Cytotoxic effects of NF-κB inhibitors in combination with anti-herpes agents on Epstein-Barr virus-positive gastric carcinoma *in vitro*

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Abstract. Epstein-Barr virus (EBV) infection in tumor cells is usually restricted to the latent form, indicating that the induction of viral lytic infection may present a novel approach for the treatment of EBV-associated tumors. By contrast, EBV lytic replication is inhibited by high-levels of nuclear factor (NF)-κB, which suggests that NF-κB inhibitors may activate lytic replication from the latent form. In the current study, the addition of NF-κB inhibitors (Bay11-7082, Z-LLF-CHO and aspirin) was observed to induce the EBV lytic genes *BZLF1, BRLF1* and *BMRF1* in EBV-positive gastric cancer (GC) cells. Both EBV-positive and -negative GC cells were treated with different concentrations of anti-herpes agents and the cytotoxic effects were measured at different time points following induction of EBV lytic replication. A marginal dose- and time-dependent reduction in cell viability was observed for EBV-positive and-negative GC cells. The cytotoxic effects of NF-κB inhibitors on EBV-positive GC cells were enhanced by the addition of the anti-herpes agents, ganciclovir, acyclovir, foscarnet and brivudine ($P<0.05$). However, there was no significant synergistic effect on EBV-negative GC cells. The combination of 5 mM aspirin and ganciclovir exhibited the highest cytotoxic effect in EBV-positive GC cells (CC_{50}=7.2 μg/ml).

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Introduction

γ-Herpesviruses, including Epstein-Barr virus (EBV), are characterized by two distinct life cycles: Latency and lytic infection, and EBV-associated malignant cells are in the latent form. In this status, the infected cells are poorly recognized by the host immune system due to the fact only certain viral gene products are expressed. Thus, the virus is allowed to persist in cells for long periods. By contrast, lytic replication is unfavorable for the virus, as the host cells cannot survive.

There are various factors, both host and viral, that regulate viral reactivation *in vivo*. Regarding the viral factor in EBV infection, latent membrane protein 1 suppresses the virus reactivation in a nuclear factor (NF)-κB dependent manner (1). EBV-encoded small RNA also mediates the NF-κB induction via reactivation of retinoic acid inducible gene I and its downstream signal pathway, the inhibitor of κB (IκB) kinase (IKK)α/IKKβ pathway (2). Brown *et al* (3), demonstrated that NF-κB inhibitors led to lytic replication in EBV-positive lymphocytes and epithelial cells, suggesting that NF-κB may be a potential target to disrupt virus latency. They additionally demonstrated the different thresholds for reactivation via inhibition of NF-κB among different cell lines.

Acetylsalicylic acid (aspirin) is a non-steroidal anti-inflammatory drug commonly used due to its known safety record and and reasonable price. It has been reported that aspirin inhibits NF-κB activity by inhibiting IKK activity and thereby blocking IκBα degradation in the cytoplasm (4,5). Liu *et al* (6) demonstrated that incubation of the EBV-positive malignant cell lines B95-8 and Raji with aspirin depleted NF-κB (p65) and resulted in EBV lytic replication, which consequently reduced the viability of EBV-positive B lymphocytes. Notably, combination treatment with an anti-herpes agent, ganciclovir, was observed to enhance the cytotoxic effects only in EBV-positive cells (6). Similar results using other NF-κB inhibitors, Bay11-7802 and Z-LLF-CHO, were also reported by the same research group (7).

Commonly used anti-herpes agents can be chemically classified into three groups: i) Nucleoside analogs, ii) nucleotide analogs and iii) pyrophosphate analogs (8). Although there are

several anti-herpes agents, ganciclovir and acyclovir, which are nucleoside analogs, have been considered as standard treatments for herpes simplex virus (HSV), varicella-zoster virus (VZV) and cytomegalovirus (CMV). These drugs are monophosphorylated by the viral-encoded protein kinase (PK) or thymidine kinase (TK), and later converted into deoxyguanosine-triphosphate (dGTP) by cellular kinases. A previous study demonstrated that ganciclovir and acyclovir are monophosphorylated by the EBV-encoded PK (EBV-PK), however not the EBV-encoded thymidine kinase (EBV-TK) prior to being converted into dGTP (9). Brivudine, an additional nucleoside analog, which is an alternative for ganciclovir and acyclovir, requires the viral-TK for both mono- and di-phosphorylation (10). For the treatment of the drug-resistant strain, foscarnet, a pyrophosphate analog, is also applied in clinical use. Foscarnet directly inhibits the pyrophosphate binding site on viral DNA polymerases without requiring activation by viral kinases (11).

EBV has been demonstrated to be a cause of gastric carcinoma called as EBV-positive gastric cancer (GC) (12-14). Due to the fact that the episomal EBV genome is detected in almost all gastric tumor cells, however not in neighboring normal epithelial cells, the combination treatment of the lytic induction strategy with cytotoxic drugs such as ganciclovir, which is converted to its active form by the lytic form of EBV infection, is expected to selectively destroy tumor cells (15). Ji Jung *et al* (16) demonstrated the lytic induction by 5-fluorouracil, cisplatin and taxol, and the enhancement of lytic replication and apoptosis with the combination of ganciclovir in an EBV-positive GC cell line, SNU-719, which is naturally infected with EBV. However, these chemotherapeutic agents are also cytotoxic for normal cells and, thus, safer agents are required for the induction of lytic replication. Furthermore, it would be beneficial to examine the effects in combination with other anti-herpes agents rather than ganciclovir, which cannot be used for the drug-resistant strain although it is the first-line agent against HSV, CMV and VZV infections. Liu *et al* (6,7) demonstrated the induction of lytic replication by NF-κB inhibitors including aspirin in EBV-positive lymphocytes, however their effects on EBV-positive GC cells remain unclear. Thresholds for reactivation via NF-κB inhibition may vary among different cell lines, thus it is warranted to confirm the induction of lytic replication by NF-κB inhibitors using epithelial cell lines (3).

To determine an effective combination of lytic inducers and anti-herpes agents leading to selective cytotoxicity of EBV-positive GC cells, the cytotoxic effects of the NF-κB inhibitors {aspirin, Bay11-7082 [(E)-3-(4-methylphenylsulfonyl)-2-propenenenitrile] and Z-LLF-CHO (benzyloxycarbonyl-t-leucyl-L-leucyl-t-phenylalanilal)} in combination with the anti-herpes agents (ganciclovir, acyclovir, brivudine and foscarnet) were examined in EBV-positive and-negative GC cells.

Materials and methods

Cell lines. The human GC cell lines, SNU-216 (EBV-negative) and SNU-719 (EBV-positive), which are moderately differentiated adenocarcinomas, were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics at 37˚C in a humidified 5% CO_2 incubator.

Chemical agents. Acetylsalicylic acid (aspirin), Bay11-7082, Z-LLF-CHO, ganciclovir, acyclovir, foscarnet, brivudine and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma-Aldrich. TPA was used as a positive control for lytic replication induction. Stock solutions of aspirin (100 mM), Bay11-7082 (20 mM), Z-LLF-CHO (20 mM), TPA (1.6 mM), and 10 mg/ml anti-herpes agents: Ganciclovir (39.2 mM), acyclovir (44.4 mM), foscarnet (79.4 mM) and brivudine (30.0 mM), were prepared in dimethyl sulfoxide (DMSO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to test cell viability (MTT Cell Proliferation Assay; American Type Culture Collection, Manassas, VA, USA).

Cytotoxicity assays. MTT assays were used to assess the effects of NF-κB inhibitors on cell viability. Briefly, EBV-positive SNU-719 and EBV-negative SNU-216 cells were plated in 96-well cell culture plates with serial dilutions of the NF-κB inhibitors for 0, 2, 4, 6 and 8 days. In addition, *in vitro* cell viability assays were performed on EBV-positive SNU-719 and EBV-negative SNU-216 cells using NF-κB inhibitors in combination with the anti-herpes agents. In the baseline experiments, different concentrations of the compounds were used. Subsequently, cells were plated in 96-well cell culture plates with doses selected from baseline experiments of aspirin (5 mM), Bay11-7082 (20 μM) or Z-LLF-CHO (5 μM). Only the most promising NF-κB inhibitors that blocked IκB kinase were selected. After 24 h, 100 μg/ml anti-herpes agents: Ganciclovir (392 μM), acyclovir (444 μM), foscarnet (794 μM) or brivudine (300 μM), were added, and cells were incubated for 2, 4, 6 and 8 days at 37˚C. In additional experiments, cells were treated with different concentrations of NF-κB inhibitors in combination with anti-herpes agents for 8 days. Cells were also treated with NF-κB inhibitors in combination with different concentrations of anti-herpes agents (10, 100 or 200 μg/ml) for 0, 2, 4, 6 and 8 days. After the indicated times, the plates were incubated with MTT solution for 4 h at 37˚C. Detergent reagent was then added, and the absorbance was measured at 570 nm using a microplate reader. The percentage of viable cells was set at 100% for untreated controls.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for BZLF1, BRLF1 and BMRF1. EBV-positive SNU-719 cells were grown to 70% confluence and treated with aspirin (1, 5 and 10 mM), Bay11-7082 (10, 20 and 30 μM), Z-LLF-CHO (1, 5 and 10 μM) or TPA (20 ng/ml, 32 nM) for 24 h. Non-treated cells and DMSO-treated cells were used as negative controls. Total RNA was extracted from $3x10^6$ cells using the the RNeasy Mini kit (Qiagen, Hombrechtikon, Switzerland), according to the manufacturer's instructions. *DNase* I (Roche Diagnostics Japan, Tokyo, Japan) treatment was performed prior to cDNA synthesis with the Advantage RT-for-PCR kit (Clontech Laboratories, Inc., Mountain View, CA, USA). RT-qPCR was performed with validated TaqMan systems for the housekeeping gene glyceraldehyde 3-phosphate

dehydrogenase (*GAPDH*; VIC/MGB Probe, primer limited; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as an endogenous control and the lytic EBV genes *BZLF1*, *BRLF1* and *BMRF1* using an ABI 7200 Cycling System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: *BZLF1*, forward: 5'-AAA TTTAAGAGATCCTCGTGTAAAACATC-3' and reverse: 5'-CGCCTCCTGTTGAAGCAGAT-3' and the probe was 5'-(FAM) ATAATGGAGTCAACATCCAGGCTTGGGC (TAMRA)-3'; *BRLF1*, forward: 5'-GAGTCCATGACAGAG GATTTGA-3' and reverse: 5'-GCAGCAGACATTCATCAT TTAGA-3' and the probe was 5'-(FAM) ATGTATCCAAGA TTTCATTAAGTTCG (TAMRA)-3'; *BMRF1*, forward: 5'-CAACACCGCACTGGAGAG-3' and reverse: 5'-GCCTGC TTCACTTTCTTGG-3' and the probe was5'-(FAM) ATC GTCGGAGGCCAGGCAGAAGCAGAAGC (TAMRA)-3'. Sequences of primers and probes were as previously described by Ryan *et al* (17) and Hilscher *et al* (18). Each TaqMan gene expression assay consisted of a fluorogenic dye-labelled probe (10 μM; 1.25 μl), two amplification primers (forward and reverse of 100 μM) 0.45 μl each, PCR master mix (25 μl) and the TaqMan endogenous control (2.5 μl). The real-time PCR reactions were run for 25 cycles using cycling conditions (94˚C for 45 sec, followed by 60˚C for 45 sec and 72˚C for 2 min, and a final extension at 72˚C for 7 min). TaqMan data were analyzed using SDS software, version 2.2 (Applied Biosystems; Thermo Fisher Scientific, Inc.), and mRNA expression was normalized to *GAPDH* mRNA. RT-qPCR data for the control vector were set to 1, and the expression of lytic genes was compared to the control. All experiments were performed in triplicate.

RT-qPCR for RelA and RelB. For RT-qPCR, total RNA was isolated from the control as well as treated cells of SNU-719 using RNeasy Mini kit (Qiagen). cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). Expressed genes were detected quantitatively using a LightCycler® 2.0 Instrument (Roche Diagnostics Japan) with LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics Japan) according to the manufacturer's instructions.

The primers for the genes were purchased from FASMAC Co., Ltd. (Atsugi-shi, Japan). PCR amplification was performed in a total volume of 20 μl containing cDNA, each primer (0.5 μM) and master mix supplied by the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics Japan). The PCR cycling conditions were as follows: 95˚C for 10 sec, followed by 45 cycles at 95˚C for 10 sec and 60˚C for 10 sec, and 72˚C for 15 sec. The fluorescent product was determined at the end of the 72˚C temperature step. All PCR assays were performed a minimum of four times. The sequences of the primers used were as follows: *GADPH*, forward 5'-GCCTCC TGCACCACCAACTG-3' and reverse 5'-GACGCCTGCTTC ACCACCTTCT-3'; *RelA*, forward 5'-CTGCCGGGATGGCTT CTAT-3' and reverse 5'-CCGCTTCTTCACACACTGGAT-3'; and *RelB*, forward 5'-TCCCAACCAGGATGTCTAGC-3' and reverse 5'-AGCCATGTCCCTTTTCCTCT-3'. The data obtained were analyzed using the LightCycler analysis software version 4.1 (Roche Diagnostics Japan). To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. Threshold cycle values of the target genes were normalized to those of the internal control genes. Values are presented as the mean ± standard error of three experiments.

Statistical analysis. All results are expressed as the mean of triplicate assays ± standard error. The results were tested for significance using the Mann-Whitney U test using Stata software, version 9.2. (StataCorp, College Station, TX, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of NF-κB inhibitors on EBV-positive and EBV-negative GC cells. The effects of different concentrations of NF-κB inhibitors on the cell viability of EBV-positive and EBV-negative GC cells were examined (Fig. 1). Aspirin, Bay11-7082 and Z-LLF-CHO reduced the cell viability of the two GC cell lines in a dose-dependent manner. No significant difference was observed between the effects observed in EBV-positive and EBV-negative cells. The cytotoxic effect of Z-LLF-CHO did not increase significantly over concentrations ranging from 1 to 10 μM or after 8 days of treatment.

Effect of NF-κB inhibitors in combination with anti-herpes agents. The combined effects of NF-κB inhibitors and anti-herpes agents on the cell viability were observed. In the baseline experiments, treatment with different doses of anti-herpes agents alone did not influence the cytotoxicity. Thus, presented are the optimal doses from the combination assays. The cytotoxic effect of 5 mM of aspirin, 20 μM Bay11-7082 and 5 μM Z-LLF-CHO on SNU-719 cells was significantly enhanced by the addition of 100 μg/ml ganciclovir (392 μM), acyclovir (444 μM), foscarnet (794 μM) or brivudine (300 μM) ($P<0.05$ for all co-treatment; Fig. 2A). To give a specific example, the combination of ganciclovir and increasing concentrations of NF-κB inhibitors reduced the cell viability of SNU-719 by 60% in a dose-dependent manner, while NF-κB inhibitors alone slightly reduced cell viability (Fig. 2B). In contrast, NF-κB inhibitors with anti-herpes agents had a negligible effect on cell viability in SNU-216 cells (Fig. 3A). Additionally, the cytotoxic effects of increasing concentrations of NF-κB inhibitors on SNU-216 cells was not significantly different with or without ganciclovir (Fig. 3B). Finally, increasing concentrations of ganciclovir enhanced the cytotoxic effects of NF-κB inhibitors in SNU-719 cells in a dose-dependent manner (Fig. 4A). The same effect was not observed in SNU-216 cells (Fig. 4B). Thus the cytotoxic effect of ganciclovir was always seen as EBV-dependent.

Cytotoxic concentrations of NF-κB inhibitors and anti-herpes agents. Cytotoxic concentrations required to reduce the growth of cells by 50% (CC_{50}) were determined for NF-κB inhibitors and anti-herpes agents in EBV-positive and EBV-negative GC cells. Aspirin alone exhibited a CC_{50}>10,000 μM in EBV-positive and EBV-negative cells (Table I). However, the addition of 100 μg/ml of an anti-herpes agents increased the cytotoxic effect, with the combination of aspirin/acyclovir resulting in the greatest effect (CC_{50}=235 μM). Bay11-7082 and Z-LLF-CHO resulted in CC_{50}>30 μM and CC_{50}>10 μM,

Table I. Cytotoxic effect of NF-κB inhibitors in combination with 100 μg/ml anti-herpes agents in EBV-positive/negative GC cells.

NF-κB inhibitor + anti-herpes agent	CC_{50}[a] (μM) of NF-κB inhibitors[b]		Selectivity index[c]
	EBV-positive GC	EBV-negative GC	
Aspirin	>10,000	>10,000	>1
Aspirin + ganciclovir (392 μM)	675±7.2	>10,000	>15
Aspirin + acyclovir (444 μM)	235±15.8	>10,000	>43
Aspirin + foscarnet (794 μM)	338±20.5	>10,000	>30
Aspirin + brivudine (300 μM)	1,230±13.2	>10,000	>8
Bay11-7082	>30	>30	>1
Bay11-7082 + ganciclovir (392 μM)	16.5±3.3	>30	>2
Bay11-7082 + acyclovir (444 μM)	14.5±4.1	>30	>2
Bay11-7082 + foscarnet (794 μM)	10.6±2.5	>30	>3
Bay11-7082 + brivudin (300 μM)	13.6±3.2	>30	>2
Z-LLF-CHO	>10	>10	>1
Z-LLF-CHO + ganciclovir (392 μM)	3.5±1.2	>10	>3
Z-LLF-CHO + acyclovir (444 μM)	2.4±0.5	>10	>4
Z-LLF-CHO + foscarnet (794 μM)	1.8±0.6	>10	>6
Z-LLF-CHO + brivudin (300 μM)	2.7±0.6	>10	>4

[a]Cytotoxic concentration following 8 days of incubation (concentration required to reduce the growth of cells by 50%). [b]Data are presented as the mean values for three independent experiments ± standard deviation. [c]Selectivity index (cytotoxicity in EBV-negative GC cells/cytotoxicity in EBV-positive GC cells). NF-κB, nuclear factor κB; EBV, Epstein-Barr virus; GC, gastric cancer.

Table II. Cytotoxic effect of anti-herpes agents in combination with NF-κB inhibitors in EBV-positive and EBV-negative GC cells.

Anti-herpes agent + NF-κB inhibitor	CC_{50}[a] (μM) of anti-herpes agent[b]		Selectivity index[c]
	EBV-positive GC	EBV-negative GC	
Ganciclovir	>784	>784	>1
Ganciclovir + Aspirin (5 mM)	28±9.0	>784	>28
Ganciclovir + Bay11-7082 (20 μM)	379±9.0	>784	>2
Ganciclovir + Z-LLF-CHO (5 μM)	543±18.0	>784	>1
Acyclovir	>888	>888	>1
Acyclovir + Aspirin (5 mM)	161±8.9	>888	>6
Acyclovir + Bay11-7082 (20 μM)	348±32.4	>888	>3
Acyclovir + Z-LLF-CHO (5 μM)	561±48.0	>888	>2
Foscarnet	>1587	>1587	>1
Foscarnet + Aspirin (5 mM)	189±32.5	>1587	>8
Foscarnet + Bay11-7082 (20 μM)	519±40.5	>1587	>3
Foscarnet + Z-LLF-CHO (5 μM)	1351±97.6	>1587	>1
Brivudine	>600	>600	>1
Brivudine + Aspirin (5 mM)	139±10.2	>600	>4
Brivudine + Bay11-7082 (20 μM)	330±45.6	>600	>2
Brivudine + Z-LLF-CHO (5 μM)	569±22.5	>600	>1

[a]Cytotoxic concentration following 8 days of incubation (concentration required to reduce the growth of cells by 50%). [b]Data are presented as the mean values for three independent experiments ± standard deviation. [c]Selectivity index (cytotoxicity in EBV-negative GC cells/cytotoxicity in EBV-positive GC cells). NF-κB, nuclear factor κB; EBV, Epstein-Barr virus; GC, gastric cancer.

respectively, and their cytotoxic effects were enhanced by the addition of an anti-herpes agent (Table I). The cytotoxic concentrations of anti-herpes agents were also determined in the two cell lines (Table II). Ganciclovir in combination with 5 mM of aspirin resulted in the greatest cytotoxic effect (CC_{50}=7.2 μg/ml, 28.2 μM).

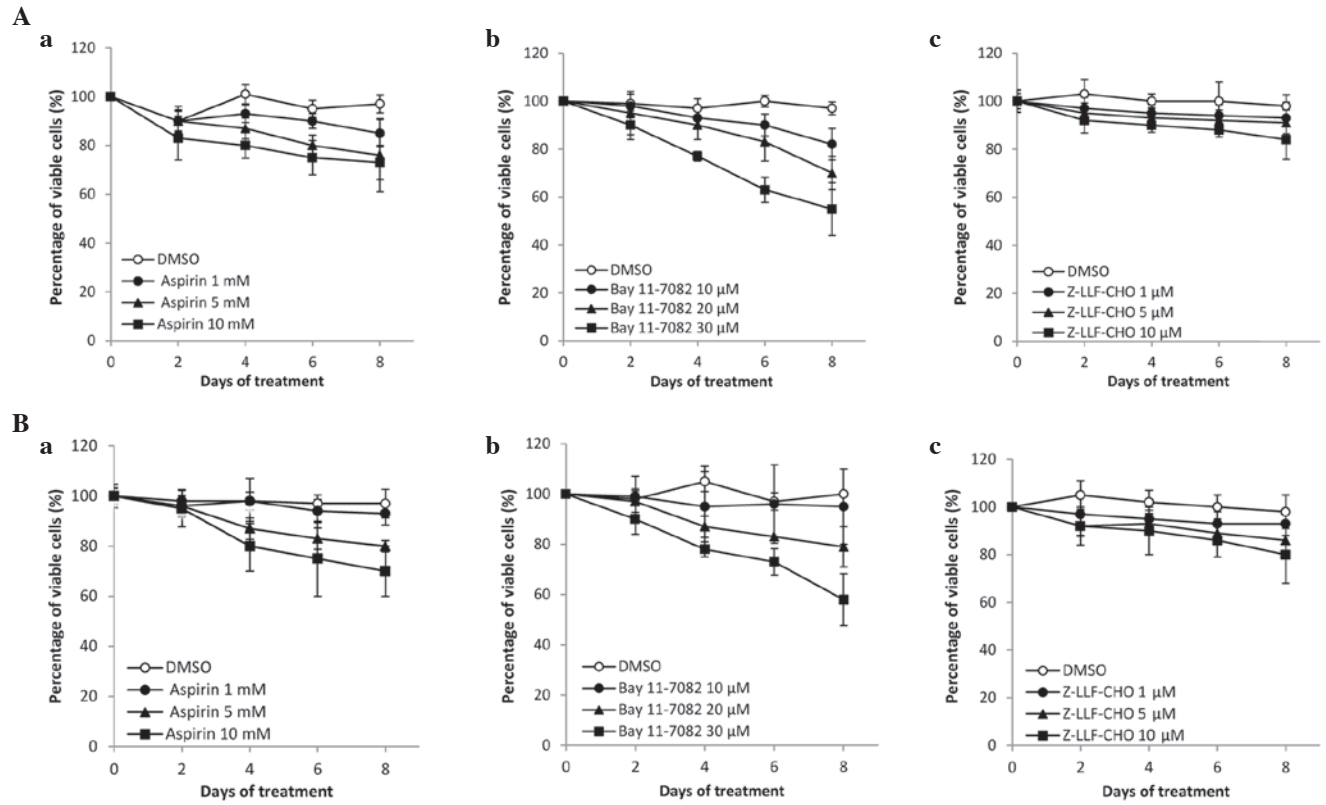

Figure 1. Effect of NF-κB inhibitors on EBV-positive and EBV-negative gastric cancer cells. (A) EBV-positive SNU-719 and (B) EBV-negative SNU-216 cells were incubated with serial dilutions of (a) aspirin, (b) Bay11-7082 and (c) Z-LLF-CHO at the indicated concentrations. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed on the indicated days of treatment. Values are presented as the mean ± standard error of three independent experiments. NF-κB, nuclear factor κB; EBV, Epstein-Barr virus; DMSO, dimethyl sulfoxide.

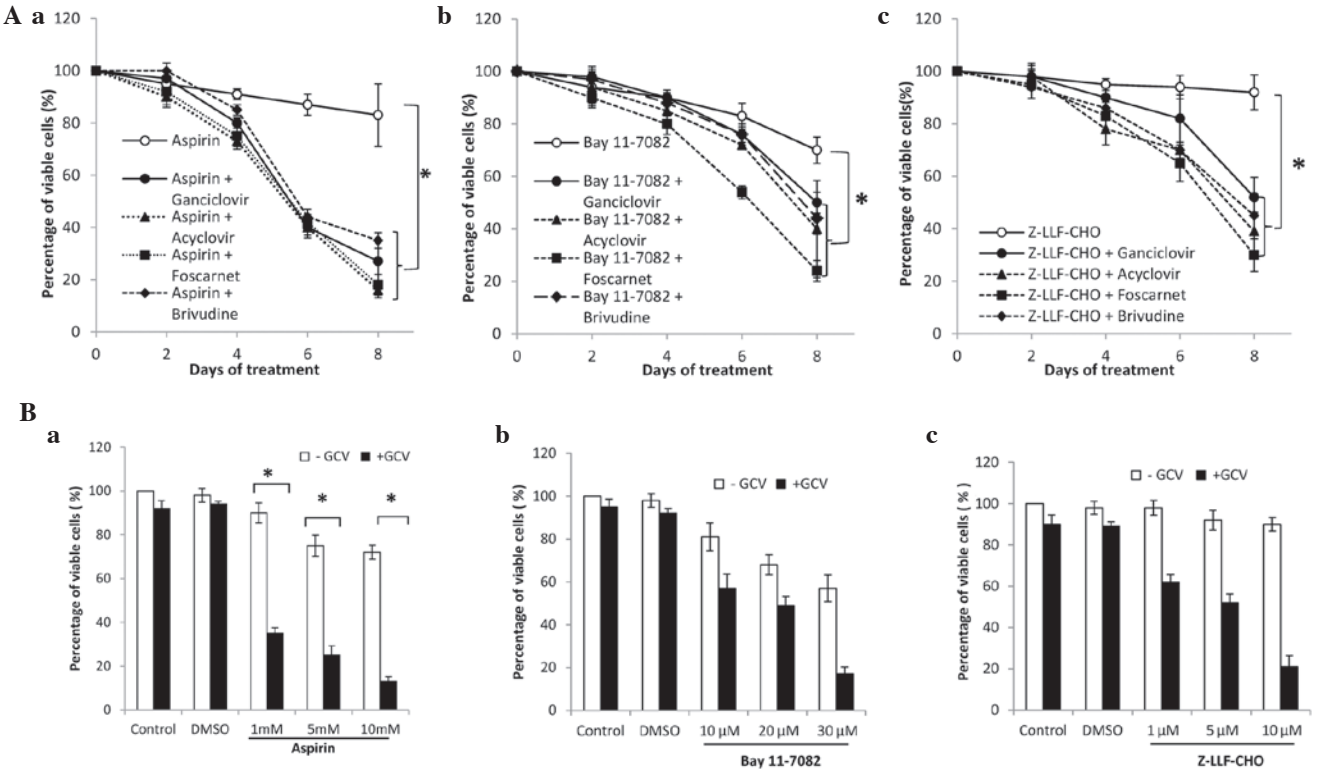

Figure 2. Effect of NF-κB inhibitors in combination with anti-herpes agents in SNU-719 cells. (A) (a) Aspirin (5 mM), (b) Bay11-7082 (20 μM) and (c) Z-LLF-CHO (5 μM) were used in combination with GCV, acyclovir, foscarnet or brivudine (100 μg/ml). An MTT assay was performed following 0, 2, 4, 6 and 8 days of treatment. (B) Cells were treated with the indicated concentrations of (a) aspirin, (b) Bay11-7082 and (c) Z-LLF-CHO in combination with anti-herpes agents (100 μg/ml). The MTT assay was performed following 8 days of treatment. The percentage of viable cells was set at 100% for untreated controls. Values are presented as the mean ± standard error of three independent experiments (*P<0.05). NF-κB, nuclear factor κB; GCV, ganciclovir; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EBV, Epstein-Barr virus; DMSO, dimethyl sulfoxide.

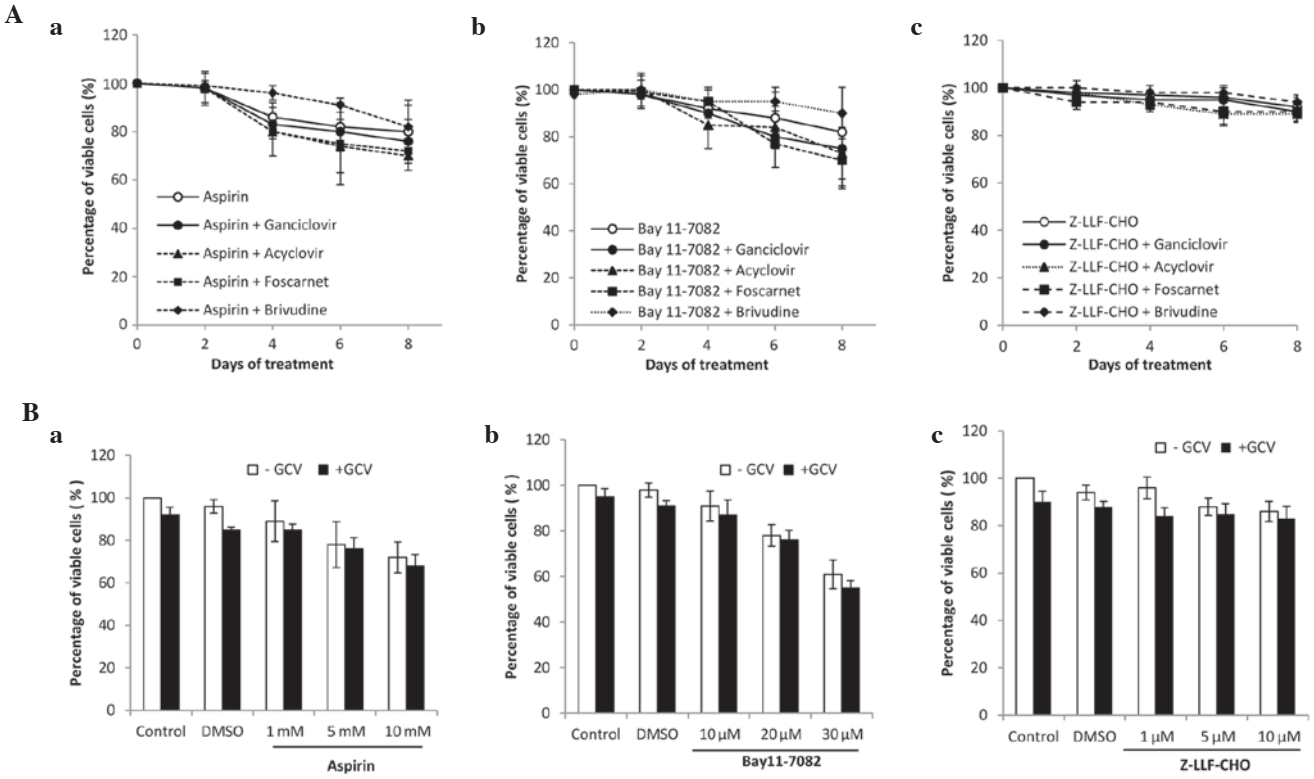

Figure 3. Effects of NF-κB inhibitors in combination with anti-herpes agents in SNU-216 cells. (A) (a) Aspirin (5 mM), (b) Bay11-7082 (20 μM) and (c) Z-LLF-CHO (5 μM) were used in combination with GCV, acyclovir, foscarnet or brivudine (100 μg/ml). The MTT assay was performed following 0, 2, 4, 6 and 8 days of treatment. (B) Cells were treated at the indicated concentrations of (a) aspirin, (b) Bay11-7082 and (c) Z-LLF-CHO in combination with anti-herpes agents (100 μg/ml). The MTT assay was performed after 8 days of treatment. The percentage of viable cells was set at 100% for untreated controls Values are presented as the mean ± standard error of three independent experiments. NF-κB, nuclear factor κB; GCV, ganciclovir; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

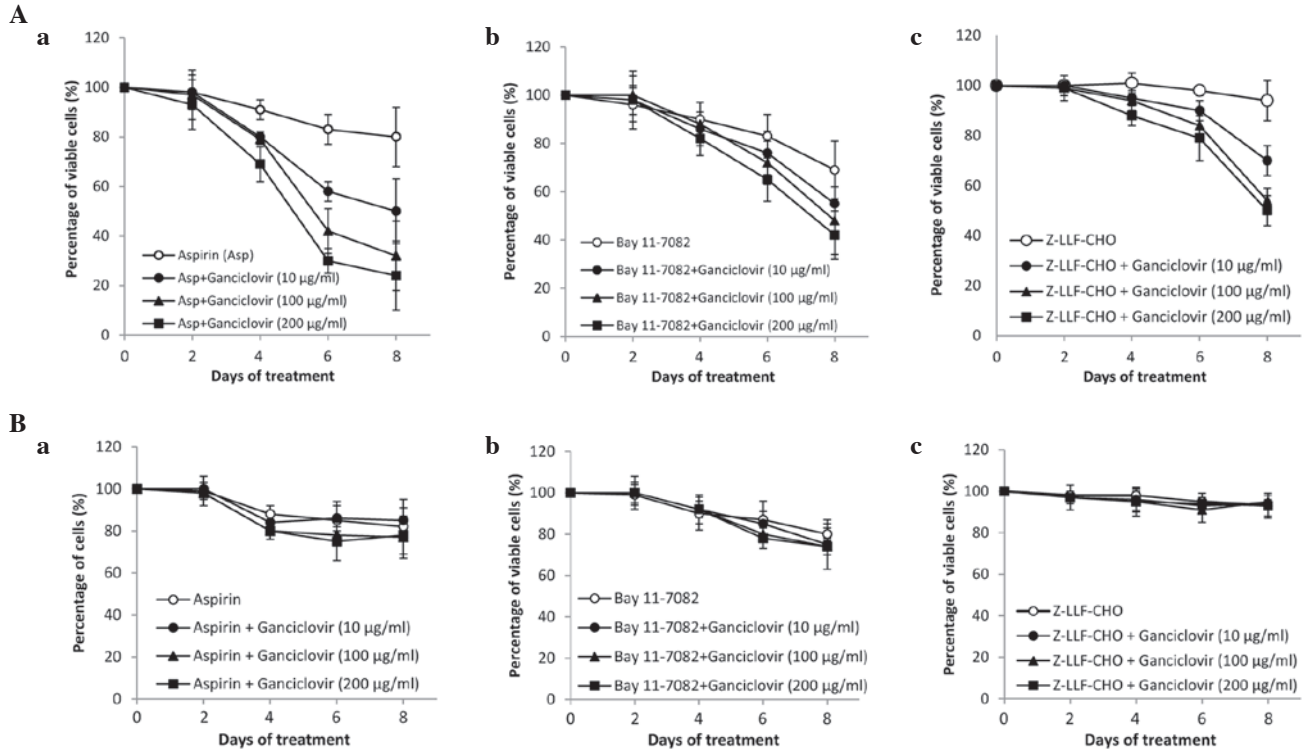

Figure 4. Effect of NF-κB inhibitors in combination with different concentrations of ganciclovir. (A) In SNU-719 cells and (B) SNU-216 cells, (a) aspirin (5 mM), (b) Bay11-7082 (20 μM) and (c) Z-LLF-CHO (5 μM) were used in combination with ganciclovir (10, 100 or 200 μg/ml corresponding to 39.2, 392 and 784 μM, respectively). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed after 0, 2, 4, 6 and 8 days of treatment. The percentage of viable cells was set as 100% for untreated controls. Values are presented as the mean ± standard error of three independent experiments. NF-κB, nuclear factor κB; Asp, aspirin; DMSO, dimethyl sulfoxide.

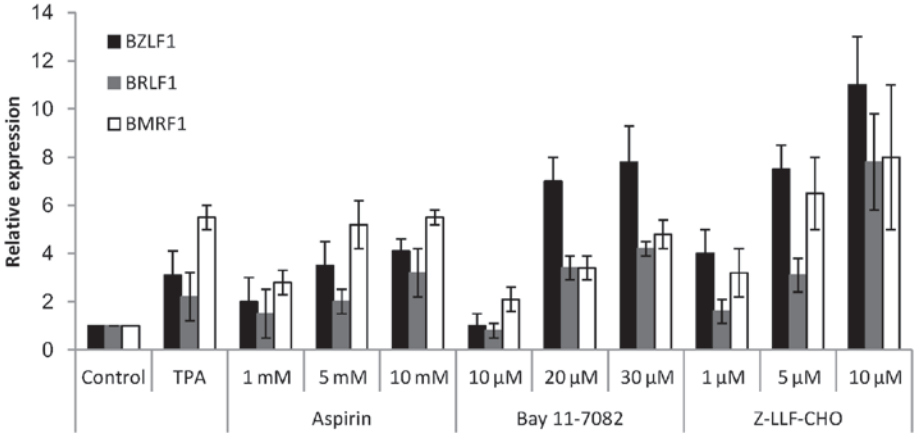

Figure 5. Activation of EBV lytic genes by NF-κB inhibitors. EBV-positive SNU-719 cells were treated with different concentrations of aspirin, Bay11-7082 or Z-LLF-CHO for 24 h. Total RNA was extracted and expression levels of the EBV lytic genes *BZLF1*, *BRLF1* and *BMRF1* were quantified by reverse transcription-quantitative polymerase chain reaction. Treatment with TPA was used as a positive control. Untreated cells and dimethyl sulfoxide-treated cells were used as negative controls. The glyceraldehyde 3-phosphate dehydrogenase gene was amplified as an internal control. Values are presented as the mean ± standard error of three independent experiments. EBV, Epstein-Barr virus; NF-κB, nuclear factor κB; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

Activation of EBV lytic genes by NF-κB inhibitors. To explain the cytotoxic effects of NF-κB inhibitors in combination with anti-herpes agents, the induction of EBV lytic genes by NF-κB inhibitors was investigated in EBV-positive GC cells. SNU-719 cells were treated with different concentrations of NF-κB inhibitors: Aspirin at 1, 5 and 10 mM; Bay11-7082 at 10, 20 and 30 μM; and Z-LLF-CHO at 1, 5 and 10 μM. Subsequent to 24 h treatment, EBV lytic reactivation was confirmed by measuring the expression levels of the lytic genes *BZLF1*, *BRLF1* and *BMRF1*. All three inhibitors induced the expression of lytic genes in a dose-dependent manner (Fig. 5). *BMRF1* expression levels subsequent to induction by NF-κB inhibitors were similar to the level induced by TPA (Fig. 5). However, induction of the immediate early genes, *BZLF1* and *BRLF1,* by NF-κB inhibitors was higher than the induction by TPA, in particular when using 10 μM Z-LLF-CHO (Fig. 5).

Inhibition of RelA and RelB. Based on the results of the current study, it was suggested that aspirin was the most promising NF-κB inhibitor in the SNU-719 cell line. To understand whether EBV reactivation occurred though NF-κB, the mRNA expression levels of *RelA* and *RelB*, which are the subunits of NF-κB, were examined. SNU-719 cells were treated with different concentrations of aspirin (0, 1, 5, 10 mM), and after 24 h the cells were harvested and examined for the gene expression using RT-qPCR. *RelA* and *RelB* activity was observed to be high prior to the addition of aspirin, and a dose-dependent reduction was observed following inhibition (Fig. 6).

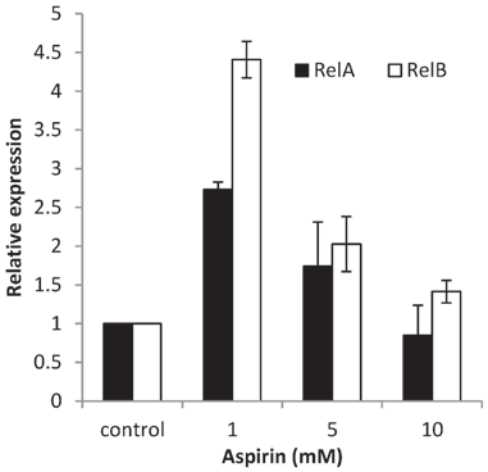

Figure 6. The mRNA expression levels of *RelA* and *RelB* following treatment with aspirin in SNU-719 cells. EBV-positive SNU-719 cells were treated with different concentrations of aspirin and then total RNA was extracted and expression levels of the *RelA* and *RelB* were quantified by reverse transcription-quantitative polymerase chain reaction.

Discussion

In the present study, the cytotoxic effects of the NF-κB inhibitors (aspirin, Bay11-7082 and Z-LLF-CHO), in combination with four anti-herpes agents; ganciclovir, acyclovir, brivudine, and foscarnet, using EBV-positive and-negative GC cells. The cytotoxic effects of NF-κB inhibitors on EBV-positive GC cells were enhanced by the addition of anti-herpes agents (Fig. 2). However, no significant alterations in cytotoxicity in EBV-negative GC cells were observed (Fig. 3). The combination of aspirin and ganciclovir resulted in the lowest CC_{50} of any anti-herpes agent, 7.2 μg/ml (28.2 μM), in EBV-positive SNU-719 GC cells (Table I). In contrast, the CC_{50} of this combination in the EBV-negative SNU-216 GC cells was greater than 200 μg/ml (>783.6 μM). These observations are consistent with the results of a previous study using EBV-positive and -negative B-lymphocytes (6). Jeong *et al* (19) reported that the inhibition of NF-κB by NF-κB/p65-specific small interfering RNA induced a near total cessation of cell proliferation in EBV-positive GC cells, however variably affected cell proliferation in EBV-negative GC cells, indicating that NF-κB inhibition may be beneficial in the treatment of EBV-positive GC.

NF-κB inhibitors reduced cell viability of EBV-positive and EBV-negative GC cells in a dose- and time-dependent

manner (Fig. 1). However, the cytotoxic effects of Z-LLF-CHO in the two cell lines were minor compared with the two other NF-κB inhibitors. Z-LLF-CHO, a reversible proteasome inhibitor, inhibits nuclear translocation of NF-κB (20). By contrast, aspirin and Bay11-7082 act on the stabilization of IκBα, resulting in reduced NF-κB expression. Bay11-7-82 is an irreversible inhibitor of IκBα phosphorylation (21), and aspirin can inhibit IκB kinase activity thereby blocking IκBα degradation (5). The weak cytotoxic effects of Z-LLF-CHO may be partially explained by the difference in the site of inhibitory function among these NF-κB inhibitors.

The cytotoxic effects of NF-κB inhibitors varied among the combinations of anti-herpes agents. Due to the fact that foscarnet, an inhibitor of viral DNA polymerase does not require activation by viral TK/PK, no increase of the cytotoxicity of the NF-κB inhibitors was expected. However, marginally increased enhancement of the cytotoxicity of NF-κB inhibitors was observed in EBV-positive GC cells with combination treatment with foscarnet (Fig. 2). Notably, similar observations have been reported in the study using BCBL-1 cells (20). The number of viable HHV-8-positive cells was further reduced by co-treatment of foscarnet and valoriate, which was more capable of inducing lytic replication of HHV-8-positive cells than that of treatment with foscarnet alone (22). Although the exact mechanism of this phenomenon remains unclear, foscarnet is suggested to act on virus-infected cells effectively in the lytic replication stage.

Regarding the cytotoxicity of aspirin, the most efficient combination with a fixed concentration of anti-herpes agent was acyclovir/aspirin (235 μM) followed by foscarnet/aspirin (338 μM), ganciclovir/aspirin (675 μM) and brivudine (1230 μM) (Table I). However, these orders were not consistent with those when anti-herpes agents were combined with higher concentrations of aspirin (5 mM) (Table II). Ganciclovir and foscarnet exhibited the greatest and the least efficient cytotoxity, respectively. Further investigation into the synergistic effects between NF-κB inhibitors and anti-herpes agents is required to explain this discrepancy.

In the present study, the expression levels of the immediate-early genes *BZLF1* and *BRLF1*, which have been reported to induce the entire program of lytic EBV gene expression, were determined (23). The expression of the early gene *BMRF1*, which has been reported to be essential for lytic virus replication, was additionally confirmed. All NF-κB inhibitors tested, including aspirin, induced the expression of *BZLF1*, *BRLF1* and *BMRF1* in the EBV-positive GC cell line SNU-719 (Fig. 5). The results of the current study on EBV-positive gastric cancer cells are in agreement with previous studies on lytic induction by NF-κB inhibitors in the EBV-positive B-lymphocyte cell lines B95-8 and Raji (6), indicating that induction of EBV lytic replication is achievable regardless of cell type.

TPA was used as a positive control of the induction of lytic replication because of its efficacious induction of EBV lytic replication in persistently infected lymphoblastoid and epithelial cells (24). In the present study, *BMRF1* expression levels susbequent to induction by NF-κB inhibitors were similar to those induced by TPA, while at the maximum dose ($<CC_{50}$), the expression levels of *BZLF1* and *BRLF1* induced by NF-κB inhibitors, particularly Z-LLF-CHO, were considerably higher than those induced by TPA (Fig. 5). Although the expression levels of these immediate-early/early genes by aspirin were lower than those of Z-LLF-CHO, the efficiency of cytotoxicity enhancement induced by anti-herpes agents between aspirin and Z-LLF-CHO was equivalent or even higher in aspirin (Fig. 2). Previous reports demonstrated that *RelA* expression is involved in the aspirin-induced EBV lytic replication (6). In addition to *RelA*, a dose-dependant reduction in the mRNA levels of *RelB* was observed seen in the current study. Thus, it is suggested that EBV reactivation can be achieved not only through p65 however additionally through *RelB*. Bren *et al* (25) demonstrated that *RelB* transcription can be induced by *RelA* activation, which indicates that aspirin-induced activation of *RelA* may have enhanced the activation of *RelB*. Further experiments are required to fully elucidate this effect.

The following limitations were identified in the current study: i) Only one EBV-positive and one EBV-negative GC cell line were analyzed, therefore the results of the NF-κB inhibitor and anti-herpes agent cytotoxicity assays are too limited to extrapolate and generalize for other epithelial cell lines; ii) the effects of NF-κB inhibitors on the expression levels of EBV-TK/-PK and NF-κB activity were not examined. Further studies are therefore necessary in order to address these issues.

In conclusion, the present study indicated that NF-κB inhibitors reactivated the EBV lytic genes *BZLF1*, *BRLF1* and *BMRF*, in the SNU-719 EBV-positive GC cell line in a time- and dose-dependent manner. Significant cytotoxicity of NF-κB inhibitors was enhanced by anti-herpes agents suggesting that induction of lytic viral transcription using NF-κB inhibitors in combination with anti-herpes agents may be an effective therapeutic strategy for treating EBV-associated GC. Further *ex vivo* and *in vivo* studies are warranted to confirm these results and to evaluate the clinical relevance of the use of NF-κB inhibitors in combination with anti-herpes agents as a therapeutic strategy for EBV-positive GC.

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