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Selective Inhibition of Human Cytomegalovirus Replication by a Novel Nucleoside, Oxetanocin G

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A novel nucleoside with an oxetanosyl-*N*-glycoside has been recently isolated from a culture filtrate from *Bacillus megaterium* and named oxetanocin A (N. Shimada, S. Hasegawa, T. Harada, T. Tomisawa, A. Fujii, and T. Takita, J. Antibiot. 39:1623–1625, 1986). In this study, we evaluated the antiherpesvirus activity of oxetanocin A and its derivatives and found that 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G) was very potent and selective in inhibiting the replication of human cytomegalovirus (HCMV) in vitro. The median effective concentration for HCMV strain AD169 was 1.0 μg/ml, and that for herpes simplex virus type 2 strain 186 was 3.5 μg/ml. The selectivity index, based on the ratio of the median inhibitory concentration for cell growth of human diploid fibroblasts to the median effective concentration for HCMV plaque formation, was more than 300. The synthesis of HCMV-induced late polypeptides such as the 150,000-molecular-weight capsid and the 68,000-molecular-weight major matrix proteins was strongly suppressed when OXT-G (5 μg/ml) was added to the cultures at the beginning of infection. At this concentration of OXT-G, the amount of HCMV DNA detected in the drug-treated infected cells was less than 1/10 of that detected in the infected control cells. The results suggest that the mode of action of OXT-G is inhibition of viral replication by impairing the viral DNA synthesis.

Human cytomegalovirus (HCMV) is an important human pathogen which causes congenital cytomegalic inclusion disease, heterophile-negative mononucleosis, retinitis, and interstitial pneumonia (3, 11). In immunocompromised individuals such as transplant recipients and patients with acquired immunodeficiency syndrome, cytomegalovirus pneumonia is a major cause of mortality (7). While several chemotherapeutic agents are clinically available for the treatment of herpes simplex virus (HSV) and varicella-zoster virus, there are no licensed drugs against HCMV infections. At present the most promising candidate is the acyclic guanosine analog 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) (1, 8), which is currently undergoing clinical trials for the therapy of severe HCMV infections in immunodeficient patients (2). However, it is essential that we search for more selective and potent agents against this pathogen.

Recently, a novel nucleoside, 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)adenine (OXT-A), was isolated from a culture filtrate of *Bacillus megaterium* NK84-0218 (12). This compound was the first natural product with an oxetanosyl-*N*-glycoside and has been found to have antiviral activities against HSV and human immunodeficiency virus (4). In this study, we investigated the effect of OXT-A and its derivatives, including 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)2-aminoadenine (2-amino-OXT-A) and 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G), against HCMV in vitro.

MATERIALS AND METHODS

Chemicals. OXT-A was obtained from a culture filtrate of *B. megaterium* NK84-0218 as described previously (13); and 2-amino-OXT-A, OXT-G, 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)hypoxanthine (OXT-H), and 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)xanthine (OXT-X) were produced from OXT-A by chemical and biological transformation. The chemical structures of some OXTs used in this study are shown in Fig. 1. 9-(2-Hydroxyethoxymethyl)guanine(ACV) and DHPG were provided by Burroughs Wellcome Co., Research Triangle Park, N.C., and Syntex Laboratories, Inc., Palo Alto, Calif., respectively. L-[^{35}S]methionine (600 Ci/mmol) and α-^{32}P (410 Ci/mmol) were purchased from Amersham Laboratories, United Kingdom.

Cell and virus. Human fibroblastic diploid cells (human embryo lung fibroblast [HEL], Detroit 551, and CCD-18Co), human lung squamous carcinoma cells (PC-1), human epidermoid carcinoma cells (KB), and human hepatoma cells (HepG2) were used in this study and were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum. HCMV strain AD169, HSV type 1 (HSV-1) strain HF, and HSV type 2 (HSV-2) strain 186 (wild type [TK$^+$] and thymidine kinase deficient [TK$^-$]) were propagated in HEL cell monolayers as described previously (9, 10).

Plaque reduction assays. Confluent monolayers of HEL cells in plastic dishes (diameter, 35 mm) were infected with 100 to 150 PFU of HCMV or HSV. After a 1-h adsorption period at 37°C, the cultures were overlaid with 2 ml of 0.5% agarose in minimal essential medium containing 3% fetal bovine serum and various concentrations of drugs. The cultures infected with HSV and those infected with HCMV were fixed and stained at 1 or 2 and 9 or 10 days after infection, respectively. In the case of HCMV-infected cultures, the second agarose overlay containing appropriate concentrations of drugs was added 5 days after infection. Plaque numbers were counted by using a dissecting microscope at ×20 magnification.

Cell growth inhibition tests. Cells were seeded at a range of between 1.0×10^3 and 3.0×10^3 cells per well in 96-well

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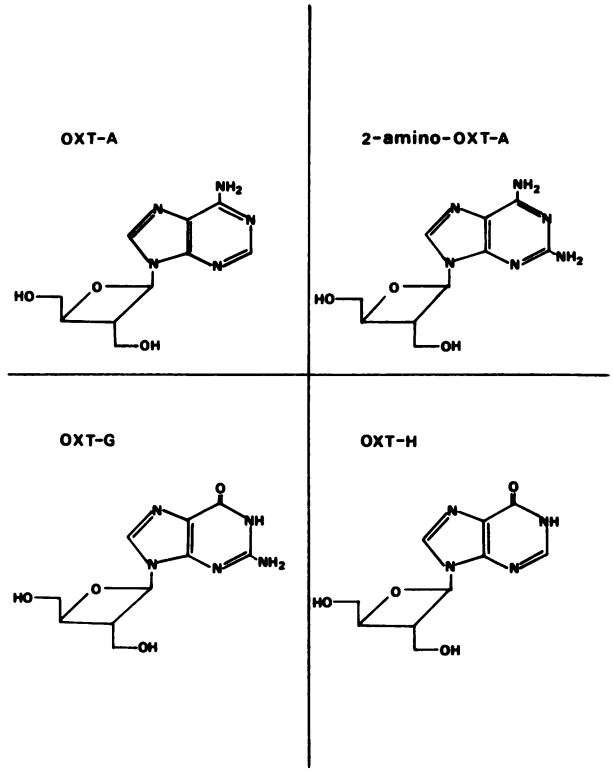

FIG. 1. Structures of OXTs.

tissue culture plates. Each of the cells was cultured at 37°C in 0.2 ml of bicarbonate-buffered minimal essential medium containing 10% fetal bovine serum in a 5% CO_2 humidified incubator. The drugs were added 24 h after cultivation to make appropriate concentrations. After a further incubation at 37°C, cell growth was usually assayed by an indirect colorimetric method with slight modifications, as described by Kobori et al. (5). In brief, after the culture medium was removed from each well, adherent cells were fixed by incubation for 2 min with 50 μl of methanol, mixed with 100 μl of 10 mM Tris hydrochloride buffer (pH 8.5) containing 0.05% methylene blue, and incubated for 30 min at room temperature. The stained cells were washed with distilled water, mixed with 200 μl of 3% HCl, and incubated for 24 h at room temperature to elute the methylene blue from the cells. The optical density of the HCl solution was measured at 660 nm with a microplate reader (model MR600; Dynatech Laboratories, Inc., Alexandria, Va.). The cultivation time was 72 h for PC-1 and 96 h for Detroit 551, CCD-18Co, and HepG2.

Polyacrylamide slab gel electrophoresis. Cells were labeled with [^{35}S]methionine (10 μCi/ml) in methionine-free minimal essential medium, and the labeled proteins were analyzed by the method of Laemmli (6). Samples were dissociated in 0.0625 M Tris hydrochloride (pH 6.8) containing 5% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue, followed by heating at 100°C for 1 min. 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.

Dot blot hybridization. Infected cells were collected by centrifugation; washed with phosphate-buffered saline; and suspended in a buffer solution (pH 8.1) containing 10 mM Tris hydrochloride, 0.1 mM EDTA, and 150 mM NaCl. Cells were lysed by the addition of 1.0% SDS and digested with pronase at a concentration of 500 μg/ml at 37°C for 3 h. The solution was extracted 3 times with a mixture of phenol and chloroform-isoamyl alcohol (24:1) and dialyzed against 2 liters of TE solution (10 mM Tris hydrochloride, 1 mM EDTA) with several changes. The sample was treated with 100 μg of DNase-free RNase per ml at 37°C for 2 h and extracted with organic solvents as described above. After dialysis, DNA was precipitated with ethanol, dissolved in TE solution, and denatured by heating. Each sample was serially diluted with SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), to yield a final volume of 100 μl, and each dilution was applied with suction to a 4-mm-diameter spot on a membrane (Gene Screen Plus; New England Nuclear Corp., Boston, Mass.). Prehybridization and hybridization were performed as described in the protocol of the supplier. Briefly, the membrane was prehybridized by treating it in 10 ml of the following solution; 50% formamide (deionized), 1% SDS, 1 M sodium chloride, and 10% dextran sulfate. The solution was added to a sealable plastic bag containing the membrane. The plastic bag was sealed and incubated with constant agitation for at least 6 h at 42°C. The solution containing denatured salmon sperm DNA (≥100 μg/ml) and denatured radioactive probe (*Xba*I fragments of HCMV DNA isolated from purified virions) was added to the bag containing the prehybridization buffer and the membrane. The plastic bag was resealed and incubated with constant agitation for 24 h at 42°C. The membrane was removed from hybridization solution, washed with SSC, allowed to dry at room temperature, and used for autoradiography.

TABLE 1. Antiviral and anticellular activities of OXTs

Virus or cell	IC_{50} (μg/ml)[a]				
	OXT-A	2-amino-OXT-A	OXT-G	OXT-H	OXT-X
Virus					
HCMV	13	2.1	1.0	18	>50
HSV-1	4.8	ND[b]	ND	>50	>50
HSV-2	10	4.2	3.5	>50	>50
Cell					
HEF (Detroit 551)	>300	>300	>300	>300	>300
CCD-18Co	>300	>300	>300	>300	>300
KB	59	>300	221	>300	>300
HepG2	23	205	198	>300	>300
PC-1	24	223	232	>300	>300

[a] Results are the average of two or three different experiments. Each experiment was carried out in duplicate or triplicate.
[b] ND, Not determined.

RESULTS

Antiviral and anticellular activities of OXTs. The antiviral and anticellular activities of OXT-A and its derivatives were measured by the plaque reduction assays and cell growth inhibition tests and are expressed in terms of the median effective concentration (EC_{50}) and the median inhibitory concentration (IC_{50}), which were defined as the drug concentrations that reduced viral and cellular replication to 50%, respectively. The results are summarized in Table 1. Among five compounds tested, OXT-G was found to have the most potent antiherpetic activity; the EC_{50}s of the compound against HCMV and HSV-2 were approximately

TABLE 2. Inhibitory effects of OXT-G, ACV, and DHPG against HCMV and wild-type and TK⁻ HSV-2

Compound	Virus	Host cell	EC_{50} (μg/ml)[a]
OXT-G	HCMV	HEL	0.75
	HSV-2 (TK^+)	Vero	2.2
	HSV-2 (TK^-)	Vero	2.0
ACV	HCMV	HEL	6.8
	HSV-2 (TK^+)	Vero	0.16
	HSV-2 (TK^-)	Vero	18
DHPG	HCMV	HEL	0.60
	HSV-2 (TK^+)	Vero	0.30
	HSV-2 (TK^-)	Vero	69

[a] Results are the averages of two experiments. Each experiment was carried out in duplicate or triplicate.

1.0 and 3.5 μg/ml, respectively. On the other hand, the IC_{50}s for uninfected human fibroblasts such as Detroit 551 and CCD-18Co were more than 300 μg/ml. It was also shown that 2-amino-OXT-G, which can be considered a prodrug of OXT-G, had potent antiviral activity and selectivity, comparable to those of OXT-G.

Comparative efficacy of OXT-G, ACV, and DHPG against HCMV and HSV-2. The antiviral activity of OXT-G was compared with those of the known antiherpesvirus drugs ACV and DHPG. The potency of OXT-G against HCMV was almost the same as that of DHPG, but it was less active against HSV-2 (TK^+) than ACV and DHPG (Table 2). Both ACV and DHPG exhibited much less potent antiviral activities against HSV-2 (TK^-). On the other hand, OXT-G was found to have about the same activities against both TK^- and TK^+ HSV-2.

Effects of OXT-G on HCMV-induced protein and DNA synthesis. To determine the effect of OXT-G on HCMV-induced protein synthesis, SDS-polyacrylamide gel electrophoresis was performed. In control cells infected with HCMV (Fig. 2), the high rate of synthesis of the 150,000-molecular-weight capsid (150K capsid) and the 68K major matrix proteins was observed. In HCMV-infected cells treated with OXT-G, however, the synthesis of these proteins was suppressed in a dose-related manner. At a concentration of 5 μg/ml, the synthesis of the 68K major matrix protein was reduced to less than one fifth of that of control cells, as measured with a densitometer. On the other hand, the synthesis of these late proteins was not affected at all, even at 50 μg of OXT-G per ml, when the drug was added 3 h before labeling.

The effect of OXT-G on HCMV DNA synthesis was determined by a dot blot hybridization technique, with ^{32}P-labeled HCMV *Xba*I fragments used as probes. The drug was added to the infected cultures 1 h after infection, and the DNA was extracted from cells at 72 h postinfection. The amount of viral DNA that accumulated in infected cells was inhibited by more than 50% at 1 μg of OXT-G per ml (Fig. 3). At a concentration of 5 μg/ml, it was reduced to less than 1/10 of that of untreated infected cells.

DISCUSSION

OXT-A, a novel nucleoside from bacteria, and its derivatives were evaluated for their antiherpesvirus activities, and OXT-G was found to be the most potent compound against HCMV and HSV. The EC_{50} for HCMV strain AD169 was approximately 1.0 μg/ml, and that for HSV-2 strain 186 was 3.5 μg/ml. On the other hand, OXT-G exhibited low toxicity in vitro to various types of cells of human origin. The selectivity index, based on the ratio of the IC_{50} for cell

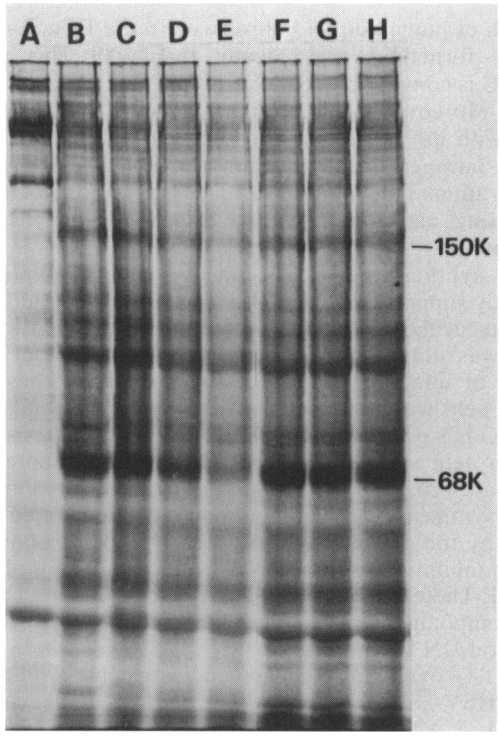

FIG. 2. Effect of OXT-G on the synthesis of HCMV-induced polypeptides. Mock-infected (lane A) or HCMV-infected (lanes B to H) HEL cells were incubated at 37°C in the absence (lanes A and B) or presence of 1 (lane C), 2 (lane D), 5 (lane E), 10 (lane F), 20 (lane G), and 50 (lane H) μg of OXT-G per ml and labeled with [^{35}S]methionine (10μCi/ml) between 72 and 73 h postinfection. The drug was added at 1 h (lanes C, D, and E) or 69 h (lanes F, G, and H) postinfection. The labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis, as described in the text.

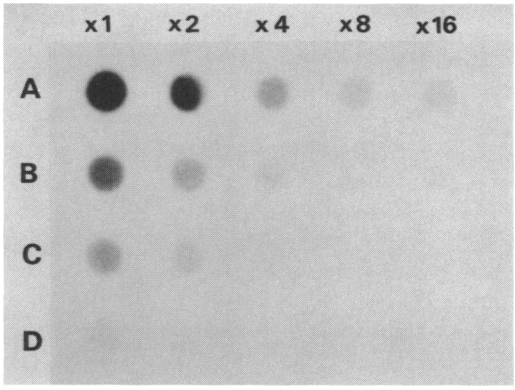

FIG. 3. Effect of OXT-G on HCMV DNA synthesis. HCMV-infected HEL cells were incubated in the absence (row A) or presence of 1 (row B), 2 (row C), and 5 (row D) μg of OXT-G per ml. The drug was added at 1 h postinfection. Infected cells were collected at 72 h postinfection; and DNA was extracted, diluted serially twofold, and then spotted onto a Gene Screen Plus membrane. Dot blot hybridization was carried out as described in the text.

growth of human diploid fibroblasts to the EC_{50} for HCMV plaque formation, was greater than 300. This value of OXT-G is comparable to those of DHPG reported previously (1, 8). Moreover, we found that the acute toxicity was very low, with its 50% lethal dose being 600 mg/kg following a single intraperitoneal injection (unpublished data). These observations indicate that OXT-G may be one of the most promising candidates as a therapeutic drug for HCMV infections.

The synthesis of HCMV-induced late polypeptides was strongly suppressed when OXT-G (5 μg/ml) was added to the cultures at the beginning of infection. If the addition of the drug was done several hours before labeling, at the late phase of infection, however, the protein synthesis of infected cells was not affected at all. These results indicate that OXT-G has no or little direct effect on the synthesis of RNA and protein synthesis, suggesting that the suppression of viral late protein synthesis is due to the inhibition of viral DNA synthesis. On the other hand, quantification of HCMV DNA by the dot blot hybridization method demonstrated a strong inhibition of viral DNA synthesis at 5 μg of OXT-G per ml. Therefore, it is suggested that the mode of action of this compound is inhibition of viral replication by impairing the viral DNA synthesis.

OXT-G exhibited equal potencies against wild-type and TK^- HSV-2. This suggests that OXT-G, unlike ACV and DHPG, is a poor substrate of HSV-2 TK. At present, the precise mechanism of action of this agent remains unelucidated. However, it may be reasonable to infer that OXT-G is phosphorylated by cellular nucleoside kinases and then that OXT-GTP inhibits HCMV-induced DNA polymerase, inhibiting viral DNA synthesis. Further studies are necessary to clarify these points.

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