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A COMPARISON OF PHOSPHONOACETIC ACID AND PHOSPHONOFORMIC ACID ACTIVITY IN GENITAL HERPES SIMPLEX VIRUS TYPE 1 AND TYPE 2 INFECTIONS OF MICE*

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The activity of phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) against four strains of herpes simplex virus type 1 (HSV-1) and four strains of HSV-2 were compared in tissue culture and in a murine model of genital herpes. In mouse embryo fibroblast cells, both drugs were three-fold more active against the HSV-1 strains than against the HSV-2 strains. In contrast, in the animal model infections, PAA appeared to be more active against the HSV-2 strains, while PFA was equally effective against both HSV types. In mice infected intravaginally with HSV-2 and treated with intravaginal 5% PAA, none of the treated mice became infected, replication of virus in the genital tract was completely inhibited, none of the infected mice died from encephalitis, and latent infection in lumbosacral ganglia of surviving animals was completely prevented. In the HSV-1 genital infection treated with PAA, 20–60% of mice became infected, replication of virus in the genital tract was strikingly reduced, none of the infected mice died, and latent infection was completely prevented. In both HSV-2 and HSV-1 genital infections, 20–70% of animals treated with 8% PFA became infected, growth of virus in the genital tract was reduced significantly but not completely suppressed, mortality was variably altered, and there was a trend towards reduction in the frequency of latent infection. These results indicate that HSV-1 strains are more sensitive to PAA and PFA in tissue culture, but the HSV-2 strains are generally more amenable to therapy in the murine model of genital herpes. Although PAA appeared to be more active than PFA in the genital infection, both drugs significantly altered the course of the infection. Since dermal toxicity associated with PAA precludes its use in humans and since PFA is already undergoing trials in patients with recurrent herpes labialis, the current results suggest that topical PFA deserves further evaluation in the treatment of mucocutaneous HSV infections, including genital herpes.

phosphonoacetic acid phosphonoformic acid herpes simplex virus genital herpes mice

INTRODUCTION

Genital herpes simplex virus (HSV) infection may be caused by either the type 2 (HSV-2) or the type 1 (HSV-1) strains of virus and is now recognized as one of the

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leading causes of sexually transmitted disease in both males and females [7,13,22,30, 32]. Attempts at treatment of these infections with a variety of antiviral agents have generally been unsuccessful [1,11,22,23,25]. Since it has been reported that HSV-2 and HSV-1 may differ in their sensitivity to antiviral compounds in vitro [20,24,29] and since in vitro susceptibility may not be representative of in vivo efficacy [14,16], it is important to compare the susceptibility of strains of both types to a potential antiviral agent, not only in tissue culture, but also in experimental infections of animals. Additionally, since the sensitivity of laboratory strains with long tissue culture-passage histories may not necessarily be representative of strains isolated from a current HSV infection, recent isolates with minimal passage in tissue culture should also be examined.

We reported previously that, in experimental genital HSV-2 infection of mice and guinea pigs, topical phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) were highly effective in inhibiting viral replication in the genital tract and in preventing the development of external genital lesions and mortality due to encephalitis if treatment was initiated early [14,16]. Other studies have shown both compounds to be active in early treatment of cutaneous HSV-1 infections of mice and guinea pigs [2,9,17] and a genital HSV-2 infection of guinea pigs [3]. Although PAA is an effective topical antiviral, it has not reached clinical trials because of unacceptable dermal toxicity [2,28]. On the other hand, PFA is presently being evaluated in treatment of mucocutaneous HSV infections in humans [27]. There is limited information on the direct comparison of PAA and PFA in the treatment of both HSV-1 and HSV-2 infections, using both in vitro and in vivo systems. The purpose of the current study was to compare the sensitivity of recent and laboratory-passaged HSV type 1 and type 2 isolates to PAA and PFA in tissue culture cells, and to determine the efficacy of the two drugs in the treatment of a genital infection of mice using these same isolates.

MATERIALS AND METHODS

Experimental infection

Groups of ten 6–8 week old Swiss Webster mice (Simonsen Laboratories, Gilroy, CA) were inoculated intravaginally with 0.05 ml of each HSV strain, using a small plastic catheter attached to a syringe. Two hours prior to viral inoculation the mice were swabbed using a dacron-tipped applicator moistened in 0.1 N NaOH to remove vaginal secretions. Each animal received approximately 1×10^4 plaque-forming units (p.f.u.) of virus.

Virus strains, media, cell cultures, and virus assays

Laboratory passaged strains of known type designated MS, X-79 and E-377 were obtained from A. Nahmias, Emory University, Atlanta, GA. All other strains utilized were isolated from patients seen in our Herpes Study Clinic from oral (HL-3,HL-34) or genital (Heeter, Turner, Wilson) lesions [4,31]. These isolates were initially typed using the chick embryo cell microtest technique [34], and confirmed by restriction endonuclease

analysis [6]. The type 1 strains were E-377, HL-3, HL-34 and Wilson and the type 2 strains MS, X-79, Heeter and Turner.

The media utilized, preparation of cell cultures, and assays for HSV have been described previously [15].

Antiviral agents

Both PAA and PFA were provided through the Antiviral Substances Program (NIAID, NIH). The PAA was supplied as a 5% cream by Abbott Laboratories, North Chicago, IL. The trisodium hexahydrate salt of PFA was synthesized in the Department of Biochemistry laboratories, Michigan State University, East Lansing, MI, and was supplied by J. Boezi. To compensate for the difference in molecular weight the PFA was prepared as an 8% suspension in 0.4% agarose just prior to administration [14]. Both agents were given to mice intravaginally in a volume of 0.1 ml. In these studies, the two compounds were not tested in the same vehicle; however, in previous experiments, PAA and PFA have been utilized at equimolar concentrations in phosphate-buffered saline against HSV-1 and HSV-2 infections. The results were similar to those obtained using agarose or the cream as vehicles.

Susceptibility of HSV strains in vitro

The sensitivity of virus strains to PAA and PFA was determined by a 50% plaque reduction assay in mouse embryo fibroblast (MEF) cells. Confluent cell monolayer cultures were inoculated with 20 – 50 p.f.u. of each strain and incubated at 37°C for 1 h. Then serial two-fold dilutions of PAA or PFA in twice concentrated minimal essential medium were mixed with an equal volume of 1.0% agarose and the mixture added to the culture plates. After incubation for 48 h, the cells were stained with neutral red and plaques counted.

Assay for HSV in vaginal secretions

Vaginal swabs for quantitation of virus were obtained from both placebo- and drug-treated with animals on days 1, 3, 5, and 7 after viral inoculation. The swabs were placed in 1.0 ml of tissue culture media, vortexed, and frozen at −70°C until assayed on fetal lamb kidney (FLK) cells for the presence of HSV. Viral titers are expressed as $\log_{10}$ p.f.u./ml of media in which the swab was placed. For each experimental group of 10 animals, the mean HSV titer of the vaginal secretion specimens collected on days 1–7 were used to calculate a mean virus titer-day area under the curve [31].

Recovery of latent HSV

Surviving mice were sacrificed 90–120 days after intravaginal inoculation. The lumbosacral spinal column was removed aseptically and the dorsal root ganglia were dissected out and removed. Eight to ten lumbosacral ganglia were recoverable from each mouse. The intact ganglia from each mouse were placed into a 35 mm Petri dish with a confluent monolayer of FLK cells. The monolayers were observed for 21 days for the appearance

of viral cytopathic effect (CPE). All virus-positive cultures were confirmed by plating 0.2 ml of supernatant onto a fresh FLK monolayer and observing for typical HSV CPE.

Statistical analysis

Differences in final mortality rates were evaluated using the Fisher Exact test. The in vitro sensitivity results and the areas under the virus titer-day curves in the genital infection were compared using the Mann–Whitney *u* test.

RESULTS

Susceptibility of type 1 and type 2 strains to PAA and PFA in vitro

The mean 50% inhibitory levels from three separate experiments for four type 1 and four type 2 strains of HSV in MEF cells are listed in Table 1. Because the two compounds are of different molecular weight, the inhibitory levels are expressed both in micrograms per milliliter (μg/ml) and in millimolar (mM) concentrations. The mean values for the type 1 strains were 4.6 ± 0.5 μg/ml with PAA and 8.7 ± 1.8 μg/ml for PFA. The type 2

TABLE 1.

Susceptibility of type 1 and 2 strains of HSV to PAA and PFA in mouse embryo fibroblast cells

Virus strain	50% inhibitory levels[a]			
	μg/ml ± S.D.		mM	
	PAA	PFA	PAA	PFA
HSV type 1				
E-377[b]	4.6 ± 2.2	8.4 ± 2.1	0.025	0.028
HL-3[c]	3.6 ± 0.9	4.9 ± 2.7	0.020	0.016
HL-34[c]	6.1 ± 4.4	13.6 ± 5.1	0.033	0.045
Wilson[c]	4.1 ± 1.6	8.0 ± 0.1	0.022	0.027
Mean[d]	4.6 ± 0.5	8.7 ± 1.8	0.025	0.029
HSV type 2				
MS[b]	15.8 ± 10.5	32.8 ± 7.5	0.086	0.110
X-79[b]	15.6 ± 8.3	31.0 ± 7.0	0.085	0.103
Heeter[c]	13.5 ± 5.0	22.5 ± 0.07	0.073	0.075
Turner[c]	12.9 ± 4.6	25.9 ± 5.4	0.072	0.086
Mean[d]	14.5 ± 0.7	28.1 ± 2.4	0.079	0.094

[a] Determined by plaque reduction assay. S.D., standard deviation.
[b] Laboratory-passaged strain.
[c] Recent clinical isolate.
[d] Mean ± standard error of the mean.

strains were less sensitive with mean inhibitory values of 14.5 ± 0.7 μg/ml for PAA and 28.1 ± 2.4 μg/ml for PFA. When the two drugs are compared on an equimolar basis, however, there is little difference in the susceptibility of either HSV type to PAA or PFA. The HSV-1 strains were two to threefold more sensitive than the type 2 strains to either compound ($P = 0.02$).

Treatment of mice inoculated intravaginally with HSV type 2 strains with PAA or PFA

Treatment with 5% PAA cream or 8% PFA in agarose was administered intravaginally twice daily for 5 days beginning 3 h after intravaginal inoculation of four strains of HSV type 2. The effect of treament on the infection and mortality rates of these mice are listed in Table 2. In the placebo-treated control groups, 50–90% of the animals became infected and most infected mice died. In the groups treated with PAA, none of the animals became infected as determined by vaginal swab for HSV and none died. In contrast, when the animals were treated with an equimolar concentration of PFA, 20–50% of mice became infected and, depending on the virus strain, the mortality rate was 0–100%. Although the final mortality of PFA-treated animals was different from the placebo-

TABLE 2

Effect of treatment with PAA or PFA on infection rates and mortality rates in mice inoculated intravaginally with HSV type 2 strains

Strain of HSV type 2	Treatment	PAA				PFA			
		Infection rate[a]		Mortality rate[b]		Infection rate[a]		Mortality rate[b]	
		No.	%	No.	%	No.	%	No.	%
MS[c]	Control	8/10	80	8/8	100	8/10	80	8/8	100
	Treated[d]	0/10	0[h]	0/0		2/10	20[f]	0/2	0[f]
X-79[c]	Control	5/10	50	4/5	80	7/10	70	6/7	86
	Treated	0/10	0[f]	0/0		5/10	50	2/5	40
Heeter[e]	Control	9/10	90	9/9	100	5/10	50	5/5	100
	Treated	0/10	0[h]	0/0		3/10	30	1/3	33
Turner[e]	Control	7/10	70	6/7	86	9/10	90	8/9	89
	Treated	0/10	0[g]	0/0		4/10	40	4/4	100

[a] No. infected/No. inoculated.
[b] No. dead/No. infected.
[c] Laboratory-passaged strain.
[d] Treatment with 0.1 ml of 5% PAA cream or 8% PFA in agarose initiated 3 h after virus inoculation continued every 12 h for 5 days.
[e] Recent clinical isolate.
[f] $P < 0.05$.
[g] $P < 0.01$.
[h] $P < 0.001$.

treated controls with three of the four strains, the therapeutic effect was less than that observed with PAA treatment.

The effect of treatment with PAA or PFA on the replication of the four HSV-2 strains in the genital tract of these animals is shown in Fig. 1. In the eight placebo-treated groups the mean virus titer-day areas ranged from 13.3 to 26.9. As noted above, intravaginal treatment with PAA completely inhibited viral replication in the genital tract with all four strains. In mice treated with PFA, many of the animals still became infected, but the mean virus titer-day areas were reduced significantly (1.7–8.9) when compared to placebo-treated mice. Although PFA effectively reduced vaginal HSV titers, the effect was less striking than that observed in PAA-treated mice.

Treatment of mice inoculated intravaginally with type 1 strains with PAA or PFA

The effect of treatment with PAA or PFA initiated 3 h after virus inoculation and continued twice daily for 5 days on infection and mortality rates of mice inoculated with four strains of HSV type 1 is shown in Table 3. In the placebo-treated control

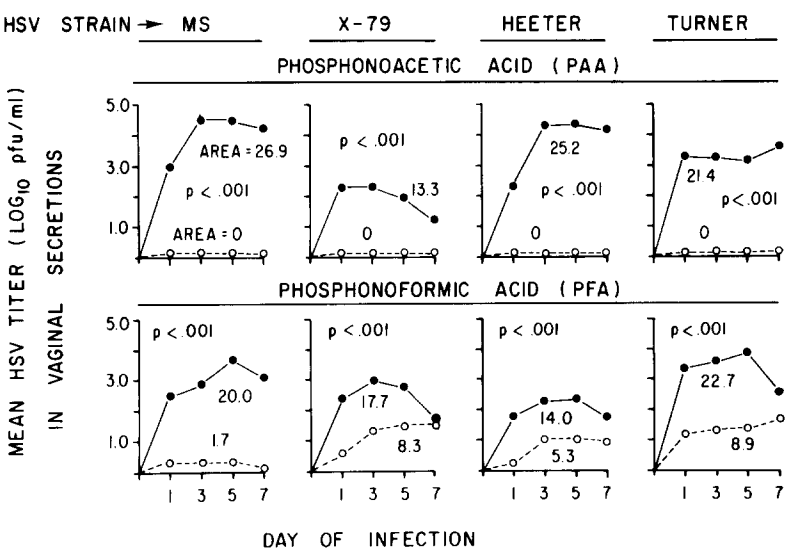

Fig. 1. Treatment of genital herpes simplex virus type 2 infection of mice with phosphonoacetic acid (PAA) or phosphonoformic acid (PFA). Treatment by the intravaginal route with 5% PAA cream or 8% PFA in agarose, twice daily for 5 days was initiated 3 h after virus inoculation. The mean virus titer in vaginal secretions is shown for days 1, 3, 5 and 7 of infection. ●–●, Placebo-treated; ○–○, drug-treated.

TABLE 3

Effect of treatment with PAA or PFA on infection rates and mortality rates in mice inoculated intravaginally with HSV type 1 strains

Strain of HSV type 1	Treatment	PAA				PFA			
		Infection rate[a]		Mortality rate[b]		Infection rate[a]		Mortality rate[b]	
		No.	%	No.	%	No.	%	No.	%
E-377[c]	Control	10/10	100	4/10	40	8/10	80	4/8	50
	Treated[d]	3/10	30[f]	0/3	0	3/10	30	0/3	0
HL-3[e]	Control	8/10	80	0/8	0	9/10	90	0/9	0
	Treated	6/10	60	0/6	0	2/10	20[f]	0/2	0
HL-34[e]	Control	9/10	90	6/9	67	8/10	80	5/8	63
	Treated	2/10	20[f]	0/2	0	5/10	50	2/5	40
Wilson[e]	Control	9/10	90	5/9	55	8/10	80	5/8	63
	Treated	4/10	40	0/4	0	7/10	70	1/7	14

[a] No. infected/No. inoculated.
[b] No. dead/No. infected.
[c] Laboratory-passaged strain.
[d] Treatment with 0.1 ml of 5% PAA cream or 8% PFA in agarose initiated 3 h after virus inoculation and continued every 12 h for 5 days.
[e] Recent clinical isolate.
[f] $P < 0.01$.

groups, 80–100% of mice became infected and final mortality ranged from 0 to 67%. It should be noted that mortality cannot always be used as a parameter of treatment efficacy, since the type 1 strains of HSV are generally less virulent than type 2 strains when inoculated intravaginally. In mice inoculated with the type 1 strains and treated with PAA, 20–60% became infected, but all infected animals survived. Twenty to seventy percent of animals treated with PFA became infected and 0–40% of these died.

The effect of treatment with PAA or PFA on the replication of the HSV type 1 strains in the genital tract is shown in Fig. 2. In the placebo-treated groups, the mean HSV titer-day areas ranged from 24.0 to 32.0 for each of the four strains. Virus in the genital tract of mice inoculated with type 1 strains replicated to higher titers than the type 2 strains, but appeared to clear faster. Treatment with PAA markedly inhibited replication of all four HSV-1 strains, where area under the curve values ranged from 2.3 to 4.2. With PFA therapy, inhibition of the E-377 and HL-3 strains was similar (areas of 3.3 and 2.4, respectively), but suppression of HL-34 and Wilson was less striking (areas of 13.0 and 12.2). Nevertheless, in both PAA- and PFA-treated mice, virus titers in genital samples were significantly lower than those from placebo-treated mice.

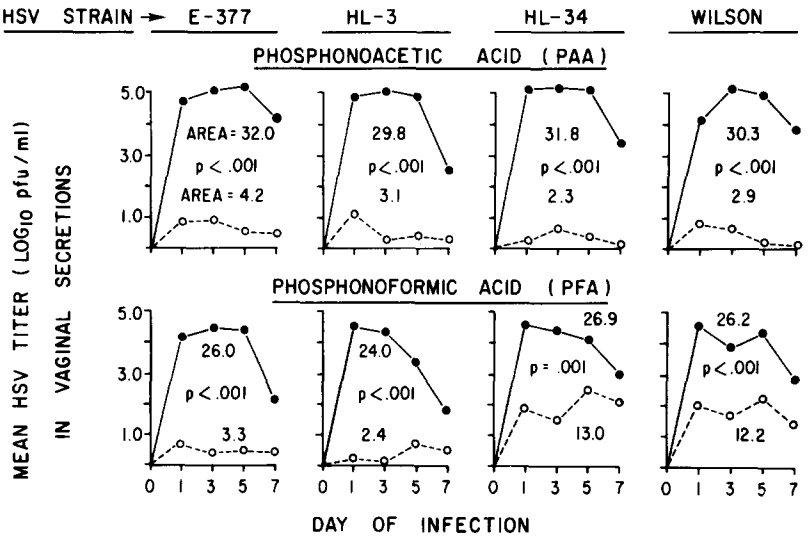

Fig. 2. Treatment of genital herpes simplex virus type 1 infection of mice with phosphonoacetic acid (PAA) or phosphonoformic acid (PFA). Treatment by the intravaginal route with 5% PAA cream or 8% PFA in agarose, twice daily for 5 days was initiated 3 h after virus inoculation. The mean virus titer in vaginal secretions is shown for days 1, 3, 5 and 7 of infection. ●–●, Placebo-treated; ○–○, drug-treated.

Isolation of HSV from spinal ganglia in placebo-, PAA- or PFA-treated mice

Since treatment with PAA or PFA reduced or prevented mortality and substantially reduced vaginal virus titers in all animals, regardless of the strain utilized, we next determined whether treatment was also effective in preventing the virus from reaching and becoming latent in the lumbosacral ganglia. At least three surviving mice from each untreated and treated group with the four HSV-2 and the four HSV-1 strains were sacrificed for examination for latent virus in ganglia. The one exception was placebo-treated mice infected with the MS strain of HSV-2, where there were only two survivors available for examination.

In HSV-2-challenged animals, latent virus was demonstrated in seven of 11 (64%) placebo-treated, but none of 12 PAA-treated ($P = 0.007$) and two of 12 (17%, $P = 0.13$) PFA-treated mice. In animals infected with HSV-1 strains, latent virus was demonstrated in six of 12 (50%) placebo-treated, but none of 12 PAA-treated ($P = 0.01$) and two of 12 (17%, $P = 0.12$) PFA-treated animals.

DISCUSSION

The susceptibility of strains of HSV to antiviral compounds in tissue culture can be variable depending on the type of assay system, the species of host cells, the pH of the medium, the use of inhibitors to prevent drug degradation, and other factors. The data presented in this report indicate that the type 1 strains are three-fold more sensitive to PAA and PFA in MEF cells than the type 2 strains. There was no difference between recent clinical isolates and laboratory-passaged strains in their susceptibility to PAA or PFA. The PAA and PFA inhibitory levels we observed for both the type 1 and the type 2 strains are similar to what has been reported previously [12,14,16,26]. Although most studies have shown little or no difference in sensitivity to antiviral agents between the type 1 and the type 2 strains of HSV [5,10,16,21,29], the type 1 strains appear to be more susceptible to idoxuridine, adenine arabinoside and bromovinyldeoxyuridine in some systems [8,10,24,29].

The greater susceptibility of the HSV-1 strains to PAA and PFA in tissue culture did not reflect the results of treating the infections in mice. Topical 5% PAA ointment appeared to be more effective against the HSV-2 than against the HSV-1 genital infection. With the HSV-2 infection none of the mice became infected, replication of virus in the genital tract was completely inhibited, none of the mice died from encephalitis, and latent infection of lumbosacral ganglia was completely prevented. In the HSV-1 genital infection treated with PAA, 20–60% of mice became infected, replication of virus in the genital tract was strikingly reduced but not completely inhibited, none of the mice died, and none had latent infection in sensory ganglia. Topical PFA appeared to be equally active against the HSV-2 and the HSV-1 genital infection. In both infections 20–70% of animals treated with topical 8% PFA became infected, replication of virus in the genital tract was reduced significantly but not completely suppressed, mortality was variably altered, and there was a trend towards reduction in the frequency of ganglionic latency. Other investigators have noted differences between in vitro susceptibility and in vivo efficacy. North and coworkers [24] compared the activity of idoxuridine and adenine arabinoside against one strain of HSV-1 and one of HSV-2 in tissue culture and in a rabbit keratoconjunctivitis model. Although the type 1 strain was more sensitive to both drugs in cell culture, the infection in rabbits with either virus type responded equally well to treatment with either drug. These combined results further substantiate the concept that, with antiviral agents, data from in vitro susceptibility testing may not be predictive of efficacy in experimental animal infections. The results also underscore the importance of testing antiviral agents in animal models of human viral disease before proceeding to clinical trials.

The results in the current study indicate that PAA was more effective than PFA in treating both HSV-2 and HSV-1 genital infections of mice. In cutaneous HSV-1 infections of mice, Klein et al. [17–19] reported that PAA treatment initiated early in the course of infection was more effective than PFA treatment in preventing the development of skin lesions and subsequent mortality. Similar results were reported in an HSV-1 skin

infection in athymic nude mice [9]. On the other hand, the two compounds were reported to be equally effective in the treatment of cutaneous HSV-1 and HSV-2 infections of guinea pigs [2]. These combined results indicate that, in general, topical PAA appears to be somewhat more effective than PFA in mucocutaneous HSV infections of experimental animals.

Prevention of latent infection in regional sensory ganglia is another important goal in the treatment of primary genital herpes infection. The results in the current study indicate that PAA was more effective than PFA in reducing the frequency of latent infection in lumbosacral ganglia of mice surviving both the HSV-2 and the HSV-1 genital infections. Wohlenberg and coworkers [33] demonstrated that treatment of mice with HSV-1 skin infection with PAA within 24 h of dermal challenge significantly reduced the frequency of latent ganglionic infection. Treatment after 24 h, however, failed to alter ganglionic infection rates, and therapy had no influence on latent infection already established in ganglia. In a dermal HSV-1 infection of hairless mice, Klein and coworkers [17–19] showed that PAA was more effective than PFA in preventing the establishment of latent ganglionic infections when treatment was begun 3 h after viral challenge. These combined studies indicate that PAA is more effective than PFA in preventing latent ganglionic infection of mice challenged in the skin or mucous membranes with either HSV-1 or HSV-2. In order to be successful, however, topical therapy must be applied very early in the course of the infection, presumably before HSV reaches sensory nerves.

The data from this study indicate that PAA is more effective than PFA in the treatment of a HSV-2 genital infection of mice and that PAA is similar to PFA in the HSV-1 genital infection. However, early therapy with both drugs significantly altered the course of infection with both HSV-2 and HSV-1 strains. Importantly, PAA has not come to clinical trials in patients because of dermal toxicity [2,28]. On the other hand, PFA is currently being evaluated in the topical therapy of herpes labialis [27]. Our results suggest that continued evaluation of PFA in mucocutaneous HSV infections of patients is warranted and that this compound may be of value in genital herpes.

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