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Suppression of Herpes Simplex Virus Infection by Phosphonoacetic Acid

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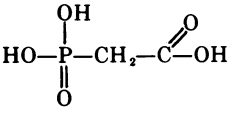
Disodium phosphonoacetate when administered orally or topically to mice experimentally infected with herpes simplex virus was able to significantly reduce the mortality associated with the agent. In addition, this compound was able to reduce herpesvirus lesions on the corneas of infected rabbits.

The literature has revealed no reports of antiviral activity associated with phosphonoacetic acid (PAA) or its analogues. PAA has been known since 1924 (24). Compounds containing a carbon to phosphorous bond are rare in nature, and until recently were thought to be nonexistent (7). Inglot et al. (17-20) reported the antiviral activity of benzyl phosphonic acid against encephalomyocarditis virus which is a picornavirus and completely unrelated to herpesvirus.

Random testing of compounds with a tissue culture screen designed to detect antiviral compounds revealed that PAA inhibited replication of herpesvirus types 1 and 2. The activity was consistently obtained at a concentration of 100 μg/ml.



Phosphonoacetic Acid

Our in vitro data suggested the possibility of achieving a favorable in vivo therapeutic index against herpesvirus. We present data on our animal studies.

MATERIALS AND METHODS

Chemicals. PAA was obtained from Bodman Chemicals. After preliminary animal experiments with the free acid, it was decided to use a salt in the animal experiments to lessen skin irritation. Since disodium phosphonoacetate in aqueous solution is approximately neutral, animal experiments were performed with this salt. Preparation was as follows. PAA (739 g, 5.28 moles) was dissolved in 500 ml of water. Sodium hydroxide (422.2 g, 10.6 moles) in 1,500 ml of water was added with stirring and cooling. The solution was concentrated to a syrup. Two liters of ethanol (3A) was added and digested on the steam bath until it became solid. The solid was filtered, washed with alcohol, and dried. A second crop was obtained by concentrating the alcohol (yield, 995 g). The calculated analysis for $C_2H_3Na_2O_5P$ was: 17.26% of phosphorus and 25.56% of sodium. The actual analysis yielded 16.84% of phosphorus and 25% of sodium.

Virus. Herpesvirus, type 1, rabbit kidney, passage-4; Wi-38, passage-1; titer of $10^{6.0}$ mean tissue culture infective dose ($TCID_{50}$) per ml. Herpesvirus, type 2, rabbit kidney, passage-1; Wi-38, passage-1; titer of $10^{7.0}$ $TCID_{50}$ per ml.

Herpesvirus types 1 and 2 were obtained from the American Medical Association Research Group, Chicago, Illinois. Tissue culture growth was carried out with Eagle medium 199 and incubation at 37 C. Virus was harvested when cytopathic effect was complete, after 3 to 4 days of incubation. Storage was −60 C.

Mouse dermatitis, pathogenesis. Twenty gram, female, CF mice, under light ether anesthesia, had a 10-mm square area of their back plucked free of hair. Herpesvirus, type 2 (0.05 ml), was applied topically to the denuded skin (28). A 24-gauge sterile hypodermic needle was used to prick the surface of the skin beneath the drop of herpesvirus. A small 1 by 1-mm herpes vesicle developed in 3 to 5 days. In 6 to 10 days the round vesicle developed further into a 2 by 10-mm band that stretched across the denuded area. In 11 to 15 days the mice developed a flaccid, posterior paralysis. Once paralyzed, death usually followed in 24 h. In untreated mice, there was a good correlation between lesion development and subsequent death. Thus, mortality was used as a measure of activity of the compound against herpes dermatitis in mice.

Mouse dermatitis, treatment. For topical application, the compound was applied to the site 2 h after the virus had been placed onto the skin and the needle pricks had been administered. The compound was then applied twice daily for 5 consecutive days at a concentration indicated in the individual experiments. An ointment containing lanolin, mineral oil, and white petrolatum was prepared. Various groups of mice were treated with either an aqueous suspension of the drug or with the ointment.

For oral administration, an aqueous suspension of the drug was prepared and administered by gavage to

the mice. The first dose was administered 2 h after infection, then twice daily per day for 5 consecutive days.

Herpes keratitis, pathogenesis. The corneas of both eyes of anaesthetized (Nembutal) New Zealand rabbits were abraded with a sterile dry swab. Four rabbits were used for each medication group. Herpes simplex virus, type 1, was applied with a second swab. The eyes of the rabbits were examined for corneal lesions with fluorescein solution and a Wood's ultraviolet light. The severity of the corneal lesions was used as an index of efficacy and was graded as follows. The size of lesions in millimeters was none, 1 to 3, 4 to 7, 8 to 10, and complete; the corresponding ratings were 0, 1, 2, 3, and 4, respectively.

Herpes keratitis, treatment. The left eye of each rabbit was used as the untreated control. The right eye was treated with various concentrations of the drug, either aqueous or incorporated into an ointment. The concentration of drug in aqueous form was 5%, and the concentration of drug in ointment was 5, 2, 1, 0.5, 0.1, and 0.05%. The topical treatments (2 drops or 0.1 ml) were started 2 h postinfection and were repeated hourly for 4 h on the day of infection, then hourly for 8 h on each of postinfection days 1 and 2. On day 3, the animals were treated every hour for 4 h. A total of 24 treatments was conducted according to the following schedule: postinfection day 0, treatment every hour for 4 h; postinfection day 1, treatment every hour for 8 h; postinfection day 2, treatment every hour for 8 h; and postinfection day 3, treatment every hour for 4 h.

Herpes keratitis, evaluation. For each individual experiment, the corneal lesion scores for treated eyes on any given day of treatment were compared with untreated control eyes. The means for each cell of four rabbits were considered to be one treatment group, and a comparison was made with the untreated control eyes. Comparison was made by the Duncan multiple range test (9). This indicates which of the means in a series of means is significantly different at the $P_{0.05}$ level.

RESULTS

Efficacy of disodium phosphonoacetate by the topical application of an aqueous or ointment preparation from 0.5% to 5.0% was demonstrated in mice experimentally infected with herpes dermatitis. Efficacy was also demonstrated when the compound was administered orally, using an aqueous suspension with a dosage range of from 800 to 1,400 mg per kg per day for 6 days (Table 1). The mice that died all had lesions typical of herpesvirus infection; normal control animals did not die. Virus-isolation studies were not carried out.

The topical application of disodium phosphonoacetic acid to the eyes of rabbits for 3 days after they were experimentally infected with herpesvirus produced the following results (Table 2).

A 5% drug-in-liquid treatment or 5, 2, 1, or 0.5% drug-in-ointment treatment reduced corneal lesions from 5 to 9 days after infection. After 10 days, the lesion scores between treated and untreated groups were not significantly different. Drug concentrations below 0.5% were not effective with the exception of one group in the 0.05% series.

Herpes keratitis is a self-limiting infection of

TABLE 1. *Effect of disodium phosphonoacetic acid on herpes dermatitis in mice*[a]

Experiment	Group	Drug			Mice	P[b]
		Route	Vehicle	Concn	No. dead/ total	
1	Virus Control	—	—	—	10/10	
	Treated	Topical	Aqueous	0.5%	2/10	0.0003
	Treated	Topical	Aqueous	1.0%	1/10	0.0000
	Treated	Topical	Aqueous	2.0%	0/10	0.0000
	Treated	Topical	Aqueous	4.0%	0/10	0.0000
2	Virus Control	—	—	—	8/10	
	Treated	Topical	Ointment	1.0%	0/10	0.0003
	Treated	Topical	Ointment	2.0%	0/10	0.0003
	Treated	Topical	Ointment	5.0%	0/10	0.0003
3	Virus Control	—	—	—	10/10	
	Treated	Oral[c]	Aqueous	800[d]	0/10	0.0000
	Treated	Oral	Aqueous	1000[d]	0/10	0.0000
	Treated	Oral	Aqueous	1200[d]	0/10	0.0000
	Treated	Oral	Aqueous	1400[d]	0/10	0.0000

[a] Herpesvirus, type 2, Wi-38; titer $10^{7.0}$ $TCID_{50}$ per ml.
[b] Fisher exact probability for proportions (11).
[c] Milligrams per kilogram per day for 6 days.
[d] Concentration in milligrams per kilogram per day.

TABLE 2. *Effect of disodium phosphonoacetic acid on herpes keratitis lesions in rabbits*[a]

Experiment	Group	Mean lesion scores		
		Days postinfection		
		5-6	7-8	9-12
1	Untreated	2.73	2.27	1.0
	5.0% Ointment	0.25 (S)[b]	0.25 (S)	0.33 (N)
	0.10% Ointment	2.50 (N)	1.50 (N)	0.75 (N)
	0.05% Ointment	2.00 (N)	0.66 (N)	0.66 (N)
2	Untreated	2.33	1.70	0.6
	5.0% Ointment	0.0 (S)	0.0 (S)	0.0 (N)
	0.50% Ointment	0.75 (S)	0.33 (N)	0.0 (N)
	0.25% Ointment	2.5 (N)	2.5 (N)	1.5 (N)
3	Untreated	3.25	2.75	1.64
	5.0% Ointment	0.00 (S)	0.00 (S)	0.00 (S)
	2.0% Ointment	1.25 (S)	1.00 (S)	0.33 (N)
	1.0% Ointment	1.00 (S)	1.25 (S)	0.75 (N)

[a] Herpesvirus, type 1, Wi-38; titer $10^{6.0}$ $TCID_{50}$ per ml.

[b] Duncan multiple range test (9). Abbreviations (at $P_{0.05}$ level): S, significant; N, not significant.

the eye. As time progresses after the infection, the eye lesions become less severe and eventually disappear. However, during the early, acute phase of the infection disodium phosphonoacetic acid in concentrations greater than 0.5% was able to reduce the lesions caused by the virus. In the experimental design of this experiment, both eyes of a rabbit were infected; the right was treated while the left acted as the infected control. Probably as a result of this design, herpesvirus was in some instances able to cause central nervous system involvement and death of the rabbits.

DISCUSSION

After a tissue culture screen revealed that PAA inhibited replication of herpesvirus types 1 and 2, several analogues were also tested in tissue culture. The structural requirements for activity were very rigorous. The triethyl ester (3) was tested and found to be devoid of activity, as were phosphonopropionic acid, disodium phosphonobutyrate, and disodium α-methyl phosphonoacetate. To be certain that the activity of the free acid was not attributable to a pH effect, acetic and phosphoric acids were also tested at equivalent molar concentrations in tissue culture. Both were inactive.

Disodium phosphonoacetic acid was chosen to test in vivo efficacy. The animal models used were herpes dermatitis in mice, utilizing type 2 virus, and herpes keratitis in rabbits, utilizing type 1 virus. The compound was found to be effective against the dermatitis when applied topically in concentrations of from 0.5 to 5.0% and when administered orally at a dosage range of from 800 to 1,400 μg per kg per day for 6 days. The corneal lesions caused by herpesvirus, type 2, were significantly reduced in severity when the compound was applied topically at concentrations of 0.5 to 5.0%.

It should be noted that in our in vivo studies, drug treatment began 2 h after virus inoculation. This model system does not allow a direct comparison with human infections where virus has been present for weeks or years in latent form. Nevertheless, in an initial animal test system every attempt should be made to permit the drug to reveal its potential. Further experiments are planned to demonstrate the effects of the drug in chronic infections in animals.

It is quite surprising that a compound as highly charged as PAA can penetrate the cell membrane. Perhaps the active moiety is not PAA but a derivative of PAA. However, determination of this requires further work.

Herpes dermatitis in man is a very common though usually minor disease for which no effective form of therapy exists (29). Better control of this condition could result in fewer cases of herpes keratitis, the single most common corneal infection in the United States (2). The great reduction in the mortality of mice infected with herpesvirus and treated with disodium phosphonoacetate suggests that this compound might be effectively used in herpes encephalitis. The nature of this condition is so serious that even a drug of low therapeutic ratio would be useful (6). A common complication of presently available immunosuppressive therapy is increased susceptibility to herpesvirus infection. Normally a benign though annoying resident of the body, the virus may become activated and produce a fatal infection in the presence of lowered host resistance (8). This compound could be useful in such situations.

Serologic and epidemiologic studies have suggested an implication of herpesviruses with certain types of cancer in animals (12, 14-16, 21, 25) including humans (1, 4-5, 10, 13, 22-23, 26-27). The use of specific herpesvirus inhibitors, such as PAA, in controlling infections may

contribute to further understanding of the relationships of these viruses to cancer.

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