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# Genipin as a novel chemical activator of EBV lytic cycle

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**Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that causes acute infection and establishes life-long latency. EBV causes several human cancers, including Burkitt's lymphoma, nasopharyngeal and gastric carcinoma. Antiviral agents can be categorized as virucides, antiviral chemotherapeutic agents, and immunomodulators. Most antiviral agents affect actively replicating viruses, but not their latent forms. Novel antiviral agents must be active on both the replicating and the latent forms of the virus. *Gardenia jasminoides* is an evergreen flowering plant belonging to the *Rubiaceae* family and is most commonly found growing wild in Vietnam, Southern China, Taiwan, Japan, Myanmar, and India. Genipin is an aglycone derived from an iridoid glycoside called geniposide, which is present in large quantities in the fruit of *G. jasminoides*. In this study, genipin was evaluated for its role as an antitumor and antiviral agent that produces inhibitory effects against EBV and EBV associated gastric carcinoma (EBVaGC). In SNU719 cells, one of EBVaGCs, genipin caused significant cytotoxicity (70 μM), induced methylation on EBV C promoter and tumor suppressor gene *BCL7A*, arrested cell-cycle progress (S phases), upregulated EBV latent/lytic genes in a dose-dependent manner, stimulated EBV progeny production, activated EBV F promoter for EBV lytic activation, and suppressed EBV infection. These results indicated that genipin could be a promising candidate for antiviral and antitumor agents against EBV and EBVaGC.**

***Keywords***: genipin, Epstein-Barr virus, lytic activation, methylation

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## Introduction

*Gardenia jasminoides* is an evergreen flowering plant belonging to the *Rubiaceae* family (Liu *et al.*, 2013). This plant originates in Asia and is most commonly found growing wild in Vietnam, Southern China, Taiwan, Japan, Myanmar, and India. *G. jasminoides* synthesizes iridoids such as genipin, geniposide, geniposidic acid, gardenoside, genipin-D-gentiobioside, gardoside, shanzhiside, 10-acetylgeniposide, deacetylasperulosidic acid methyl ester, scandoside methyl ester, geniposidic acid, 6-hydroxygeniposide, and gardeniside A-C (Ueda *et al.*, 1991). Diverse members of the iridoids family are found in many medicinal plants, indicative of their possible role in several pharmaceutical activities. The bioactivities of iridoids include cardiovascular, antihepatotoxic, choleretic, hypoglycemic, analgesic, anti-inflammatory, antimutagenic, antispasmodic, antitumor, antiviral, immunomodulatory, and purgative activities (Dinda *et al.*, 2007; Tundis *et al.*, 2008). A research study conducted on rats with rheumatoid arthritis demonstrated that *G. jasminoides* significantly lowered serum IL-1β and TNF-α levels, with its effect having a close relationship with the inhibitory development of rheumatoid arthritis (Tseng *et al.*, 1995; Dai *et al.*, 2014). In addition, the livers of rats fed with geniposide, an iridoid glycoside that is abundantly present in the fruit of *G. jasminoides*, exhibited enhanced glutathione content. The antioxidant glutathione is an important factor in the immune system because it helps modulate the immune response by cytokine production.

Genipin, an aglycone derived from geniposide, is known to be an excellent natural cross-linker for proteins, collagen, gelatin, and chitosan and is also used for its choleretic action, which aids in liver disease control (Liu *et al.*, 2013). In addition, a study conducted by Chen *et al.* demonstrated an enhanced binding affinity of genipin to influenza M2 channel activity, indicating that it could be a potent M2 inhibitor (Lin *et al.*, 2011). Select molecular dynamic simulations of the M2-derivative complexes showed a stable hydrogen bond interaction between genipin derivatives and the M2 residues Ser10 and Ala9. In addition, a patent has been registered for the use of genipin's strong anti-hepatitis B virus effect (Moon, 1997).

Members of the *Herpesviridae* family are well represented in nature and can be found in many different species across the animal kingdom. Herpesviruses have a double-stranded DNA genome (124–230 kb) enclosed in an icosahedral capsid (~125 nm in diameter) composed of 162 capsomeres. Based on their biological properties such as host range, replication cycle, and cell tropism, these viruses are classified into the alpha-, beta-, and gamma-herpesviridae subfamilies (Roizman *et al.*, 1981). Gammaherpesviruses include Epstein-Barr virus (EBV, also known as HHV4) and Kaposi's sar-

coma-associated herpesvirus (KSHV, also known as HHV8) (Blake, 2010). EBV causes infectious mononucleosis and is a causative agent of Burkitt's lymphoma, nasopharyngeal, and gastric carcinoma, and multicentric Castleman's disease. KSHV infection is associated with Kaposi's sarcoma (KS) and certain B-cell malignancies such as an AIDS-related form of non-Hodgkin lymphoma called primary effusion lymphoma (PEL), and multicentric Castleman's disease. In spite of the fact that EBV causes a wide range of human cancers, no effective antiviral agents that target specifically EBV are available (Moore *et al.*, 2001; Young and Rickinson, 2004).

Antiviral agents can be categorized as virucides, antiviral chemotherapeutic agents, and immunomodulators (Son *et al.*, 2013). These agents affect actively replicating viruses, but not their latent forms. It would be impossible to prevent host cells from being transformed unless the antiviral agents disturb the latent viral replication process. Thus, novel antiviral agents must be active on both the replicating and the latent forms of the virus. With the aim of identifying a new antiviral drug, we tested genipin for its antiviral activity against replicating and latent viruses and demonstrated that genipin produces strong antiviral activity against EBV life cycle by stimulating the viral lytic replication cycle.

## Materials and Methods

### Preparation of genipin

Genipin (purity ≥98%) was obtained from Sigma-Aldrich (USA) and was also obtained as a gift from C. Jeong in Duksung Women's University. Genipin was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and a 200-mM stock solution was prepared. This was then filtered through 0.22-μm filters (Sartorius Stedim Biotech) and stored at -20°C until use.

### Cell cultures

SNU719 (EBV genome-associated gastric carcinoma cell line) cells were cultured in RPMI1640 (Welgene), supplemented with 10% fetal bovine serum (FBS, Welgene), 1% antibiotics/antimycotics (Gibco), and 1% GlutaMAX (Gibco), and incubated at 37°C, under conditions of 5% $CO_2$ and 95% humidity in a $CO_2$ incubator. HEK293-EBV-GFP cells are HEK293 cells transfected with the EBV-GFP bacmid, selected with hygromycin B. HEK293-EBV-GFP (EBV latently infected endothelial cell line) cells were cultured in the same media used to culture iSLK-BAC16 cells except using low concentration of hygromycin B (50 μg/ml) (Waco Chemicals).

### Cytotoxicity assay

The cytotoxic effects of genipin against SNU719 cells was evaluated by performing a cellular cytotoxicity assay, using Cell Counting Kit-8 (CCK-8) (Dojindo), as described previously (Ishiyama *et al.*, 1997). Briefly, 100 μl of cell suspension ($1 \times 10^4$ cells) was seeded into each well of a 96-well plate. The cells were then treated with genipin at a series of concentrations on the following day and were incubated for 48 h. In total, 10 μl of CCK-8 solution was added to each well, following which the cells were further incubated for 3 h, and absorbance was subsequently read at 450 nm by using an ELISA reader. All steps of the procedure were conducted according to the manufacturer's recommended protocol.

### Apoptosis analysis

The apoptotic activity of genipin was verified using fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection assay. These assays were conducted on SNU719 cells that were treated with DMSO (negative control) and 70 μM genipin ($CD_{50}$ value) for 48 h. In this assay, 5 ml of SNU719 cell suspension ($1 \times 10^6$ cells) was seeded onto a 6-cm plate, followed by treatment with 70 μM genipin ($CD_{50}$ value) on the following day. The cells were stained with FITC-Annexin V Apoptosis Detection Kit I (BD Pharmingen) at 48 h post treatment, and were then subjected to analysis by using FACSAria III (BD Biosciences) within 1 h. Positive controls used in these experiments were HDAC inhibitors such as sodium butyrate (NaB, 3 mM) and 12-O-tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml).

### Membrane integrity analysis

To determine whether genipin caused any effects on the membrane integrity of SNU719 cells, FITC-Annexin V Apoptosis Detection assay was conducted on cells treated with DMSO (negative control) and 70 μM genipin ($CD_{50}$ value) for 48 h. The FITC-Annexin V Apoptosis Detection assay was performed as mentioned above.

### Methylation-specific PCR

To determine if genipin affects tumor suppressor gene methylation in SNU719 cells, a methylation-specific PCR assay was performed using DNA subjected to bisulfite conversion as described previously (Haruhiko, 2002). Following lysis and sonication by using a Bioruptor sonicator (5 min, 30 sec on/off pulses), genomic DNA was extracted from SNU719 cells treated with DMSO (negative control) and genipin 70 μM ($CD_{50}$ value). We used the CpGenome DNA Modification Kit (Millipore) for sodium bisulfite conversion of the DNA. The sequences of the *BCL7A* primers used are shown in Table 1. Each 25-μl reaction contained 5 μl of bisulfite-treated DNA template, 5 μl of 5× reaction mix (NanoHelix), 5 μl of 5× TuneUp solution (NanoHelix), 1 μl of Taq-plus polymerase (NanoHelix), and 2.5 μl of 10 μM forward/reverse primer. Primers were specific for methylated and unmethylated *BCL7A*. The primer pairs specific for regions upstream and downstream of C promoter and W promoter (Cp/Wp) as well as upstream and downstream of F promoter and Q promoter (Fp/Qp) are listed in Table 1. The following cycle conditions were used: 95°C for 3 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; followed by 72°C for 10 min. The reactions were performed using a TaKaRa PCR Thermal Cycler and then run on a 1.5% agarose/TBE gel.

### Cell-cycle analysis

The effect of genipin on the progress of the SNU719 cell cycles was assessed by the following protocol. The cells were treated with DMSO (negative control), 70 μM genipin ($CD_{50}$

**Table 1.** Primers for methylation-specific & EBV promoter usage polymerase chain reaction

| Genomic DNA | | Primer sequence (5′→ 3′) | | Primer sequence (5′→3′) |
|---|---|---|---|---|
| *BCL7A* | MF | GGTAGGCGACGTTTTAGTTC | UF | TGGGGTAGGTGATGTTTTAGTTT |
| | MR | GAATTAAAAACACCGATTCG | UR | CCAAATTAAAAACACCAATTCAA |
| Upstream of EBV Cp/Wp region | MF (11147-11259) | TTTAAAGTGGTAATAATATTAGGCGG | UF | TTAAAGTGGTAATAATATTAGGTGG |
| | MR (11148-11259) | CTACATTTTTCAAATCGTAAACGAA | UR | CTACATTTTTCAAATCATAAACAAA |
| Downstream of EBV Cp/Wp region | MF (18971-19084) | GTTTTTTAGAGGAATTAGGGATTTC | UF | TTTTTTAGAGGAATTAGGGATTTTG |
| | MR (18972-19087) | TCAAACATTCTTTAAATTTAACGAA | UR | CCCTCAAACATTCTTTAAATTTAACA |
| Upstream of EBV Fp/Qp region | MF (45005-45106) | TTTGGGGTATGGTATATTTAGTAGC | UF | TGGGGTATGGTATATTTAGTAGTGT |
| | MR (45007-45107) | AACCTAATTCTTAACTCGTTCGAC | UR | AAACCTAATTCTTAACTCATTCAAC |
| Downstream of EBV Fp/Qp region | MF (53255-53371) | ATTGTTTTATTTAGTTGGTGGTGTC | UF | TGTTTTATTTAGTTGGTGGTGTTGA |
| | MR (53257-53372) | CAAAATTTCCTAACTTTTTACGAA | UR | ACAAAATTTCCTAACTTTTTACAAA |
| EBV Cp/Wp | F (36134-36152) | TGCCTGAACCTGTGGTTGG | R (95664-95654/55361-55348) | CATGATTCACACTTAAAGGAGACGG |
| EBV Qp | F (50152-50168) | GTGCGCTACCGGATGGC | R (95664-95654/55361-55348) | CATGATTCACACTTAAAGGAGACGG |
| EBV Fp | F (50099-50115) | GGGTGAGGCCACGCTTT | R (55326-55304) | CAGGTCTACTGGCGGTCTATGAT |

* indicates genomic location in EBV genome (NCBI Reference Sequence: NC_007605.1)

value) and stained with propidium iodide (PI) solution for 48 h post treatment. Cell cycle analysis was performed using FACSAria III (BD Bioscience). Briefly, $3 \times 10^6$ cells were seeded onto 6-cm culture dishes. On the following day, when the cells reached 70% confluence, they were treated with genipin; SNU719 cells were treated with DMSO and genipin 70 μM. These were harvested using trypsin at 72 h and 48 h post treatment, respectively. The cells were then washed with cold Phosphate buffered saline (PBS), fixed in 95% ethanol for at least 1 h, and then treated with 300 μg of RNase A to remove all traces of RNA. The cells were stained with 10× PI solution and analyzed for cell-cycle progress with FACSAria III (BD Biosciences).

### FACS analysis

The effect of genipin on EBV latency was evaluated using FACSAria III (BD Biosciences). HEK293-EBV-GFP cells (Tempera *et al.*, 2010) treated with DMSO were used as internal negative controls. HEK293-EBV-GFP cells were subsequently treated with genipin 50 and 100 μM for 48 h. $0.5 \times 10^6$ cells resultant from initial $1.0 \times 10^6$ cells were fixed with 2% paraformaldehyde (PFA) in PBS solution and subjected to FACS analysis. The events from $1 \times 10^4$ cells were acquired by gating live cells based on forward and side scatter profiles, and the expression of green fluorescence protein (GFP) was measured using the FITC filter.

### Real time–quantitative polymerase chain reaction (RT-qPCR)

The effect of genipin on EBV transcription was demonstrated using the RNeasy Mini Kit (Qiagen). The kit was used to extract total RNA from SNU719 cells that were treated with DMSO (negative control), genipin 50 μM and 100 μM. cDNA was then synthesized using Superscript II Reverse Transcriptase (Invitrogen) and was diluted 50 times for the analysis of latent and lytic genes of EBV. The effect of genipin on EBV transcription was then evaluated by RT-qPCR assay. EBV latent gene primers were specific for *EBNA1*, *EBNA2*, *EBNA3A*, *EBNA3C*, *LMAP1*, *LMP2A*, and *EBER* and the lytic gene primers were specific for *BNRF1*, *BCRF1*, *BLLF1*, *BZLF1*, and *BRLF1*. The internal control gene primers were specific for GAPDH and GFP. RT-qPCR was performed using the iQ SYBR Green reagent (Bio-Rad) in Real-Time qPCR CFX96 (Bio-Rad). Each sample was analyzed in triplicate for EBV gene expression. Although all primer set sequences have been previously published (Tempera *et al.*, 2010, 2011; Kang *et al.*, 2011), these sequences are available upon request.

### Western blot analysis

The effect of genipin on EBV protein synthesis was assessed using western blot analysis. These assays were performed on SNU719 cells treated with DMSO (negative control), genipin 70 μM ($CD_{50}$ value). Positive controls used in these experiments was HDAC inhibitors such as sodium butyrate (NaB, 3 mM) and 12-O-tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml). The treated SNU719 cells were harvested using trypsin at 48 h post treatment. In total, $10 \times 10^6$ cells were lysed using 100 μl of reporter lysis buffer (Promega), supplemented with 1 μl of proteinase inhibitor (Sigma-Aldrich) and 10 μl of phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Cell lysates were further fractionated by sonication, using the Bioruptor sonicator (Diagenode) for 5 min, with 30-sec on/off cycles. If necessary, the cell lysates were snap-frozen in liquid nitrogen and stored at −80°C. Cell lysate (25 μl) was resolved on a 7% SDS-PAGE gel and further subjected to western blot analysis using antibodies developed against EBV proteins. EBV EBNA1, EBV BZLF1, EBV LMP2A, DNMT1, DNMT3A, SAPK/JNK, phospho-SAPK/JNK, STAT3, phospho-STAT3, and PARP were tested using beta-actin or GAPDH as an internal control. Following antibodies were used: anti-EBV EBNA1 (Santa Cruz Biotechnology), anti-EBV BZLF1 (Santa Cruz Biotechnology), anti-EBV LMP2A (Santa Cruz Biotechnology), anti-DNMT1 (Santa Cruz Biotechnology), anti-DNMT3A (Santa Cruz Biotechnology), anti-SAPK/JNK (Cell Signaling Technology), anti-phospho-SAPK/JNK (Cell Signaling Technology), anti-beta-actin (Santa Cruz Biotechnology), anti-GAPDH (Cell Signaling Technology) were detected. Horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosiences), Horseradish peroxidase-

conjugated donkey anti-rabbit IgG (Amersham Biosiences), Horseradish peroxidase-conjugated goat anti-rat IgG (Bethyl Laboratories) were used as secondary antibodies.

### Quantification of intracellular and extracellular EBV genomic DNA copy number

Lysis and sonication (5 min with 30-sec on/off cycles) procedures were followed by genomic DNA (gDNA) extraction from SNU719 cells treated with DMSO (negative control), genipin 50 μM and 100 μM. Fifty nanograms of the resultant gDNA was subjected to RT-qPCR, and the relative amount of EBV gDNA was determined, using an internal control such as GAPDH or DMSO (negative control). Intracellular EBV copy number was considered the relative amount of EBV gDNA in total gDNA. The relative extracellular EBV copy number was measured using 20 ml of each culture medium collected from SNU719 cells treated with DMSO and genipin 70 μM. The culture media was filtered through a 0.45-nm syringe filter (Sartorius Stedim Biotech), loaded onto a 20% sucrose cushion in PBS solution, and subjected to ultracentrifugation (CP100WX, Hitachi) at 27,000 rpm for 90 min. The viral pellet was lysed in 100 μl of FA lysis buffer [EDTA (1 mM, pH 8.0), HEPES-KOH (50 mM, pH 7.5), and NaCl (140 mM)] and sonicated in the Bioruptor for 5 min with 30-sec on/off cycles, which was followed by a general DNA extraction process. The viral DNA extracted was resolved in 100 μl of RNase-free water and subjected to quantitative PCR (qPCR) analysis for quantification, using primer sets specific for either EBV *EBNA1*.

### Promoter usage detection assay

The effects of genipin on the selection of EBV promoter usage was determined by conventional PCR, using cDNA isolated from SNU719 cells treated with DMSO (negative control), genipin 70 μM ($CD_{50}$ value). Positive controls used in these experiments was HDAC inhibitors such as sodium butyrate (NaB, 3 mM) and 12-O-tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml). Total RNA was extracted from SNU719 cells treated with DMSO, genipin 70 μM and TPA/NaB, using RNeasy Mini Kit (Qiagen) and then synthesized into cDNA by using the Superscript II Reverse Transcriptase (Invitrogen). Comparative control cDNAs were synthesized from total RNA extracted from KEM1 and KEM3 cells, which were EBV latency type 1 and 3 EBV-Burkitt's lymphoma cell lines, respectively (Hughes *et al.*, 2011). The primer sequences selected included actin, EBV Q promoter, EBV C/W promoter, and EBV F promoter, which have been previously published (Tempera *et al.*, 2010) and listed in Table 1. cDNA was amplified in a 25 μl reaction solution containing 5 μl of 5× reaction mix (NanoHelix), 5 μl of 5× TuneUp solution (NanoHelix), 1 μl of Taq-plus polymerase (NanoHelix), and 2.5 μl of 10 μM Forward/Reverse primer. The cycling conditions were as follows: 95°C for 3 min, 30 cycles of 95°C for 10 sec, 55°C for 30 sec, and 72°C for 10 min, and 72°C for 10 min. The reactions were carried out in the TaKaRa PCR Thermal Cycler (TaKaRa Bio Inc.), and the samples were subsequently run on a 1.2% agarose/Tris-borate-EDTA (TBE) gel.

### EBV infection assay

The effect of genipin on EBV infection of AGS (gastric carcinoma) cells was determined by cell-to-cell coinfection of AGS and LCL-EBV-GFP (B-cell lymphocyte) cells. Cell to cell coinfection assay using LCL-EBV cells (EBV positive) and AGS cells (EBV negative) was designed to test if genipin could reinforce AGS cells against EBV infection. In total, $0.625 \times 10^6$ AGS cells were seeded per well in a 6-well plate (Corning). The following day, the AGS cell culture medium (RPMI 1640 (HyClone), supplemented with 10% FBS (HyClone), antibiotics/antimycotics (Gibco), and GlutaMAX (Gibco), was replaced with fresh medium. Then, LCL-EBV-GFP cells ($1.25 \times 10^6$/ml) were overlaid onto AGS cells, and both cell lines were treated with DMSO (negative control) and genipin 70 μM for 72 h. At 72 h post coinfection, the medium was completely removed and the cells were washed 2 times with PBS in order to retain only AGS-EBV-GFP cells. The infected AGS-EBV-GFP cells were overlaid with 1.5% Bacto agar [with 2× RPMI 1640 (HyClone), supplemented with 20% FBS (HyClone), antibiotics/antimycotics (Gibco), and GlutaMAX (Gibco)], and further incubated for 72 h. Post incubation, the GFP foci formed on the AGS cells treated with genipin 70 μM or DMSO (negative control) were counted.

## Results

### Genipin cytotoxicity

Initially, we determined whether genipin causes cytotoxicity in the EBV associated gastric carcinoma cell line SNU719 cells. This was determined by a cellular toxicity assay using CCK 8, where a 50% cytotoxicity dose ($CD_{50}$) of genipin was

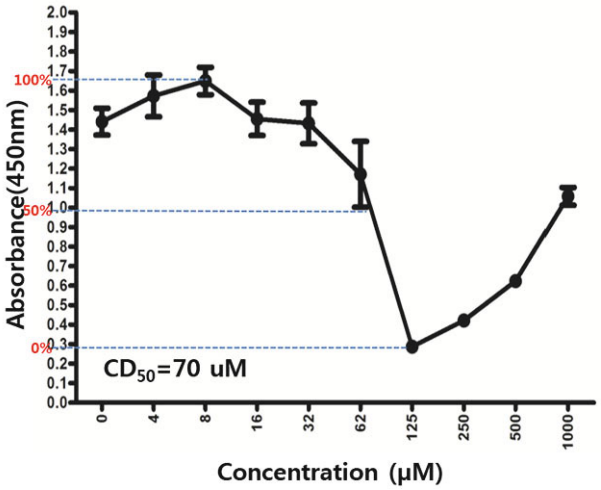


**Fig. 1. Effect of genipin on induction of cytotoxicity.** Cytotoxicity assay was conducted to determine whether genipin causes cytotoxicity Genipin produced cytotoxicity in SNU719 cells, which were latently infected with EBV. 50% cytotoxicity dose ($CD_{50}$) of genipin on SNU719 cells was determined to be 70 μM. Each measurement was repeated in three times. Averages and standard errors of measurements were displayed on graphs. Percentages indicate percentages of survival of SNU719 cells treated with genipin at a series of concentrations.

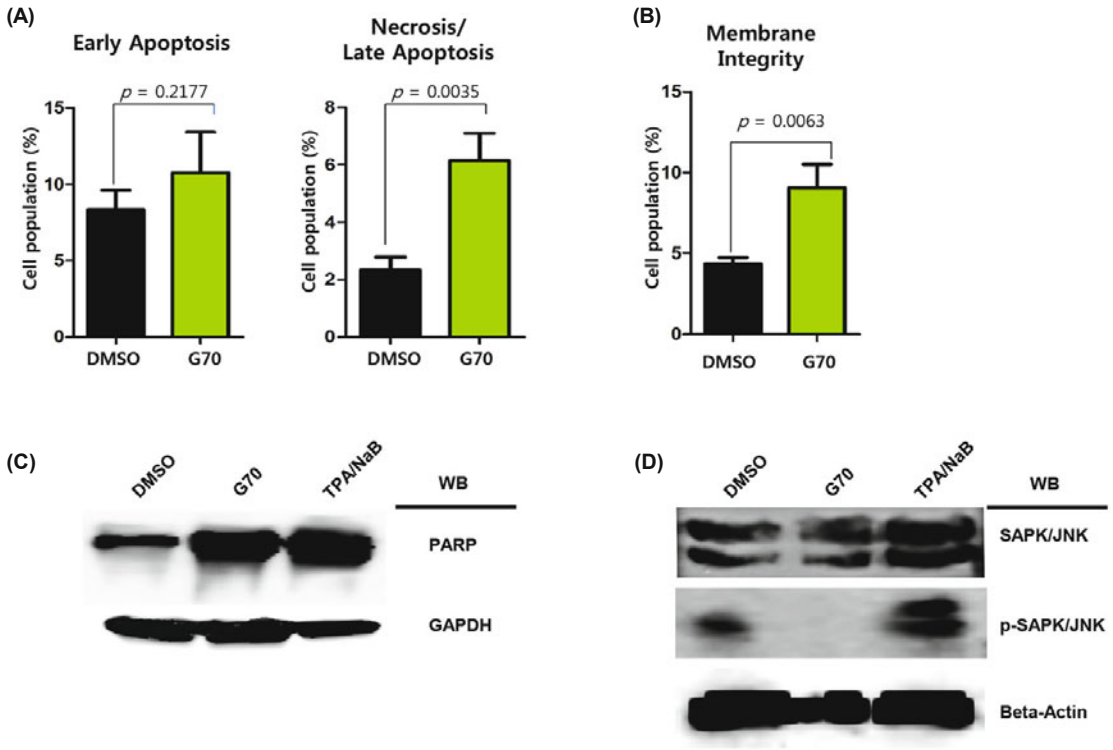


**Fig. 2. Effect of genipin on induction of apoptosis and membrane integrity.** The effect of genipin on apoptosis induction was investigated by FITC-Annexin V Apoptosis Detection Assay. (A) FITC-Annexin V Apoptosis Detection assay demonstrated that SNU719 cell populations undergoing necrosis/late stage apoptosis were significantly enhanced by genipin treatment at 70 μM. (B) FITC-Annexin V Apoptosis Detection assay was performed to investigate the effect of genipin on membrane integrity of SNU719. Effect of genipin on membrane integrity of SNU719 cells determined using FITC-Annexin V Apoptosis Detection assay. Genipin was seen to increase the membrane integrity of SNU719 cells. DMSO and G70 stand for DMSO (blank) and genipin 70 μM treatment, respectively. (C) Western blot analysis using anti-PARP antibody was used to determine whether genipin treatment impacts on the cleavage of PARP in SNU719 cells. Compared to DMSO treatment (negative control), genipin treatment did not promote PARP cleavage. (D) Western blot analysis using anti-SAPK/JNK and anti-phospho-SAPK/JNK antibodies was used to determine whether a $CD_{50}$ dose (70 μM) of genipin impacts on the apoptosis. Compared to DMSO treatment (negative control), genipin treatment completely eliminated phosphor-SAPK/JNK. DMSO, G70, and NaB/TPA stand for DMSO, genpin 70 μM, NaB (3 mM)/TPA (1 ng/ml) treatments, respectively. WB stands for Western blot analysis using indicated antibodies. Beta-actin and GAPDH was used as an internal control in Western blot analysis.

injected against SNU719 cells. CCK-8 allows for sensitive colorimetric assay determination of the number of viable cells in cell proliferation and cytotoxicity assays. $CD_{50}$ of genipin against SNU719 was determined to be 70 μM. This indicated that genipin caused a strong cytotoxicity in latently infected host cell EBV (Fig. 1).

### Effect of genipin on apoptosis and membrane integrity

Considering the strong cytotoxicity exhibited by genipin, the mechanisms underlying its cytotoxicity in SNU719 cells at $CD_{50}$ doses needed to be identified. Assuming that, in most cases, cytotoxicity occurs by either apoptosis or necrosis (Bonfoco *et al.*, 1995), FITC Annexin V Apoptosis detection kit I was used to investigate genipin-induced cytotoxicity in SNU719 cells (treated with genipin at $CD_{50}$ dose for 48 h). Compared to DMSO-treated cells, genipin-treated SNU719 cells showed an increase of 28% in early apoptosis and an increase of approximately 163% in late apoptosis/necrosis (Fig. 2A). This analysis indicated that genipin caused cytotoxicity by the induction of necrosis in SNU719 cells, rather than early apoptosis. The effect of cytotoxicity on the integrity of the plasma or endosomal membranes in SUN719 cells was investigated. The FITC Annexin V Apoptosis detection kit I was also used to determine whether a $CD_{50}$ dose (70 μM) of genipin disrupts the membrane integrity of SNU719 cells. Compared to the membrane integrity of DMSO-treated cells, the membrane integrity of genipin-treated SNU719 cells displayed an increase of approximately 208% (Fig. 2B). This result indicated that the disruption of membrane integrity of SNU719 cells may contribute to the cytotoxicity of genipin on SNU719 cells. As PARP is known to help cells to maintain their viability (Oliver *et al.*, 1998), cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver *et al.*, 1998). Therefore, we conducted Western blot analysis using anti-PARP antibody to determine if genipin induces apoptosis by cleaving PARP in SNU719 cells. Compared to DMSO treatment (negative control), genipin treatment did not showed significant increase of cleaved PARP although it promoted to remarkably increase intact PARP. These results also indicated that genipin does not induce apoptosis that requires caspase-3 mediated PARP cleavage (Fig. 2C). Last, Western blot analysis using anti-SAPK/JNK antibodies was conducted to determine if genipin affects JNK signaling pathway that is known to induce apoptosis (Papa *et al.*, 2006). Compared to DMSO

treatment (negative control), genipin treatment completely eliminated phospho-SAPK/JNK (Fig. 2D). This result indicated that the loss of phospho-SAPK/JNK may contribute to the weak induction of late apoptosis by genipin.

### Effect of genipin on the methylation of EBVaGC cells

The effect of cytotoxicity on the methylation in SUN719 cells was investigated. Western blot analysis using DNMT1 and DNMT3A antibodies was used to determine whether a $CD_{50}$ dose (70 μM) of genipin impacts on the methylation. Compared to the methylation of DMSO-treated cells, both DNMT1 and DNMT3A expression of genipin-treated SNU719 cells displayed an increase (Fig. 3A). This result was interesting that the induction of DNMT1 and DNMT3A expression by genipin may contribute to the activation of EBV early lytic viral promoter through BZLF1, as described in a previous study (Wille *et al.*, 2013). As STAT3 pathway is known to upregulate *DNMT1* (Hino *et al.*, 2009), these results suggested that genipin might inhibit STAT3 signaling pathway. To test this hypothesis, Western blot analysis using anti-STAT3 and anti-phospho-STAT3 antibodies was conducted. Compared to DMSO treatment, genipin treatment significantly induced phospho-STAT3 expression. This results indicated that genipin activates the STAT3 signaling pathway and consequently upregulated DNA methyltransferases (DNMTs) (Fig. 3B). Next, it was tested that genipin affected methylation on EBV genomic locuses using methy-

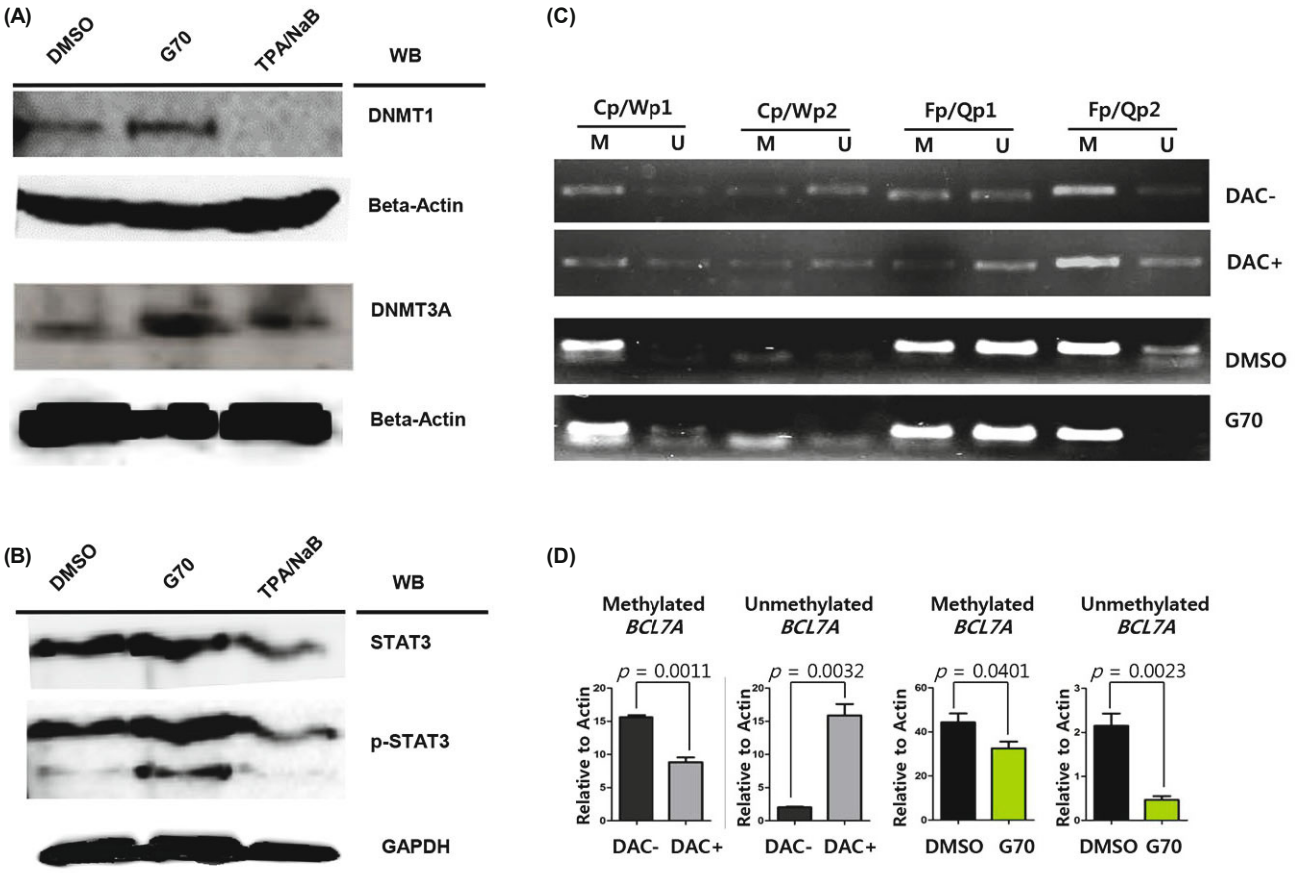


**Fig. 3. Effect of genipin on methylation.** Effect of genipin on methylation in SNU719 cells was determined using Western blot analysis. (A) Western blot analysis using DNMT1 and DNMT3A antibodies was used to determine whether a $CD_{50}$ dose (70 μM) of genipin impacts on the methylation. Compared to the methylation of DMSO-treated cells, both DNMT1 and DNMT3A expression of genipin-treated SNU719 cells displayed an increase. DMSO, G70, and NaB/TPA stand for DMSO, genipin 70 μM, NaB (3 mM)/TPA (1 ng/ml) treatments, respectively. WB stands for Western blot analysis using indicated antibodies. Beta-actin and GAPDH was used as an internal control in Western blot analysis. (B) Western blot analysis using anti-STAT3 and anti-phospho-STAT3 antibodies was used to determine whether genipin treatment affects the upregulation of DNMTs. Compared to DMSO treatment (negative control), genipin treatment significantly enhanced phosphor-STAT3 production. (C) A methylation-specific PCR assay was performed to determine if genipin affected methylation of EBV genomic loci in SNU719 cells treated with genipin (70 μM: G70). As expected, treatment of 10 μM 5-aza-2′-deoxycytidine (DAC), HDAC inhibitor enhanced methylation on downstream region of F/Q promoter and unmethylation on both regions (up and downstream) of F/Q promoter. Genipin treatment showed to enhance methylation of EBV genomic loci (both regions of up and downstream) near C/W promoters, whereas it reduced unmethylation around F/Q promoter. Specifically, regions downstream of the F/Q promoters were highly unmethylated in response to genipin treatment. Cp/Wp1 and Cp/Wp2 are EBV genomic loci upstream and downstream of C/W promoters, respectively. Fp/Qp1 and Fp/Qp2 are EBV genomic loci upstream and downstream of F/Q promoters, respectively. M, methylation-specific primers; U, unmethylation-specific primers. (D) Methylation-specific quantitative PCR (qPCR) was performed on genipin-treated (70 μM) SNU719 cells to determine if genipin affected methylation of *BCL7A*, a tumor suppressor gene. Compared to the mock treatment, 10 μM 5-aza-2′-deoxycytidine (DAC) treatment highly suppressed methylation and induced unmethylation (left two panels). The methylation-specific qPCR assay showed that genipin treatment (70 μM) reduced *BCL7A* methylation by up to 27% and suppressed unmethylation by up to 80% in SUN719 cells (right two panels). *P*-values <0.05 (95% confidence) were considered statistically significant.

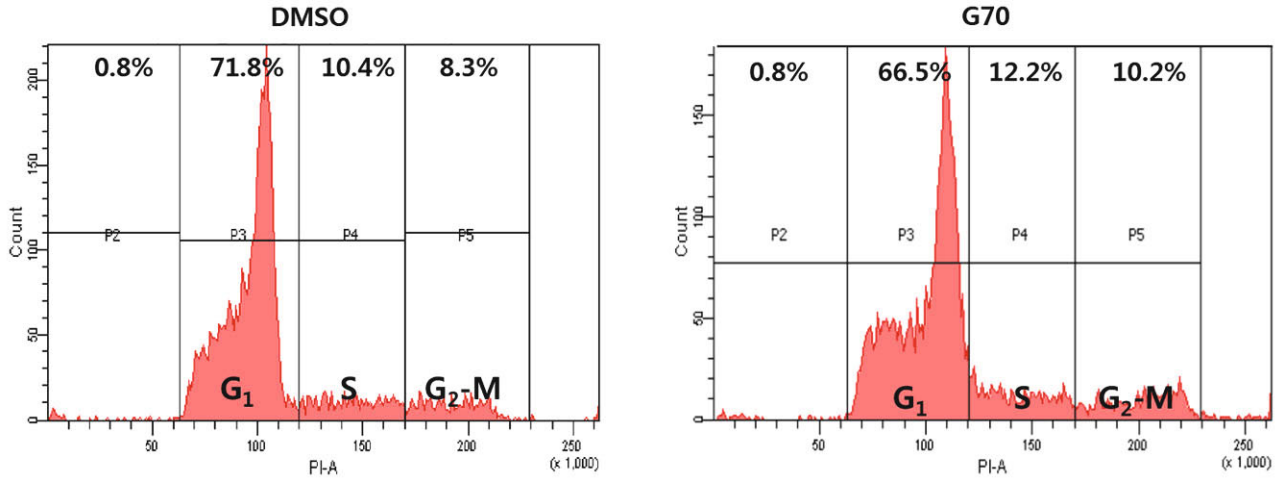


**Fig. 4. Effect of genipin on cell cycle progress.** Effect of genipin on cell cycle progress was determined using PI staining and FACs analysis. Genipin was observed to arrest cell cycle of SNU719 at S and G2/M phases. Genipin was treated to SNU719 cells at 70 μM (G70).

lation-specific PCR assay (Fig. 3C). As expected, treatment of 10 μM 5-aza-2′-deoxycytidine (DAC), HDAC inhibitor enhanced methylation on downstream region of F/Q promoter and unmethylation on both regions (up and downstream, Table 1) of F/Q promoter. Genipin treatment showed to enhance methylation of EBV genomic loci (both regions of up and downstream) near C/W promoter, whereas it reduced unmethylation around F/Q promoter. Specifically, regions downstream of the F/Q promoter was highly unmethylated in response to genipin treatment. These results indicated that genipin induced methylation on regions close to EBV major latent promoters such as C promoter and Q promoter, rather than EBV lytic promoter such as F promoter. Last, it was determined if genipin affected methylation on cellular genomic locus. *BCL7A*, tumor suppress gene was tested if methylation on its locus was affected by genipin using RT-qPCR assay (Fig. 3D). This assay showed that unmethylation was significantly decreased on *BCL7A* while methylation was also a little decreased. Actually, compared to the mock treatment, 10 μM 5-aza-2′-deoxycytidine (DAC) treatment highly suppressed methylation and induced unmethylation (left two panels). Genipin treatment (70 μM) suppressed *BCL7A* unmethylation by up to 80% and suppressed methylation by up to 26% in SUN719 cells (right two panels). Taken these results together, genipin was suggested to play as a regulator in transcription of cellular or viral genes by inducing methylation on these genes.

### Effect of genipin on cell cycle

The effect of genipin on the cell cycles of SNU719 cells was evaluated. Propidium iodide (PI) staining followed by FACS analysis was conducted on both sets of genipin-treated cells. PI stains cellular genomic DNA, enabling an evaluation of the cell viability, or DNA content, for cell cycle analysis. Genipin activity in SNU719 slices arrested at S and G2/M phases, suggested that genipin causes serious defects in DNA synthesis and chromatin segregation (Fig. 4).

### Effect of genipin on EBV gene expression

The effect of genipin on EBV gene expressions was investigated in response to genipin-impingement on cell proliferation. RT-qPCR assay was performed for the quantification of transcripts of EBV genes expressed in SNU719 cells treated with genipin. The 100 μM genipin treatment to SNU719 cells showed that EBV lytic genes *BNRF1*, *BCRF1*, *BLLF1*, *BZLF1* and EBV latent genes *LMP1*, *LMP2A*, *EBNA2* in SNU719 cells were upregulated, while EBV latent genes *EBER1*, *EBNA3A*, *EBNA3C*, *EBNA1*, and EBV lytic gene *BRLF1* were downregulated (Fig. 5A). The 50 μM genipin treatment to SNU719 cells showed that EBV lytic gene *BZLF1* only was upregulated, while EBV latent genes *LMP1*, *LMP2A*, *EBER1*, *EBNA2*, *EBNA3A*, *EBNA3C*, *EBNA1*, and EBV lytic genes *BNRF1*, *BCRF1*, *BLLF1*, *BRLF1* were downregulated (Fig. 5A). These results indicate that genipin plays a dual role in regulating most EBV genes in a dose-dependent manner, which genipin activates EBV gene expression at its low concentration but suppresses the expression at its high concentration. As genipin was demonstrated to induce the maximum EBV gene expression at a concentration of 100 μM, the effect of genipin on the selection of EBV latency promoters was investigated. The KEM1 control cells which are specific to EBV type I, showed a high frequency of use of Q and F promoters (Fig. 5B), whereas KEM3 control cells specific to EBV type III, demonstrated a high frequency of use of C/W promoter (Fig. 5B). Genipin was observed to intensively increase the frequency of use of EBV-F promoter and weakly decrease that of EBV-Q promoter at 100 μM (Fig. 5B). However, 50 μM genipin did not significantly affect the selection of EBV latency promoters (Fig. 5B). These findings are consistent with the results of a previous study, which observed that most early lytic viral promoters are preferentially activated by the *BZLF1* promoter (EBV-immediate early protein) in the methylated form. These results also indicated that genipin plays a dual role by increasing the frequency of use of F promoter to undergo EBV lytic replication depending on its concentration. The effect of genipin on EBV and KSHV protein ex-

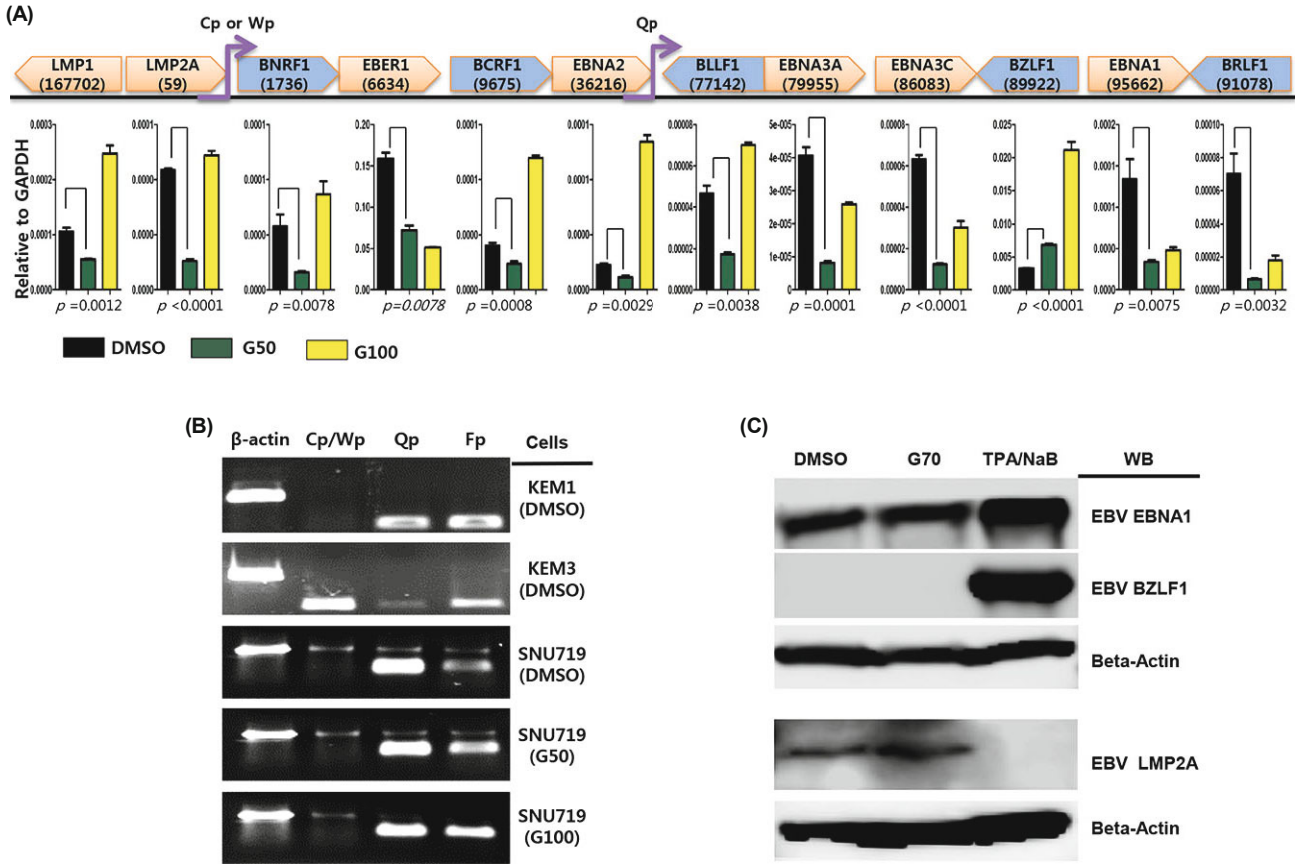


**Fig. 5. Effect of genipin on EBV gene expression.** RT-qPCR assay was conducted to demonstrate the effect of genipin on EBV transcription in SNU719 cells. (A) cDNA synthesized from RNA in SNU719 cells treated with genipin (0, 50, 100 μM) was subjected to RT-qPCR assay. This was followed by determination of relative transcription levels of EBV latent genes and lytic genes. Compared with the DMSO treatment, transcripts from *EBV LMP1*, *EBER1*, *BLLF1*, and *BZLF1* were produced in greater quantity by 50 μM genipin, and *BRLF1* was produced less. However, treatment with 100 μM genipin appeared to enhance transcriptions of all EBV genes tested. Cp, Wp, and Qp stand for EBV promoters activated depending on EBV latency types (Tempera *et al*., 2010). EBV latent and lytic genes were in pink and blue colors, respectively. Statistical significance is when the *P*-value is < 0.05 (95% confidence). RT-PCR assay was conducted to determine the effect of genipin on the selection of EBV latency promoters in EBV associated gastric carcinoma cells (SNU719). (B) KEM1 control cells specific to EBV type I showed high frequency of Q and F promoter usage. KEM3 control cells specific to EBV type III demonstrate a high frequency of C/W promoter usage. Genipin intensively increased the frequency of F promoter usage, but weakly decreased that of Q promoter usage at 100 μM in SNU719 cells. However, genipin did not significantly affect the selection of EBV latency promoters at 50 μM. Cp, Wp, Qp, and Fp stand for EBV promoters activated depending on EBV latency types (Tempera *et al*., 2010). Western blot assay was performed to define the effect of genipin on EBV translation in SNU719 cells. Total proteins were extracted from SNU719 cells treated with genipin and subjected to Western blot analysis. (C) Compared to DMSO treatment, EBV EBNA1 synthesis was not affected, but EBV LMP2A synthesis was slightly enhanced by genipin treatment. DMSO, G70, and NaB/TPA stand for DMSO, genpin 70 μM, NaB (3 mM)/TPA (20 ng/ml) treatments, respectively. Beta-actin was used as an internal control in Western blot analysis.

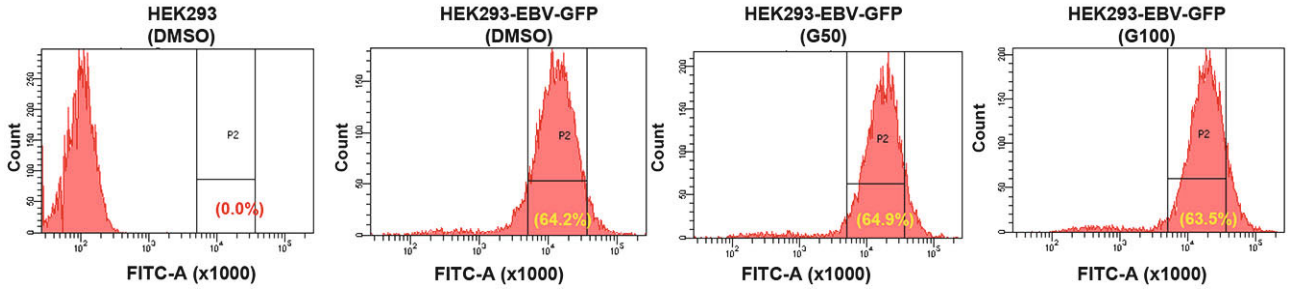


**Fig. 6. Effect of genipin on EBV latency.** Effect of genipin on EBV latency was determined using *in vitro* HEK293-EBV-GFP infection system and FACS analysis. HEK293-EBV-GFP is HEK293 cells transfected with the EBV-GFP bacmid, selected with hygromycin B. HEK293-EBV-GFP system was subjected to the EBV latency test by using a fluorescence microscope. Genipin did not affect the EBV latency at 50 μM (G50), but barely affected the EBV latency at 100 μM (G100).

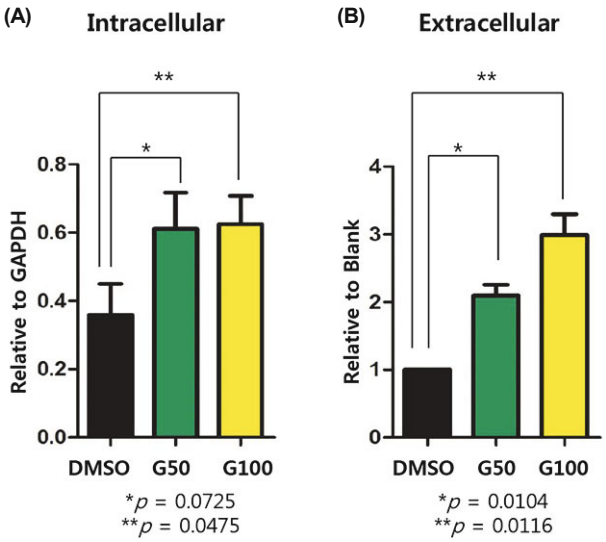


**Fig. 7. Effect of genipin on the production of EBV.** In order to define the effect of genipin on the production of EBV, intracellular (A) and extracellular (B) genome copy numbers of EBV were determined. EBV intracellular and extracellular genome copy numbers in SNU719 were significantly increased by treatment with genipin at 50 μM and 100. DMSO, G50, and G100 stand for DMSO (blank), genipin 50 μM, and genipin 100 μM treatments, respectively. Intracellular and extracellular stand for relative intracellular (relative to GAPDH) and extracellular (relative to blank treatment) EBV genome copy numbers, respectively. Statistical significance is when the *P*-value is < 0.05 (95% confidence).

pression was tested. Western blot analysis was performed on genipin-treated SNU719 cells to analyze EBV protein expression. Unlike the transcription of viral genes, the assay showed that genipin did not greatly affect EBV protein expression (Fig. 5C). EBNA1 and LMP2A expression in SNU719 cells was slightly more when compared with control treatments, while BZLF1 expression remained unchanged by genipin (Fig. 5C). These results indicated that genipin plays a critical role in EBV translation and reactivation of EBV lytic cycle without expression of BZLF1 in a dose-dependent manner.

### Effect of genipin on latency maintenance

The physiological effects produced by genipin on host cells raises the question as to whether genipin causes any defects in EBV latency. Measurement of the FITC emission from FACS Aria III helps determine the cytometric profile of EBV latency in HEK293-EBV-GFP cells treated with genipin. The assay showed that genipin treatment was getting to slightly lose EBV latency in HEK293 cells dependent on genipin concentrations (Fig. 6). These results suggested that EBV latency is not abruptly dismantled by genipin to derive EBV lytic replication.

### Effect of genipin on the production of EBV progeny

The effect of genipin stimulation on EBV progeny production was tested. The intracellular and extracellular EBV genome copy numbers were determined using SNU719 cells, by following methods previously described. Extracellular and intracellular EBV genome copy numbers significantly increased up to 209% and 170% at genipin 50 μM, respectively, and up to 299% and 173% at genipin 100 μM, respectively. These results indicated that genipin efficiently stimulates the production of EBV progeny in a dose-dependent manner.

### Effects of genipin on EBV infection

The strong stimulation provided by genipin for EBV progeny production raised the question as to whether genipin reinforces virus-susceptible cells against EBV infection. LCL-EBV-GFP and AGS cell to cell coinfection assay was designed to test if genipin could reinforce AGS cells against EBV infection. EBV could transfer from LCL-EBV cells to AGS cells using cell to cell coculture infection system. AGS cells infected EBV-GFP virus were in attached form and GFP florescent (Fig. 8A). Treatment of genipin at 70 μM reduced the frequency of EBV infection from LCL-EBV cells to AGS cells (Fig. 8B). These results demonstrate that genipin inhibits the infection of AGS with EBV, indicating that efficient EBV infection requires other unknown factors.

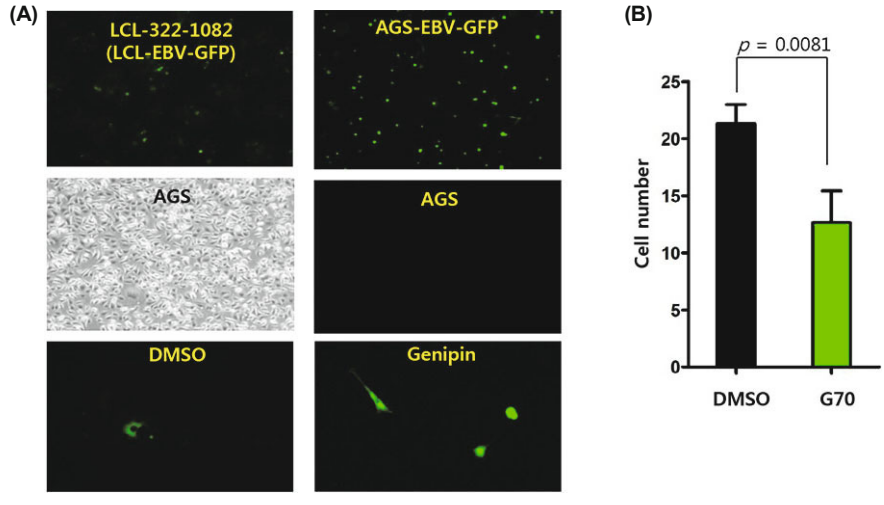


**Fig. 8. Effect of genipin on EBV infection.** The effect of genipin in reinforcing EBV infection to the viral susceptible cells was investigated. Cell to cell coinfection assay using LCL-EBV cells (EBV positive) and AGS cells (EBV negative) was designed to test if genipin could reinforce AGS cells against EBV infection. EBV could transfer from LCL-EBV cells to AGS cells using cell to cell coculture infection system. AGS cells infected EBV-GFP virus were in attached form and GFP florescent (A). Treatment of genipin at 70 μM significantly reduced the frequency of EBV infection from LCL-EBV cells to AGS cells. Statistical significance is when the *P*-value is < 0.05 (95% confidence) (B).

## Discussion

In this study, we showed that genipin isolated from *Gardenia jasminoides* has distinguished antitumor or antiviral activities against gastric carcinoma and EBV. Given our observations, antitumor and antiviral effects of genipin were followed. $CD_{50}$ values of geipin were 70 μM against SNU719 cells (Fig. 1). Genipin induced a strong necrosis/late apoptosis and caused to lose membrane integrity of SNU719 cells (Fig. 2). Genipin showed upregulation of DNMTs and enhanced methylation on cellular and EBV genomes (Fig. 3). Genipin significantly arrested S/G2 transition of SNU719 cells (Fig. 4). Genipin upregulated noticeably EBV latent and lytic genes in dose-dependent manner (Fig. 5). EBV latency was not weakened by genipin (Fig. 6). However, genipin stimulated to produce EBV progeny viruses from SNU719 cells (Fig. 7). Interestingly, infection of EBV from lymphocyte to gastric adenocarcinoma cells were significantly inhibited by genipin (Fig. 8).

Our study shows that genipin stimulates the conversion of latent to lytic replication and the production of EBV progeny. Genipin increased the copy number of the intracellular viral genome, which suggested that genipin causes EBV to enter the lytic replication stage for extracellular viral particles. This feature is observed when histone deacetylase (HDAC) inhibitors such as sodium butyrate (NaB) and 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulate the production of extracellular gammaherpesviral particles from host cells latently infected with gammaherpesvirus (Forrest and Speck, 2008). In the gammaherpesviral life cycle, the increase in intracellular copy number is associated with the high production of extracellular viral particles. Thus, unlike the extracts of most natural products, genipin might target specific factors rather than cellular factors.

It was interesting that genipin upregulated DNMT1 and DNMT3A expression. Our results were not consistent with previous study reported that DNMT3A might methylate the replicated viral genome and support to establish the latent infection (Leonard *et al.*, 2011). In our study, genipin showed to upregulate DNMTs at 70 μM and EBV latent/lytic genes at 100 μM. Resultant DNMTs induction showed to enhance methylation on regions of EBV latent promoters such as C promoter and Q promoter, rather than F promoter. Activation of F promoter is highly linked to EBV lytic replication (Tempera *et al.*, 2010). Further study about effect of genipin on selection of EBV promoters showed that genipin promotes the frequency of use of F promoter rather than Q promoter in a dose-dependent manner, suggesting that genipin derives EBV lytic replication. This trend of promoter selection by genipin might contribute to downregulate EBV latent genes and upregulate EBV lytic genes. Taken together, we speculated that genipin might play dual roles such as a transcriptional activator for EBV lytic genes and suppressor for EBV latent genes in a dose-dependent manner.

Presence of viral progenies at high concentrations in the extracellular environment may cause difficulties in avoiding an immune attack; eventually inducing the host immune system to inactivate the viral progenies or destroy host cells producing viral progenies (Liang *et al.*, 2008; Brulois and Jung, 2014). Since genipin mechanically turns on lytic replication and turns off latent replication in a dose-dependent manner, it is expected to enhance the possibility of production of viral particles that have exhibited defects in viral genome replication and infection. Such a high ratio of defective viral particles may result in the reduction of infectious virus particles in the extracellular environment. In addition to EBV gene regulation, as genipin is known to be an excellent natural cross-linker for proteins (Liu *et al.*, 2013), it is possible that extracellular genipin might bind to EBV attachment proteins such as EBV gp350 and gp42, cause to malfunction these proteins in recognizing cellular EBV receptors such as CD21 and CD35, and finally prevents EBV infections (Young and Rickinson, 2004). At the same time, extracellular genipin binds to cellular EBV receptors and may cause to block cellular EBV receptors from EBV attachment proteins. From these speculations, we proposed at least two mechanisms to produce antitumor and antiviral activities by genipin. One is to disturb EBV transcriptional regulation and the other is to harass EBV recognition for host cells. However, these proposals are required to test by further studies.

Genipin produced cytotoxicity in SNU719 cells latently infected with EBV. This cytotoxicity was not a result of early apoptosis, but that of necrosis or late apoptosis instead. Induction of necrosis or late apoptosis can be assumed to convert viral replication from latent to lytic phase. The SNU719 cells are supposed to die during the EBV lytic replication stage, as lytic replication suspends host cellular metabolism (Kitano *et al.*, 2006). Conversely, viral lytic replication might lead to the induction of necrosis or latent apoptosis in host cells, eventually leading to their death. Indeed, owing to the harsh cellular conditions caused by necrosis induced by genipin, EBV was assumed to quickly trigger lytic replication to produce progeny viruses as well as to escape or release progeny viruses from the dying host cells. However, further studies are needed to clarify this speculation.

Fruit from *G. jasminoides* is most popularly used as tea for treatment of certain disorders. This is because the fruit is known to contain rich reserves of genipin, which affect a diverse range of bioactivities (Liu *et al.*, 2013; Son *et al.*, 2013). Our study demonstrates that genipin acts as an antiviral and antitumor agent against EBV and EBVaGC. Thus, the fruit of *G. jasminoides* can be used to protect host cells from EBV infection, as well as the development of disorders due to EBV infection. Future studies must focus on the development of the fruit of *G. jasminoides* into a medicinal food for the prevention of gastric carcinoma or lymphoma.

This novel study highlights a fundamental possibility of the development of *G. jasminoides* as a medicinal food in order to prevent EBV infections and the cancers caused by such infections.

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