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Efficacy of 2′-nor-cyclicGMP in treatment of experimental herpes virus infections

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Summary

9-[(2-Hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]guanine *P*-oxide (2′-nor-cGMP), the cyclic phosphate of 2′-nor-deoxyguanosine (2′-NDG) was synthesized by phosphorylation of 2′-NDG and evaluated for antiherpetic activity in cell cultures and in animal protection studies. 2′-nor-cGMP was effective in cell culture against both thymidine kinase deficient and wild-type herpes simplex virus type 1 strains and also against herpes simplex virus type 2. The anti-herpes activity of 2′-nor-cGMP against thymidine kinase deficient HSV-1 was confirmed by animal protection studies. Also, in comparative cell culture protection studies, the ED_{50} (μM) of 2′-nor-cGMP was approximately 10-fold lower than that of 2′-NDG against three strains of varicella zoster virus. In addition, 2′-nor-cGMP was effective orally in preventing HSV-1 orofacial infection and HSV-2 genital infection of mice. Topical therapeutic applications of 2′-nor-cGMP prevented orofacial HSV-1 lesion development in mice and development of HSV-2 genital lesions in guinea pigs. Subcutaneous application of 2′-nor-cGMP to intracerebral HSV-1 challenged weanling mice significantly prolonged survival. These studies indicate that 2′-nor-cGMP is not dependent on viral thymidine kinase for its antiviral activity and is highly effective in preventing experimental HSV infections.

2′-nor-cyclicGMP; thymidine kinase; HSV infections; in vivo activity

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Introduction

A series of acyclic nucleosides have now been established as having the capacity to specifically inhibit the replication of herpes group viruses. These include acyclovir (ACV) [9,19], 2′-nor-deoxyguanosine (2′-NDG) [1,11], also referred to as BW759 [18], BIOLF-62 [21] and DHPG [15], and 9-(2,3-dihydroxypropoxymethyl)guanine (iNDG) [2]. Each of these compounds acts as a prodrug, requiring phosphorylation in situ by viral and cellular kinases to the respective triphosphate, which inhibits viral DNA polymerase.

As part of our studies of antiherpetic acyclonucleosides, we synthesized the cyclic phosphate of 2′-NDG, 9-[(2-hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]guanine *P*-oxide (2′-nor-cGMP) and discovered its surprisingly broad spectrum anti-DNA viral activities against herpes group viruses, vaccinia, adenovirus, and bovine papilloma virus, but not against RNA viruses in cell culture [23]. In addition, we found that the anti-herpetic activity of 2′-nor-cGMP was not dependent on the action of herpes simplex virus thymidine kinase (HSV-TK) indicating that the mechanism of activity was not dependent on dephosphorylation to 2′-NDG followed by viral dependent rephosphorylation [23].

In the present studies, we describe a new synthesis of 2′-nor-cGMP and demonstrate its antiviral activity in cell culture against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and against TK deficient HSV-1 mutants. In addition, we demonstrate that 2′-nor-cGMP is more effective against three strains of varicella zoster virus (VZV) than either 2′-NDG or ACV. The efficacy of 2′-nor-cGMP is also demonstrated in protection studies in vivo against TK deficient HSV-1 infection of mice. Finally, we demonstrate that 2′-nor-cGMP is effective given orally or topically against experimental HSV-1 and HSV-2 infections in mice and guinea pigs.

Materials and Methods

Viruses

HSV-1 strain KOS and strain PFA_{2a}, a DNA polymerase mutant of KOS were provided by Dr. Y.C. Cheng [5]. HSV-1 ACG_9, a TK-deficient mutant of strain KOS, was provided by P.A. Schaffer [6]. HSV-1 strains Schooler and S and HSV-2 strain Curtis were from our virus stock preparations and have been previously described [8,11]. HSV-1 (NDG_{R1}), a TK-deficient mutant of HSV-1 Schooler, was isolated for resistance to 2′-NDG [23]. VZV strains KMcC, Oka, and simian (Delta herpes virus) were provided by B.J. Neff and have been previously described [10,11].

All viruses were prepared using plaque purified isolates. Stocks were prepared as 10% freeze-thaw extracts from infected cell cultures and stored at −70°C.

Animals

Six-week-old female hairless mice (HRS/J) were purchased from Buckshire Farms, Perkasie, PA. ICR/Ha mice were provided by Merck Sharp & Dohme. Hartley female guinea pigs (250–300 g) were purchased from Millbrook Farms, Amherst, MA. All animals were housed for two weeks in our laboratories for adaptation prior to experimental use.

Preparation of 9-[(2-hydroxy-1,3,2,-dioxaphosphorinan-5-yl)oxymethyl]guanine P-oxide (2'-nor-cGMP)

To a stirred, chilled (0°C) mixture of sieve-dried triethylphosphate (60 ml) and freshly distilled phosphorus oxychloride (2.20 ml, 23.56 mmol) was added 5.91 g (23.15 mmol) of 2'-NDG. This suspension was allowed to rise to room temperature and stirring was continued for 5 h, during which time dissolution occurred. The mixture was clarified by filtration and the filtrate was added to hexane (600 ml). The precipitate so formed was collected and washed (hexane) by decantation and then dried in vacuo to give a 15.9 g residue. After dissolution of this material in water (800 ml) and pH adjustment to 7 with aqueous KOH, the solution was filtered and the filtrate lyophilized to give 9.25 g of a white solid. This was dissolved in water (1 l), clarified by filtration, and passed onto a Bio-Rad AG 1X8(HCO_3^-) column (5 × 23 cm), packed in H_2O. A linear gradient of 0.05–0.5 M $KHCO_3$ (2 l/reservoir) was then commenced, followed by 0.5 M $KHCO_3$ as eluent until UV absorbing components were removed from the column. Fractions 450–540 (20 ml each) contained the required product and were pooled and stirred with Bio-Rad AG 50WX8(H^+) resin (600 ml), under house vacuum. The resin was removed by filtration, and the filtrate concentrated to 150 ml in vacuo. The title compound (as the free acid) precipitated and was filtered and dried in vacuo to give 0.445 g (a second crop gave an additional 0.046 g); total yield = 1.55 mmol (7%).

To convert this material to the sodium salt, the free acid was suspended in H_2O, the pH adjusted to 6.6 using aqueous NaOH, and the solution lyophilized to give a white powder. Analysis: calculated for $C_9H_{11}N_5O_6PNa \cdot 0.5\ H_2O$: C, 31.04; H, 3.48; N, 20.11; P, 8.89; Na, 6.60. Found C, 31.31; H, 3.58; N, 20.01; P, 9.24; Na 6.69. NMR (200 MHz, D_2O): δ7.99 (s, 1H, H8), δ5.64 (s, 2H,–NCH_2O–), δ4.39 (d of d of d, 2H,–$CH_{ax}OP$, ^2J_H–HHgem = 12.5 Hz, $^3J_{PHax}$ = 5.0 Hz, $^3J_{HHax}$ = 1.8 Hz), δ4.25 (d of d of d, 2,–$CH_{eq}OP$, ^{2}JHHgem = 12.5 Hz, ^{3}JPHeq = 19.5 Hz, ^{3}JHHeq = 2.2 Hz), δ3.94 (m, 1H –CH–). UV (0.1 M phosphate, pH 7): λ_{max} 252 nm (11 772), infl. 275 nm (7781).

A similar preparation, but utilizing Bio-Rad 1X2($HCOO^-$) for separation, and a gradient of formic acid (0–5 N), followed by ammonium formate (1 and 2 M) for elution, accounted for 98.5% recovery of the purine chromophore by UV and showed the major product (73.4%) of this reaction to be 9-(1,3-dihydroxy-2-propoxymethyl)-guanine bisphosphate.

Determination of antiviral efficacy in cell culture

Monolayers of MRC-5 cells in 60-cm^2 culture dishes were washed and infected using the viruses indicated in Table 1, adsorbed for 1.5 h at 37°C, and refed with maintenance medium (MEM plus 2% fetal calf serum and 2% methyl cellulose) containing serial 2-fold dilutions of drug from 100 μg/ml. Cultures were incubated at 37°C for 5 days in 5% CO_2 prior to plaque or cytopathology determination on the fixed and stained monolayers. All drug concentrations were assayed in duplicate. Each drug was assayed at least twice. For all viruses, except HSV-2, the ED_{50} was determined as the least drug concentration which reduced plaque numbers by 50% in the treated cultures compared to untreated infected cell cultures. For HSV-2 infected cells, the ED_{50} determinations represented the drug concentrations which prevented development of viral cytopathology in half of quadruplicate treated cultures.

Preparation of drugs for animal treatment

2′-NDG and ACV were synthesized at Merck Sharp & Dohme Research Laboratories (MSDRL), Rahway, NJ by methods previously described [1,11,19]. For oral dosage, 2′-NDG and 2′-nor-cGMP were solubilized in saline at pH 11 and 7, respectively, and were soluble at all doses tested. For topical treatment of mice, 2′-nor-cGMP was prepared in a hydroalcoholic vehicle containing 5% polyvinylpyrrolidone, 52.5% propylene glycol, 7.5% absolute ethanol, and 35% water. For topical treatment of guinea pigs, 2 mm diameter vaginal inserts of 2′-nor-cGMP at the concentrations indicated were prepared by dissolving 2′-nor-cGMP in polyvinyl alcohol (PVA), Gelvatol 40–10®, Monsanto. 50 μl drops were dried overnight at ambient temperature followed by 2 h at 35–40°C in vacuo. 2′-nor-cGMP content in the inserts was evaluated by UV absorption (252 nm) of aqueous solution of the inserts to yield the following results. 1% inserts (154 ± 9 μg), 0.25% inserts (31 ± 1.5 μg), 0.06% inserts (6.7 ± 1.7 μg), 0.02% inserts (below detection). 2′-nor-cGMP was freely soluble at all concentrations used. Placebo treatments were phosphate-buffered saline (PBS) at pH 11 or inserts prepared with PBS.

Infection and evaluation of HSV-1 and HSV-2 infections

Orofacial skin infections were established and evaluated using the procedures described by Klein et al. [13]. Briefly, the snout skin of HRS/J mice was abraded using a 20-gauge needle and infected by applying 0.05 ml of saline containing 2×10^5 PFU of HSV-1 (strain S); this challenge resulted in a sublethal, localized disease in 70–100% of infected animals. Inflammation developed by day 5 and progressed to vesicles, which were evaluated on day 7. Lesion severity was determined using a scoring system of 0 (for no lesions) to 4 (for most severe lesions). The average lesion score was calculated as the mean severity for all animals in a treatment group, as well as for only lesion-bearing animals in that group. To evaluate latent ganglionic infection, the mice were killed by cervical dislocation 28–35 days after the initial infection. Trigeminal ganglia were removed, minced, and cocultivated on MRC-5 cell monolayers. These cultures were observed for 14 days for herpes cytopathology development. Selected virus isolates were confirmed to be HSV-1 by neutralization with antiserum specific for HSV-1.

Vaginal infection of 30 g ICR/Ha mice was accomplished according to the methods of Nahmias et al. [16]. Mice were vaginally swabbed with a cotton swab and then infected with a cotton tampon carrying approximately 10 LD_{50} of HSV-2 (2×10^4 PFU). Tampons were removed after one day. Only infected animals developed vaginitis after 4 days, followed by posterior paralysis and death after day 7. Mice were evaluated daily for vaginitis (infection) and survival. Development of vaginitis (inflammation of the genital area) was a reliable measure of infection and usually progressed to paralysis and death. Those mice which did not develop vaginitis developed insufficient infection to stimulate production of circulating neutralizing antibodies, determined at 4 weeks after infection. Vaginal infection of female Hartley guinea pigs was accomplished by depositing 0.2 ml of HSV-2 (2×10^4 PFU in 0.3% bovine serum albumin; approx. 10 ID_{50}) in the vaginal vault 1 h following vaginal swabbing with a saline-soaked cotton swab [7]. Lesion severity was evaluated on a scale of 0 (for no inflammation or external lesions) to 4 (for large herpetic ulcers and maceration or

urine retention with swelling) [20]. Only guinea pigs which developed herpetic lesions also developed serum neutralizing antibodies, determined 4–5 weeks after infection.

Ether anesthetized 3-week-old ICR/Ha mice of either sex were intracerebrally inoculated using 10^2 PFU (approx. 100 LD_{50}) of HSV-1 (strain Schooler) in 50 µl of 0.3% BSA. Mock infected mice (inoculated with 50 µl 0.3% BSA) all survived, whereas infected animals began to die on day 4.

Animal treatments

Infected animals were randomized into groups of 10 (for mice) and 8 (for guinea pigs). Oral or subcutaneous drug treatments in doses of 0.1 ml of either 2′-NDG or placebo saline (pH 11) or 2′-nor-cGMP or placebo (pH 7) were administered to unanesthetized mice twice daily at an 8-h interval. Topical treatments of (a) orofacially infected mice were by application of 10 µl of drug in the hydroalcoholic vehicle or the hydroalcoholic vehicle alone to the infected skin areas, (b) vaginally infected guinea pigs were by insertion of polyvinyl alcohol wafers into the vaginal vault. Mice were treated topically 4 times per day at 3-h intervals, whereas guinea pigs received two topical treatments each day at 8-h intervals.

Analysis of data

Treatment groups were compared on rate of lesion development and frequency of ganglionic infection by Fisher's exact test [3]. Lesion scores, duration of lesions, time-to-infection, and survival times were evaluated for statistical significance relative to placebo-treated animals by Duncan analysis [4]. Parallel line analyses were performed on dose-response in survival times and lesion scores, to generate relative potencies [12].

Survival times and time-to-infection were treated to a negative exponential transformation [14] before analysis. In that transformation, animals that had survived beyond the study period were assumed to have died the day after the study was completed, or survived thereafter. This will cause estimates of average survival time and time-to-infection that exceed the study duration.

Results

In vitro antiherpetic activity

The results of comparative in vitro efficacy studies of 2′-nor-cGMP, 2′-NDG, and ACV are shown in Table 1. The ED_{50} (µM) for 2′-NDG was approximately 10-fold less than for 2′-nor-cGMP against HSV-1 strains KOS and Schooler, but were approximately equivalent against HSV-2 strain Curtis. Like 2′-NDG, but in contrast to ACV, 2′-nor-cGMP was similarly effective against both HSV-1 KOS and PFA_{2a}. However, both 2′-NDG and ACV were at least 10-fold less active against HSV-1 strain ACG^r_9 and HSV-1 (NDG_{R1}) (both TK^- viruses) compared to their antiviral activities against the respective parent strains (HSV-1 KOS and Schooler). The antiviral activity of 2′-nor-cGMP was approximately the same against either the TK^- or wild-type viruses.

2′-nor-cGMP has enhanced efficacy compared to 2′-NDG or ACV against VZV strain KMcC [23]. These observations have now been expanded to include human

TABLE 1

In vitro efficacy of 2′-nor-cGMP, 2′-NDG, and ACV against strains of HSV-1, HSV-2 and VZV

Virus	Ed_{50} (μM)[a]		
	2′-nor-cGMP	2′-NDG	ACV
HSV-1 strain KOS	18	0.7	4
HSV-1 strain PFA_{2a}	18	0.7	57
HSV-1 strain ACG^{r}_{9}	3–6	22–93	57–114
HSV-1 strain Schooler	10–37	0.7–3.0	0.9–1.8
HSV-1 (NDG_{R1})	37	93	114
HSV-2 strain Curtis[b]	1–2	1–2	7
VZV strain KMcC	0.6–1.2	22	27
VZV strain Oka	0.3–0.6	9–18	14
Simian Varicella	2.3–4.7	22–93	45[c]

[a] ED_{50}'s were determined by plaque reduction assays on monolayers of MRC-5 cells with test drug added to the maintenance medium following viral adsorption. Plaque determinations were made after incubation for 5 days at 37°C.

[b] Determined by microtiter limit dilution assay for prevention of viral cytopathology.

[c] From Soike et al. [22]

VZV strain Oka and simian varicella, each of which were at least 10-fold more sensitive to 2′-nor-cGMP than to either 2′-NDG or ACV (Table 1).

Monolayers of MRC-5 maintained in 2′-nor-cGMP at 300 mM retained 95% viability as determined by Trypan blue exclusion. However, cell growth rates were decreased at 2′-nor-cGMP concentrations of 150 μM.

HSV antiviral activity is HSV-TK independent

The observations that, unlike 2′-NDG and ACV, 2′-nor-cGMP is active in protecting cell cultures against either TK^{+} or TK deficient HSV-1 have been documented in Table 1 and by Tolman et al. [23]. The observations have also been demonstrated in animal protection studies. Preliminary experiments had indicated that determining drug dose-response curves for protection against wild-type HSV-1 (Schooler) and TK deficient HSV-1 (NDG_{R1}) at precisely matched viral challenge (LD_{50}) was not possible due to biological variation. Instead, a single concentration of each drug (3.1 mg/kg per day given as half doses subcutaneously at an 8-h interval) was employed for treatment of groups of mice challenged using a range of virus concentrations. Drug efficacy was then compared in treated animals which had received equivalent virus challenges, defined by equivalent survival times in a parallel titration in placebo-treated mice. Interestingly, increased virus challenge over a 1000-fold dose range ($10–10^4$ LD_{50}), resulted in a modest, but not significant reduction of survival time for either wild-type or TK^{-} virus challenge, respectively. Thus, a broad range of 'equivalent virulence' based on survival times for wild-type and TK^{-} HSV-1 (NDG_{R1}) infection was useful for comparison of 2′-NDG and 2′-nor-cGMP efficacies. The data are presented in Table 2. 2′-NDG was significantly less effective in prolonging survival time of TK deficient HSV-1 (NDG_{R1}) than in prolonging survival time of HSV-1 (average survival

TABLE 2

Protective efficacy of 2′-nor-cGMP and 2′-NDG in mice infected with HSV-1 or TK deficient HSV-1 NDG_{R1}

Virus challenge[a]	Treatment regimen[b]					
	Placebo		2′-NDG		2′-nor-cGMP	
	Survivors / total	Survival time (d)	Survivors / total	Survival time (d)	Survivors / total	Survival time (d)
HSV-1	0/60	5.9	37/60[c]	12.6[c]	32/60[c]	12.7[c]
HSV-1 (NDG_{R1})	4/60	7.2	12/60	9.1	45/60[c]	13.4[c]

[a] Six groups of 10 ICR/Ha mice were challenged intraperitoneally either with HSV-1 (Schooler) or HSV-1 (NDG_{R1}) using increments of severity of challenge from 10 to $10^4 LD_{50}$.

[b] Virus infected mice were treated twice daily subcutaneously for 4 days, starting immediately following infection, with PBS or 3.1 mg/kg per day of 2′-NDG or 2′-nor-cGMP. Deaths were recorded daily for 15 days and no. survived/total was recorded for each group.

[c] Significantly different from placebo treated infected mice ($P<0.05$).

time = 9.1 and 12.6 days, respectively, $P<0.0001$). By contrast, 2′-nor-cGMP was equivalent in prolonging survival times for either TK^- or TK^+ virus (average survival time = 13.4 and 12.7 days respectively, $P = 0.43$). Compared to the respective placebo-treated infected mice, only 2′-NDG treatment of TK deficient HSV-1 (NDG_{R1}) infection failed to significantly extend survival time.

Efficacy of oral administration of 2′-nor-cGMP against HSV-1 and HSV-2 infections of mice

The efficacy of 2′-nor-cGMP, delivered by oral gavage, was demonstrated by dose-response titration and delay of treatment studies for protection against herpetic infections of mice. Against orofacial infection of HRS/J mice using HSV-1 (strain S), 2′-nor-cGMP, given twice daily for 5 days was effective using 3.1 mg/kg per day (8.3 μmol/kg per day) in preventing development of severe acute lesions. Administration of 2′-nor-cGMP as either two half doses or as one daily dose resulted in apparent equivalent efficacy (Table 3), causing a substantial reduction in severity of acute HSV-1 skin lesions.

Oral treatment of vaginal HSV-2 infection of mice using 2′-nor-cGMP was also evaluated by dose-response titration (Table 4). Effective increase in survival time of mice treated with 2′-nor-cGMP in half daily doses for 10 days was achieved using 0.8 mg/kg per day (2.1 μmol/kg per day). Furthermore, the time to development of symptoms of infection (vaginitis) was significantly increased using 50 mg/kg per day, and both 50 and 12.5 mg/kg per day treatments resulted in a significant increase in animals that survived HSV-2 infection. The efficacy was comparable to that achieved using 2′-NDG in parallel evaluations, e.g., 2′-NDG treatment with 0.8 mg/kg per day (3.0 μmol/kg per day) significantly increased survival time.

The therapeutic efficacy of oral 2′-nor-cGMP against HSV-2 infection in mice is also shown in Table 4. Although 12.5 mg/kg per day did not consistently protect mice from

TABLE 3

Titration of oral 2'-nor-cGMP administered once or twice daily for prevention of acute orofacial HSV-1 infection in mice

Drug	Treatments[a] per day	Daily[b] dose	No. of mice with lesions[c] total	Average (±S.D.) lesion score[c] All mice	Lesion-bearing mice
2'-nor-cGMP	2	12.5	8/10	2.3[d] (± 1.57)	2.9[d] (± 1.13)
		3.1	10/10	2.5[d] (± 1.32)	2.5[d] (± 1.32)
		0.8	10/10	3.6 (± 0.97)	3.6 (± 0.97)
		0.2	10/10	3.9 (± 0.32)	3.9 (± 0.32)
Placebo		-	10/10	4.0	4.0
2'-nor-cGMP	1	50	3/10[d]	0.2[d] (± 0.35)	0.7[d] (± 0.29)
		25	5/10[d]	0.5[d] (± 0.58)	1.0[d] (± 0.35)
		12.5	8/10	1.0[d] (± 0.62)	1.3[d] (± 0.38)
		6.3	10/10	2.9[d] (± 1.29)	2.9[d] (± 1.29)
	2	50	4/10[d]	0.4[d] (± 0.53)	0.9[d] (± 0.48)
		25	6/10	1.3[d] (± 1.18)	2.2[d] (± 0.52)
		12.5	9/10	1.4[d] (± 1.01)	1.5[d] (± 0.78)
		6.3	9/10	2.5[d] (± 1.37)	2.8[d] (± 1.12)
Placebo		-	10/10	3.8 (± 0.48)	3.8 (± 0.48)

[a] Administered daily either as two half doses 8-h apart or as one dose beginning 3-h after infection for 5 days.

[b] 2'-nor-cGMP was given at the doses indicated (mg per kg) in 0.1 ml PBS by gavage; placebo was 0.1 ml PBS.

[c] Evaluated 7 days after infection.

[d] Significantly different from placebo-treated infected mice ($P < 0.05$).

development of genital inflammation (infection), this dose did effectively increase survival time even with initiation of treatment delayed up to 72 h after infection.

ICR/Ha mice (20 g, mixed sex) treated for 5 days using oral doses of 50 mg 2'-nor-cGMP/kg per day did not show evidence of toxicity (reduced physical activity or appetite) over a 2-week period of observation.

Efficacy of topical administration of 2'-nor-cGMP against HSV-1 and HSV-2 infection of mice and guinea pigs

Protection resulting from topical treatment using 10 µl of 0.02, 0.06, 0.25 or 1.0% 2'-nor-cGMP applied 4 times daily to the infected orofacial area of mice beginning 3 h after infection and continuing for 3 days is shown in Table 5. Significant reduction in both the number of mice exhibiting herpetic lesions and the severity of lesions recorded on day 7 was achieved using 0.06% of the drug. The therapeutic efficacy using 1.0% 2'-nor-cGMP is shown in Table 6. Delay of topical treatment to 48 h after infection resulted in a signficant reduction in lesion severity, while delay to 24 h resulted in a significant increase in apparent lesion-free mice. However, ganglionic infection occurred in all infected groups, suggesting that subclinical HSV-1 infection had occurred.

A similar dose-response titration for topical treatment of HSV-2 infected guinea

TABLE 4

Efficacy of oral 2'-nor-cGMP for prevention of acute HSV-2 vaginal infection of mice

Treatment regimen[a]					
Drug	Daily dose (mg/kg)	Treatment initiation (h)	No. of mice surviving / total	Average time (days)[b] To symptoms of infection	For survival
2'-nor-cGMP	50	0	10/10[c]	18.0[c] (11.7-i)	27.3[c] (22.2-40.1)
	12.5		9/10[c]	11.1 (6.6-20.2)	24.5[c] (19.8-34.6)
	3.1		4/10	9.3 (5.3-16.7)	15.7[c] (11.9-22.4)
	0.8		4/10	8.8 (5.7-13.6)	15.2[c] (11.2-22.4)
	0.2		2/10	6.4 (3.7-10.3)	10.5 (7.8-14.6)
2'-NDG	50	0	10/10[c]	27.3[c] (22.2-40.1)	27.3[c] (22.2-40.1)
	12.5		7/10[c]	20.3[c] (14.7-37.4)	25.1[c] (20.7-34.0)
	3.1		8/10[c]	10.1 (6.1-17.4)	24.5[c] (19.9-34.0)
	0.8		4/10	7.7 (4.6-12.5)	16.5[c] (12.8-22.9)
	0.2		1/10	5.2 (3.4- 7.3)	10.7 (8.9-12.9)
Placebo			3/10	7.3 (4.0-12.4)	11.1 (7.9-16.0)
2'-nor-cGMP	12.5	0	8/10[c]	8.2 (5.4-12.2)	22.3[c] (17.1-35.8)
		24	3/10	7.6 (4.9-11.5)	14.9[c] (11.8-19.5)
		48	2/10	5.2 (4.4- 6.1)	12.4 (9.6-16.5)
		72	3/10	5.4 (2.5- 9.7)	14.0[c] (10.5-19.7)
Placebo		0	2/10	4.9 (1.9- 9.2)	9.2 (6.6-13.0)

[a] Administered daily in two half doses 8 h apart beginning immediately after infection (0 h) or at the times indicated and continued for 10 days.

[b] Animals were observed for vaginal inflammation and survival for 21 days. Data are presented as average of transformed times with the 95% confidence limits.

[c] Statistically different from placebo-treated infected mice ($P<0.05$), by Duncan analysis.

i, indeterminate.

TABLE 5

Titration of topical 2'-nor-cGMP for prevention of acute HSV-1 orofacial infection of mice

Treatment regimen[a]		No. of mice with lesions / total[b]	Average (± S.D.) lesion score[b] All mice	Lesion-bearing mice
Drug	Conc. (%)			
2'-nor-cGMP	1.0	1/10[c]	0.1[c] (± 0.32)	1.0[c] (± 0.0)
	0.25	4/10[c]	0.5[c] (± 0.67)	1.3[c] (± 1.20)
	0.06	5/10[c]	1.4[c] (± 1.63)	2.7[c] (± 0.91)
	0.02	9/10	2.9 (± 1.33)	3.2 (± 1.31)
Placebo		10/10	3.9 (± 0.47)	3.9 (± 0.47)

[a] Administered at 3-h intervals beginning 3 h after infection with three treatments on the first day and four treatments on the next 2 days.

[b] Evaluated on day 7 after infection at the time of peak lesion development.

[c] Significantly different from placebo-treated infected mice ($P<0.05$).

TABLE 6

Therapeutic efficacy of topical 2'-nor-cGMP on acute and latent HSV-1 orofacial infection of mice

Treatment regimen[a]		No. of mice with lesions[b]	Average (± S.D.) lesion score[b]		No. of mice[c] with infected ganglia
Drug	Treatment initiation	total	All mice	Lesion bearing mice	total examined
2'-nor-cGMP (1%)	3	0/10[d]	0.0[d]		4/10
	8	2/10[d]	0.2[d] (± 0.11)	0.8[d] (± 0.25)	3/10
	24	2/10[d]	0.3[d] (± 0.21)	1.5[d] (± 0.50)	7/10
	48	6/10	1.1[d] (± 0.34)	1.8[d] (± 0.28)	6/10
	72	10/10	3.3 (± 0.36)	3.3 (± 0.36)	8/8
Placebo	3	10/10	3.9 (± 0.15)	3.9 (± 0.15)	6/8

[a] Administered at 3-h intervals at the times indicated after infection, with three treatments on the first day and four treatments on the next 2 days.
[b] Evaluated on day 7 after infection.
[c] Evaluated 29–31 days after infection.
[d] Statistically different from placebo-treated infected animals ($P<0.05$).

pigs using vaginal inserts containing 0.02, 0.06, 0.25 or 1.0% 2'-nor-cGMP is shown in Table 7. 2'-nor-cGMP treatments (1.0, 0.25 and 0.06% inserts) resulted in a significant reduction in the number of animals which developed lesions, in average lesion severity, and average lesion duration. In addition, serum neutralizing anti-HSV-2 titer determinations for animals surviving acute HSV-2 challenge showed that animals which were free of external herpetic lesions did not develop detectable antibody titers, indicating that suppression of external lesion development was a valid indication of inhibition of more generalized HSV-2 infection.

TABLE 7

Titration of topical 2'-nor-cGMP for prevention of acute genital HSV-2 infection of female guinea pigs

Drug	Nominal conc. (%)	No. with lesions total	Average (± S.D.) lesion score for all guinea pigs	Duration of lesions (days)	No. with HSV-2 Ab total	GMT
2'-nor-cGMP	1.0	1/8[b]	0.1[b] (± 0.33)	0.5[b] (± 1.41)	1/8[b]	< 16[b]
	0.25	0/8[b]	0.0[b]	0.0[b]	0/8[b]	< 16[b]
	0.06	2/8[b]	0.3[b] (± 0.66)	1.2[b] (± 2.55)	2/7[b]	43
	0.02	8/8	2.2 (± 1.18)	6.2 (± 2.25)	6/6	456
Placebo		8/8	2.6 (± 1.04)	6.9 (± 1.64)	4/4	362

[a] Vaginal inserts (see Materials and Methods) with the concentrations indicated were administered twice daily at 8 h intervals starting 3 h after intravaginal infection with HSV-2 (Curtis). Treatments were continued for 10 days, and lesion development was evaluated as described daily for 10 days to determine the average lesion score. Survivors at 4 weeks after infection were bled for determination of serum neutralizing titer against HSV-2 infection of MRC-5 cell monolayers. GMT = geometric mean serum neutralizing titer.
[b] Statistically different from placebo-treated infected animals ($P<0.05$).

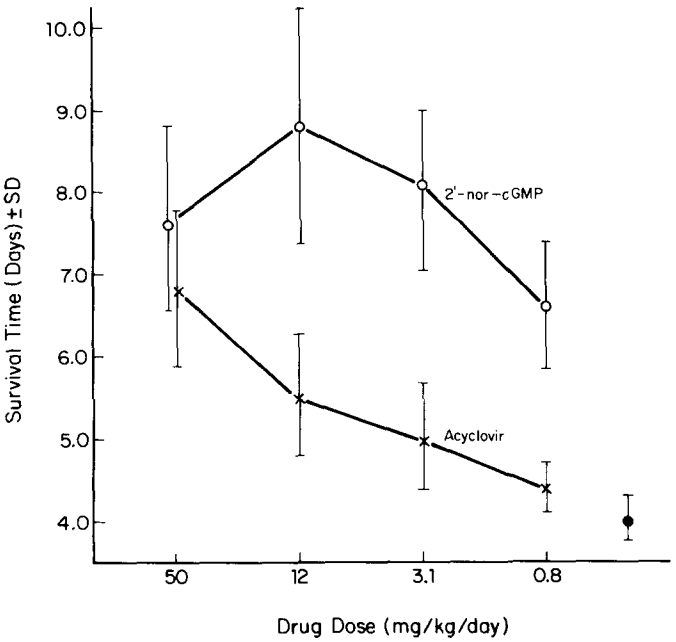

Fig. 1. Efficacy of 2'-nor-cGMP and ACV against intracerebral HSV-1 infection of mice. Weanling (3 week) ICR/Ha mice were infected under ether anesthesia with 0.05 ml of HSV-1 (100 LD_{50}, Schooler) by intracerebral inoculation. Infected mice were treated in groups of 10, twice daily s.c. for 4 days at an 8-h interval with acyclovir (×) or 2'-nor-cGMP (○) at the doses indicated. Placebo-treated mice (●) received vehicle (PBS, pH 11). Animal survival was recorded daily for 15 days. Similar placebo treatment of uninfected mice was well tolerated. Results are presented as average survival times with indicated standard deviations.

2'-nor-cGMP applied topically at 1% to mice or guinea pigs did not cause any inflammation in the treated area or alteration of appetite or activity.

Efficacy of 2'-nor-cGMP against intracerebral HSV-1 infection of mice

Weanling ICR/Ha mice infected intracerebrally using 100 LD_{50} HSV-1 were treated subcutaneously twice daily at an 8-h interval using 0.8, 3.1, 12.5 or 50 mg/kg per day of either ACV or 2'-nor-cGMP. Significant increases in survival time (but not overall survival) were achieved using 0.8 mg/kg per day or larger doses of 2'-nor-cGMP or 50 mg/kg per day of ACV (Fig. 1). The infected weanling mice receiving 50 mg/kg per day of 2'-nor-cGMP experienced weight loss and apparent drug-related death. Lower drug concentrations had no apparent ill effects.

Discussion

9-[(2-Hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]guanine *P*-oxide (2'-nor-cGMP) has been synthesized from 2'-NDG by phosphorylation using phosphorus

oxychloride and the cyclic phosphate was isolated as a free acid by ion-exchange chromatography. Unlike 2′-NDG, 2′-nor-cGMP inhibits a diverse number of unrelated DNA viruses at concentrations which do not effect cell growth. Several interesting features of these antiviral activities of 2′-nor-cGMP should be noted.

First, HSV-1 strains KOS and Schooler were less sensitive to 2′-nor-cGMP than to 2′-NDG or ACV in cell culture protection studies. Yet these antiviral drugs appeared nearly equivalent in efficacy against HSV-2 strain Curtis. These observations, however, do not seem to translate to animal protection. From our data presented here and previously published [8] and unpublished observations, it appears that 2′-nor-cGMP and 2′-NDG have near equivalent capacities to protect against HSV-1 infections in mice.

Second, the antiviral activity 2′-nor-cGMP is not HSV-TK dependent. 2′-nor-cGMP was equally effective against TK deficient or TK positive HSV-1 both in cell culture and in animal protection studies. These observations extend those previously reported by Tolman et al. [23] and agree with the cytotoxicity studies of Oliver et al. [17]. These observations suggest that activation of 2′-nor-cGMP is uniquely different from other nucleoside analogs such as ACV and 2′-NDG, and that the antiherpes activity of 2′-nor-cGMP does not result from dephosphorylation of 2′-NDG and subsequent HSV-TK dependent rephosphorylation. The antiherpes activity 2′-NDG correlates well with the concentration of its triphosphate produced in the infected cell. This is not true for 2′-nor-cGMP. At their respective ED_{50} concentrations, there is 10-fold less 2′-NDG triphosphate produced from 2′-nor-cGMP than from 2′-NDG [23]. These findings indicate that 2′-nor-cGMP may inhibit viral replication through a mechanism different from that observed for known acyclonucleosides.

Third, 2′-nor-cGMP was equivalently active in protecting cells against either HSV-1 KOS or the mutant PFA_{2a}. This mutant is altered at the DNA polymerase locus and is less sensitive to ACV than is HSV-1 KOS, but is unaltered in sensitivity to 2′-NDG. Thus, 2′-nor-cGMP and 2′-NDG may have a common site(s) of inhibition on the DNA polymerase as ultimate target for the phosphorylated molecules.

Lastly, 2′-nor-cGMP is approximately 10-fold more active in cell culture against strains of VZV than are either 2′-NDG or ACV. This observation has yet to be tested in animal protection studies and the mechanism of this enhanced activity is not yet known.

We have demonstrated using the HSV-1 and HSV-2 infection models in mice and guinea pigs that 2′-nor-cGMP can effectively be used orally, topically or by subcutaneous injection. It is important now to obtain detailed safety studies to fully evaluate the potential utility of this interesting antiviral drug.

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