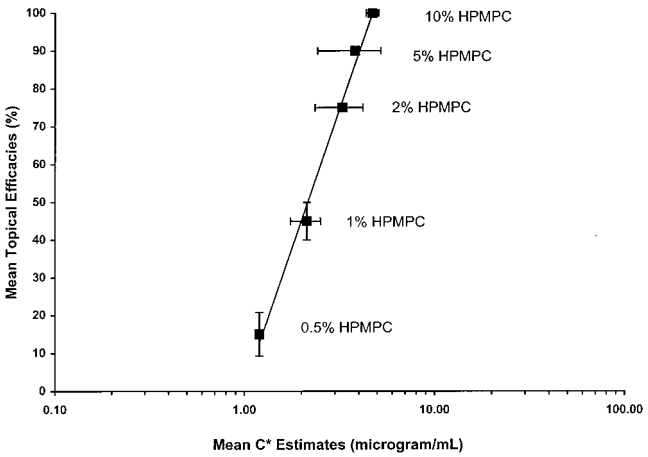

Figure 4—The topical efficacy of HPMPC as a function of *C**. The mean *C** estimates for different concentrations of HPMPC were obtained from combined in vivo–in vitro experiments ($n = 3$). The in vivo efficacies were obtained using protocol coded 1A ($n = 1$–6) (see text for details).

Another difference between ACV and HPMPC is their rate of cellular uptake and rate of conversion to the active metabolites. In cell culture studies (for ACV), the intracellular levels of ACVppp reach a maximum after 8 h of exposure of the cells to ACV.[11] For HPMPC, the intracellular levels of HPMPCpp continued to rise for 24 h after the cells were exposed to HPMPC.[13,14] More notable is the fact that the clearance of the metabolites of HPMPC from the cells is extremely slow. Various studies reported the intracellular half-life of HPMPCpp to be in the order of 17−24 h.[12−14] The long intracellular half-life of HPMPCpp after the extracellular drug is removed would be expected to lead to persistence of antiviral activity. The intracellular half-life of HPMPCp−choline adduct was reported to be >48 h. The long intracellular half-life of this adduct may produce an intracellular reservoir of drug from which the active metabolite is slowly released. This may contribute to the long duration of antiviral action of HPMPC. These facts further explain the protective effects seen with HPMPC pretreatment in Figure 3A. Also, because of the slow build-up of the metabolites and their slow elimination, it is expected that longer duration of exposure would result in higher levels of the active species and a correspondingly higher efficacy. This pattern is clearly seen in Figures 2A and 3A.

It must be noted that as for ACV, the intracellular concentration of active species of HPMPC, and in turn its in vivo efficacy, is also a function of its extracellular concentration or *C**. It was reported in cell culture studies that the HPMPCpp concentration inside the cell increased in response to increased extracellular HPMPC concentrations, and it was speculated that the antiviral efficacy should show dose responsiveness.[12] Results obtained for the treatment of HSV-1 infection with HPMPC in a murine model support this theory.[15] In a separate series of experiments we observed that for a fixed protocol coded 1A, increasing the dose of HPMPC from 0.5% to 10% resulted in an increase in the *C** values from 1.2 μg/mL to 4.7 μg/mL, which in turn resulted in an increase in topical efficacy from 17% to 100% (Figure 4). These results are very similar to those obtained with ACV.[2] However, for HPMPC, although efficacy is a function of *C**, it is not a single-valued function of *C**. Apart from *C**, the rate of its cellular uptake, rate of conversion to its active metabolites, and rate of elimination of active metabolites all have an effect on the efficacy. In conclusion, for ACV, because of the rela-

tively rapid pharmacokinetics, the intracellular level of the active metabolites as well as the in vivo efficacy may be time-independent and a single-valued function of the *C**. In contrast, for HPMPC, the in vivo efficacy is strongly time dependent and is not a single-valued function of *C** because of slow cellular kinetics of the drug and its active metabolites.

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