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Analysis of Phosphorylation Pathways of Antiherpesvirus Nucleosides by Varicella-Zoster Virus-Specific Enzymes

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The inhibitory activities of acyclovir (ACV), 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU), ganciclovir (GCV), 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G), and (+)-9-[(1*R*,2*R*,3*S*)-2,3-bis(hydroxymethyl)cyclobutyl]guanine (cOXT-G) on the replication of wild-type and thymidine kinase (TK)-negative strains of herpes simplex virus types 1 and 2 and varicella-zoster virus (VZV) and the wild-type strain of human cytomegalovirus were tested to clarify whether the phosphorylation of these compounds is catalyzed by viral TK or other enzymes. ACV and BV-araU had little effect on the replication of TK-negative virus strains. On the other hand, GCV, OXT-G, and cOXT-G inhibited the replication of TK-negative VZV at concentrations 10 times higher than those at which they inhibited wild-type VZV, indicating that a kinase other than TK phosphorylates GCV and OXT-G in VZV-infected cells. GCV phosphorylation activity was not detected in VZV-infected cell lysates; therefore, this activity was evaluated in COS 1 cells expressing viral TK and viral protein kinase (PK). The COS 1 cells expressing VZV TK were shown to be susceptible to all compounds tested. In contrast, VZV PK-expressing COS 1 cells were susceptible to only GCV, OXT-G, and cOXT-G. These results suggest that VZV PK phosphorylates some nucleoside analogs, for example, GCV, OXT-G, and cOXT-G. This phosphorylation pathway may be important in the anti-VZV activities of some nucleoside analogs.

Herpesviruses are common parasites of humans that manifest a variety of clinical symptoms. Many efforts have been made to develop safe, effective antiherpesvirus drugs, because some herpetic infections are severe, prolonged, and life-threatening. Both acyclovir (ACV) and ganciclovir (GCV) have been successful in the clinical management of these diseases.

The mechanism by which these compounds inhibit herpes simplex virus (HSV) replication is the selective phosphorylation of ACV and GCV by a virus-encoded thymidine kinase (TK) (5, 7). The number of case reports describing the isolation of TK-negative ACV-resistant strains of HSV, mainly from immunocompromised patients, is increasing (2, 3, 22). Therefore, the development of new drugs which have different mechanisms of antiherpesvirus activity and which are effective against ACV-resistant TK-negative HSV strains is required.

On the other hand, GCV is effective against human cytomegalovirus (HCMV), although this virus does not have the gene that encodes TK. Recent studies have shown that the enzyme which catalyzes GCV phosphorylation is an HCMV-encoded protein kinase (PK) (11, 16). This suggests that there are phosphorylation pathways for nucleoside analogs other than TK in herpesvirus-infected cells. This means that new antiherpesvirus drugs which would be more potent against ACV-resistant virus and which would have a broader spectrum of activity than that of ACV could be developed.

In the study described here, we investigated the phosphorylation activities of GCV, 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G), and (+)-9-[(1*R*,2*R*,3*S*)-2,3-bis(hydroxymethyl)cyclobutyl]guanine (cOXT-G) by varicella-zoster virus (VZV)-encoded TK and PK (open reading frame 47) (12, 13), which is homologous to HCMV PK (UL97) (4), and clarified the nucleoside phosphorylation mechanisms of VZV.

MATERIALS AND METHODS

Compounds. ACV was supplied by Japan Wellcome Co. Ltd., Osaka, Japan. 1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU) was synthesized by Yamasa Shoyu Co. Ltd., Choshi, Japan. GCV and [³H]GCV were provided by Syntex Research, Palo Alto, Calif. OXT-G and cOXT-G were gifts from N. Shimada, Nippon Kayaku Co. Ltd., Tokyo, Japan.

Cells and viruses. Human embryo lung (HEL) fibroblasts were prepared and maintained in our laboratory. COS 1 cells were supplied by H. Tanaka, Asahikawa Medical College, Asahikawa, Japan. These cells were cultured in Eagle's minimum essential medium (MEM) containing 10% newborn calf serum (MEM-CS10). The VR-3 strain of HSV type 1 (HSV-1) and the UW-268 strain of HSV-2 were supplied by the American Type Culture Collection, Rockville, Md. The TK-negative mutants of VR-3 and UW-268 strains (VRTK⁻ and UWTK⁻, respectively) were isolated in our laboratory (21). Genetic analysis of the VRTK⁻ strain revealed that a single nucleotide substitution changed amino acid 281 (arginine; CGA) to a termination codon (TGA) (18). In the case of the UWTK⁻ strain, a single nucleotide insertion at codon 186 causes a frame shift and premature termination at codon 229 (18). The YS strain of VZV and its TK-negative mutant, the YSR strain, were isolated in our laboratory (15). Sequence analysis of the TK gene of strain YSR revealed a one-nucleotide deletion at codon 164, and this leads to the synthesis of a half-size TK polypeptide, which consists of 170 amino acids, of the wild-type VZV TK (10). None of the TK-negative mutants has TK activity (15, 18, 21). Cell-free virus stocks of VZV were prepared by sonication of VZV-infected cells in SPGA solution (0.218 M sucrose, 0.0038 M KH₂PO₄, 0.0072 M Na₂HPO₄, 0.0049 M sodium glutamate, 1% bovine serum albumin) as described previously (8). The AD169 strain of HCMV was supplied by S. Chiba, Sapporo Medical College, Sapporo, Japan.

Plaque reduction assay. The antiviral activities of the compounds tested against herpesviruses (HSV, VZV, and HCMV) in HEL cells were evaluated by a plaque reduction assay as described previously (20). Briefly, HEL cell monolayers were cultured in 24-well microplates and were inoculated with about 20 PFU of virus in 0.2 ml. After an adsorption period of 60 min, the inocula were removed, and infected cell sheets were incubated in MEM-CS10 containing 0.5% methylcellulose and serially diluted antiviral compounds at 37°C for 2, 7, and 14 days for HSV, VZV, and HCMV, respectively. The cell sheets were fixed with formaldehyde and were stained with 0.05% crystal violet. The concentration required to inhibit plaque formation by 50% was determined graphically.

Assay of GCV phosphorylation activity. Phosphorylation activity in VR-3-, YS-, and AD169-infected HEL cells was evaluated as follows. The cells were suspended in 25 mM Tris hydrochloride (pH 7.5)–5 mM 2-mercaptoethanol and were disrupted in a sonicator (Kontes, Vineland, N.J.) for 30 s at full power. The sonicated cells were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was used as the crude enzyme material.

The reaction mixture contained 13 mM ATP, 50 mM Tris hydrochloride (pH 7.5), 1 μCi of [³H]GCV, and 10 mM MgCl₂ for TK or 10 mM MnCl₂ and 50 mM

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TABLE 1. Antiviral and anticellular activities of nucleosides against herpesviruses and HEL cells[a]

Virus (strain) or cell	IC$_{50}$ and EC$_{50}$ of the following compounds (μg/ml)[b]:				
	ACV	BV-araU	GCV	OXT-G	cOXT-G
HSV-1 (VR-3)	0.15	0.022	0.018	11.0	0.023
HSV-1 (VRTK⁻)	>100	>100	6.8	4.5	8.6
HSV-2 (UW-268)	0.68	34.2	0.22	20.7	0.051
HSV-2 (UWTK⁻)	>100	>100	68.8	33.5	11.6
VZV (YS)	0.53	0.00015	0.35	0.024	0.025
VZV (YSR)	33.5	>100	2.18	0.19	0.14
HCMV (AD169)	13.2	69.9	0.16	0.062	0.19
HEL cell	>200	>200	45.3	133.5	19.8

[a] Results are the averages of two different experiments.
[b] IC$_{50}$, 50% inhibitory concentration for plaque formation of herpesviruses; EC$_{50}$, 50% effective concentration for cell growth inhibition.

KCl for PK, plus 40 μl of enzyme extract in a total volume of 100 μl. After an appropriate incubation period at 37°C, 20 μl of the mixture was added to 2 μl of cold 100% trichloroacetic acid, and the reaction was terminated. Of this mixture, 20 μl was spotted onto Whatman DE-81 paper disks, which were then washed twice with 1.5 mM ammonium formate, once with distilled water, and once with 100% ethanol. The radioactivity retained on the disks was measured with a liquid scintillation counter.

Plasmids. The VZV TK gene was recloned from pkk-VZTK, which is the VZV TK gene inserted into multiple cloning sites of the prokaryotic expression vector pkk223-3 (19), into the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, Calif.), creating pRc-VZTK. The VZV PK gene was cloned from the genomic DNA of the YS strain into pRc/CMV by PCR with the oligonucleotides VZ47Xb and VZ47Ap as primers. The sequences of the primers were 5′-GTT Atc TAg ACA ATG GAT GCT GAG GAC A-3′ for VZ47Xb and 5′-TAC AAA AGG Gcc CTG TAG ACC TCC C-3′ for VZ47Ap (capital letters represent the viral sequence). PCR was performed with 100 ng of YS genomic DNA as the template and 100 pmol of each primer in a 100-μl reaction mixture. The initial denaturation at 99°C for 5 min was followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min). The PCR product was cleaved with *Xba*I and *Apa*I and was ligated into the *Xba*I and *Apa*I sites of pRc/CMV (pRc-VZPK). The sequence of pRc-VZPK was confirmed by double-stranded DNA sequencing (24).

Transfection. COS 1 cells (10⁵) were seeded onto 30-mm dishes 24 h before transfection. The cells were first washed twice with serum-free Dulbecco's modified Eagle medium (DMEM) and then 1 μg of DNA (pRc-VZPK or pRc-VZTK) was mixed gently with diluted Lipofectin (GIBCO, Grand Island, N.Y.), and the mixture was then added to the COS 1 cells in serum-free DMEM. After a 24-h incubation, the DMEM was exchanged with MEM-CS10. After a 48-h incubation, the medium was changed to selection medium (MEM-CS10 supplemented with neomycin to a final concentration of 500 μg/ml). The expression of VZV TK or VZV PK polypeptides in COS 1 cells (COS-TK or COS-PK) was confirmed by direct immunofluorescence.

Determination of inhibitory effects of the compounds on cell growth. COS 1, COS-TK, and COS-PK cells were seeded in 24-well tissue culture microplates at a density of 2 × 10⁴ cells per well. After 1 day, the cell cultures were replenished with medium containing an appropriate amount of the test compound. After a 2-day incubation, the cells were dispersed with trypsin and the viable cell numbers were counted. The 50% effective concentration for cell growth was determined graphically.

RESULTS

Antiviral activities of compounds in HEL cells. The 50% inhibitory concentrations of ACV, BV-araU, GCV, OXT-G, and cOXT-G for the herpesviruses are given in Table 1. ACV and BV-araU were effective against the wild types of HSV-1 and VZV, and ACV was effective against the wild type of HSV-2 but BV-araU was not. However, these two compounds had little effect on the replication of HCMV and the TK-negative mutants of HSV and VZV. In contrast, GCV, OXT-G, and cOXT-G were efficient against TK-negative VZV and HCMV compared with their activities against TK-negative HSV-1 and HSV-2. These results suggest that VZV TK activity is not essential for the anti-VZV activities of GCV, OXT-G, and cOXT-G and that there is another phosphorylation pathway of these compounds in addition to that which is dependent on viral TK in VZV-infected cells.

GCV phosphorylation activity of VZV-infected cell lysate. To analyze the phosphorylation pathway of GCV in VZV-infected cells, the GCV phosphorylation activity in crude extracts of VR-3-infected, YS-infected, and AD169-infected cells was measured. Phosphorylation activity was detected in HSV-1-infected cells (Fig. 1A) and HCMV-infected cells (Fig. 1B) but not in VZV-infected cells, although the YS strain was susceptible to GCV. In order to enhance the sensitivity of the experiment, GCV phosphates in VZV-infected cells and mock-infected cells which were cultivated with medium containing 5 μg of [³H]GCV per ml for 24, 48, or 72 h were analyzed by polyethyleneimine-cellulose thin-layer chromatography as described previously (20). However, differences were not observed between VZV-infected cells and mock-infected cells. These results suggest that the level of phosphorylated GCV may be low in VZV-infected cells.

Expression of VZV TK or VZV PK in mammalian cells and cell growth inhibition. Because GCV phosphorylation was not detected in YS-infected cells, VZV TK and VZV PK were expressed in COS 1 cells. The expression of these VZV pro-
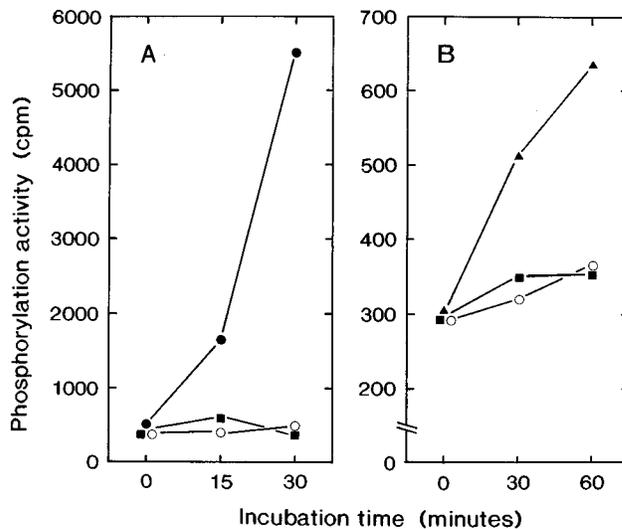

FIG. 1. Phosphorylation of GCV in extracts of HEL cells infected with HSV-1 VR-3, VZV YS, and HCMV AD169. (A) The activity of TK was assayed in the mixture (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 13 mM ATP, 1 μCi of [³H]GCV). (B) The activity of PK was assayed in the mixture (50 mM Tris-HCl [pH 7.5], 10 mM MnCl₂, 50 mM KCl, 13 mM ATP, 1 μCi of [³H]GCV). ●, VR-3-infected cells; ■, YS-infected cells; ▲, AD169-infected cells; ○, mock-infected cells.

TABLE 2. Cell growth inhibition of compounds against COS cells expressing VZV PK or TK

Cell	EC$_{50}$ (μg/ml)[a]				
	ACV	BV-araU	GCV	OXT-G	cOXT-G
COS 1	129	422	232	69.9	66.0
COS-PK	108	333	34.9	8.1	22.5
COS-TK	26.6	4.5	18.6	0.2	1.9

[a] EC$_{50}$, 50% effective concentration for cell growth inhibition.

teins was confirmed by direct immunofluorescence with biotinylated anti-VZV TK and anti-VZV PK rabbit antibodies. Specific TK and PK signals were detected in the cytosols and nuclei, respectively (data not shown).

The phosphorylation activities of VZV TK and VZV PK for nucleoside analogs were evaluated by measuring the level of susceptibility of COS-TK or COS-PK to these nucleosides compared with that of control COS 1 cells. The COS-TK cells became susceptible to all compounds, but the COS-PK cells did so only to GCV, OXT-G, and cOXT-G (Table 2). These results suggested that ACV and BV-araU were phosphorylated by TK only but that GCV, OXT-G, and cOXT-G were phosphorylated not only by TK but also by PK or some cellular factors activated by PK.

DISCUSSION

The results of the present study show that GCV, OXT-G, and cOXT-G are effective against TK-negative mutants of VZV and HCMV, although ACV and BV-araU had little effect against these two viruses. During TK-negative virus infection, enzymes other than TK must phosphorylate antiviral compounds, because phosphorylation of nucleoside analogs is necessary for the inhibition of viral replication. It has been reported that HCMV PK (UL97) is related to GCV phosphorylation (11, 16), although it is not clear whether HCMV PK phosphorylates GCV directly or by the activation of another cellular or viral protein. This suggests that other herpesvirus PKs, which have some homology with HCMV PK (4), also participate in the phosphorylation of nucleosides. To clarify this pathway in VZV-infected cells, the antiherpesvirus activities of five nucleoside analogs were studied by using mutant virus strains and COS 1 cells expressing viral TK or PK.

The susceptibilities of COS 1 cells expressing VZV TK or VZV PK to antiviral agents changed, indicating that there are some nucleoside analogs which are phosphorylated by the VZV PK directly or some other factors activated by the VZV PK. These results indicate that nucleoside analogs against VZV can be classified into two groups by their phosphorylation pathway dependence on viral enzymes; the phosphorylation of one group depends only on TK, as for ACV and BV-araU, and that of the other group depends on TK and PK, as for GCV, OXT-G, and cOXT-G. A previous study has reported that ACV can also be phosphorylated by cellular 5′-nucleotidase, indicating that other phosphorylation pathways of GCV, OXT-G, and cOXT-G by cellular enzymes cannot be eliminated (9).

ACV and BV-araU are phosphorylated by VZV TK (1, 23), but the affinities between these nucleosides and VZV TK are quite different. The K_i value of ACV for VZV TK is >500 μM, and that of BV-araU is 0.26 μM (17). Moreover, the affinities of these nucleoside triphosphates for cellular DNA polymerase also differ, and the K_i values of ACV triphosphate and BV-araU triphosphate are 2.1 μM (6) and 0.007 to 0.14 μM (14),

respectively. These explain why the ratio of the BV-araU susceptibility of COS 1 cells to that of COS-TK cells was much greater than that for ACV (Table 2). Therefore, various factors, such as the affinity of the nucleoside triphosphate for DNA polymerase, affected the results presented in Table 2.

Recently, several ACV-resistant virus infections have been described, especially in immunocompromised hosts (2, 3, 22). Thus, new antiherpesvirus drugs with a mechanism of antiviral activity different from that of ACV and with a broader spectrum of activity than that of ACV are urgently required. In the present study, we showed that GCV, OXT-G, and cOXT-G can be phosphorylated by VZV PK directly or indirectly. Further detailed analyses are required to understand the mechanism of antiviral activity and the target enzymes so that new compounds can be designed.

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