

ANTIVIRAL EFFECT OF OXETANOCIN G AGAINST GUINEA PIG
CYTOMEGALOVIRUS INFECTION *IN VITRO* AND *IN VIVO*NAOHIKO YAMAMOTO, YOSHINARI YAMADA, TOHRU DAIKOKU, YUKIHIRO NISHIYAMA,
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The antiviral activity of a new guanosine analog, 9-(2-deoxy-2-hydroxymethyl- β -D-erythro-oxetanosyl)guanine (OXT-G), against guinea pig cytomegalovirus (GPCMV) was evaluated both *in vitro* and *in vivo*. In the plaque reduction assay, the median effective concentration (EC₅₀) of OXT-G, ganciclovir (DHPG) and acyclovir (ACV) against GPCMV was 0.03, 6.4 and 52 μ g/ml, respectively. The selectivity index, based on the ratio of the median inhibitory concentration for cell growth of guinea pig embryo fibroblasts to the median effective concentration for GPCMV plaque formation, was about 100-fold higher than that of DHPG. In an *in vivo* study, Hartley guinea pigs infected with GPCMV were treated with OXT-G or DHPG (20 mg/kg/day) for 2 weeks, and it was found that virus titers in the salivary gland were 70-fold lower in OXT-G-treated guinea pigs than in DHPG-treated animals. The results indicate that OXT-G was more potent and selective against GPCMV infection than DHPG.

Human cytomegalovirus infection is a major cause of mortality to immunocompromised individuals, including bone marrow and other transplant recipients, and patients with acquired immunodeficiency syndrome^{1,2}. Ganciclovir (DHPG), a potent inhibitor against human cytomegalovirus (HCMV) *in vitro*, has been shown to have a significant antiviral activity against HCMV infections in these patients^{3,4}. However, the therapeutic efficacy is limited by its toxicity, ganciclovir-induced myelosuppression^{5,6}. Recently we have reported that one of the oxetanocins⁷, 9-(2-deoxy-2-hydroxymethyl- β -D-erythro-oxetanosyl)guanine (OXT-G), has a potent and selective activity against HCMV *in vitro*⁸, and against herpes simplex virus type 2 (HSV-2) and murine cytomegalovirus (MCMV) infections in mice⁹. Because of the strict species specificity demonstrated by HCMV, testing of potential antiviral agents for activity against HCMV in animals is not possible. The guinea pig model of CMV infection is well suited for testing antiviral agents because the pathogenesis of the viral infection in this animal model closely resembles that in human host^{10,11}. In the present study, we examined the effects of OXT-G on the replication of guinea pig cytomegalovirus (GPCMV) in cell cultures, and its therapeutic effect against GPCMV in guinea pigs.

Materials and Methods

Chemicals

OXT-G was synthesized as described previously⁷. 9-(2-Hydroxyethoxymethyl)guanine (ACV) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) were provided by Burroughs Wellcome Co., Research

Triangle Park, N.C., and Syntex Laboratories, Inc., Palo Alto, Calif., respectively. For use *in vivo* studies, OXT-G and DHPG were dissolved in phosphate buffered saline (PBS) at 2 mg/ml. L-[³⁵S]Methionine (600 Ci/ml) was purchased from Amersham Laboratories, United Kingdom.

Cell Cultures, Viruses and Infectivity Assay

Human embryo lung fibroblasts (HEL) and guinea pig embryo (GPE) cells used in this study were grown in EAGLE's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). HCMV strain AD169 was prepared as described previously^{12,13} and GPCMV strain 22122 was prepared from the salivary gland homogenates of GPCMV-infected guinea pigs. The infectivities of HCMV and GPCMV were measured in HEL and GPE cells, respectively. For plaque formation, infected cells were overlaid with MEM containing 0.5% agarose and 2% FBS, and incubated at 37°C. After 10~14 days, cells were fixed with 5% formalin and stained with 0.5% crystal violet solution. Plaque numbers were counted by using a dissecting microscope at ×20 magnification.

Plaque Reduction Assays

Confluent monolayers of HEL or GPE cells in plastic dishes (diameter, 35 mm) were infected with 100 to 150 PFU of HCMV or GPCMV. After a 1-hour adsorption period at 37°C, the cultures were overlaid with 2 ml of MEM containing 0.5% agarose, 2% FBS and various concentrations of drugs, and plaques were enumerated as described above.

Cell Growth Inhibition Tests

Cells were seeded at approximately 2×10^4 cells per well in 24-well tissue culture plates, and cultured at 37°C in 1.0 ml of bicarbonate-buffered MEM containing 10% FBS in a 5% CO₂ humidified incubator. Various concentrations of drugs were added 24-hour after cultivation, and the number of viable cells was then counted on day 3 post-seeding of cells.

Polyacrylamide Slab Gel Electrophoresis

Cells were labeled with [³⁵S]methionine (10 μCi/ml) in methionine-free MEM, and the labeled proteins were analyzed by the method of LAEMMLI¹⁴. Samples were dissolved in 0.0625 M Tris-hydrochloride (pH 6.8) containing sodium dodecyl sulfate (SDS) 5%, 2-mercaptoethanol 2%, glycerol 10%, and bromophenol blue 0.001% followed by heating at 100°C for 1 minute. The acrylamide concentrations were 8.5% for the separating gel and 3% for the stacking gel. After electrophoresis, the gels were fixed, dried, and then exposed to film (Royal X-Omat; Eastman Kodak Co., Rochester, N.Y.) at -80°C.

Infection and Drug Administration in Guinea Pigs

For experiments *in vivo*, mature Hartley guinea pigs (300~400 g) were used. Guinea pigs were inoculated intraperitoneally with 1 ml of 1×10^4 PFU of GPCMV, and were sham-treated with PBS or treated with OXT-G or DHPG with a dose of 5 mg/kg twice daily for a week or 10 mg/kg twice daily for 2 weeks, starting 6 hours post-infection. Animals were checked daily and their body weights were recorded.

Isolation of GPCMV from Tissues of Guinea Pigs

Infected guinea pigs were anesthetized with diethyl ether and sacrificed on day 7, 14, 21, 28 post-inoculation. Various organs were removed aseptically for virus titration, and made into 10% (w/v) homogenate and assayed for GPCMV in GPE monolayers.

Results

Activity of OXT-G against GPCMV In Vitro

The antiviral and anticellular activity of OXT-G, DHPG and ACV against GPCMV and GPE cells were measured in plaque reduction assays and cell growth inhibition tests, and were expressed in terms of the median effective concentration (EC₅₀) and the median inhibitory concentration (IC₅₀); drug concentrations that reduced viral and cellular replication to 50%, respectively (Table 1). Antiviral activity

Table 1. Antiviral and anticellular activities of OXT-G, DHPG and ACV.

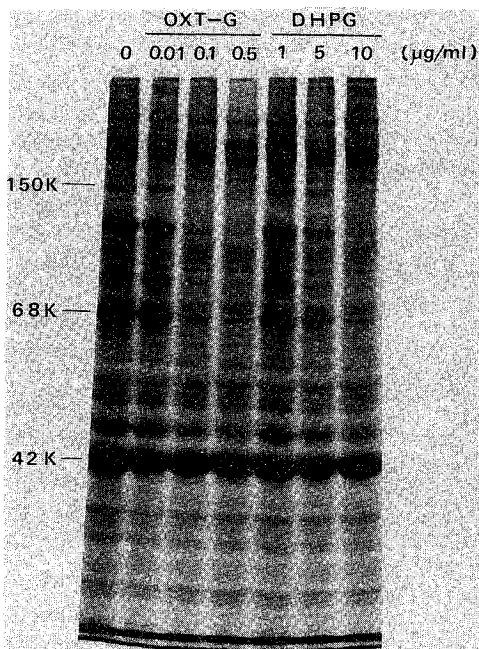
Compound	EC ₅₀ (μg/ml) ^a		IC ₅₀ (μg/ml) ^b	Selectivity ^c index
	Anti-HCMV ^b activity	Anti-GPCMV ^b activity	Anti-GPE cell activity	
OXT-G	0.75	0.03	55	1,850
DHPG	0.60	6.36	100	15.7
ACV	6.8	51.6	114	2.2

^a Results were the average of two or three different experiments. Each experiment was carried out in duplicate or triplicate.

^b Anti-HCMV or anti-GPCMV activities were measured by the plaque reduction assays in HEL or GPE cell culture, respectively.

^c The selectivity index was calculated from the ratio of the IC₅₀ for GPE cell growth to the EC₅₀ for GPCMV plaque formation.

Fig. 1. Effect of OXT-G or DHPG on the synthesis of GPCMV-induced polypeptides.

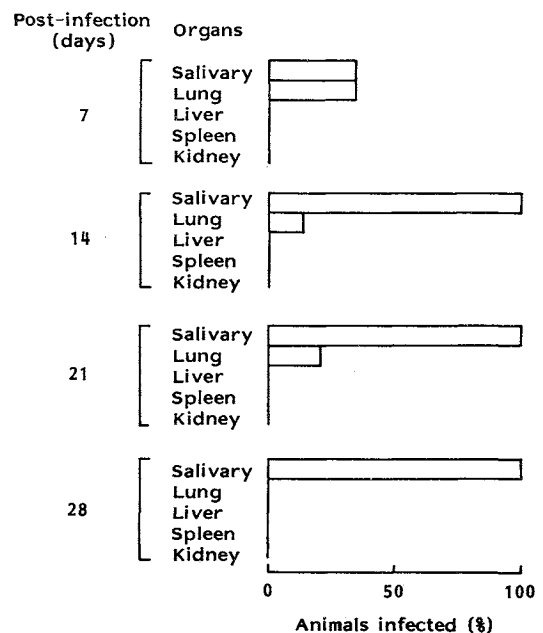


GPCMV-infected GPE cells were incubated at 37°C in the absence or presence of drugs and labeled with [³⁵S]methionine (10 μCi/ml) between 72 and 73 hours post-infection. The labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis, as described in the text.

of OXT-G against HCMV was almost the same as that of DHPG, as described previously⁸⁾, but the EC₅₀ of OXT-G against GPCMV was about 200-fold lower than that of DHPG. Thus the selectivity index of OXT-G, based on the ratio of the IC₅₀ for cell growth of GPE cells to the EC₅₀ for GPCMV plaque formation, was about 100-fold higher than that of DHPG.

The high potency of OXT-G against GPCMV was also confirmed by examination of its effect on virus-induced protein synthesis. After 1 hour virus adsorption, appropriate concentrations of the drug were added and cells were labeled with [³⁵S]methionine between 72 and 73 hours post-infection. In control

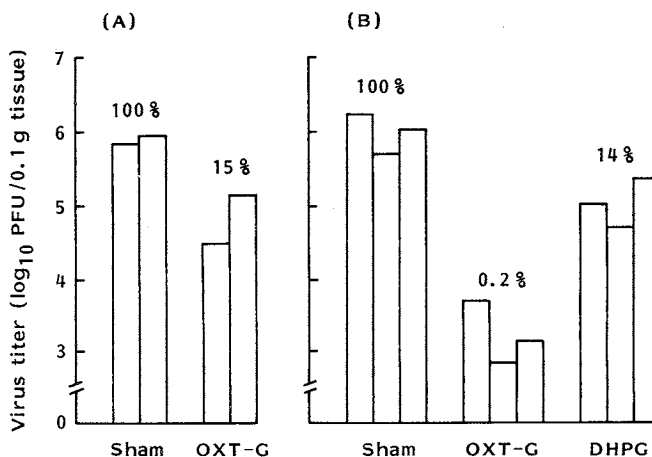
Fig. 2. Distribution of GPCMV in different organs of infected guinea pigs after intraperitoneal inoculation.



Twenty animals were tested in total as described in Materials and Methods.

of OXT-G against HCMV was almost the same as that of DHPG, as described previously⁸⁾, but the EC₅₀ of OXT-G against GPCMV was about 200-

Fig. 3. Virus titers in the salivary gland of guinea pigs untreated or treated with OXT-G or DHPG.

(A) 10 mg/kg/day \times 1 week, (B) 20 mg/kg/day \times 2 weeks.

The infected animals were sacrificed on day 14 post-inoculation, and the virus titers in the salivary gland were assayed as described in Materials and Methods.

cells infected with GPCMV, the high rate of synthesis of GPCMV-specific late proteins such as 150K and 68K proteins was observed (Fig. 1). In GPCMV-infected cells treated with 0.1 μ g/ml of OXT-G, however, the synthesis of these proteins was strongly suppressed. DHPG required much higher concentrations to suppress the synthesis of GPCMV-specific late proteins than OXT-G.

GPCMV Distribution in Different Tissues of Guinea Pigs Inoculated Intraperitoneally

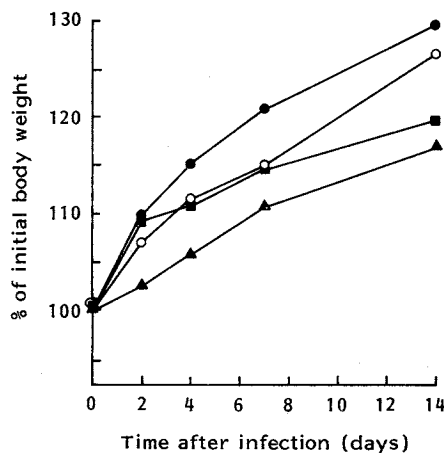
Guinea pigs infected intraperitoneally with 1 ml of $1 \times 10^4 \sim 4 \times 10^5$ PFU of GPCMV were sacrificed on day 7, 14, 21 and 28 post-inoculation to determine the distribution of GPCMV. Various tissues obtained from sacrificed animals were made into 10% (w/v) homogenate and assayed for GPCMV in GPE monolayers (Fig. 2). On day 7, GPCMV was detected in the salivary glands and lungs of about 30% of infected animals. On day 14, 21 and 28, viruses were recovered from the salivary glands of 100% of infected guinea pigs. However, GPCMV could not be detected from liver, spleen and kidney.

Effect of OXT-G on GPCMV Infection in Guinea Pigs

Because viruses were detected from the salivary glands of all of infected guinea pigs on day 14, we examined the antiviral activity of OXT-G by measuring the titer of GPCMV in the salivary glands at 14 days post-infection. When infected guinea pigs were treated with OXT-G at a dose of 10 mg/kg/day for 1

Fig. 4. Effect of OXT-G or DHPG on body weight of guinea pigs during GPCMV infection.

○ Uninfected and sham-treated, ● infected and sham-treated, ▲ GPCMV-infected and DHPG-treated, ■ GPCMV-infected and OXT-G-treated.



Three to six animals per group were tested.

week, the mean titer of GPCMV in salivary glands of OXT-G-treated animals were $5.0 \log_{10}$ PFU/0.1 g tissue, and was about 15% of those of sham-treated ones. However, animals treated with 20 mg/kg/day of the drug for 2 weeks, showed a much greater reduction of GPCMV titers in the salivary gland. The mean virus titer of the treated animals was reduced to 0.2% of that of sham-treated infected animals. In animals treated with 20 mg/kg/day of DHPG for 2 weeks, the reduction of virus titer in salivary glands was less marked than that observed in OXT-G treated animals. Both OXT-G and DHPG, administered at 20 mg/kg/day, were tolerated by guinea pigs although increase of body weight was slightly suppressed by administration of the drugs (Fig. 4).

Discussion

The present study indicates that OXT-G was about 200-fold more active and also 100-fold more selective *in vitro* than DHPG as an inhibitor of GPCMV. In addition, our studies have shown that OXT-G was highly effective against GPCMV infection in an animal model. At present we do not know the reason why OXT-G was so active and selective against GPCMV *in vitro*. Previous studies indicated that OXT-G was not a good substrate for viral thymidine kinase of herpes simplex virus and suggested that this nucleoside analog was phosphorylated by a host cell-derived nucleoside kinase⁹). Although we can not rule out the possibility that OXT-G is activated by a GPCMV-encoded kinase, the most likely explanation is that the high potency of OXT-G against GPCMV may be dependent on the properties of GPCMV DNA polymerase.

When a dosage of 20 mg/kg/day of OXT-G was administered for 2 weeks, virus titer in salivary glands was reduced by 99.8% compared with that of sham-treated animals. By comparison, treatment with DHPG at a dosage of 20 mg/kg/day for 2 weeks decreased the virus titer only to 14% of that of sham-treated animals. However, there was no significant difference between OXT-G and DHPG in toxicity as far as could be assessed from measurements of the body weights of animals. When a dosage of 20 mg/kg/day of either drug was administered for 2 weeks, the increase of the body weights of infected animals was suppressed, but the toxicity of both drugs was tolerated, as demonstrated by constant and gradual increase of body weights. These data indicate that OXT-G was more potent and selective against GPCMV infection than DHPG.

In a study of the efficacy of OXT-G and DHPG in a murine model of CMV infection, it has been shown that DHPG is more potent and selective against MCMV than OXT-G⁹). Since GPCMV and HCMV infections have more features in common^{10,11}), it is believed that the guinea pig model of CMV infection is more suited for testing anti-HCMV drugs than murine models^{15~18}). However, it seems difficult to determine which of the two drugs is most active for treatments of HCMV infection. With regard to their subacute toxicity in animals, preliminary studies have shown that OXT-G is more toxic in rats and mice, but less toxic in dogs than DHPG. In dogs, the former agent was considerably less toxic against hematopoietic tissue than the latter (unpublished observations). Studies are now in progress to characterize the toxicological features of OXT-G in animals.

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