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Potentiation of ganciclovir toxicity in the herpes simplex virus thymidine kinase/ganciclovir administration system by ponicidin

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The herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) administration system is commonly used in gene therapy trials. We have evaluated the effect of ponicidin, a diterpenoid isolated from a plant, *Rabdosia ternifolia*, on the cell-killing activity of the anti-herpes drugs acyclovir (ACV) and GCV. Ponicidin preferentially activated HSV-1-specific TK but not cellular kinases. In HSV-infected cells, ponicidin significantly accumulated the phosphorylated metabolites of GCV and suppressed the extracellular release of GCV. These data suggested that the cytotoxicities of ACV and GCV in HSV-TK-expressing cells might be potentiated by ponicidin. After transfected with the HSV-1 *TK* gene, COS-1 and several human cancer cells became highly sensitive to the cytotoxic properties of the nucleoside analogs. When ponicidin at the concentration without antiviral activities (0.2 μg/mL) was combined with ACV or GCV, the cytotoxic levels in HSV-TK-expressing cells were enhanced by 3- to 87-fold and 5- to 52-fold, respectively, compared with the nucleoside alone. When the stability of the bioactivity of ponicidin in the blood of mice was evaluated, the substance showed relatively long-lasting effects on the potentiation of the anti-herpetic and cytotoxic activities of GCV after intravenous administration. These data suggest that the combined use of ponicidin with GCV will be effective for cancer gene therapy, because high cytotoxicity in viral TK-expressing cells should yield more rapid and enhanced tumor elimination.
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In herpes simplex virus (HSV)-infected cells, an anti-herpes drug, acyclovir (ACV), must first be phosphorylated to the monophosphate by a virus-coded thymidine kinase (TK) for it to inhibit viral replication.[1–3] Next, the monophosphate is further phosphorylated to the diphosphate and triphosphate by host cellular kinases.[4,5] Another antiherpes drug, ganciclovir (GCV), is converted to the monophosphate by the same HSV-TK that catalyzes the phosphorylation of ACV, and the resultant monophosphate is also metabolized into the diphosphate and triphosphate (GCV-DP and GCV-TP) derivatives by cellular kinases.[6–10] ACV-triphosphate and GCV-TP are the fully activated metabolites that are toxic to the virus. Their mechanisms of action appear to be selective inhibition of the viral DNA polymerase and termination of viral DNA chain elongation.[11,12]

In gene therapy trials, the HSV-*TK* gene is used as a "suicide gene."[13–17] That is, the transfer of the HSV-*TK* gene combined with GCV administration has become a popular method for treating solid tumors such as brain tumors, in which TK-activated GCV kills only dividing tumor cells while normal brain tissue should not be affected by the treatment.

During screening for the compounds that have synergism with ACV and GCV in HSV-1 replication, we found that ponicidin showed potent synergistic antiviral action in combination with the nucleoside analogs. Ponicidin is a diterpenoid isolated first from *Rabdosia japonica* (Labiatae) as a minor constituent; its structure was determined by Fujita et al.[18] This compound has also been isolated from *Rabdosia rubescens*,[19] *Rabdosia rosthornii*,[20] and *Rabdosia ternifolia*,[21] and is known to exhibit the prolonged effect for the life span of mice inoculated with Ehrlich ascites, carcinoma cells, and hepatoma cells.[20] In this study, we first evaluated the effects of ponicidin on the potentiation of ACV and GCV antiviral toxicities and TK enzyme activities. Ponicidin activated HSV-TK without showing any marked enhancement in host cellular kinases. The triphosphates of the nucleoside analogs play a key role in HSV-infected cells, that is, in HSV-TK-expressing cells. Thus, the effect of ponicidin on the persistence of the active metabolite of GCV was also determined. Because the

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suicide gene trials rely upon the ability to remove HSV-TK-expressing cells, we have studied the cytotoxic effects of ponicidin in the HSV-TK/GCV and HSV-TK/ACV systems using various *in vitro*-cultured cell lines. We have also examined in animal experiments whether the therapeutic levels of ponicidin could be achieved.

MATERIALS AND METHODS

Chemicals

The ponicidin used in this study was isolated from *R. ternifolia*.[21] ACV was obtained from Japan Wellcome (Osaka, Japan). GCV was purchased from Syntex (Palo Alto, Calif). [Methyl-^{3}H]thymidine (specific activity, 50 Ci/mmol) was obtained from ICN Biomedicals (Costa Mesa, Calif), and [8-^{3}H]ACV (specific activity, 27 Ci/mmol) and [8-^{3}H]GCV (specific activity, 20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, Calif).

Cells and virus

Simian virus 40-transformed African green monkey kidney (COS-1) cells, human bladder carcinoma (EJ-1) cells, human glioblastoma (PKG-1) cells, and human cervical carcinoma (HeLa 229) cells were obtained from Human Science Research Resources Bank (Osaka, Japan). HEp-2 cells from a tumor of the larynx and Vero cells from African green monkey kidney cells have been maintained in our laboratory. Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS); other cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. The HSV-1 HF strain, which is one of the standard strains and causes massive cell fusion by syncytial formation, was grown on Vero cells.

Antiviral activity

The plaque yield reduction assays for anti-HSV-1 activity have been described previously.[22] Vero cell monolayers were infected with virus at 5 plaque-forming units (PFU)/cell for 1 hour at room temperature and refed with maintenance medium (MEM plus 2% FBS) containing different concentrations of ponicidin. Virus yields were determined by plaque assay at 1 day of incubation. The 50% inhibitory concentration for antiviral activity (IC_{50}) was obtained from dose-response curves.

Assay of TK activity

Confluent Vero cells were mock-infected or infected with HSV-1 at 5 PFU/cell. After a 1-hour adsorption period, the monolayers were incubated in the absence or presence of ponicidin and harvested at 16 hours postinfection. The cell pellet was washed twice in cold phosphate-buffered saline (PBS), washed once in 10 mM tris(hydroxymethyl)aminomethane/5 mM 2-mercaptoethanol/2 mM $MgCl_2$, sonicated for 60 seconds, and centrifuged at 10,000 $\times$ *g* for 30 minutes. The supernatant fluid was used for the TK assay. Cellular and viral TK activities, which were determined using [^{3}H]thymidine as a substrate, were assayed according to the method of Jamieson et al,[23] except that 200 μM of thymidine and 100 μg of bovine serum albumin per milliliter were added to the reaction mixture. When viral TK activity was measured using [^{3}H]ACV as a substrate, assays were carried out as reported by Taylor et al,[24] except that 200 μM of ACV was added to the reaction mixture. TK activity was calculated and normalized for the amount of protein present in each sample as determined by the method of Lowry et al[25] with bovine serum albumin for the standard curve.

Plasmid construction

The plasmid pTK-1 contains the *Bam*HI Q fragment of HSV-1 DNA inserted into the *Bam*HI site of pBR322.[26] The *Bam*HI Q fragment is a 3.6-kb DNA fragment including the HSV-1 *TK* gene (1.3-kb coding region) but not the gene for ribonucleotide reductase. pSV2-HSTK-1 and pRc-HSTK-1 were constructed by digesting pTK-1 with *Bam*HI and then reinserting the *Bam*HI fragment into the expression vectors pSV2-neo[27] and pRc/CMV (Invitrogen, San Diego, Calif), respectively.

Transfection

COS-1 and human cancer cells ($1–2 \times 10^5$) were inoculated in 35-mm dishes, cultured for 24 hours at 37°C, and transfected with pSV2-HSTK-1 and pRc-HSTK-1, respectively, as follows: a total of 1 μg of plasmid DNA was mixed with diluted Lipofectamine (Life Technologies, Gaithersburg, Md), and the mixture was overlaid onto the cells rinsed with FBS-free DMEM. After a 24-hour incubation at 37°C, the medium was replaced with DMEM supplemented with 5% FBS. At 72 hours posttransfection, the medium was changed to selective medium containing 400 μg/mL geneticin (Life Technologies).

Inhibition of proliferation of cells expressing viral TK

The cells expressing HSV-1 TK and wild-type cells without expression of the enzymes were inoculated in 24-well plates at a density of 5×10^4 cells/well. After 1 day, the cells were replenished with medium containing varying concentrations of the test compounds. The cells were allowed to proliferate for 5 days, and the viable cell numbers were counted by trypan blue exclusion. The IC_{50} was determined graphically.

Determination of the phosphorylation rate of [^{3}H]GCV in HSV-1-infected cells

Vero cell monolayers in 35-mm dishes were infected with 1 PFU of HSV-1 per cell for 1 hour at 37°C, washed three times with PBS, and incubated in the medium containing 10 μM [^{3}H]GCV (5 μCi/dish) in the absence or presence of 0.2 μg/mL ponicidin. At 2, 4, 6, 8, 10, and 12 hours postinfection, the monolayers were washed five times with ice-cold PBS, harvested by scraping, and sonicated for 60 seconds. After centrifugation at 10,000 rpm for 1 hour, aliquots of the supernatants were spotted onto DE81 discs. To determine the [^{3}H]GCV-phosphates, the discs were dried, rinsed once with distilled water for 5 minutes, rinsed twice with 70% ethanol/2 mM ammonium acetate (pH 7.5) for 5 minutes, and rinsed once with 95% ethanol for 5 minutes. The discs were then dried and assayed for radioactivity in a liquid scintillation cocktail.

Persistence of [^{3}H]GCV-phosphates in HSV-1-infected cells

Vero cells were infected with 1 PFU of HSV-1 per cell and exposed to 10 μM [^{3}H]GCV (5 μCi/dish) in the absence or presence of 0.2 μg/mL ponicidin for 8 hours. The cells were then washed five times with PBS and replenished with fresh

Table 1. Effect of Ponicidin Treatment on Cellular and HSV-1-Induced Kinase Activities

	Enzyme activity (pmol of nucleoside incorporated/min/mg of protein)*		
	In uninfected cells	In infected cells	
Drug treatment	[^{3}H]thymidine	[^{3}H]thymidine	[^{3}H]ACV
None	626 ± 59 (100)†	985 ± 96 (100)	443 ± 20 (100)
Ponicidin (5 μg/ml)	423 ± 32 (68)‡	842 ± 55 (85)	590 ± 22 (133)§
Ponicidin (0.2 μg/ml)	621 ± 67 (99)	966 ± 182 (98)	546 ± 41 (123)§

*Cellular and viral kinase activities were measured in uninfected or HSV-infected Vero cells, respectively, after 16 hours of treatment with ponicidin. Each value is the mean ± SD of triplicate assays.

†Percent activity compared with untreated control cells.

‡Significant decrease compared with untreated cells on the basis of the Student *t* test ($P < .01$).

§Significant increase compared with untreated cells on the basis of the Student *t* test ($P < .05$).

medium containing the same concentration of unlabeled GCV with or without ponicidin. At 0, 1, 2, 4, 6 and 8 hours, the medium and the cells were harvested separately for analysis of extracellular and intracellular radioactivities as described above.

Quantitative analysis of ponicidin in mouse serum

Female ddY mice (6 weeks of age, 25.6 ± 0.64 g) obtained from Sankyo Labo Service (Shizuoka, Japan) received an intravenous administration of ponicidin (2 mg/kg). The blood samples were taken individually from the carotid artery under ether anesthesia at 0.5, 1, 2, 6, and 10 hours postinjection. The serum concentration of the drug was determined by high performance liquid chromatography (HPLC). The serum sample (0.2 mL) was mixed with 0.5 mL of methanol and centrifuged at 5000 rpm for 3 minutes. The supernatant was evaporated to dryness. The residue was dissolved in 2 mL of chloroform and applied to a Bond Elut (Silica Cartridge, Varian Sample Preparation Products, Calif). After eluting with 3 mL of chloroform, the column was further eluted with 5 mL of 5% methanol in chloroform. The second eluate was then evaporated to dryness, and the residue was dissolved in 0.1 mL of methanol containing methylester of scopadulcic acid B (20 μg/mL) as an internal standard. This solution was subjected to HPLC. A Shimazu LC-6A HPLC system (Kyoto, Japan) equipped with an ultraviolet detector (Shimazu, model SPD-MIA) was used. A Cosmosil 5C18-AR (Waters type) Packed Column (150 × 4.6 mm inner diameter Nacalai tesque, Kyoto, Japan) was used and was maintained at 30°C. The mobile phase consisted of methanol-H_2O (3:1), and its flow rate was 1.0 mL/minute. The eluent was monitored at 230 nm.

Ex vivo antiviral and cytotoxic assays

Female ddY mice (6 weeks of age, 25.9 ± 0.63 g) obtained from Sankyo Labo Service received an intravenous administration of ponicidin (10 mg/kg). The blood samples were taken individually from the carotid artery under ether anesthesia at 0.5, 1, 2, 6, and 10 hours postinjection. For the evaluation of the anti-HSV-1 activities of the serum-GCV combination, an aliquot of the serum was diluted with MEM to a final 50-fold dilution and added to the medium in the combination of GCV immediately after virus infection. Antiviral activities were assessed under *in vitro* conditions by plaque yield reduction assay and were expressed as the IC_{50} values of GCV in the presence of the serum. The final 50-fold diluted serum samples were also subjected to the assays of cytotoxicity against COS-1 cells expressing HSV-1 TK (COS-HSTK-1) in the presence of different concentrations of GCV to determine the IC_{50} values.

Data analysis

Data are presented as the mean ± SD. The significance of differences between the two treated groups was determined with Student's *t* test; $P < .05$ is considered to be significant.

RESULTS

Antiviral and synergistic actions of ponicidin

In plaque yield reduction assays, ponicidin showed a dose-dependent inhibition of HSV-1, with an IC_{50} of 3.2 ± 0.70 μg/mL. At 0.2 μg of ponicidin per mL, which is the concentration tested in the study on the cell-killing effects of ACV and GCV, the compound exerted ≤5% inhibition of HSV-1 replication (data not shown). When the interaction between ponicidin and ACV/GCV was investigated in HSV-1-infected cells, ponicidin was found to significantly decrease the IC_{50}s of both ACV and GCV as evaluated by isobologram (data not shown).[28]

Effects of ponicidin on cellular and viral TK

The phosphorylation of ACV and GCV to their triphosphates, which is essential for their antiviral activity, is considered to be dependent upon the activities of HSV-specific TK and host cell kinases. Thus, the potentiation of ACV/GCV by ponicidin might involve changes in the activities of these kinases. To determine the effects of ponicidin on the kinases, uninfected or HSV-infected Vero cells were treated with ponicidin (Table 1). Ponicidin at 5 μg/mL significantly reduced ($P < .01$) the cellular kinases in uninfected cells. The inhibition of the enzyme activity was well correlated with the cytotoxicity, because at the concentration of 5 μg/mL, ponicidin showed a 25–30% inhibition of Vero cell growth (data not shown). At the lower concentration of 0.2 μg/mL, ponicidin showed no inhibitory effect on cellular enzyme activity.

The level of TK activity in HSV-infected cells was

Table 2. Intracellular Distribution of [^{3}H] After Incubation of HSV-Infected Vero Cells in the Medium Containing [^{3}H]GCV and/or Ponicidin*

Time after infection (hours)	[^{3}H]GCV (pmol/10^9 cell)		[^{3}H]GCV + ponicidin (pmol/10^9 cell)	
	Total [^{3}H]†	[^{3}H]GCV-phosphates‡	Total [^{3}H]	[^{3}H]GCV-phosphates
2	42 ± 2.4	26 ± 3.6	52 ± 2.7§	34 ± 4.6§
4	118 ± 7.8	92 ± 4.6	126 ± 8.5§	102 ± 2.3§
6	202 ± 18	158 ± 14	221 ± 12§	175 ± 4.9§
8	303 ± 62	220 ± 31	376 ± 71§	271 ± 51
10	403 ± 21	282 ± 19	459 ± 48§	346 ± 33§
12	443 ± 12	310 ± 12	598 ± 22§	456 ± 25§

*Each value is the mean ± SD from triplicate assays. The specific radioactivity of [^{3}H]GCV is 0.172 pmol/1000 cpm.
†[^{3}H]-containing metabolites, presumably a mixture of GCV, GCV-MP, GCV-DP, and GCV-TP.
‡[^{3}H]GCV-phosphates formed in infected cells exposed to 10 μM [^{3}H]GCV (5 μCi/dish).
§$P < .05$ as compared with the corresponding values from the treatment with GCV alone.

measured at 16 hours postinfection using either thymidine or ACV as the substrate. When infected cells were treated with ponicidin at 5 or 0.2 μg/mL, the enzyme activity detected using ACV as a substrate was increased by 33% and 23%, respectively, whereas no significant change was seen in the activity measured by the phosphorylation of thymidine. These data indicated that HSV-specific TK rather than cellular kinases was selectively activated by ponicidin. Because the compound was inhibitory for the cellular functions at 5 μg/mL but still exerted a stimulating effect on HSV-TK activity without cytotoxicity at 0.2 μg/mL, ponicidin was used at the concentration of 0.2 μg/mL in the following studies.

Effect of ponicidin on the phosphorylation of [^{3}H]GCV in HSV-infected cells

We determined whether the stimulation of HSV-TK activity by ponicidin might be reflected in the level of intracellular GCV-phosphates. As shown in Table 2, intracellular radiolabeled compounds were quantified after 2- to 12-hour incubations of HSV-infected cells with [^{3}H]GCV in the absence or presence of ponicidin. Treating the cells with the combination of ponicidin and GCV produced a significant increase in the concentration of ^{3}H-containing metabolites (presumably a mixture of GCV, GCV-MP, GCV-DP and GCV-TP) throughout the incubation period when compared with the concentration in the cells treated with GCV alone. Importantly, the amount of [^{3}H]GCV-phosphates found in the cells treated with ponicidin and GCV was significantly higher than that found in GCV-treated cells at 4 hours postinfection. Thus, ponicidin proved to be effective in maintaining the active metabolite of GCV to a high level.

Effect of ponicidin on intra- and extracellular GCV levels

As mentioned above, ponicidin was believed to increase the GCV-phosphate level by stimulating HSV-TK activity. However, there is another factor that might contribute to a high intracellular GCV-phosphate level: suppression of the extracellular release of GCV from the cells by the dephosphorylation of GCV-phosphates. Thus, the effect of ponicidin was studied with regard to its influence on the metabolic fate of GCV-phosphates once they have formed in HSV-infected cells. After virus-infected cells were exposed to 10 μM [^{3}H]GCV for 8 hours in the absence or presence of ponicidin and subsequently incubated with the medium containing the same concentrations of ponicidin and unlabeled GCV, the changes in both intracellular [^{3}H]GCV-phosphate and extracellular [^{3}H]GCV concentrations were investigated for an additional 8 hours. In the cells treated with GCV alone, a rapid decrease in the intracellular GCV-phosphate level during the first 2 hours followed by a slower drop was observed (Table 3). A plateau was reached by 6 hours in the presence of 10 μM of GCV in the medium. Corresponding to the decrease in intracellular [^{3}H] radioactivity, the level of [^{3}H] detected in the medium was gradually increased over 8 hours. Because GCV-phosphates should not be able to diffuse through the cell membrane before dephosphorylation, the extracellular radioactivity seemed to be due to the leakage of dephosphorylated [^{3}H]GCV and due in part to the catabolism of [^{3}H]GCV. When ponicidin was used with GCV, a gradual degradation of GCV-phosphates was observed in HSV-infected cells during an 8-hour incubation. A high level of intracellular GCV-phosphates was maintained throughout the incubation, and the concentration of [^{3}H] left the cells persisted at a significantly low level when compared with the cell cultures treated with GCV alone.

*Cytotoxic activity of compounds against HSV-*TK *gene-transfected COS-1 cells*

The data reported above indicated that concomitant use of ponicidin resulted in a high level of toxic metabolites of the nucleoside analogs in HSV-infected cells. Thus, it was expected that ponicidin could potentiate the susceptibility of the cells expressing only HSV-1 TK to the toxic action of ACV and GCV.

At first, ACV and GCV were evaluated for their inhibitory effects on the growth of COS-1 and HSV-TK-expressing COS-HSTK cells in the absence or presence

Table 3. Disappearance of Phosphorylated [^{3}H]GCV from HSV-Infected Vero Cells After Exposure to Fresh Medium Containing the Same Concentrations of Unlabeled GCV and/or Ponicidin*

Time after exposure (hours)	[^{3}H]GCV (pmol/10^9 cells)			[^{3}H]GCV + ponicidin (pmol/10^9 cells)		
	Intracellular		Extracellular	Intracellular		Extracellular
	Total [^{3}H]†	[^{3}H]GCV-phosphates‡	[^{3}H]	Total [^{3}H]	[^{3}H]GCV-phosphates	[^{3}H]
0	317 ± 27	239 ± 9.0	0	343 ± 25§	265 ± 22§	0
1	273 ± 32	194 ± 35	16 ± 3.1	321 ± 3.5§	243 ± 9.5§	10 ± 3.1§
2	249 ± 45	177 ± 22	41 ± 13	289 ± 26	223 ± 16§	24 ± 4.9§
4	249 ± 51	192 ± 53	50 ± 8.0	282 ± 41§	214 ± 19	27 ± 4.9§
6	199 ± 38	147 ± 26	87 ± 9.2	250 ± 30§	183 ± 16§	67 ± 12§
8	195 ± 6.1	146 ± 15	118 ± 13	271 ± 24§	204 ± 9.0§	94 ± 6.5§

*Each value is the mean ± SD from triplicate assays.
†[^{3}H]-containing metabolites, presumably a mixture of GCV, GCV-MP, GCV-DP, and GCV-TP.
‡[^{3}H]GCV-phosphates formed in infected cells exposed to 10 μM [^{3}H]GCV (5 μCi/dish).
§$P < .05$ as compared with the corresponding values from the treatment with GCV alone.

of ponicidin. COS-1 cells were not markedly inhibited by ACV (IC_{50}, 923–935 μM) and GCV (IC_{50}, 767–818 μM) (Table 4). In contrast, the proliferation of COS-HSTK cells was inhibited by ACV and GCV, with IC_{50} values of 133 and 1.6 μM, that is at concentrations that are 7- and 580-fold, respectively, lower than those found to be inhibitory to the corresponding wild-type COS-1 cells (Table 4). GCV was ~80-fold more potent an inhibitor of COS-HSTK cell growth than ACV.

Ponicidin at a concentration of ≤0.2 μg/mL showed no inhibitory effect on the growth of COS-1 cells when administered alone (data not shown). The compound showed no potentiation of cytotoxicity of both ACV and GCV against COS-1 cells (Table 4). However, the combination of ACV with ponicidin was inhibitory to COS-HSTK cells, with an IC_{50} of 4.2 μM. This concentration was 32-fold lower than the concentration of ACV alone that was required to inhibit COS-HSTK cell growth (Table 4). Also, the combination of GCV plus ponicidin lowered the concentration of GCV required for a 50% inhibition of the growth of COS-HSTK cells by 18-fold (Table 4). The cytotoxicity produced by the combination of GCV with ponicidin was ~50-fold more potent than that produced by ACV plus ponicidin as assessed by the IC_{50} values. Thus, ponicidin proved to potentiate the cytotoxic activities of ACV and GCV against HSV-1 gene-transfected cells but not wild-type cells.

*Effect of compounds on the proliferation of HSV-*TK *gene-transfected human cancer cells*

The combinatory effects of ponicidin and ACV/GCV were further evaluated for their cytotoxicities on various human cancer cell lines, including HEp-2, EJ-1, HeLa 229, and YKG-1 cells. Wild-type cancer cells, which were not transfected with the HSV-1 *TK* gene, were not markedly inhibited by ACV and GCV, with IC_{50}s of 1917–3147 μM and 423–760 μM, respectively (Table 5). In the cells expressing HSV-TK, however, the cytotoxic effects of ACV and GCV were intensified by 10- to 26-fold and 9- to 311-fold, respectively, compared with the IC_{50} values of the corresponding wild-type cells. GCV exhibited much stronger toxicity on HSV-TK-expressing cancer cells than ACV as observed in COS-1 cells.

Ponicidin (0.2 μg/mL) alone exerted no toxicity on these cells (data not shown). The compound also showed no augmentation of the toxic action of ACV and GCV on wild-type cancer cells (Table 5). In contrast, strong cytotoxicity was observed in HSV-TK-expressing cells treated with the combination of ponicidin and ACV, with the IC_{50}s being 1.9–68 μM. These levels were 3- to 87-fold lower than those required by ACV alone to inhibit each HSV-TK-expressing cell line. Similar results were obtained in the cell cultures treated with the combination of ponicidin and GCV, where the IC_{50}s

Table 4. Cell Growth Inhibition of ACV and GCV Against COS-1 Cells Expressing HSV-1 TK in the Absence or Presence of 0.2 μg/mL Ponicidin

Cell	IC_{50} (μM)*			
	ACV	ACV + ponicidin	GCV	GCV + ponicidin
COS-1	935 ± 23†	908 ± 56	767 ± 48	763 ± 45
COS-HSTK	133 ± 24	4.2 ± 0.82	1.6 ± 0.17	0.09 ± 0.020

*IC_{50}, 50% inhibitory concentration of ACV or GCV for cell growth inhibition.
†Each value is the mean ± SD of triplicate assays.

Table 5. Cell Growth Inhibition of ACV and GCV Against Human Cancer Cells Expressing HSV-1 TK in the Absence or Presence of 0.2 μg/mL Ponicidin

Cell line	Cytotoxicity (IC_{50}; μM)*			
	ACV	ACV + ponicidin	GCV	GCV + ponicidin
HEp-2	1947 ± 328†	2233 ± 25	760 ± 62	627 ± 72
HEp-2-HSTK	119 ± 11	2.9 ± 0.70	6.5 ± 2.1	0.20 ± 0.01
EJ-1	1917 ± 150	2580 ± 62	423 ± 63	442 ± 46
EJ-1-HSTK	185 ± 38	68 ± 4.9	49 ± 6.3	9.9 ± 2.3
HeLa 229	3147 ± 434	3200 ± 356	490 ± 26	660 ± 70
HeLa 229-HSTK	166 ± 9.1	1.9 ± 0.46	3.4 ± 0.4	0.066 ± 0.0067
YKG-1	2193 ± 156	2010 ± 168	497 ± 85	627 ± 15
YKG-1-HSTK	83 ± 5.1	2.8 ± 0.65	1.6 ± 0.42	0.067 ± 0.0095

*IC_{50} of ACV or GCV for cell growth inhibition.
†Each value is the mean ± SD of triplicate assays.

were reduced by 5- to 52-fold compared with those of GCV alone. Again, the susceptibility of different human cancer cell lines to HSV-TK/ACV- and HSV-TK/GCV-mediated cytotoxic effects was potentiated by the concomitant administration of ponicidin. All cell lines were sensitive to the analogs, but the sensitivity varied among the cell lines to some extent; their toxic concentrations in EJ-1 cells were higher than those in HEp-2, HeLa, and YKG-1 cells.

Evaluation of ex vivo biological activities

For the HPLC analysis of ponicidin in mouse serum, the retention times of ponicidin and some components present in the serum sample were superimposed. This made it impossible to directly measure the quantity of ponicidin in the serum. Instead, in this study, the potentiation of the antiviral and cytotoxic activities of GCV was examined by the sera from mice treated intravenously with ponicidin (Table 6). The serum samples were obtained from each mouse at 30 minutes to 10 hours thereafter. When the changes in the anti-HSV-1 activity of GCV were determined by treating the infected cells with the combination of GCV and 50-fold diluted serum samples, the potentiation of the antiherpetic effect of GCV could be detected in the blood even at 6 hours after ponicidin administration. The cell-killing effect of GCV on the HSV-TK-expressing cells was also stimulated by the serum samples obtained at 0.5 to 6 hours postadministration. Both of these activities were undetectable by 10 hours postinjection. No mouse treated with doses of 10–20 mg of ponicidin per kg showed evidence of toxicity such as a change in activity or appetite.

Table 6. Antiviral and Cytotoxic Activities in Sera Obtained from Mice Treated Intravenously with Ponicidin*

Time after administration (hours)	Anti-HSV-1 activity† (IC_{50} of GCV; μM)	Cytotoxicity‡ against COS-HSTK-1 cells (IC_{50} of GCV; μM)
0.5	0.038 ± 0.014	0.081 ± 0.013
1	0.041 ± 0.011	0.11 ± 0.066
2	0.075 ± 0.057	0.27 ± 0.074
6	0.21 ± 0.18	0.52 ± 0.35
10	0.39 ± 0.087	1.7 ± 0.15
Untreated control	0.46 ± 0.10	1.7 ± 0.62

*Each value is the mean ± SD from three mice.
†Antiviral activity was evaluated by adding 50-fold diluted serum samples to the medium in combinations of different concentrations of GCV immediately after infection and calculating the IC_{50} of GCV.
‡Cytotoxicity was expressed as the IC_{50} of GCV for growth inhibition of COS-HSTK-1 cells in the presence of 50-fold diluted serum samples.

DISCUSSION

In the present study, ponicidin showed synergistic antiviral interaction with both ACV and GCV. The mechanism by which ponicidin augments the action of these nucleoside analogs could be accounted for by the selective stimulation of HSV-specific TK activity and the suppression of extracellular release of degradated metabolite of the nucleoside analogs. These actions should result in high intracellular levels of phosphorylated metabolites as observed in HSV-infected cells treated with ponicidin.

In cancer gene therapy, the potent cytotoxicity of GCV-TP has been ingeniously used to kill cancer cells. The increase in the amount of GCV-TP by ponicidin might mean that the diterpenoid could be a candidate for the potentiator of the efficacy of GCV in gene therapy. Therefore, to evaluate the possible value of ponicidin in gene therapy with the HSV-TK/GCV system, we first determined the effects of ponicidin on the phosphorylation activity of HSV-1 TK for ACV and GCV by measuring the level of susceptibility of COS-1 cells transfected with the corresponding gene to these drugs in the absence or presence of ponicidin. At the concentration of 0.2 μg/mL tested, ponicidin *per se* exerted no marked toxicity against cell proliferation, whereas the compound increased the sensitivity of HSV-*TK*-transfected cells to both ACV and GCV, which might reflect the significant increase of their toxic metabolites. In these cytotoxicity assays, GCV was much

more potent an inhibitor of HSV-*TK* gene-transfected cell growth than ACV as measured by IC_{50}. There are several reports in which HSV-infected cells phosphorylated GCV more favorably than ACV when evaluated by kinase activities.[6,8,10]

A total of 200 ng of ponicidin per mL, for example, enhanced the cytotoxicity of GCV against HSV-*TK* gene-transfected COS-1 cells by at least 20-fold. This means that less than one-twentieth the therapeutic dose of GCV in combination with ponicidin may produce the same efficacy as its therapeutic dose in cancer gene therapy. A low level of expression of therapeutic genes sometimes limits the effectiveness of gene therapy trials.[28] However, even if exogenous HSV-*TK* genes are expressed only at lower levels in the target cells, the combined administration of GCV and ponicidin would likely enhance the cell-killing effect of GCV well enough to produce therapeutic efficacy because of the increased level of intracellular GCV-TP. ACV is a less toxic drug than GCV, and is normally not effective against HSV-*TK* gene-transduced cells because of a high IC_{50}. In combined use with ponicidin, however, ACV administration resulted in efficacy that was equivalent to GCV administration. These results suggest that ACV might be effective in the therapeutic range.

Because phosphorylated GCV plays a key role in the mechanism of its biological action, its persistence at high levels as observed in HSV-infected cells treated with ponicidin should be closely related to the differences in the cytotoxicities in HSV-TK-expressing cells treated with GCV alone and GCV plus ponicidin.

Evaluation of the suicide effect by animal experiments may be important to predict whether the applied approach has the potential to eliminate tumor cells in clinical patients. In the present study, the inhibitory effect of ponicidin-administered blood of mice on HSV-*TK*-transfected cells was maintained for a long time, with a slow decline of bioactivity by 10 hours postadministration. Thus, the therapeutic levels of ponicidin might be expected to be achievable *in vivo*.

Finally, the combined use of ponicidin in the HSV-TK/GCV system was shown to provide high cytotoxicity in viral *TK*-expressing cells. by virtue of an increased level of GCV phosphates.

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