

# Contribution of a Combination of Ponocidin and Acyclovir/Ganciclovir to the Antitumor Efficacy of the Herpes Simplex Virus Thymidine Kinase Gene Therapy System

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## ABSTRACT

We have previously reported that ponocidin (PND), isolated from *Rabdosia ternifolia*, potentiates the cell-killing activity of antiherpes prodrugs acyclovir (ACV) and ganciclovir (GCV) in human cancer cells expressing herpes simplex virus thymidine kinase (HSV-TK). To extend these *in vitro* results to *in vivo* situations, HSV-TK-expressing HeLa cells were injected into nude mice. The *in vivo* growth of TK<sup>+</sup> HeLa cells was significantly inhibited by coadministration of PND and ACV, or of PND and GCV, compared with single use of ACV or GCV in spite of lower doses of 1 or 0.25 mg/mouse, respectively. These results indicate that there is a good correlation between this *in vivo* efficacy and previously reported *in vitro* efficacy. Because of the insufficiency of incorporation of genes into tumors, bystander cell killing has attracted special interest. In the present study, we determined the ability of PND to potentiate the bystander effects of ACV and GCV in both *in vitro* and *in vivo* systems. *In vitro* combined use of PND with ACV or GCV rendered tumor cells more sensitive to the prodrugs, demonstrating a 1.8- to 97-fold or 2.8- to 26-fold reduction in IC<sub>50</sub> compared with ACV or GCV only, respectively, in 1 to 20% of HSV-TK<sup>+</sup> cells. In the *in vivo* experiments using nude mice injected with 3 or 10% HSV-TK<sup>+</sup> cells, tumor volume was lower in mice treated with a combination of PND and ACV/GCV than in those treated with ACV or GCV only. No toxicity of PND was seen in mice even at a dose 10-fold higher than that used in the *in vivo* experiments. These novel strategies could provide benefit to ablative cancer gene therapy by making it feasible to use toxic GCV at lower doses and relatively nontoxic ACV.

## OVERVIEW SUMMARY

With the advent of gene therapy, the herpes simplex virus-specific thymidine kinase (HSV-TK) gene has attracted much interest as a suicide gene for cancer ablation. Many means to improve the overall efficacy of prodrug (mainly ganciclovir [GCV] and acyclovir [ACV]) activation induced by the *tk* gene, and to reduce GCV toxicity, have been reported. Consistent with previous *in vitro* results, we report that a combined use of ponocidin (PND) and the prodrugs demonstrates a significant *in vivo* tumor growth inhibition. The bystander effect, that is, the killing of nonexpressing neighboring cells by HSV-TK-expressing cells, produces an important therapeutic effect in tumors with a low efficiency of gene transfer. Concomitant use of PND also results in the

enhancement of the bystander effect by ACV and GCV. These findings have considerable value with respect to the use of GCV at lower doses and less toxic ACV.

## INTRODUCTION

WE HAVE REPORTED that ponocidin (PND), a naturally occurring diterpenoid isolated from a plant (*Rabdosia ternifolia*), potentiates the cytotoxicity of antiherpes prodrugs acyclovir (ACV) and ganciclovir (GCV) in human cancer cells expressing herpes simplex virus-specific thymidine kinase (HSV-TK) (Hayashi *et al.*, 2000). HSV-TK is a crucial enzyme that converts ACV and GCV to their monophosphates (Elion, 1982; Cheng *et al.*, 1983). PND activates this enzyme but not

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cellular kinases. This selective action of the compound was suggested to contribute to the enhanced toxicities of ACV and GCV in HSV-TK-positive cells. In the present study, we determined whether PND could lead to the promoted elimination of HSV-TK<sup>+</sup> tumor tissues by ACV or GCV in *in vivo* situations. PND was shown to improve significantly the antitumor effects of relatively lower doses of both ACV and GCV.

In the HSV-TK/prodrug administration system, an interesting aspect from a therapeutic point of view is that the prodrug treatment eliminates not only the tumor cells or actively dividing cells that incorporate and express the HSV-*tk* gene (TK<sup>+</sup> cells), but also neighboring TK<sup>-</sup> tumor cells. Such propagation of toxicity from TK<sup>+</sup> cells to TK<sup>-</sup> cells is known as the "bystander effect" (Freeman *et al.*, 1993). Because current methodologies for incorporating genes into the target *in vivo* usually permit only a small portion of tumor cells to express HSV-TK, the bystander effect for tumor cell killing is critical for clinical success and the elimination of tumors (Roth and Cristiano, 1997). Thus, enhancement of the bystander effect may contribute to improvement of the clinical application of the HSV-TK/GCV or HSV-TK/ACV administration system. On the basis of our previous studies, we expected that the combination of PND and ACV/GCV would enhance bystander killing. In this study, we evaluated the ability of PND to potentiate bystander effects in both *in vitro* and *in vivo* systems. Even when only 1% of the cells were HSV-TK positive, elevated *in vitro* toxicities were observed with the combination of PND and ACV/GCV. These results were also proved by antitumor assay in an animal model.

## MATERIALS AND METHODS

### Chemicals

PND was isolated from *R. ternifolia* as previously reported (Takeda *et al.*, 1990). ACV was obtained from Japan Wellcome (Osaka, Japan). GCV was purchased from Syntex (Palo Alto, CA).

### Cell type

Human cervical carcinoma (HeLa 229) cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS).

### Plasmid

The plasmid pSV2-HSTK-1 contains the *Bam*HI Q fragment of HSV-1 inserted into the expression vector pSV2-neo (Southern and Berg, 1982) as reported previously (Hayashi *et al.*, 2000).

### Transfection

HeLa cells ( $1-2 \times 10^5$ ) were placed in 35-mm dishes and cultured for 24 hr at 37°C. Plasmid DNA was transfected with LipofectAMINE (Life Technologies, Gaithersburg, MD) according to the manufacturer direction. At 72 hr after transfection,

the medium was changed to selective medium containing geneticin (400  $\mu$ g/ml; Life Technologies). The transfected cells were cloned three times under selection in geneticin (800  $\mu$ g/ml) to obtain stably HSV-TK-expressing cell lines. HSV-TK enzymatic activities of the transfected cell clones were measured with [<sup>3</sup>H]ACV (Moravek Biochemicals, Brea, CA) as a substrate as reported previously (Hayashi *et al.*, 2000).

### *In vitro* bystander effects

Parental HeLa cells were admixed with HSV-TK-expressing HeLa cells at various percentages (0, 1, 5, 10, 20, and 100%). The mixtures were dispensed in triplicate at a total of  $2 \times 10^4$  cells/well into 48-well plates and grown overnight. The cells were then exposed to ACV- or GCV-containing medium at the concentrations of 0, 2, 20, 200 and 1000  $\mu$ M, or at 0, 0.01, 0.1, 1, 10, and 100  $\mu$ M, respectively, in the absence or presence of PND at 0.5  $\mu$ g/ml. The medium was replenished every 3 days. On the seventh day of exposure, cells were trypsinized and counted to determine the 50% inhibitory concentration (IC<sub>50</sub>) values.

In studies to determine the requirement for cell-cell contact to achieve the bystander effect, Transwell chambers (Costar, Cambridge, MA) with the upper and lower compartments separated by a polycarbonate membrane filter (pore size, 3  $\mu$ m) were used. HeLa-TK<sup>+</sup> cells or no cells were placed in the upper compartments. At the same time, either HeLa-TK<sup>+</sup> or HeLa-TK<sup>-</sup> cells were placed in the lower compartments. After 24 hr, drugs were added to the upper compartments, and the viable cells in the lower compartments were counted after 7 days of incubation.

### *In vivo* experiments in nude mice

Five-week-old female BALB/c athymic mice were purchased from Charles River Japan (Yokohama, Japan). The mice were maintained in accordance with institutional guidelines of the Toyama Medical and Pharmaceutical University Animal Care and Use Committee. Mice were injected subcutaneously with  $5 \times 10^6$  cells that had been resuspended in 200  $\mu$ l of phosphate-buffered saline (PBS). The following HSV-TK<sup>+</sup>/HSV-TK<sup>-</sup> ratios of HeLa cells were injected into the backs of the mice: 100, 10, 3, and 0%. After 8 days, when the tumors had reached a volume of more than 100 mm<sup>3</sup>, 10 to 12 tumors per group were treated intraperitoneally twice a day for 7 days either with ACV (1 mg/mouse per day), or with GCV (0.25 mg/mouse per day), or with PND (0.1 mg/mouse per day), or with ACV in combination with PND, or with GCV in combination with PND. Control group was treated with PBS. Tumor size was measured once weekly up to 35 days from the start of drug administration. Tumor volume was calculated according to the formula  $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$ , as previously reported (Fan *et al.* 1993).

### Data analysis

Data are presented as means  $\pm$  standard deviation (SD). The significance of differences between two treated groups was determined by Student *t* test, and  $p < 0.05$  was considered significant.

## RESULTS

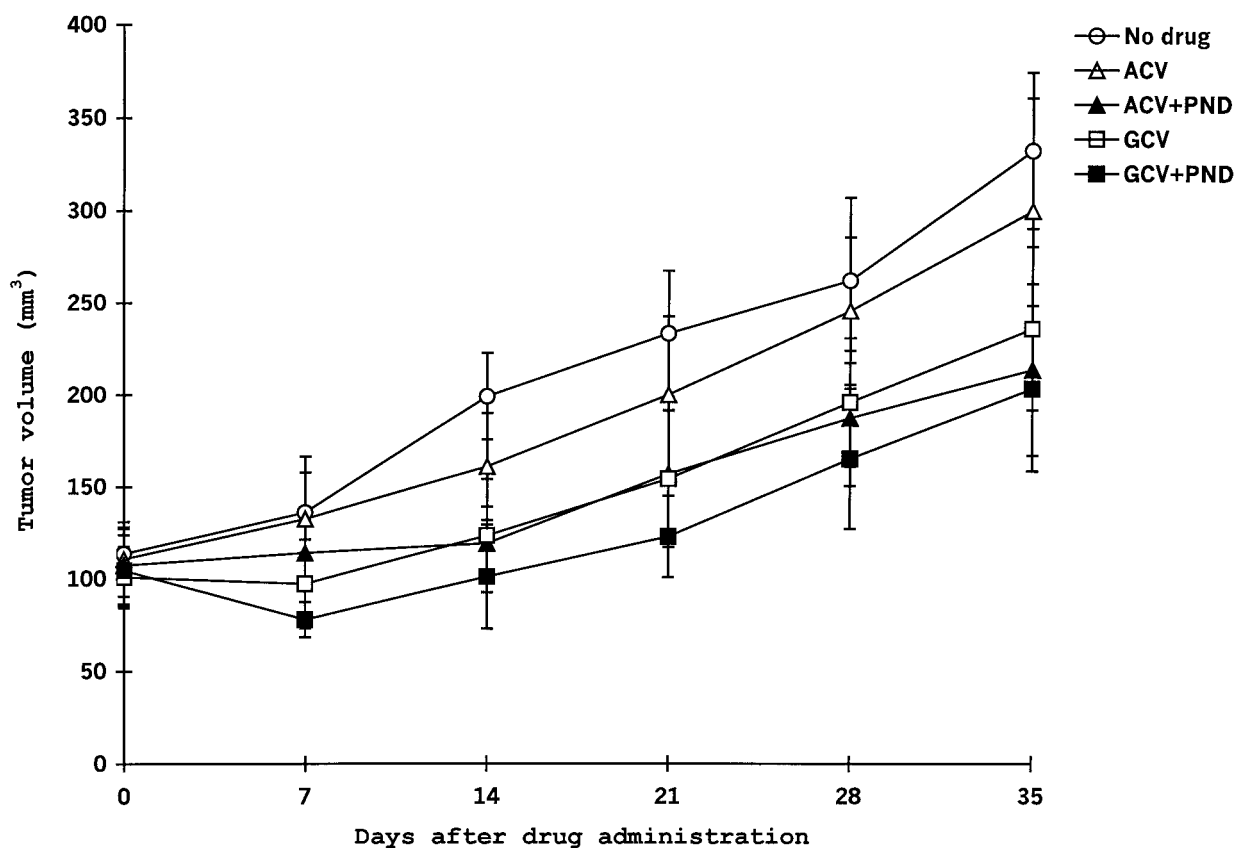
*In vivo effect of PND on antitumor activity of ACV and GCV*

A human tumor cell line, the HeLa cell line, was transfected with pSV2-HSTK-1 and selected with geneticin as described previously (Hayashi *et al.*, 2000). After cloning three times, several clones were found to express HSV-TK stably by testing for TK enzymatic activity. One of these clones was used as HSV-TK<sup>+</sup> HeLa cells in further experiments to evaluate whether *in vitro* potentiation of ACV and GCV cytotoxicities by PND (Hayashi *et al.*, 2000) would correlate with *in vivo* effects.

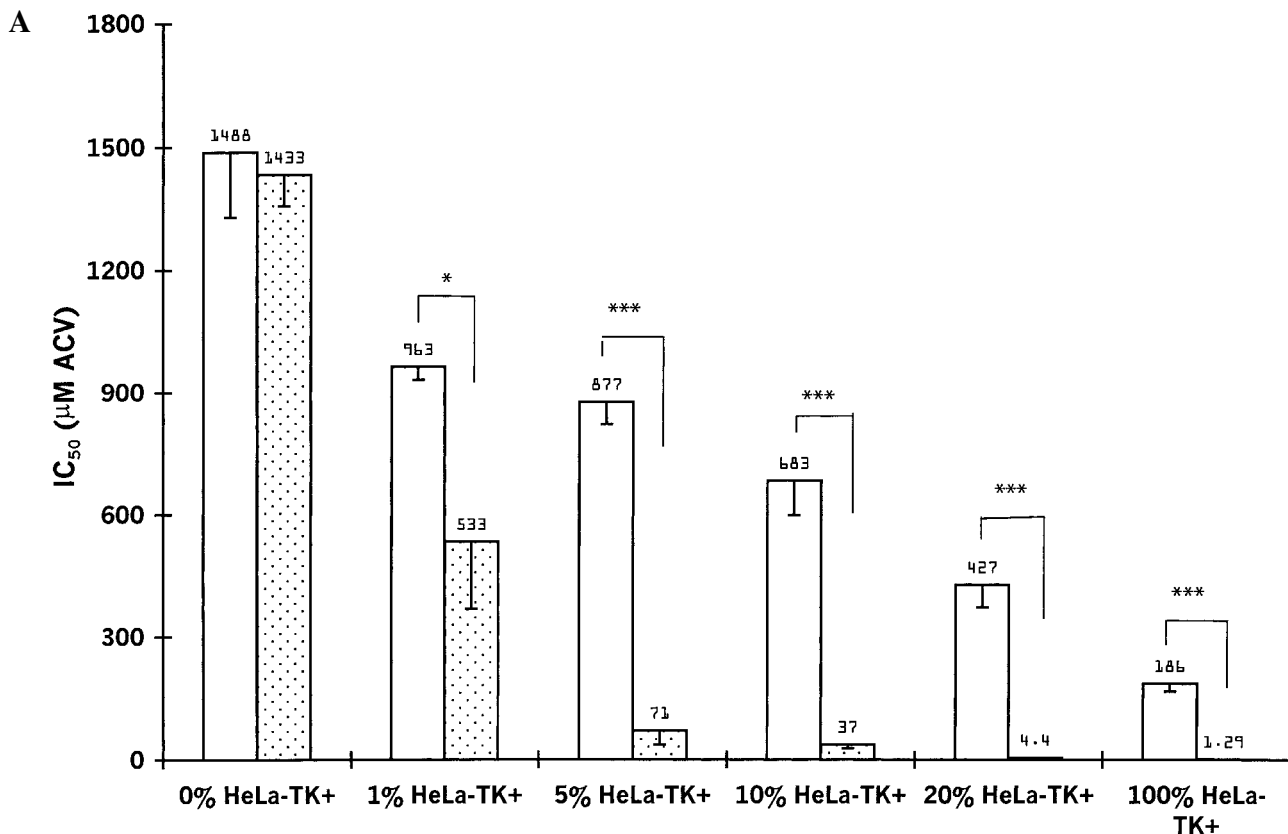
HSV-TK<sup>+</sup> cells were injected subcutaneously and drugs were administered for 7 days, beginning 8 days after cell inoculation. Tumor was measured 7, 14, 21, 28, and 35 days after the start of drug treatment. In preliminary experiments, different doses of ACV, GCV, and PND were studied for the growth of tumors induced by HSV-TK<sup>+</sup> HeLa cells in nude mice (data not shown). On the basis of these data, we selected those doses of the prodrugs that are insufficient for the complete eradication of HSV-TK<sup>+</sup> tumors and permit the constant growth of tumors over at least 5 weeks. As a result, the selected dose of

ACV was four times higher than that of GCV because ACV has been reported to be used at 4.5 times higher doses than GCV without significant side effects (Tong *et al.*, 1998). As shown in Fig. 1, ACV treatment at a dose of 1 mg/mouse showed statistically significant differences in tumor growth in comparison with the no-drug control treatment at days 14 ( $p < 0.01$ ) and 21 ( $p < 0.05$ ). PND at a dose of 0.1 mg/mouse exerted an effect on tumor growth similar to that of the no-drug control over the observation period (data not shown). A combination of PND and ACV produced significant reduction in tumor growth throughout the entire period of observation as compared with the no-drug control. Importantly, PND potentiated significantly the antitumor effects of ACV at days 14 ( $p < 0.001$ ), 21 ( $p < 0.05$ ), 28 ( $p < 0.01$ ), and 35 ( $p < 0.01$ ) in comparison with the group treated with ACV alone.

In the GCV-treated group, the growth of tumors was significantly slowed on days 7 ( $p < 0.001$ ), 14 ( $p < 0.001$ ), 21 ( $p < 0.001$ ), 28 ( $p < 0.001$ ), and 35 ( $p < 0.001$ ) by GCV at a dose of 0.25 mg/day. The combination of PND with GCV showed the most marked decrease in tumor volume over the observation period of 35 days. There were significant differences between a combination of PND plus GCV and GCV alone at days 7 ( $p < 0.001$ ) and 21 ( $p < 0.05$ ).



**FIG. 1.** *In vivo* effects of ACV and GCV on tumors induced by HSV-TK-expressing HeLa cells in the absence or presence of PND. HSV-*tk*-transfected cells were injected into nude mice. Eight days after the cell injection, the mice were treated intraperitoneally with ACV (1 mg/mouse) alone, GCV (0.25 mg/mouse) alone, a combination of ACV and PND (0.1 mg/mouse), or a combination of GCV and PND, twice a day for 7 days. The size of tumors (mm<sup>3</sup>) was estimated every 7 days for 5 weeks. Symbols and error bars represent means  $\pm$  SD of 12 determinations.



**FIG. 2.** *In vitro* bystander effects of PND and ACV/GCV on the sensitivity of HeLa cell cultures. HSV-TK<sup>+</sup> and parental cells were cocultured at a ratio of 100:0, 20:80, 10:90, 5:95, 1:99, or 0:100 in the presence of PND (0 µg/ml [open columns] or 0.5 µg/ml [dotted columns]) and ACV (0, 2, 20, 200, and 1000 µM) (A) or GCV (0, 0.01, 0.1, 1, 10, and 100 µM) (B). After 7 days of incubation, surviving cells were counted, and the IC<sub>50</sub> values for ACV and GCV were calculated. Columns and bars represent means ± SD of three determinations. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

#### *In vitro* potentiation of bystander effects of ACV and GCV by PND

We have already demonstrated that PND enhanced *in vitro* ACV and GCV cytotoxicities in cultures consisting solely of HSV-TK-expressing cells (100% HSV-TK-expressing cells) (Hayashi *et al.*, 2000). In this study, the ability of PND to potentiate the cytotoxicities of these agents was investigated in cocultures of TK-expressing and -nonexpressing (bystander) cells. PND at a concentration of 0.5 µg/ml was not toxic to either parental or HSV-TK-expressing cells (data not shown). The bystander effect was evaluated in cell mixtures containing 1, 5, 10, and 20% HSV-TK-expressing cells. In the absence of PND, the IC<sub>50</sub> of ACV ranged from 963 to 427 µM in cocultures with 1 to 20% HSV-TK<sup>+</sup> cells (Fig. 2A). The simultaneous addition of PND (0.5 µg/ml) to these ACV-treated cocultures significantly decreased the IC<sub>50</sub> of ACV from 963 to 533 µM (1.8-fold), from 877 to 71 µM (12-fold), from 683 to 37 µM (18-fold), or from 427 to 4.4 µM (97-fold) in cocultures containing 1, 5, 10, or 20% HeLa-TK<sup>+</sup> cells, respectively.

GCV showed much more potent cytotoxicity than ACV in cocultures with 1 to 20% HSV-TK<sup>+</sup> cells, its IC<sub>50</sub> ranging from 54 to 1.3 µM (Fig. 2B). A combination of GCV and PND (0.5

µg/ml) decreased the IC<sub>50</sub> of GCV depending on the ratio of HSV-TK<sup>+</sup> cells, that is, from 54 to 19 µM (2.8-fold), from 20 to 1.7 µM (12-fold), from 10 to 0.38 µM (26-fold), or from 1.3 to 0.078 µM (17-fold) in cocultures containing 1, 5, 10, or 20% HSV-TK<sup>+</sup> cells, respectively.

To determine whether the increase in bystander effect in the presence of PND requires direct cell-cell contact, TK-expressing and TK-negative HeLa cells were grown separately in Transwell chambers. When HeLa-TK<sup>+</sup> cells in upper compartments were treated with ACV or GCV, the growth of HeLa-TK<sup>+</sup> cells in lower compartments was inhibited to an extent similar to that of the culture systems in which no cells were present in upper compartments (Table 1). This means that ACV and GCV could pass freely through the membrane filters that separate the two cell monolayers. Under these conditions, PND added in upper compartments increased the cytotoxicity of ACV and GCV in lower compartments. On the other hand, even the combination use of PND and ACV/GCV in HeLa-TK<sup>+</sup> cell-grown upper compartments showed no cytotoxicity against HeLa-TK<sup>-</sup> cells in lower compartments. These results indicated that the culture supernatant itself had no suppressive effect on TK-negative cells and that cell-cell contact was required for the bystander effect by ACV/GCV and PND.

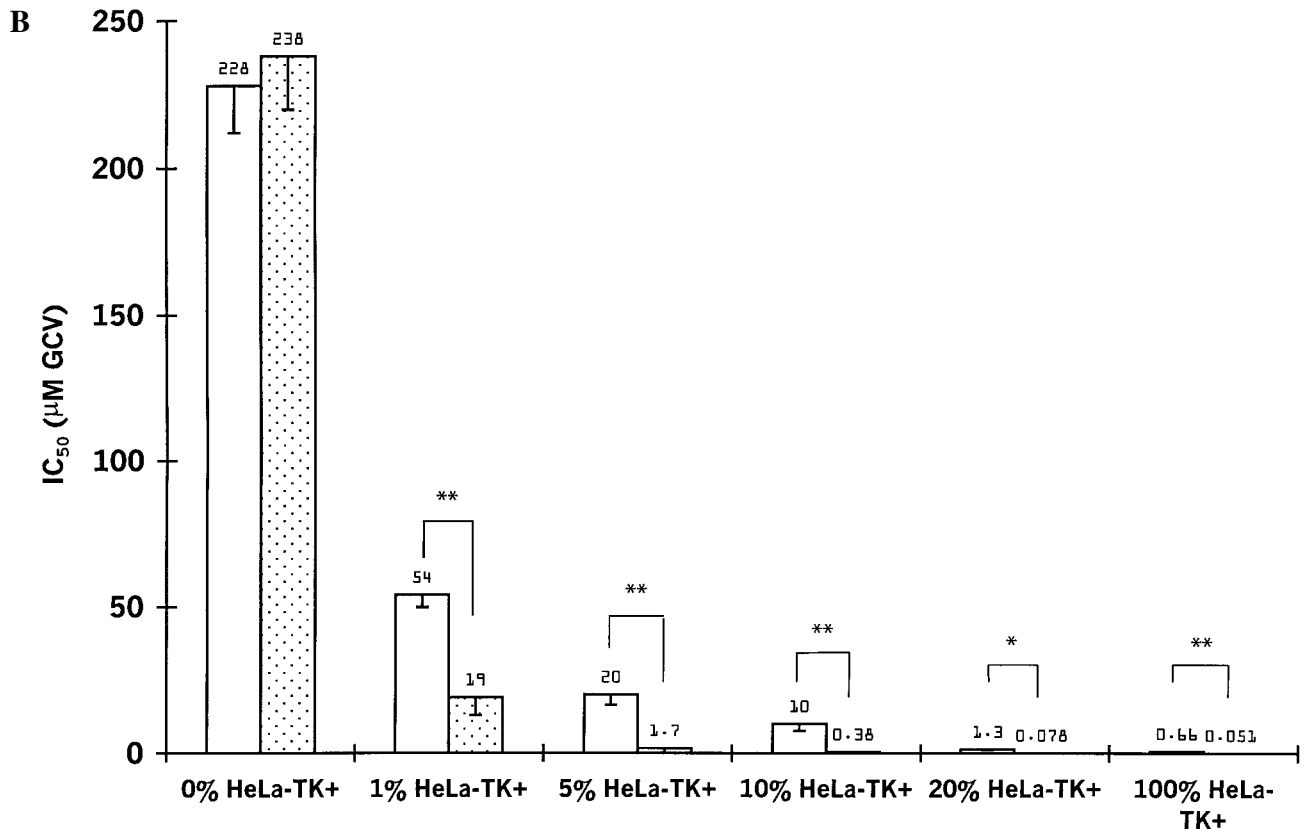


FIG. 2. Continued.

#### *In vivo* potentiation of bystander effects of ACV and GCV by PND

To extend the *in vitro* results described above to *in vivo* situations, cell mixtures consisting of 0, 3, or 10% HSV-TK<sup>+</sup> HeLa cells with parental HeLa cells were prepared. These mixtures were injected into nude mice, and ACV or GCV was administered alone or in combination with PND 8 days later for seven successive days (Fig. 3). Single administration of PND (0.1 mg/mouse) showed no significant effect in the groups injected with 0, 3, and 10% HSV-TK<sup>+</sup> cells (data not shown). When 10% HSV-TK<sup>+</sup> cells were injected, a single administration of ACV (1 mg/mouse) produced no statistically significant

reduction in tumor growth in comparison with the no-drug control over the 35 days of observation (Fig. 3A). Combined use of ACV and PND resulted in significant suppression of tumor growth as compared with control on days 14 ( $p < 0.05$ ), 21 ( $p < 0.05$ ), 28 ( $p < 0.05$ ), and 35 ( $p < 0.01$ ). Under these experimental conditions, PND enhanced significantly the inhibitory effects of ACV on day 35. Single treatment with GCV (0.25 mg/mouse) also showed significant differences in tumor growth on days 21 ( $p < 0.05$ ) and 35 ( $p < 0.01$ ), as compared with control. PND potentiated significantly these antitumor effects of GCV on days 7 ( $p < 0.01$ ), 14 ( $p < 0.001$ ), 21 ( $p < 0.01$ ), and 28 ( $p < 0.01$ ).

When the percentage of HSV-TK<sup>+</sup> cell population was de-

TABLE 1. EFFECT OF DRUG TREATMENT OF CELLS IN UPPER COMPARTMENTS ON CELL GROWTH IN LOWER COMPARTMENTS<sup>a</sup>

| Upper compartment    | Lower compartment    | Percent cell growth in lower chamber after treatment with: |           |            |            |            |            |
|----------------------|----------------------|--|-----------|------------|------------|------------|------------|
|                      |                      | No drug  | PND       | ACV        | ACV + PND  | GCV        | GCV + PND  |
| HeLa-TK <sup>+</sup> | HeLa-TK <sup>+</sup> | 100 ± 6.7  | 103 ± 6.2 | 39.8 ± 1.7 | 28.0 ± 2.9 | 5.9 ± 0.49 | 2.5 ± 0.17 |
| HeLa-TK <sup>+</sup> | HeLa-TK <sup>-</sup> | 100 ± 4.5  | 100 ± 3.4 | 101 ± 5.6  | 100 ± 2.6  | 102 ± 5.9  | 102 ± 1.7  |
| No cells             | HeLa-TK <sup>+</sup> | 100 ± 7.9  | 96 ± 6.1  | 39.8 ± 3.9 | 24.8 ± 1.9 | 4.9 ± 0.54 | 2.1 ± 0.37 |
| No cells             | HeLa-TK <sup>-</sup> | 100 ± 3.1  | 96 ± 6.0  | 97 ± 8.5   | 96 ± 9.2   | 100 ± 7.3  | 97 ± 9.1   |

<sup>a</sup>HeLa-TK<sup>+</sup> cells or no cells grown on membrane filters of Transwell chambers were treated with PND (0.5 µg/ml), ACV (100 µM), GCV (1 µM), PND + ACV, PND + GCV, or no drug. After 7 days of incubation, the growth of cells in the lower compartments was determined and expressed as a percentage of the cell number. Each value is the mean ± SD of quadruple assays.

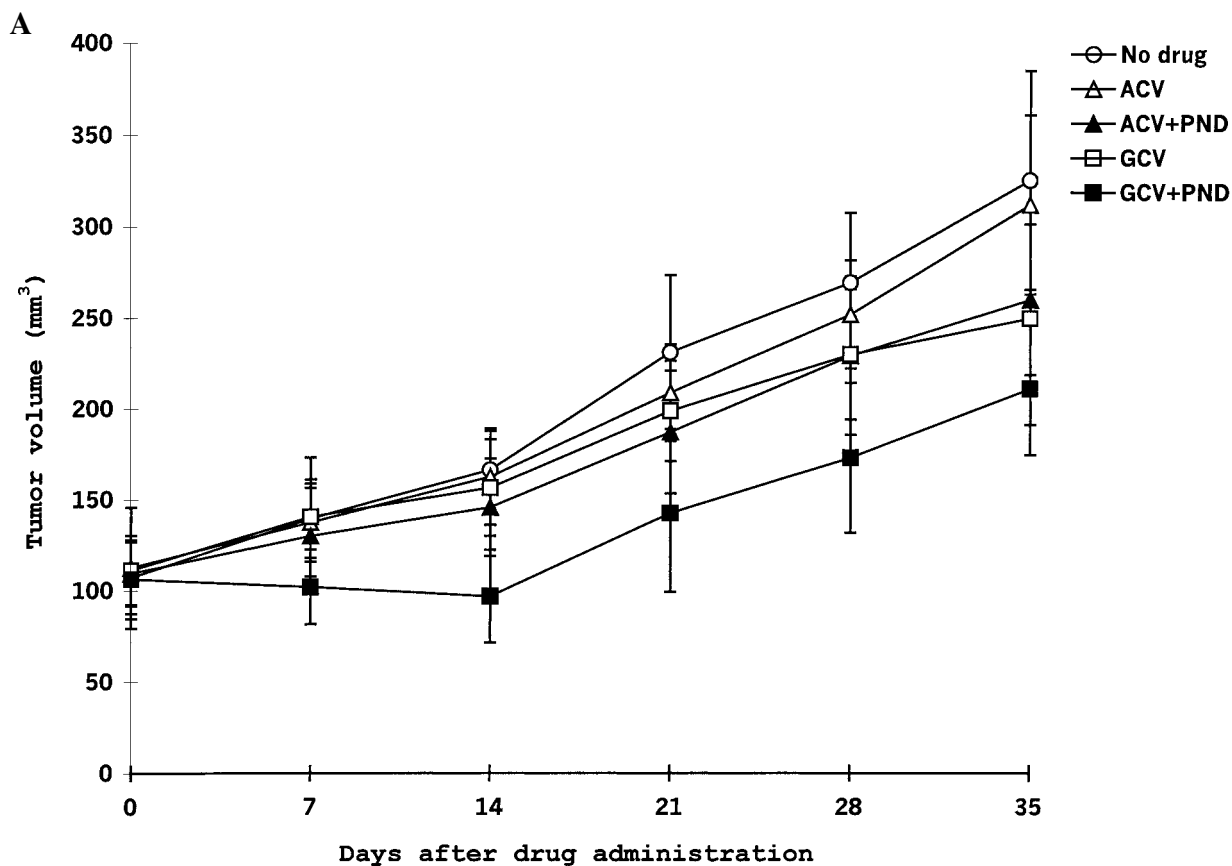
creased to 3% in tumor tissues, there was a significantly enhanced antitumor effect of PND plus ACV in comparison with ACV alone on day 28 ( $p < 0.05$ ), and consistent reduction in tumor volume over 35 days (Fig. 3B). Significant potentiation of the antitumor effect of GCV was also observed on days 14 ( $p < 0.001$ ), 21 ( $p < 0.001$ ), and 28 ( $p < 0.05$ ) in an animal group coadministered PND. In the mice injected with cultures composed only of HSV-TK-negative cells, both single administration of ACV or GCV and combined administration with PND produced no significant reduction in tumor growth as compared with control (Fig. 3C).

## DISCUSSION

We have evaluated the ability of PND to enhance cytotoxicity and bystander killing with the HSV-TK/prodrug administration system. Previously, we reported that the combination of PND and GCV provides *in vitro* high toxicity in HSV-TK-expressing cancer cells by virtue of an increased level of phosphorylated GCV metabolites. In the present study, we have con-

firmed the significantly enhanced toxicity of the prodrug in *in vivo* situations. Some attempts to augment the efficacy of HSV-TK/GCV gene therapy have been reported by adding other genes, such as connexin genes (Elshami *et al.*, 1996; Mesnil *et al.*, 1996, 1997) and the cytosine deaminase gene (Mullen *et al.*, 1992). We have demonstrated the increased antitumor efficacy of suicide gene therapy without requiring additional gene expression.

The HSV-TK/GCV gene therapy system relies on the expression of exogenous HSV-*tk* gene in cancer tissues. One major and serious limitation of this strategy is the low *in vivo* efficiency of current gene-transducing systems using various viral vectors or liposomes. Importantly, however, HSV-TK-expressing cells can induce the killing of nonexpressing neighbors (bystanders), which is called the bystander effect (Culver *et al.*, 1992; Freeman *et al.*, 1993). Thus, enhancing the efficiency of bystander killing may offer new strategies for improving the clinical application of HSV-TK/ACV or HSV-TK/GCV gene therapy. In this study, the ability of PND to enhance ACV and GCV cytotoxicities was evaluated in cocultures of HSV-TK-expressing and nonexpressing cells, using both *in vitro* and *in*



**FIG. 3.** *In vivo* bystander effects of ACV and GCV on tumors induced by mixtures of HSV-TK<sup>-</sup> and HSV-TK<sup>+</sup> HeLa cells in the absence or presence of PND. HeLa cell mixtures were prepared and injected into nude mice. Eight days after the cell injection, the mice were treated intraperitoneally with ACV (1 mg/mouse) alone, GCV (0.25 mg/mouse) alone, or ACV or GCV in combination with PND (0.1 mg/mouse), twice a day for 7 days. The size of the tumors (mm<sup>3</sup>) was estimated every 7 days for 5 weeks. The size of tumors is shown for HSV-TK<sup>-</sup> cells mixed at different ratios with HSV-TK<sup>+</sup> cells: 10% (A), 3% (B), and 0% (C) HSV-TK<sup>+</sup> cells. Symbols and error bars represent means  $\pm$  SD of 12 (A and B) or 10 (C) determinations.

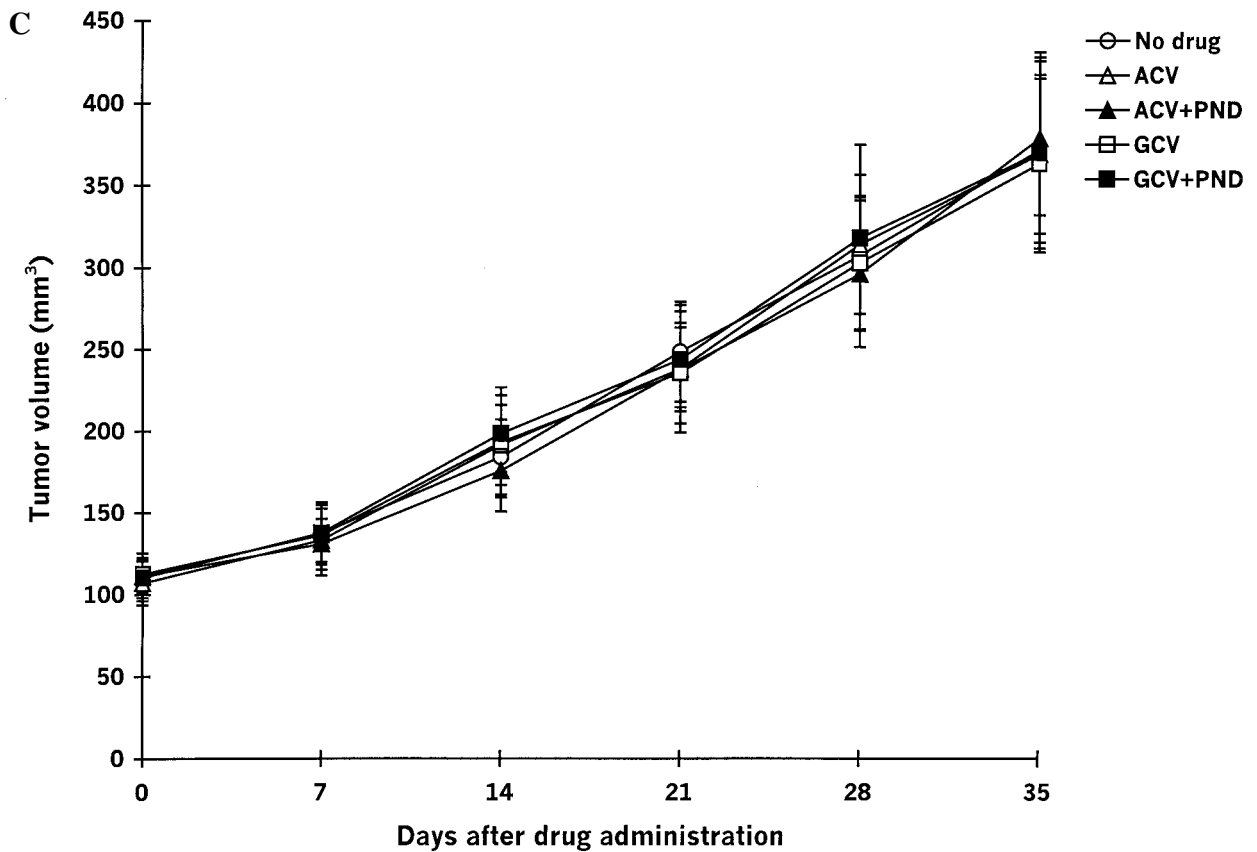
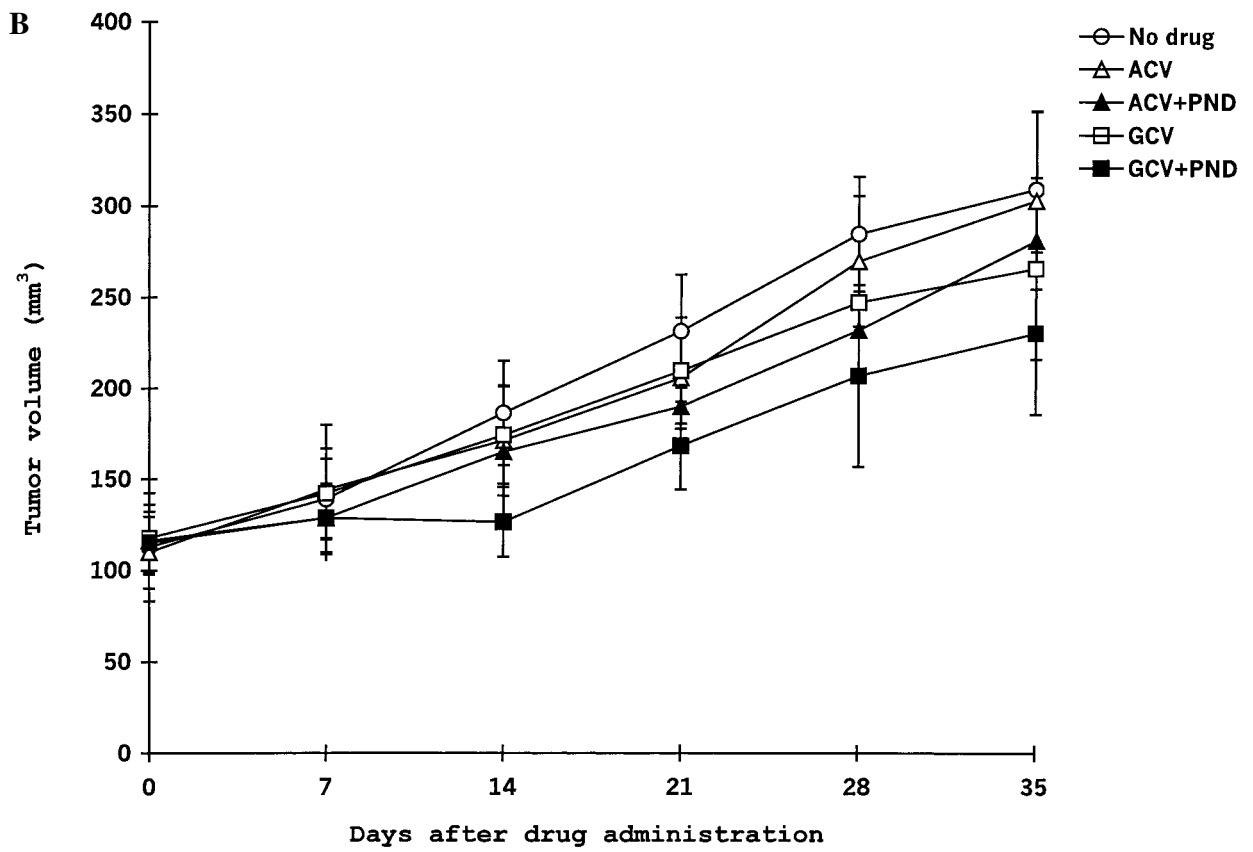


FIG. 3. Continued.

*in vivo* assay systems. The enhancement of ACV toxicity by PND seems to be weaker than that of GCV toxicity because the combination of PND and GCV suppressed tumor growth more potently in comparison with the combination of PND and ACV in both 3 and 10% HSV-TK<sup>+</sup> HeLa cell tumor-bearing animals. To evaluate the *in vivo* subacute toxicity of this compound, mice received intraperitoneal injections of a 10-fold higher dose of PND (1 mg/mouse) for 7 days. All animals survived the treatment schedule without any reduction in body weight (data not shown). We conclude that a high dose of PND does not cause any signs of toxicity. Thus, there should be room for further improvement of bystander efficacy if the doses of the drugs and duration of drug treatment were to be investigated.

So far, transfer of cytotoxic phosphorylated nucleotides between tumor cells has been shown to occur through gap junctions (Gilula *et al.*, 1972), and such a phenomenon has been thought to explain the bystander effect involved in HSV-TK/GCV gene therapy (Bi *et al.*, 1993; Fick *et al.*, 1995; Elshami *et al.*, 1996; Mesnil *et al.*, 1996). In fact, in cells with poor gap junctional intercellular communication (GJIC), a bystander effect by GCV was enhanced after transfection and expression of gap junction protein genes including that encoding connexin 43 (Mesnil *et al.*, 1996). The HeLa cells used in the present study do not communicate well by gap junction, and thus the bystander effect is limited (Dufлот-Dancer *et al.*, 1998). In spite of poor GJIC, both ACV and GCV exerted dose-dependent bystander killing in HeLa cells in the present study. There is another proposed theory that explains the bystander killing effect: phagocytosis by HSV-TK-negative cells of apoptotic vesicles containing toxic GCV metabolites from HSV-TK-positive cells (Freeman *et al.*, 1993). PND strengthened further the bystander effects of these prodrugs. At present, it is not clear whether PND would increase GJIC and/or phagocytosis of bystander cells. Because PND markedly accumulates the phosphorylated ACV or GCV metabolites and suppresses the extracellular release of GCV as confirmed in the present study and a previous study (Hayashi *et al.*, 2000), the resulting intracellular high levels of the toxic metabolites could contribute to potentiated bystander effects of the prodrug by PND. These effects of PND were found to require direct cell-cell contact as shown in the experiments using two-compartment culture systems. If tumor tissues were composed of cells with significant GJIC or if GJIC function could be enhanced by treatment with specific chemicals such as retinoids (Mehta *et al.*, 1989; Park *et al.*, 1997; Watanabe *et al.*, 1999), PND should augment the efficacy of HSV-TK/ACV or HSV-TK/GCV therapy.

ACV and GCV are known to share the same mechanism of selective cell killing in HSV-TK-expressing cells. In antitumor suicide gene therapy, GCV is a better agent for inhibiting the growth of cells expressing HSV-TK than ACV. For example, it has been reported that GCV is 10-fold more potent than ACV as an inhibitor of the growth of HSV-*tk* gene-transduced cancer cells (Balzarini *et al.*, 1993). In the present study, at the same concentration, lower cell killing efficacy and bystander effects were observed with ACV rather than GCV. In *in vivo* experiments, a four times higher concentration of ACV (1 mg/mouse) produced less effective antitumor activity than GCV (0.25 mg/mouse) (Fig. 1). Although these data suggest that GCV is the prodrug of choice for use with HSV-TK, this approach is limited in part by its toxicity. In general, administration of ACV for treating viral infections caused much less ad-

verse reactions even when used at a higher concentration than GCV, the margin of safety being greater than that of GCV. In this connection, there are reports of clinical trials applying the HSV-TK/ACV administration system in patients with recurrent ovarian cancer (Hasenburg *et al.*, 2000) or in patients with prostate cancer (Koeneman *et al.*, 2000). Therefore, the replacement of GCV with ACV in HSV-TK gene therapy may increase the antitumor effect without increasing toxicity. In this study, we evaluated the potential of HSV-TK/ACV/PND drug combination therapy in treating tumors; the combination resulted in markedly increased tumor cell killing in an *in vivo* assay and in increased bystander effects in both *in vitro* and *in vivo* assays. These results show that the use of ACV in HSV-TK gene therapy might produce a level of antitumor efficacy comparable to that of GCV when combined with PND.

Because of the toxicity of the prodrugs and insufficient delivery of the suicide gene into target tissues, many drug combination trials are underway to reduce the side effects and enhance the bystander killing effects of the prodrugs. Tumor cell killing was enhanced by increased tumor cell apoptosis through the combined use of inducers of apoptosis with GCV (McMasters *et al.*, 2000). The efficacy of HSV-TK/GCV treatment was also improved by using an inducer of gap junctions (Robe *et al.*, 2000), a polycation that induces adenovirus-mediated gene transfer (Lanuti *et al.*, 1999), and thymidylate synthase inhibitors including 5-fluorouracil (Wildner *et al.*, 1999). In clinical trials, topotecan, an antitumor agent, was used in HSV-TK/ACV therapy of patients with cancer (Hasenburg *et al.*, 2000). Boucher *et al.* (2000) have reported that the addition of a ribonucleotide reductase inhibitor, hydroxyurea, decreased cellular dGTP pools and increased the accumulation of GCV-TP levels in HSV-TK-expressing cells, resulting in an enhanced HSV-TK/GCV-mediated bystander effect in an *in vitro* model. Ponocidin, the diterpenoid used in the present study, has been shown to be directly connected with the activity of the HSV-1 TK enzyme (Hayashi *et al.*, 2000). Stimulating TK enzyme activity and elevating the levels of phosphorylated metabolites by this agent seems to be an attractive approach in HSV-TK/prodrug-mediated gene therapy.

In summary, these results prove the ability of PND to pharmacologically enhance HSV-TK/ACV- and HSV-TK/GCV-mediated tumor cell and bystander killing, and show that it may have an important therapeutic effect in tumors with a low efficiency of gene transfer.

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