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

Shin, Koyano; Tatsuo, Suzutani; Itsuro, Yoshida; Masanobu, Azuma
The inhibitory activities of acyclovir (ACV), 1-β-D-arabinofuranosyl-β-E-5-(2-bromovinyl)uracil (BV-araU), ganciclovir (GCV), 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G), and (+)-9-(1R,2R,3S)-2,3-bis(hydroxymethyl)cyclobutylguanine (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.

The reaction mixture contained 13 mM ATP, 50 mM Tris hydrochloride (pH 7.5), 5 mM 2-mercaptoethanol and was disrupted in a sonicator (Kontes, Vineland, N.J.) for 30 s at full power. The sonicated cells were centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was used as the crude enzyme material.

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KCl for PK, plus 40 μg 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 sequence of the primers were 5'-GTT Atc TAg ACA ATG GAT GCT GAG GAC A-3' for VZ47Xb and 5'-TACAAAAGGGccCTGTAGACCTCCC-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 1 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 AseI and ApalI and was ligated into the AseI and ApalI sites of pRc/CMV (pRc-VZPK). The sequence of pRc-VZPK was confirmed by double-stranded DNA sequencing (24).

**Transfection.** COS 1 cells (106) 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.), diluted Eagle medium (DMEM) and then 1 μg of plasmid DNA, 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 cells were replated 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-

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**TABLE 1. Antiviral and antacellular activities of nucleosides against herpesviruses and HEL cells**

<table>
<thead>
<tr>
<th>Virus (strain) or cell</th>
<th>IC50 and EC50 of the following compounds (μg/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACV</td>
</tr>
<tr>
<td>HSV-1 (VR-3)</td>
<td>0.15</td>
</tr>
<tr>
<td>HSV-1 (VRTK)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HSV-2 (UW-268)</td>
<td>0.68</td>
</tr>
<tr>
<td>HSV-2 (UWTK)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VZV (YS)</td>
<td>0.53</td>
</tr>
<tr>
<td>VZV (YSR)</td>
<td>33.5</td>
</tr>
<tr>
<td>HCMV (AD169)</td>
<td>13.2</td>
</tr>
<tr>
<td>HEL cell</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

* Results are the averages of two different experiments.

† IC50, 50% inhibitory concentration for plaque formation of herpesviruses; EC50, 50% effective concentration for cell growth inhibition.

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**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

<table>
<thead>
<tr>
<th>Cell</th>
<th>ACV EC50 (µg/ml)</th>
<th>BV-araU EC50 (µg/ml)</th>
<th>GCV EC50 (µg/ml)</th>
<th>OXT-G EC50 (µg/ml)</th>
<th>cOXT-G EC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS 1</td>
<td>129</td>
<td>422</td>
<td>232</td>
<td>69.9</td>
<td>66.0</td>
</tr>
<tr>
<td>COS-PK</td>
<td>108</td>
<td>333</td>
<td>349</td>
<td>8.1</td>
<td>22.5</td>
</tr>
<tr>
<td>COS-TK</td>
<td>26.6</td>
<td>4.5</td>
<td>18.6</td>
<td>0.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*EC50, 50% effective concentration for cell growth inhibition.

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 antiviral activity of five nucleoside analogs was 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<sub>e</sub> 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<sub>e</sub> 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 antiviral 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.

**REFERENCES**


