Antiviral activity of a D-glucosamine derivative against herpetic ulcers (HSV type 2) in rabbit cornea

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Abstract. Although most herpetic ocular infections in adults are caused by herpesvirus hominis type 1, several cases of culture proved HSV-2 ocular infection in adults have been described, with more severe and prolonged disease. In a screening for new antiherpetic compounds, we investigated the efficacy in vivo of a new compound, nitroderivative of D-glucosaminhydrochloride (GN-11) in comparison with D-glucosaminhydrochloride (GN), Acyclovir (ACV) and placebo against herpetic keratitis of herpes simplex type 2 in 4 × 4 eyes from 4 × 4 rabbits, respectively. ACV and GN-11 showed similar results. The treatment with GN-11 retarded the appearance of herpetic lesions, which were small and diffuse in comparison with the placebo group. A total recovery was obtained on the 12th day of the treatment. In the ACV treated group, a minimal number of small lesions appeared, but the eyes recovered normality on the 7th day of treatment. The appearance of acute herpetic keratitis was prevented by GN-11. Placebo and GN treated groups showed similar evolution, with lost vision and neurological involvement on the 7th day of infection.

Key words: antiherpetic activity – experimental herpes keratitis – D-glucosamine.

Herpes simplex virus is an important etiological agent of ocular infection and a major cause of blindness in industrialised countries. Following an initial episode of herpetic keratitis, approximately 40% of all patients experience one or more recurrences within 2 years. Latently infected ganglia have been thought to be the source of virus for recurrent herpetic diseases in humans (Kaufman 1981). The repeated recrudescence of latent herpes virus results in active keratitis and corneal damage sufficiently severe to cause visual impairment in many patients. Although most herpetic ocular infections in adults are caused by herpesvirus hominis type 1 (HSV-1) and the majority of genital disease is secondary to herpesvirus hominis type 2 (HSV-2) exceptions to these rules occur. Several cases of culture-proved HSV-2 ocular infection in adults have been described (Neumann et al. 1978). The patients with ocular HSV-2 appear to have had a more severe and prolonged disease than the average patient with ocular HSV-1. There is experimental evidence to support this clinical observation.

The conjunctivitis, corneal ulceration, pannus and iritis that resulted from dropping HSV-2 on intact and abrased rabbit corneas, were more pronounced and of longer duration than the changes produced by HSV-1 (Oh Jo et al. 1972; Oh Jo & Stevens; Stevens & Oh Jo 1973; Garcia et al. 1979). The majority however, of antiviral drugs are tested in vivo only against HSV-1 corneal infection. In an attempt to identify new compounds with antiherpetic activity we have tested the ability of several sugar analogs to inhibit the replication of HSV-2 in Vero cells (data in preparation). Because many times in antiviral research the promising results in cultured cells are ineffective when proved in animals or gives rise to hypersensitivity and toxic manifestations, the present study pretends to evaluate the in vivo activity of compound, nitroderivative of D-glucosamine, which is an effective inhibitor of herpes simplex-2 replication in vitro.

Materials and Methods

Virus

The work was carried out with the Lovelace strain of herpes simplex type 2, obtained from the National Research Centre of Virology, Inmunology and Farmacology, Majadahonda, Spain. The virus was grown in cultures of Vero cells to yield a titer of 1×10^9 plaque forming units (PFU) per ml. Aliquots of the stock solution were stored at −70°C until use. For the infection of rabbits an aliquot of the stock solution was diluted to a virus concentration of around 10^6 pfu/ml.

Animals

New Zealand white rabbits weighing between 2.0 and 3.0 kg were obtained from Biocenter, Barcelona, Spain. They were examined for pre-inoculation ocular abnormalities and housed several days prior to the experiment.

Compounds tested

The nitroderivatives of D-glucosamine were electrolytically synthesized by A. Martin employing D-glucosamine (Merck) catalytically diazoted with ion Fe^{+2} in the form of $Fe\,SO_4\,7\,H_2$. The procedure is a modification of the patent number 554 727 (1986) Consejo Superior de Investigaciones Cientificas (C.S.I.C.) Spain, which will be published in brief. The stucture of the resulting nitroderivative is not completely elucidated yet.

D (−) glucosaminhydrochloride (Merck) (GN) and transformed D (−) glucosaminhydrochloride (GN-11), were prepared as aqueous solutions in concentrations of 10%.

Aciclover (Zovirax) was kindly supplied by Gayoso-Wellcome Laboratories, Alcalá de Henares, Spain, and administered as aqueous suspension in a concentration of 3%. Some drops (3. approximately 100 µl) of DMSO were added to obtain better diluted suspension.

Method of infection and observations

The corneal epithelium of the right eye anaesthetized with 0.5% tetracaine clorhydrate (Lab. Cusi SA, Barcelona, Spain) was scratched with a 25-gauge needle making 6 criss-cross strokes. The eyes were then inoculated with 50 µl of HSV-2 suspension (10^6 PFU/ml), closed and gently massaged for 15 sec. Fluorescein stained corneas were examined daily by the same observer, using a slit-lamp biomicroscope (adapted to rabbits management, with a modification designed by R. Esteso Melero, Centro de Investigaciones Biológicas, Spain) and the number of lesions or the extent of corneal epithelial involvement was scored on a scale of 0 to 4 based on the Alenius et al. (1980) valoration.

Treatment and experimental design

A) Toxicity. An experiment to evaluate eye irritancy and ocular toxicity was carried out in 2×4 eyes from 2×4 rabbits, with 5% and 10% of an aqueous solution of the compound GN-11 and was administered in drops using a Gilson automatic syringe (100 µl). Treatments were given 5 times daily during 10 days. No signs of toxicity were revealed.

B) Viral infectivity. Preliminary experiments to evaluate the most adequate concentration of HSV-2 inoculum were carried out with two groups of four rabbits which were inoculated in one eye with 10^8 UFP/ml and 10^6 UFP/ml, respectively and observed for 17 days to study the viral evolution.

C) Infection and Treatment. Four groups of 4 rabbits, housed and allowed to acclimatize to their new environment for at least one week, were unilaterally eye infected (right eye) with a concentration of virus around 10^6 PFU/ml. Five hours after virus inoculation, therapy was initiated in both eyes to have an additional control for toxicity throughout the experiment. Animals in this study were divided randomly into four different treatment groups (4 rabbits per group).

The first group received topical 10% GN-11 solution diluted in saline and stored at 4°C until used. Second 4 rabbits received 10% solution of GN, and for comparison another 4 animals received drops of a 3% acicloguanosine solution (µl).

A saline placebo treatment was given on the same schedule.

Treatment was carried out four times a day for 10 consecutive days.

The average score of each group was calculated from day 0 to 14 th.

Results

Preliminary experiments were carried out with 2 groups of 4 rabbits which were inoculated in one eye with different HSV-2 suspensions. As shown in Fig. 1, the evolution of the herpetic keratitis was similar in the 2 groups of rabbits inoculated with

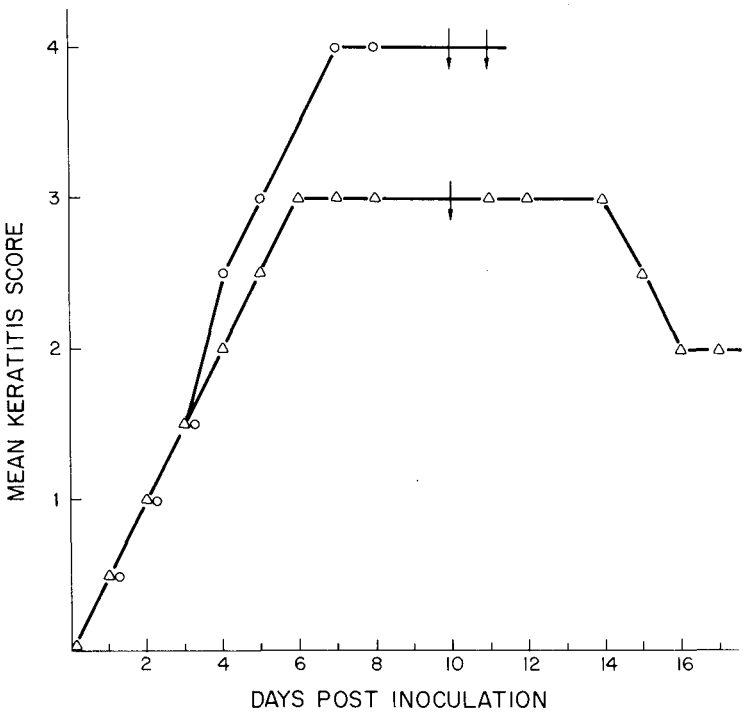


Fig. 1.

Comparison of herpetic keratitis evolution in rabbits inoculated with two different suspensions of HSV-2. Inoculum 1 × 10^8 PFU/ml (○) induced 100% of mortality while 1 × 10^6 PFU/ml (△) produced only a 50% of deaths (arrows). Two groups of four rabbits were inoculated.

different HSV-2 concentrations, but in the first group (10^8 UFP/ml inoculum) all the animals died on the 11th day of post-inoculation. The other group (10^6 UFP/ml inoculum) showed neurological involvement with hemiparalysis, but 50% survived the experiment.

We concluded that 10^6 UFP/ml was the most adequate concentration of HSV-2 inculum to insure a quick evolution of keratitis with enough percentage of survivors in the placebo treated group.

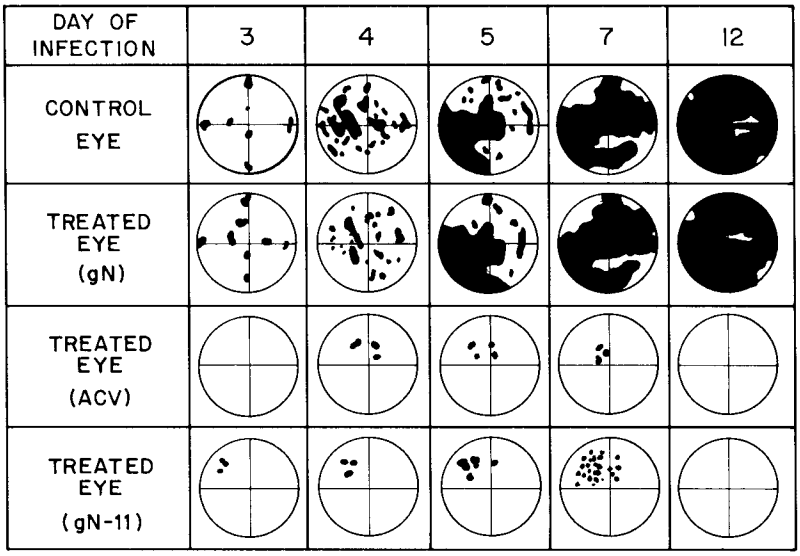
Fig. 2 shows the differences between both treated and placebo groups. The treatment with GN-11 retarded the appearance of herpetic lesions for 48 h, and they were small and diffuse showing slow evolution without conjuntivitis. A quick recovery began on the 9th day of treatment and progressed until the 12th day, on which the animals had normal eyes without lesions. No corneal clouding was present.

In comparison with the ACV treated group, these animals showed a minimal number of countable lesions on the second day of infection, and the same number remained constant until the 7th day of treatment when they disappeared and the eyes recovered normality. There were no manifestations of neurological diseases at this time.

Treatment with compound GN lightly retarded the appearance of the first scores, but they developed the same as in the control virus group and reached the maximum on the 7th day of infection. The animals lost their vision, and neurological involvement was evident. One rabbit died in this group.

On the 7th day of the experiment, GN treated rabbits did not present differences with the control group of animals. By contrast, treatment with GN-11 showed a high viral inactivation, but less than the ACV treated group. The comparison was better on the 12th day of the experiment on which both ACV and GN-11 treatment obtained the complete disappearance of herpetic keratitis scores.

In the placebo treated group, two of the animals



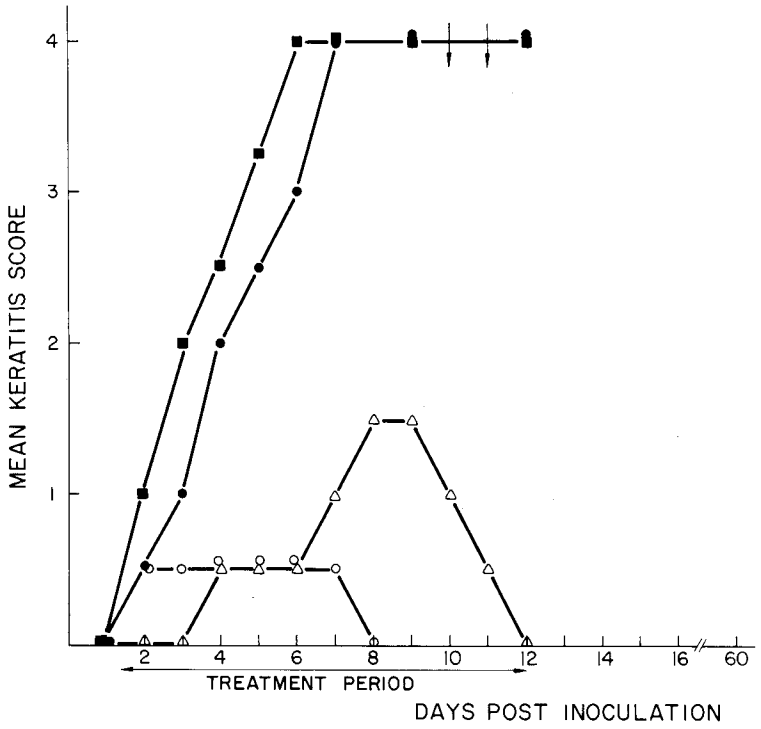


Fig. 2.

Degree of severity of keratitis score observed in rabbits following inoculation with 10^6 PFU/ml of HSV-2. Beginning 5 h after inoculation, eyes were treated topically 4 times/day with GN, GN-11, ACV and placebo (saline), (4 rabbits/group). Treatment was continued for 10 consecutive days. Arrows indicate the date of deaths. Two animals died in the placebo treated group, one died in the GN treated group. All the survivors stayed under observation for 60 days. GN treated group (●), placebo treated group (■). ACV (○) and GN-11 treated group (△). In the ACV treated group one of the four rabbits showed an anomalous regression 20 days after the treatment was finished. Shaded areas indicate corneal ulceration. Completed protocol form based in D. J. Bauer et al. (Br J Ophthalmol, 1979, 63: 429–435).

Table 1.
Recovery of HSV-2 from rabbit trigeminal ganglia. Culture with Vero cell monolayer.

No. of culture day	Trigeminal ganglia removed from inoculated rabbits 60 days post topical treatment				
	ACV	GN11	GN	Placebo	Control cells
N	3	4	2	2	
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	±	±	0
4	0	0	+	+	0
5	0	0	+	2+	0
7	0	0	2+	4+	0
9	0	0	3+	4+	0
13	0	0	4+		0

0 = no cytopathic effects. 4+ = maximum cytopathic effects. N = number of rabbits.

died on the 10th day and 11th day after inoculation. In the group treated with GN one rabbit died on the 11th day.

The survivors were kept in observation for 60 days more. In the ACV treated group one of the animals showed on the 32nd day post-infection, signs of virus re-activation, with punctual and diffuse corneal scores and light conjunctivitis. Four days later, the animal presented 1/4 of the infected eye with confluent lesions (grade 2.5 of Alenius et al.) and also hemiparalysis and convulsions. The other rabbits in the ACV treated group, and the GN-11 treated rabbits, were healthy and presented normal eyes.

The surviving animals in the placebo and GN treated groups lost their vision and showed hemiparalysis.

When the experiments were concluded, the rabbits were sacrificed, and some trigeminal ganglia were removed to be processed as described by Bubel et al. (1986). Infectious virus was recovered from trigeminal ganglion of both placebo and GN treated rabbits (2 rabbits processed per group) but not from GN-11 treated animals (all the animals were processed), nor from the ACV treated group that survived without infection for 60 days (3 rabbits) (Table 1).

Data would be interesting enough to be repeated with a major number of trigeminal ganglia.

Discussion

It has been recorded that the rabbit model system in herpetic corneal infections closely resembles those of humans, and the efficacy of drug therapy in rabbits has correlated well with that in humans (Kibrik & Laibson 1971). Because of this, the present study was developed in such an animal model using in the treatment one of the most active nitroderivatives of 20 different sugars, obtained by electrosynthesis, the antiviral activity of which had been determined in previous in vitro assays (data in preparation).

No virucidal activity was observed. That means that GN-11 would act in one or more steps of the virus replication process, not directly against the virion particle, as an antiseptic does.

The results demonstrated that GN-11 can prevent the appearance of acute herpetic keratitis (HSV-2). In comparison with ACV, under our experimental conditions, the results are similar. Compound GN-11 produces, the same as ACV, 100% absence of herpetic damage in the treated eyes on the 12th day post-infection, and prolongs these effects when the treatment is finished.

The compound GN is precursor of GN-11 and showed activity against HSV-1 in vitro (Knowles & Person 1976). It has also been assayed in vivo against Rous sarcoma and human influenza virus at very high concentrations.

We have studied (data not shown) the in vitro activity of GN against HSV-2, but the compound failed when proved in the rabbit ocular model, in which the appearance of herpetic lesions is not prevented, even by a concentration of 10% in aqueous solution, with good penetrability. The group of animals treated with GN did not show differences with the placebo treated group throughout the experiment.

By contrast the in vivo results with GN-11 were comparable to that of ACV in our experimental conditions. It confirms the in vitro activity of this complex of compounds against HSV-2.

Although the HSV-2 ocular infection is not the most extended, it presents singularly interesting characteristics.

In the newborn the HSV-2 infection is usually acquired at birth, and the source of the virus is the infected birth canal of the mother. Infection by the virus in some infants appears to be localized: skin, eyes or oral cavity. Eye involvement may be seen

alone or in combination with the infection of the CNS. Conjuntivitis and keratitis may lead to corneal scarring with blindness. On the other hand, in adults the virus may be transmitted endogenously from the genital lesion. Some interesting clinical reports suggested hematogenous spread of HSV-2 in healthy adults (Sumers et al. 1980).

All the mentioned possibilities of particular HSV-2 eye infections make the appearance and study of new antiherpetic compounds more interesting. GN-11 has a good solubility and penetration.

It promises to be a compound of therapeutic interest, the knowledge and purification of which are in progress to determine its exact formula and mechanisms of action.

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