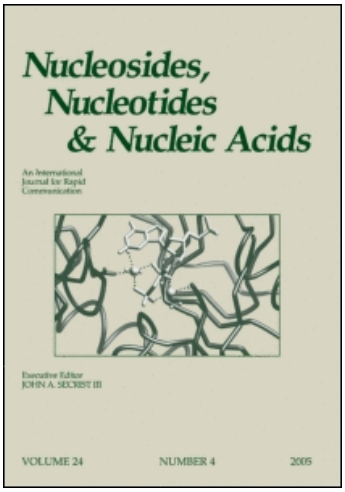

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APOPTOTIC EFFECT OF GANCICLOVIR ON PRIMARY EFFUSION LYMPHOMA CELLS INFECTED WITH KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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□ *We evaluated the cytotoxic and apoptotic effects of two purine nucleoside analogues, acyclovir (ACV) and ganciclovir (GCV), on lymphoma cells stably harboring Kaposi's sarcoma-associated herpesvirus (KSHV). Colorimetric caspase assay, flow cytometry, and immunoblotting with antibodies against apoptosis-related molecules revealed that GCV has cytotoxic activity toward KSHV-infected primary effusion lymphoma cells, while ACV has weak or little activity. In addition to the GCV-induced cytotoxicity, apoptosis* via *caspase-7/8, cleavage of poly(ADP-ribose) polymerase, and accumulation of p53 and p21 were induced by GCV treatment. In contrast, neither ACV nor GCV have cytotoxicity- or apoptosis-inducing activities toward uninfected cells.*

Keywords Epstein-barr virus (EBV); Kaposi's sarcoma-associated herpesvirus (KSHV); Human herpesvirus 8 (HHV-8); Acyclovir (ACV); Ganciclovir (GCV)

INTRODUCTION

Human lymphotropic herpesviruses, including Epstein–Barr virus (EBV, also known as human herpesvirus 4) and the more recently discovered Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8), are well known to be responsible for a wide range of diseases, such as tumors and lymphoproliferative disorders.[1] The majority of the human population carries asymptomic infection of EBV. In the case of KSHV, the seropositivity rate in the normal adult population is only about 1–30%,

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.
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and it shows a specific tropism for people of Mediterranean and sub-Sahara African countries.[2,3]

KSHV and EBV are oncogenic viruses with a long latency period in healthy hosts, and they will reactivate from dormancy when the hosts are immunosuppressed. Primary infections with these viruses in the immunocompetent host are generally asymptomatic. The neoplastic potentials of these two viruses have been well established, especially within the context of immunosuppressed patients who are undergoing bone-marrow transplant or are co-infected with human immunodeficiency virus, HIV.[4] The viral diseases of EBV and KSHV range from lymphocytic leukemia to Burkitt's lymphoma and from primary effusion lymphoma to erythromatous endothelial angiosarcoma, respectively.

KSHV was initially identified in Kaposi's sarcoma, an endothelial neoplasm that is commonly associated with AIDS in homosexual men. KSHV is also associated with primary effusion lymphoma and plasmablastic variant multicentric Castleman's disease.[5] The latency-associated nuclear antigen (LANA) is encoded by the ORF73 gene of the KSHV genome[6] and is consistently expressed in KSHV-associated tumors. LANA is a multifunctional protein that ensures association of the viral genome with the human genome via a tethering mechanism to facilitate viral DNA replication and effective segregation during cellular division.[7] This protein also contributes to KSHV-associated oncogenesis through manipulation of cellular gene expression; it interacts with tumor suppressor Rb[8] and abolishes p53-mediated apoptosis[9] as well as up-regulating the Wnt signaling pathway.[10,11] On the other hand, EBV infects more than 90% of the adult population worldwide.[12] EBV is transmitted via saliva in an oral-fecal route of transmission, and it infects B lymphocytes as well as certain epithelial cells. EBV is linked to a number of human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphoproliferative diseases in immunocompromised patients.

Currently, there is no proven effective antiviral therapy or cancer chemotherapy available for treatment of individuals infected with the above-mentioned viruses, but a few studies have been carried out to examine the effects of a limited number of antiviral drugs on these viruses[13–19]: It was found that the most widely used anti-herpesvirus drugs, ACV (an acyclic guanine analogue) and GCV (an acyclic guanine analogue), are weakly effective or not at all effective in inhibiting KSHV and EBV replications *in vitro*. While cellular pharmacological study of ACV and GCV actions has been extensively carried out, very little is known about the mechanisms by which these drugs actually lead to the death of virus-infected cells. Therefore, elucidation of the cytotoxic mechanisms of actions of the above-mentioned anti-herpesvirus drugs is important for the development of novel and effective antiviral prophylactic agents.

In this study, we evaluated the cytotoxic and apoptotic effects of ACV and GCV on lymphoblastoid cells stably harboring KSHV. By colorimetric caspase assay, flow cytometry, and immunoblotting with antibodies against apoptosis-related molecules, we found that caspases and their up- and downstream molecules are involved in GCV-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture

KSHV-mediated primary effusion lymphoma (PEL) cells (KSHV-positive, EBV-negative BC3 cells and KSHV-positive, EBV-positive BC2 cells), EBV-positive Burkitt's lymphoma cells (Raji and Akata cells), and herpesvirus-negative Burkitt's lymphoma cells (DG75 cells) were grown in 10% FBS RPMI medium 1640 at 37°C.

Cell Viability Assay

Cells were seeded in 96-well plates at 7×10^4 cells/well in 100 μl of growth medium with or without various concentrations of nucleoside analogues, ACV and GCV, and were incubated at 37°C for 5 days in a humidified CO_2-controlled atmosphere. Cytotoxic effects of the test compounds were assessed by colorimetric WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] dye reduction assay using a cell counting kit-8 (Dojindo, Osaka, Japan). WST-8 is converted into a water-soluble formazan product with an absorbance at 450 nm through the actions of mitochondrial enzymes. The optical density in ACV/GCV-treated cells was measured at 450 nm with a microplate spectrophotometer and expressed as percentage of the value in untreated cells (defined as 100%). The value of CC_{50}, 50% cytotoxic concentration of the drug, was calculated from the plot of drug concentration versus percentage of live cells. Data are shown as the mean value ± S.E.M. of three independent experiments.

Flow Cytometry

Drug-induced apoptosis was monitored by flow cytometry using propidium iodide (PI)-stained apoptotic cells. After induction of apoptosis by drug treatment, cells were harvested at various periods and fixed with 70% ethanol. The fixed cells were then treated with 0.5 mg/ml ribonuclease A at 37°C for 30 min, stained with PI (50 μg/ml) at room temperature for 10 min, and analyzed by a FACSort (Becton Dickinson, San Jose, CA, USA) with CellQuest™ software (Becton Dickinson, San Jose, CA, USA).

Caspase-3/7 Assay

Cells were subjected to ACV and GCV treatments for 72 and 120 h. Activities of caspase-3/7, -8, and -9 in the cell lysate were measured by a caspase-Glo assay kit using luciferin-conjugated DEVD, LETD, and LEHD polypeptide substrates, respectively, according to the instructions of the manufacturer (Promega, Madison, WI, USA). Luminescence was detected by an AB-2000 luminescencer-PSN (Atto, Tokyo, USA).

Antibodies and Western Blotting

Mouse monoclonal antibodies, anti-caspase-7, anti-PARP, anti-BAX, anti-p53, and anti-$p21^{Cip1}$ antibodies, were obtained from Transduction Laboratories. Anti-LANA rat monoclonal and anti-β-actin rabbit polyclonal antibodies were purchased from Advanced Biotechnologies, Inc. and Sigma, respectively.

For Western blotting, 2×10^5 cells were lysed in SDS-PAGE sample buffer containing 1 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin and 5 μg/ml aprotinin, boiled for 5 min, and sonicated for 30 s with an immersible tip-type sonicater in order to shear the chromosomal DNA. The resulting lysate was subjected to SDS-PAGE on a 10% polyacrylamide gel followed by Western blot analysis. The molecular mass standards, Pre-Stained protein ladder and BenchMark protein ladder, were purchased from Invitrogen (Carlsbad, CA, USA). Detection was performed using an enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA), and the bands were visualized with X-ray film.

RESULTS AND DISCUSSION

Cytotoxic Effects of ACV and GCV on KSHV-Infected and Uninfected Lymphoma Cells

First, we assayed the cytotoxic effects of ACV and GCV on B-cell lines isolated from a patient suffering from KSHV infection (BC3 cells), from a patient suffering from EBV-positive Burkitt's lymphoma (Raji and Akata cells), from a patient suffering from both KSHV and EBV infections (BC2 cells), and from a patient with no known EBV/KSHV infections (DG75 cells). These B-lymphoma cells were treated with 1, 5, 15, 50, and 100 μM ACV and GCV, and the cytotoxicity was assessed by analyzing viability rates of ACV/GCV-treated versus untreated cells by cell viability assay (Figure 1). There was no significant difference between the viability of KSHV- and EBV-negative DG75 cells previously treated with drugs and that of untreated cells. In contrast, the numbers of KSHV-infected BC2 and BC3 cells were decreased by treatment with 15–100 μM GCV. In addition, GCV had cytotoxicic activity

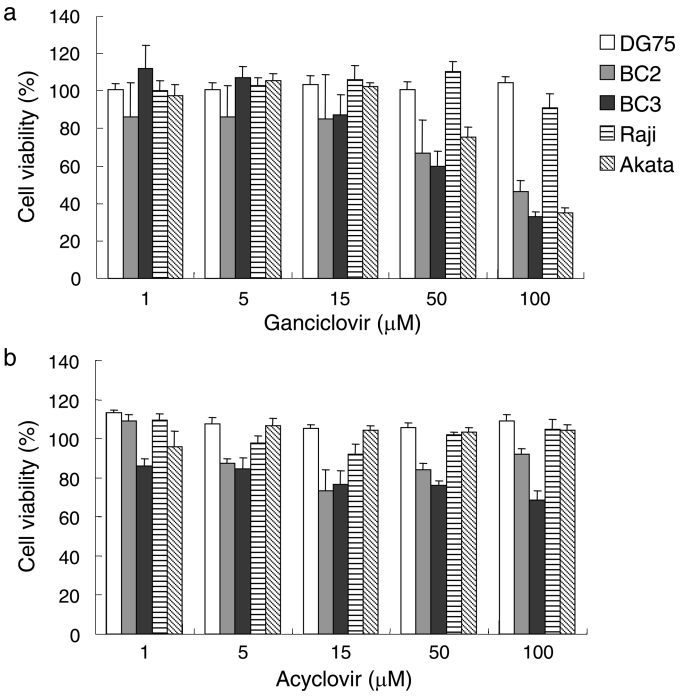

FIGURE 1 Cytotoxic effects of ACV and GCV on herpesvirus-infected lymphoma cells. KSHV-infected PEL cells (KSHV-positive, EBV-negative BC3 cells and KSHV-positive, EBV- positive BC2 cells), EBV-positive Burkitt's lymphoma cells (Raji and Akata cells), and herpesvirus-negative B-lymphoma cells (DG75 cells) were incubated with various concentrations (1, 5, 15, 50, and 100 μM) of GCV (a) and ACV (b) for 120 h and were then subjected to cell viability assays. In each experiment, viability was assessed in six replicate wells. The optical densities at 450 nm in untreated respective cells are defined as 100%. The means ± standard deviations determined in three separate experiments are indicated by error bars.

toward not only KSHV-positive PEL cells but also EBV-positive Akata cells. On the other hand, ACV had a weak cytotoxic activity against PEL cells but little activity toward Akata and Raji cells. As shown in Table. 1, GCV was active against BC2, BC3, and Akata cells with CC_{50} values of 81, 70, and 81 μM, respectively, while ACV was inactive against these cells ($CC_{50} > 100$ μM) (see Figure 1b). Raji cells were insensitive to ACV ($CC_{50} > 100$ μM) and GCV ($CC_{50} > 100$ μM) (see Figure 1).

Thymidine kinases (TKs) encoded by herpes simplex virus type 1 (HSV-1), type 2 (HSV-2) and varicella zoster virus (VZV) and a protein kinase (UL97) encoded by cytomegarovirus (CMV) are capable of monophosphorylating purine nucleoside analogues, including ACV and GCV, whereas cellular TKs have little such activities. This difference in substrate specificity between virus-encoded and cellular TKs has been utilized to design antiviral drugs such as ACV and GCV. Monophosphate of the drug formed by the action of virus-encoded TK is further phosphorylated by either cellular or viral kinase to produce the respective triphosphate, which inhibits viral

TABLE 1 Cytotoxic Effects of GCV on B-Lymphoma Cells

Cell	CC_{50} (μM)[a]
DG75	>100
BC2	81.0 ± 30.1
BC3	70.3 ± 7.7
Raji	>100
Akata	81.5 ± 2.8

[a]The CC_{50} value was calculated from the plot of drug concentration versus percentage of live cells. Data are shown as the mean value ± S.E.M. of three independent experiments.

DNA polymerase and is incorporated into cellular or viral DNA, leading to premature chain termination. As described above, we found that PEL cells and Burkitt's lymphoma cells are more sensitive to GCV than uninfected lymphoma DG75 cells. This result might be explained by the action of either an enzyme that is predicted to be encoded by ORF 21 of the KSHV genome and is similar to TKs of other herpesviruses or by another enzyme that is predicted to be encoded by ORF 36 and has a homology with other herpesvirus phosphotransferases. Cannon et al. demonstrated that the protein encoded by ORF 21 has a GCV kinase activity capable of phosphorylating GCV and that GCV induces cell death.[16] However, Gustafson et al. provided experimental evidence that human cellular TK-deficient cells expressing KSHV-TK are insensitive to GCV and that KSHV-TK is unable to phosphorylate GCV.[17] Moreover, it has been reported that GCV is not phosphorylated by KSHV-TK in an *in vitro* kinase assay.[18]

GCV Induces Apoptotic Cell Death in KSHV-Infected Lymphoma PEL Cells

Since GCV selectively decreased the viability of PEL cells, we next investigated whether this effect of GCV is due to apoptotic cell death. To monitor drug-induced apoptosis, flow cytometry using propidium iodide (PI)-stained apoptotic cells was performed. KSHV-infected PEL cells (BC3) and uninfected DG75 cells were treated with GCV and 48 and 72 h later the treated cells were stained with PI and were then subjected to flow cytometric analysis to determine the number of cells in the sub-G_1 stage (Figure 2) because the cells in the sub-G_1 stage accumulate as a result of DNA fragmentation that is indicative of apoptosis. Treatments with increasing concentrations of GCV led to incremental accumulation of the sub-G_1 fraction (apoptotic cells) in KSHV-infected BC3 cells: The percentages were calculated to be 1.76% in untreated cells and 3.29 and 5.73% in the cases of 48-h treatments with 50 and 100 μM GCV, respectively, while the values were 1.45% in untreated cells and 3.77 and 4.26% in the cases of 72-h treatments with 50 and 100 μM GCV, respectively. In contrast, the percentages of sub-G_1 fractions in uninfected

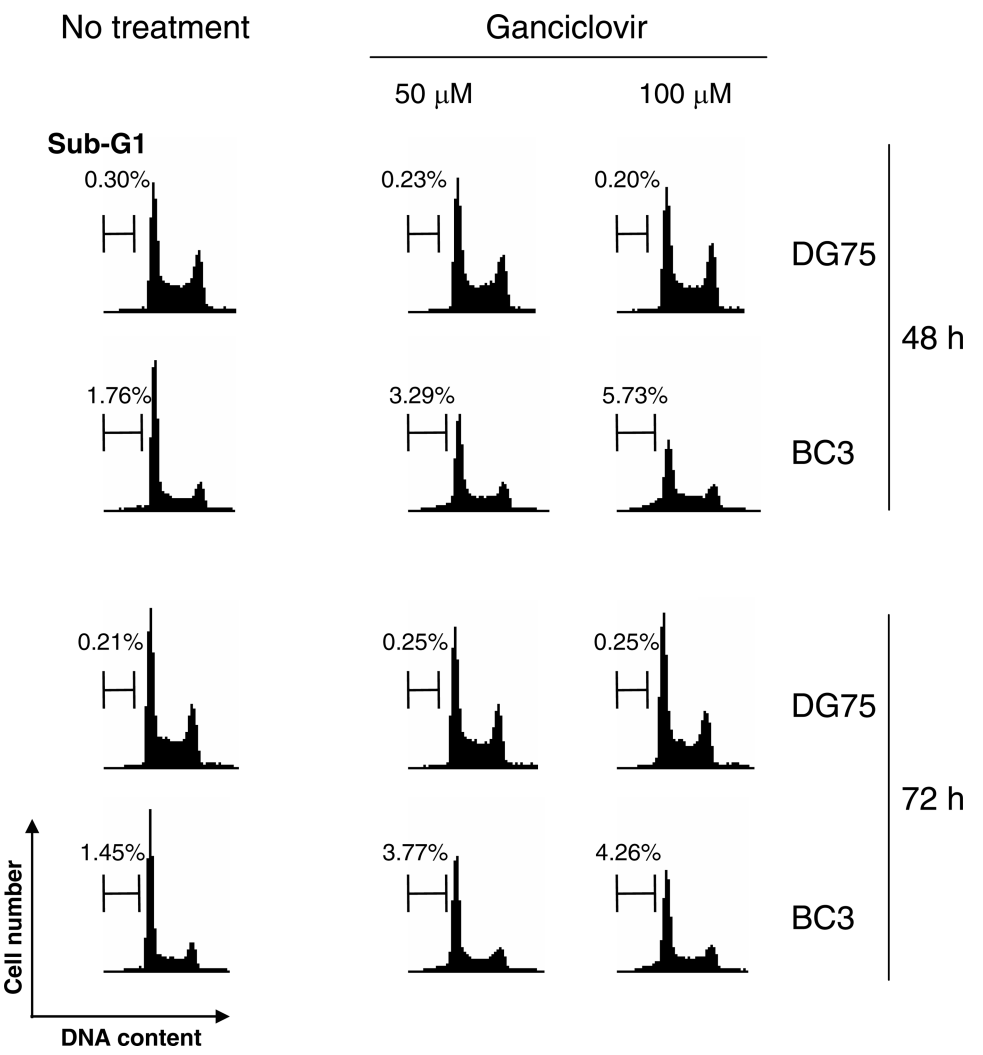

FIGURE 2 Apoptosis-inducing effects of GCV on KSHV-infected PEL cells (BC3 cells) and uninfected DG75 cells. Cells were cultured in the presence and absence of GCV (50 and 100 μM) for 48 and 72 h, and the cells were fixed and stained with PI. The percentage of apoptotic cell population was estimated by the sub-G_1 fraction analyzed by flow cytometry. The percentage of the sub-G_1 fraction is indicated in each histogram.

DG75 cells remained unchanged regardless of GCV concentration (50 and 100 μM) and incubation time (48 and 72 h).

Activation of Caspase and Expression of Apoptosis-Related Molecules in GCV-Treated PEL Cells

Apoptosis is induced by executioner caspases, such as caspase-3, -6, and -7, which have been previously activated through either the intrinsic pathway,

in which mitochondrial activation and the resulting caspase-9 activation are involved, or the extrinsic pathway, in which death-inducing signaling complex formation and the resulting caspase-8 activation are involved. Caspase-7 is cleaved and converted into its active form by the actions of caspase-9, -8, and -3. Caspase-7 activation is accompanied by the cleavage of PARP, a known endogenous substrate for caspase-7, into 28-kDa N-terminal and 85-kDa C-terminal fragments.

To assess the involvement of caspases and their up- and down-stream molecules in GCV-induced apoptosis, we measured peptidase activities of caspase-3/7 and -8 in KSHV-infected lymphoma cells pretreated with 100 μM ACV and GCV by a colorimetric assay (Figure 3). As shown in Figure 3a, a 1.25-fold increase of caspase-3/7 activity was detected in BC3 cells incubated with GCV for 72 h. The activity was increased up to 1.75-fold as incubation time was prolonged (120 h) (Figure 3b). In BC2 cells, peptidase activity of caspase-3/7 was slightly increased by GCV treatment. On the other hand, the increase in caspase-3/7 activity was scarcely detected in the case of ACV treatment. With respect to caspase-8, an initiator caspase in the extrinsic pathway, the activity was elevated in BC3 cells by 72-h treatment with both GCV and ACV (Figure 3c): It should be noted that caspase-9, another initiator caspase in the intrinsic pathway, was not activated under the same conditions (data not shown).

To obtain further evidence for the activation of caspase-3/7, we monitored the cleavages of both caspase-7 and the caspase substrate PARP by

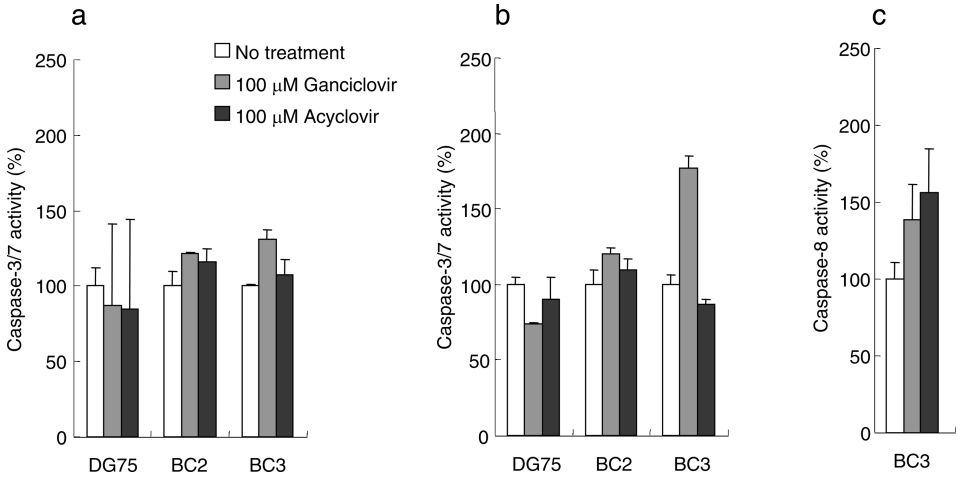

FIGURE 3 Changes in activities of caspase-3/7 and -8 in lymphoma B-cell lines treated with GCV and ACV. DG75, BC2 and BC3 cells were cultured in the presence and absence of 100 μM ACV and GCV for 72 (a, c) and 120 h (b) and were then harvested. Activities of caspase-3/7 (a), (b) and caspase-8 (c) were assayed using luciferin-conjugated DEVD and LETD polypeptide substrates, respectively. The caspase activities in untreated respective cells are defined as 100%. The error bar indicates the standard deviation.

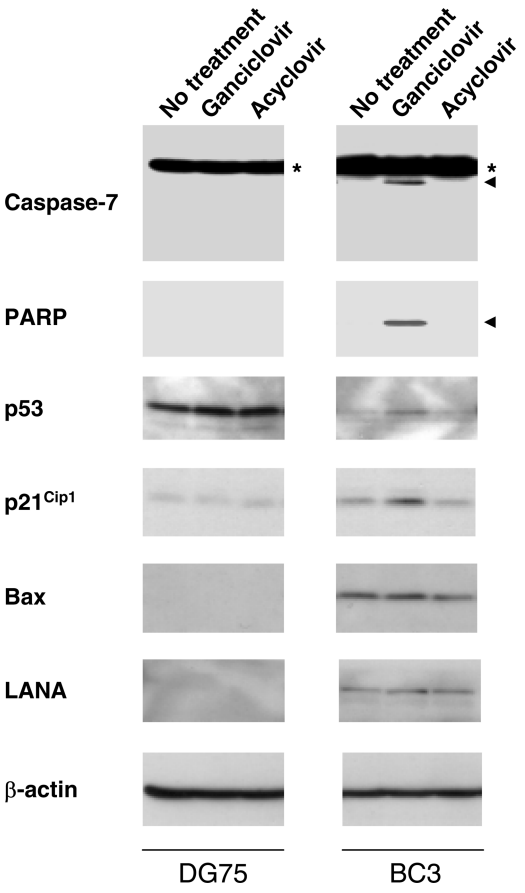

FIGURE 4 Cleavages of caspase-7 and PARP in KSHV-infected PEL cells (BC3 cells) and uninfected DG75 cells previously treated with ACV and GCV. Cells were exposed to 100 μM ACV and GCV for 72 h. To detect cleavages of caspase-7 and PARP and also expressions of p53, $p21^{Cip1}$, BAX, and LANA, Western blotting with anti-caspases-7, anti-PARP, anti-p53, anti-$p21^{Cip1}$, anti-BAX, and anti-LANA antibodies, respectively, was performed using an anti-β-actin antibody as control. Blotting with an anti-LANA antibody was performed to confirm the expression of LANA in KSHV-infected PEL cells (BC3 cells). Note that the 30-kDa cleavage product of pro-caspase-7 (35 kDa) and the 28-kDa N-terminal fragment of PARP, indicated by arrows, were produced in BC3 cells treated with GCV: The full-length caspase-7, pro-caspase-7, is indicated by an asterisk.

Western blotting using the lysates of PEL (BC3) and DG75 cells previously exposed to 100 μM ACV and GCV (Figure 4). As a result, we obtained additional evidence supporting the above assumption: The 30-kDa cleavage product of pro-caspase-7 (35 kDa) and the 28-kDa N-terminal fragment of PARP were detected in BC3 cells pretreated with GCV. In contrast, neither fragment was detected in GCV-treated DG75 cells.

DNA damage-inducible anti-cancer drugs, such as doxorubicin and cisplatinum, up-regulate p53 expression, leading to p53-mediated up-regulation of expressions of $p21^{Cip1}$ and Fas/CD95 death receptor.[20] GCV is

thought to induce DNA damage, leading to premature termination of DNA chain elongation. To determine whether GCV-induced apoptosis, induced by DNA damage, in BC3 cells is accompanied by activation of p53 signaling pathway, Western blotting with anti-p53 and anti-$p21^{Cip1}$ antibodies was performed. As shown in Figure 4, GCV treatment of BC3 cells resulted in the elevation of p53 and $p21^{Cip1}$ protein levels, suggesting that the p53 activation and signaling pathway is involved in GCV-induced apoptosis. Thus, we assume that DNA damage caused by GCV treatment induces activation of p53, leading to p53-dependent transcription of downstream molecules such as $p21^{Cip1}$ and Fas/CD95 death receptor, a component of the death-inducing signaling complex in the extrinsic apoptotic pathway, the complex formation of which induces activation of initiator caspase, caspase-8, followed by activation of executioner caspases, caspase-7/3.

To our knowledge, this is the first report on apoptotic mechanisms of GCV action in PEL cells. We demonstrated that PEL cells including Akata cells are sensitive to GCV. In addition, we found that the p53 signaling pathway is involved in GCV-mediated apoptosis. The ultimate goal of our study is to provide a rationale for the action mechanism of GCV in order to develop effective antiviral prophylactic agents.

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