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9-(4-hydroxybutyl)-N^2-phenylguanine (HBPG), a thymidine kinase inhibitor, suppresses herpes virus reactivation in mice

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Abstract

In cells of the nervous system, which have little or no cellular thymidine kinase, the pharmacologic inhibition of viral thymidine kinase may prevent the reactivation of herpes virus, which requires phosphorylated thymidine for replication. We tested a newly synthesized inhibitor of viral thymidine kinase, 9-(4-hydroxybutyl)-N^2-phenylguanine (HBPG) for its capacity to suppress the reactivation of herpes simplex virus type 1 (HSV-1) in vivo. Mice, latently infected with McKrae strain HSV-1, were treated with intraperitoneal injections of HBPG in a corn oil vehicle (200 mg/kg every 3 h for a total of ten doses), and subjected to hyperthermic stress to stimulate viral reactivation immediately before the third treatment. Three h after the last treatment, the mice were sacrificed, and the presence of infectious virus was determined by culture of ocular surface swabs and trigeminal ganglionic homogenates. Additionally, viral DNA in ganglionic extracts was analyzed by quantitative PCR. Controls included latently infected, stressed animals receiving injections of corn oil vehicle only, and latently infected, drug- and vehicle-treated, unstressed animals. HBPG had a statistically significant inhibitory effect on hyperthermia-induced viral reactivation. Homogenates of trigeminal ganglia and ocular surface swabs from HBPG-treated animals were less likely to contain infectious virus than those of infected, vehicle-treated, stressed controls ($P < 0.005$, ANOVA). Unstressed controls showed no reactivation. Quantitation of viral DNA in ganglionic extracts demonstrated a 100-fold reduction in the amount of viral DNA in the ganglia of HBPG-treated animals, compared with vehicle-treated controls ($P < 0.05$, ANOVA). The results indicate that HBPG has an inhibitory effect when given systemically for the suppression of herpes virus reactivation in mice.

Keywords: Herpes simplex virus; Virus reactivation; Recurrent herpetic disease; HBPG; Latency; Mouse

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1. Introduction

Herpes simplex virus type 1 (HSV-1) infects epithelial surfaces and enters the nerve endings that innervate these tissues, thereby gaining entry to the peripheral and central nervous systems (Corey and Spear, 1986; Mertz, 1990; Roizman and Kaplan, 1992; Straus et al., 1985), where viral latency is established (Roizman and Kaplan, 1992; Stevens and Cook, 1971; Whitley, 1990). In mice, ocular HSV-1 infection is characterized by acute infection in the corneal epithelial cells, resulting in foci of damaged and dead cells (Cook, 1992; Harding, 1993). During acute infection, the virus enters the nerve endings that interdigitate the epithelial cells. Retrograde transport carries the virus to neurons in the trigeminal ganglion, where the virus remains in the latent state (Roizman and Kaplan, 1992; Stevens and Cook, 1971). Resolution of the ocular surface infection results in the return of a clear, smooth, epithelial surface. However, in many clinical cases, viral reactivation in the ganglion and recurrent infection at the ocular surface by reactivated virus cause more severe disruption of the epithelial cell layer than that seen during the primary infection (Kaufman and Rayfield, 1988). Recurrent disease leads to permanent scarring of the cornea, which can result in loss of vision. Two major goals of studies of HSV-1 biology are an understanding of the mechanisms of viral reactivation and the development of therapeutic approaches that can prevent viral reactivation and recrudescence of disease.

One of the first antiviral compounds identified was idoxuridine, a drug found to be effective in the treatment of herpes simplex keratitis (Kaufman, 1962; Kaufman, 1963a; Kaufman, 1963b; Kaufman, 1993). Studies with this antiviral agent revealed that a substituted nucleoside that inhibits viral DNA polymerase can effectively inhibit virus multiplication. Subsequently, more selective substituted nucleosides, such as acyclovir and bromovinyldeoxyuridine, were developed (Becker and Hadar, 1980; De Clercq, 1984a; De Clercq, 1984b; Lapucci et al., 1993; Mansuri and Martin, 1991). These drugs are phosphorylated and activated by viral thymidine kinase, not cellular thymidine kinase. Therefore, only virus-infected cells contain active phosphorylated drug, and these drugs are relatively non-toxic to other cells.

We have adopted a different approach by developing selective inhibitors of viral thymidine kinase (Wright, 1994; Xu et al., 1995). Because non-neuronal cells contain endogenous thymidine kinase, which can provide a source of phosphorylated thymidine for the virus, viral thymidine kinase inhibitors should be non-toxic to and ineffective in these cells. However, in the nervous system, the neurons contain only small amounts of endogenous thymidine kinase; because the neurons do not replicate, they are not dependent on the presence of this enzyme for survival. Viral thymidine kinase expression has been implicated in reactivation of virus from the latent state (for a review, see Tenser, 1991). Therefore, inhibitors of viral thymidine kinase could act specifically in the nervous system to prevent viral reactivation from latency with little or no effect on the host cells.

We have recently been successful in synthesizing 9-(4-hydroxybutyl)-N^2-phenylguanine (HBPG), which exhibits potent inhibition of HSV-1 thymidine kinase in vitro (Xu et al., 1995). HBPG is a non-substrate, competitive inhibitor of HSV-1 thymidine kinase ($K_i = 50$ nM), and has no effect on cellular thymidine kinase. Pharmacokinetic studies of HBPG in mice have demonstrated that significant plasma concentrations can be achieved following intraperitoneal (IP) or intravenous (IV) administration (Xu et al., 1995). In this study, we tested the capacity of HBPG to inhibit viral reactivation following hyperthermic stress in latently HSV-1-infected mice.

2. Materials and methods

2.1. Virus

The McKrae strain of HSV-1 was propagated in Vero cells (American Type Culture Collection, Rockville, MD), and titrated on CV-1 (ATCC) cells for the determination of plaque-forming units (PFU). Batches of virus were titered and stored frozen at −70°C until use. BALB/c strain, female mice (The Jackson Laboratory, Bar Harbor, ME) were used. The animals were housed

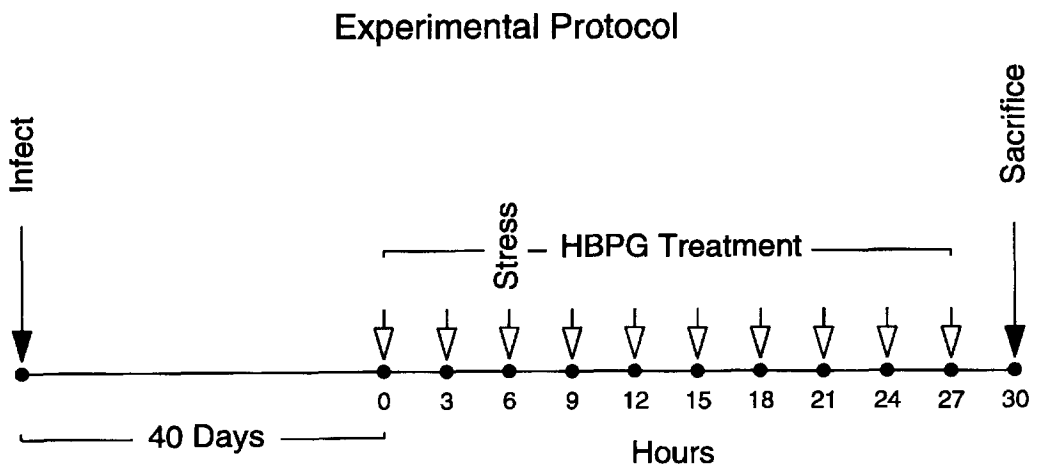

Fig. 1. Sequence of steps in the experimental protocol. Forty days after infection, groups of mice were given ten injections of either HBPG or the corn oil vehicle alone at 3-h intervals. The animals were exposed to hyperthermic stress immediately before the third injection, and were sacrificed 24 h later.

in an AAALAC-accredited animal care facility, and provided with food and water ad libitum.

2.2. Experimental design

Mice were infected by the scarification/topical application technique. A sterile syringe and needle containing the viral suspension at 1×10^5 PFU per ml were used to infect the corneas. The mice were anesthetized with a ketamine/xylazine mixture. Four cross-hatched, superficial scratches were created on both eyes of each animal, after which a 5 μl drop of the virus suspension was applied to each eye, and the eyes of the animals were held closed for 5 s. The animals were then returned to their cages.

All animals were observed with a slit lamp biomicroscope on days 3, 5, and 7 after infection. Successful infection was documented by the appearance of epithelial lesions in the central cornea. On day 5, the eyes of all animals were swabbed, and the swabs were placed into tissue culture with CV-1 cells to establish the presence of HSV-1 on the ocular surface. Animals with negative viral cultures were excluded from the study. Animals with lesions and positive cultures were housed for 40 days to permit the establishment of latency.

The experimental protocol is shown in Fig. 1. On the 40th day after infection, mice were given an intraperitoneal injection of an HBPG suspension in a corn oil vehicle at a concentration of 20 mg/ml; each 20 g mouse received 4 mg of the drug at each treatment interval (200 mg/kg). Injections were repeated every 3 h for a total of ten injections. Immediately before the third injection, the animals were exposed to hyperthermic stress, as described by Sawtell and Thompson (1992). The mice were immersed up to their necks in water (43°C, 10 min), then rapidly dried, given the third HBPG injection, and returned to their cages. At 24 h after the hyperthermic stress (3 h after the last injection), the mice were sacrificed, the ocular surfaces swabbed, and the trigeminal ganglia removed for viral cultures and viral DNA quantitation.

Control groups included latently infected mice treated on the same schedule with injections of corn oil vehicle only, and subjected to hyperthermic stress as described above; latently infected mice treated with injections of either HBPG or corn oil vehicle only, but not subjected to hyperthermic stress; and uninfected, untreated mice subjected to the stress protocol only.

2.3. Viral culture

A non-quantitative assay for infectious virus was used. CV-1 cells were grown to confluence in RPMI-1640 culture medium, supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), in 24-well tissue culture plates. Ocular surface swabs from all mice were placed in individual wells and swirled gently in the culture medium. Individual trigeminal ganglia of the mice were homogenized (Pellet Pestle®, Kontes Chemistry and Life Sciences Products, Vineland, NJ) in 0.2 ml of tissue culture medium. This process ruptures cells and nuclear membranes, as determined by microscopic examination. Infectious virus and viral DNA were liberated from the tissue matrices by this procedure.

The homogenates were centrifuged at 12 000 × *g* for 5 min at 4°C. This procedure provided a clear supernatant containing infectious virus for co-culture and a pellet from which viral DNA was extracted and amplified. (The goal of this separation procedure was to provide a particulate-free supernatant for the infectious virus assay and a pellet from the same tissue which could be used for viral DNA analysis.) Aliquots of the supernatant (0.1 ml) from each ganglion homogenate were then pipetted into individual wells of the 24-well plates. All cultures were incubated for 30 days and examined daily for the appearance of virus-induced cytopathic effect (CPE).

2.4. Quantitation of viral DNA

A quantitative polymerase chain reaction (Q-PCR) was used to analyze viral DNA in the trigeminal ganglia of HBPG- and vehicle-treated mice. The insoluble pellets from the ganglionic homogenates of HBPG-treated and control mice were processed using a commercial DNA extraction system (Qiagen Inc., Chatsworth, CA). Briefly, the homogenates were suspended in a DNA extraction buffer containing 0.01% Triton-X detergent and 0.25% Proteinase K in Tris EDTA buffer, pH 6.5. The suspensions were incubated at 60°C for 10 min, after which the samples were diluted into a mixture of Tris EDTA and isopropyl alcohol. The suspensions were transferred to filter cartridges and centrifuged for 1 min at 8 000 × *g*, after which the trapped DNA was washed three times with isopropyl alcohol. The DNA was eluted from the filter cartridge with 0.1 ml of water at 60°C and quantitated by spectrophotometry at 260 nm.

Each ganglionic homogenate was tested in triplicate by Q-PCR. The PCR system was used according to the vendor's instructions (Perkin Elmer/Applied Biosystems, Foster City, CA). Briefly, triplicate 0.5 ml tubes, each containing the appropriate amounts of 10xPCR buffer, dNTPs, Taq polymerase, and a pair of oligonucleotide primers complementary to the termini of a 476-base pair sequence of the viral DNA polymerase gene, were prepared. Next, known amounts of the competitor viral DNA sequence were added. Thus, in a typical amplification, triplicate tubes containing 10^8, 10^7, 10^6, 10^5, 10^4 10^3, 10^2, 10^1, 10^0, and 10^{-1} molecules of competitor viral DNA were prepared. To each of these tubes, 1 ng of the DNA extracted from each ganglion was added. All tubes were subjected to an amplification profile consisting of 95°C, 1 min, 72°C, 2 min, 60°C, 1 min; the cycles were repeated 35 times. Quantities (20 μl) of the amplified samples were resolved on 2% agarose gels in Tris borate EDTA buffer (TBE), pH 8.0. The DNA bands were stained with ethidium bromide and analyzed using a video densitometry system (Eagle Eye II, Stratagene, La Jolla, CA). The point of equivalence, at which the competitor viral DNA and the amplified viral DNA from a ganglion were equal in staining intensity, was used to determine the quantity of viral DNA in the ganglion sample. In all, 17 ganglion DNA samples from HBPG-treated animals and 14 ganglion samples from vehicle-treated animals were analyzed.

2.5. Statistical analysis

The data obtained were subjected to statistical scrutiny by analysis of variance (ANOVA) using a SAS program (Statistical Analysis Systems, Cary, NC).

Table 1
Effect of HBPG[a] on hyperthermia-induced viral reactivation[b]

Treatment	Number of mice	Infectious virus/no. of samples[c]	
		Ganglion	Ocular surface
Stressed			
HBPG-treated	10	7/18[d]	3/20[d]
Vehicle-treated	10	12/19[d]	8/17[d]
Not stressed			
HBPG-treated	5	0/8	0/9
Vehicle-treated	5	0/10	0/10

[a] HBPG = 9-(4-hydroxybutyl)-N^2-phenylguanine.
[b] HSV-1 latent mice were treated with HBPG in corn oil (200 mg/kg) every 3 h, stressed by immersion in water at 43°C for 10 min, and sacrificed 24 h later.
[c] Ganglion homogenates and ocular surface swabs were co-cultured on indicator cells and the appearance of cytopathic effect recorded.
[d] χ^2 analysis of the numbers of ganglion samples and ocular surface samples with infectious virus from HBPG-treated and vehicle-treated animals was performed. Statistical significance ($P = 0.033$) between the values for the ocular surface samples of the HBPG- and vehicle-treated groups was found. Similarly, statistical significance ($P = 0.033$) between the values for the ganglion samples of the HBPG- and vehicle-treated groups was noted.

3. Results

3.1. Infectious virus at the ocular surface and in the trigeminal ganglion

Latently infected, vehicle-treated, stressed control mice had a high frequency of infectious virus, both on the ocular surface and in the trigeminal ganglia (Table 1). By contrast, latently infected, HBPG-treated, stressed mice had statistically significant reductions in infectious virus on the ocular surface and in the ganglia 24 h after application of the stress (Table 1). Infected, drug- or vehicle-treated controls not subjected to hyperthermic stress showed no infectious virus on the ocular surface or in the trigeminal ganglia (Table 1). Uninfected, untreated controls, subjected only to the hyperthermic stress protocol, also showed no infectious virus in any ocular surface or ganglionic cultures (data not shown). The assay used for infectious virus did not yield reliable quantitative information regarding the amount of virus present in the ocular tissues and ganglia; therefore, we resorted to a semi-quantitative measure of viral DNA.

3.2. Viral DNA in the trigeminal ganglion

Q-PCR analysis of ganglionic homogenates from HBPG-treated, stressed animals and vehicle-treated, stressed animals was performed. This Q-PCR technique permits quantitation of the copies of a particular DNA segment in a sample under study. Examples of Q-PCR results obtained with ganglionic DNA from an HBPG-treated animal and ganglionic DNA from a vehicle-treated animal are shown in Fig. 2. The 530-base pair competitor fragment appears as a distinct band, which decreases in intensity with dilution of the competitor. The presence of viral DNA is seen as a 476-base pair band. The point at which the two bands, one derived from the competitor sequence and the other derived from the ganglionic extract DNA, appear equivalent is equal to 1×10^3 copies of the viral DNA segment (Fig. 2A). Q-PCR of ganglionic DNA from an HBPG-treated animal failed to reveal viral DNA in this sample even though the competitor DNA was detected at a dilution of 10^1 (Fig. 2B).

In all, 17 ganglionic extracts from the HBPG-treated and 14 ganglionic extracts from the vehicle-treated animals were analyzed. The amount of viral DNA in the ganglia of vehicle-treated ani-

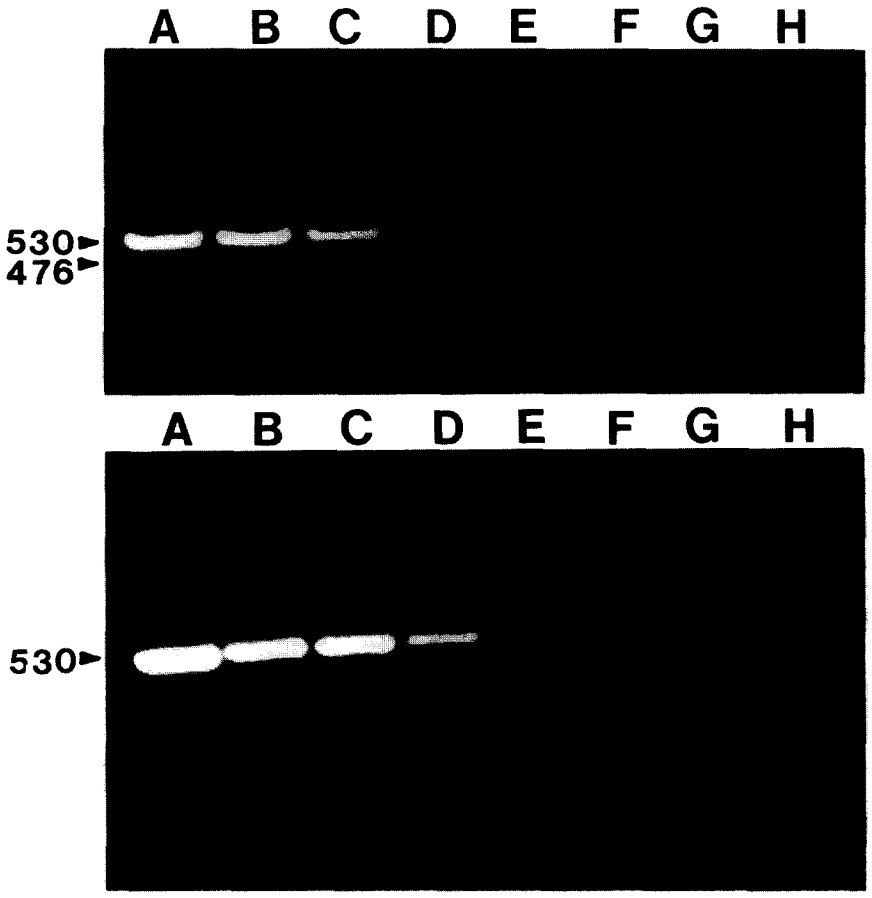

Fig. 2. Agarose gel showing the results of Q-PCR analysis of ganglionic DNA. Lanes A-G, Q-PCR samples containing 10^7, 10^6, 10^5, 10^4 10^3, 10^2, and 10^1 molecules of competitor DNA (530-base pair) and 1 ng of ganglionic DNA (476-base pair). Lane H, DNA molecular weight standards. (A) Treatment with vehicle alone allows expression of viral DNA following stress-induced reactivation; the competitor 530-base pair DNA band and the 476-base pair viral DNA band are equal at 1×10^3 molecules (lane E). (B) Treatment with HBPG prevents the expression of viral DNA following stress; only the competitor DNA bands are seen.

mals ranged from 10^4 to 10^6 copies, whereas the amount of viral DNA in the ganglia of HBPG-treated animals ranged from undetectable to 10^2 copies (Table 2).

4. Discussion

The results of this study indicate that an HSV-1 thymidine kinase inhibitor, HBPG, can inhibit viral reactivation and viral DNA synthesis in nervous system tissue, such as the trigeminal ganglia of mice, following a reactivation stimulus. These results are consistent with results of in vitro (explant co-cultivation) experiments with related compounds (Leib et al., 1990) and with the hypothesis that thymidine kinase expression is required for virus reactivation (Tenser, 1991).

The search for antivirals that target viral metabolism and do not interfere with host cell metabolic processes is continuing. Important factors in the development of an antiviral compound are drug toxicity and pharmacokinetics (Beyer et al., 1989; Leib et al., 1990). Initial studies with HBPG in mice revealed that plasma concentrations of 10 μM can be maintained when the drug is administered intraperitoneally as a suspension, every 3 h (Xu et al., 1995). A volume of distribu-

tion value in mice of approximately 3 l/kg was achieved after intravenous injection, indicating that HBPG is distributed widely in the body (Xu et al., 1995). In the present investigation, no overt toxicity was noted in the mice over the treatment period. Further studies are in progress to assess the toxicity levels of the compound.

The development of an effective antiviral that can be administered prophylactically to patients who are prone to undergo viral reactivation is a central focus of many investigations (Kaufman and Rayfield, 1988). Further study with compounds such as HBPG should lead to the development of a nontoxic, targeted, specific, and effective antiviral which can be used in the prophylaxis of herpes virus infections in humans. Studies with HBPG in a primate model of HSV-1 reactivation are in progress.

The use of viral thymidine kinase inhibitors to prevent recurrences may offer unique advantages (Kaufman et al., 1991). Such drugs should be selective for the inhibition of viral reactivation in the nervous system and should prevent the emergence of viral DNA in non-replicating cells, e.g., neurons. It may be that drugs such as acyclovir, which inhibit recurrences of genital and labial herpes (Lapucci et al., 1993; Mansuri and Martin, 1991; Rooney et al., 1993; Spruance et al., 1988), act primarily by inhibiting the peripheral viral multiplication necessary to produce a visible lesion and have a minimal effect on ganglionic reactivation. Acyclovir appears to reduce viral shedding and, therefore, can substantially reduce the risk of transmission of infection from person to person in genital herpes (Lapucci et al., 1993; Mansuri and Martin, 1991), but it is not known whether this effect is mediated peripherally, centrally (in the mouse system), or both. However, acyclovir is effective in the treatment of encephalitis, indicating a role in inhibition of central nervous system replication of the virus. In ocular HSV reactivation, we believe that a major component of viral disease is due to replication at the periphery and, therefore, are trying to identify compounds, such as HBPG, which will inhibit reactivation in the site of latency. If ganglionic reactivation can be suppressed by drugs, such as specific viral thymidine kinase inhibitors, perhaps effective inhibition of recurrence, as well as transmission of infection, can be achieved. Similarly, since herpes zoster reactivates from previous varicella zoster virus (VZV) infection, it may well be that drugs targeted to VZV thymidine kinase can prevent the emergence of shingles. We believe these studies are important, not only because they offer the possibility of a new drug, but also because they provide supportive evidence for the validity of a new kind of pharmacologic approach to the prevention of recurrences of disease caused by the herpes viruses.

Table 2
Effect of HBPG[a] on hyperthermia-induced viral DNA expression[b]

Treatment	No. of ganglia analyzed	Viral DNA[c]
Stressed		
HBPG-treated	17	0 to 1×10^2 [d]
Vehicle-treated	14	1×10^4 to 1×10^6 [d]

[a] HBPG = 9-(4-hydroxybutyl)-N^2-phenylguanine.
[b] HSV-1 latent mice were treated with HBPG in corn oil (200 mg/kg) every 3 h, stressed by immersion in water at 43°C for 10 min, and sacrificed 24 h later.
[c] Ganglionic DNA was amplified in quantitative, competitive polymerase chain reaction (Q-PCR), and the copy number of the viral gene present was determined at the equivalence point.
[d] The mean numbers of viral gene DNA copies in the HBPG-treated and vehicle-treated samples are statistically significantly different ($P < 0.005$).

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