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Sangivamycin induces apoptosis by suppressing Erk signaling in primary effusion lymphoma cells

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ABSTRACT

Sangivamycin, a structural analog of adenosine and antibiotic exhibiting antitumor and antivirus activities, inhibits protein kinase C and the synthesis of both DNA and RNA. Primary effusion lymphoma (PEL) is an aggressive neoplasm caused by Kaposi's sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients and HIV-infected homosexual males. PEL cells are derived from post-germinal center B cells, and are infected with KSHV. Herein, we asked if sangivamycin might be useful to treat PEL. We found that sangivamycin killed PEL cells, and we explored the underlying mechanism. Sangivamycin treatment drastically decreased the viability of PEL cell lines compared to KSHV-uninfected B lymphoma cell lines. Sangivamycin induced the apoptosis of PEL cells by activating caspase-7 and -9. Further, sangivamycin suppressed the phosphorylation of Erk1/2 and Akt, thus inhibiting activation of the proteins. Inhibitors of Akt and MEK suppressed the proliferation of PEL cells compared to KSHV-uninfected cells. It is known that activation of Erk and Akt signaling inhibits apoptosis and promotes proliferation in PEL cells. Our data therefore suggest that sangivamycin induces apoptosis by inhibiting Erk and Akt signaling in such cells. We next investigated whether sangivamycin, in combination with an HSP90 inhibitor geldanamycin (GA) or valproate (valproic acid), potentiated the cytotoxic effects of the latter drugs on PEL cells. Compared to treatment with GA or valproate alone, the addition of sangivamycin enhanced cytotoxic activity. Our data thus indicate that sangivamycin may find clinical utility as a novel anti-cancer agent targeting PEL.

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1. Introduction

Sangivamycin, 4-amino-5-carboxamide-7-(D-ribofuranosyl) pyrrolo [2,3-d] pyrimidine, is an adenosine deaminase-resistant nucleoside analog and 7-deazaadenosine antibiotic produced by *Streptomyces rimosus*. Sangivamycin has antiviral activity [1,2] and inhibits the proliferation of a variety of human cancer cells, including leukemia cells and lung, breast, and colon carcinoma cells [3–5]. Mechanistically, sangivamycin inhibits DNA and RNA synthesis by competitively inhibiting DNA polymerase and RNA polymerase II. Additionally, sangivamycin, a potent inhibitor of protein kinase C (PKC) family, competes with the binding of ATP to the catalytic fragment [6]. Recently, sangivamycin has been shown to induce apoptosis in leukemia cells via the caspase-dependent cleavage of Bid [7], and to induce mitochondrion-mediated apoptosis via the activation of c-Jun N-terminal (JNK) in a protein kinase C (PKC)delta-dependent manner [4]. However, the mechanism of the antitumor activity of sangivamycin remains unknown.

Primary effusion lymphoma (PEL; also termed body cavity-based lymphoma) is classified as a non-Hodgkin's B cell lymphoma developing in immunocompromised patients such as those with AIDS or patients who have undergone organ transplantation [8,9]. In general, PEL presents as a lymphomatous effusion in body cavities. PEL cells are infected with Kaposi's sarcoma-associated herpesvirus (KSHV; also termed HHV-8) and often also with Epstein-Barr virus (EBV). KSHV is the causative agent of Kaposi's sarcoma and lymphoproliferative disorders associated with AIDS such as PEL and multicentric Castlemans disease [10]. During a latent infection, the KSHV genome circularizes to form a double-stranded episome in the nucleus of PEL cells. Establishment of a latent KSHV infection in PEL cells is associated with the expression

Abbreviations: KSHV, Kaposi's sarcoma-associated herpesvirus; HHV-8, human herpes virus-8; PEL, primary effusion lymphoma.

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of several viral proteins, including LANA; vFLIP; vIRF3; kaposins A, B, and C; and 12 microRNAs. These proteins and microRNAs manipulate cellular signaling pathways, including p53, Wnt, NF-κB, Akt, and Erk, to maintain the malignant phenotype and to ensure PEL cell survival [11–13]. In particular, KSHV activates MEK-Erk signaling, allowing the establishment of a KSHV infection [14], the survival of infected cells [15], and viral replication [16]. The activation of Akt and Erk signaling is essential for the survival and growth of KSHV-infected PEL cells. Any anti-cancer effect of sangivamycin against PEL remains unknown; we therefore investigated whether sangivamycin kills PEL cells and the underlying molecular mechanism thereof.

2. Materials and methods

2.1. Cell lines and inhibitors

KSHV- and EBV-positive PEL cell lines (HBL6 and BC2), and KSHV-positive, EBV-negative PEL cell lines (BC3 and BCBL1), were derived from patients with KSHV-mediated PEL. All PEL cells and KSHV-negative lymphoma cell lines (Ramos and DG75) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. Sangivamycin, an Akt inhibitor (1L6-hydroxymethyl-chiro-inositol-2-[R]-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate), and a MEK inhibitor (U0126), were purchased from Merck (Whitehouse Station, NJ) and dissolved in dimethyl sulfoxide.

2.2. Cell viability assay

Cells were seeded in 96-well plates at 3×10^4 cells/well in 100 μl of medium with or without sangivamycin at various concentrations and incubated at 37 °C for 32 h. Viable cell numbers were estimated using a Cell-Counting Kit-8 (Dojindo, Kumamoto, Japan) [17]. The optical density of each sample was measured at 450 nm employing a microplate spectrophotometer and expressed as a percentage of that of untreated cells (defined as 100%). All data are the means ± SEMs from three independent experiments.

2.3. Western blotting, immunofluorescence (IF), and antibodies

Western blotting and IF were performed as described previously [18]. The primary antibodies used detected IκBα, caspase-7, cleaved caspase-7, cleaved caspase-9, cleaved PARP, and PARP (Cell Signaling Technology, Beverly, MA); S473-phospho-Akt, T202/Y204-phospho-Erk1/2, Erk1, T180/Y182-phospho-p38, T183/Y185-phospho-JNK, and $p21^{Cip1}$ (BD Biosciences, Franklin Lakes, NJ); and β-actin and K-bZIP (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. IF analysis

Prior to IF, BC3 PEL cells were treated with 0.1 μM sangivamycin for 3 or 12 h and fixed in methanol on glass slides followed by incubation with primary antibodies for 1 h. After washing, the cells were incubated with Alexa Fluor 594 donkey anti-rabbit (or -mouse) IgG (Invitrogen, Carlsbad, CA). To stain the nucleus, cells were incubated in 2.5 μg/ml DAPI in PBS during binding of the secondary antibody. Immunofluorescent images were obtained via fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

2.5. Caspase assay

A total of 5×10^5 cells were incubated with sangivamycin for 3 h and the activities of caspase-8 and -9 in cell lysates were measured using a Caspase-Glo Assay kit according to the manufacturer's instructions (Promega, Madison, WI). Luminescence was detected with the aid of an AB-2000 instrument (ATTO, Tokyo, Japan). The caspase activities of untreated cells were defined to be relative light units (RLUs) of unity.

2.6. Measurement of viral DNA by real-time PCR

Real-time PCR was performed as described previously [19]. Briefly, BCBL1 cells (8×10^5) were treated with or without 1.5 mM *n*-butyrate (sodium butyrate) for 24 h to induce production of KSHV particles. Viral DNA was purified and extracted from 200 μl of DNase I-treated medium using a QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands). To quantify viral DNA, SYBR green real-time PCR was performed using specific primers amplifying the KSHV ORF50 gene (5′-GATGACAAGGTAAAGATCGACCT-3′ and 5′-GGTCAAGTACACCGAACACTTAA-3′). The expression levels of ORF50 were normalized to that of *GAPDH*.

3. Results and discussion

3.1. The cytotoxic effects of sangivamycin on PEL cells

To address the potential clinical use of sangivamycin, we evaluated the cytotoxic effect of the drug on PEL cell lines using cell viability assays based on the WST-8 proliferation assay. We used two types of cells: KSHV-infected lymphoma (PEL) (i.e., HBL6, BCBL1, BC2, and BC3 cells) and KSHV-uninfected lymphoma cell lines (i.e., Ramos and DG75 cells). B lymphoma cells were cultured in the presence of sangivamycin for 32 h, and cytotoxicity was assessed by measuring the viabilities of sangivamycin-treated and -untreated cells. Sangivamycin significantly decreased the viability of KSHV-infected PEL cells compared to uninfected cells (Fig. 1). Interestingly, sangivamycin prevented the proliferation of all PEL cell lines at lower concentrations than were required to inhibit the proliferation of KSHV-uninfected Ramos and DG75 cells. The cytotoxic effects of sangivamycin on B lymphoma cells are summarized in Table 1. Sangivamycin was active against BC3 cells with the CC_{50} values of 10 nM, while Ramos and DG75 cells were insensitive to sangivamycin ($CC_{50} > 500$ nM). To the best of our knowledge, this is the first report to show the specific inhibition by sangivamycin of PEL cell proliferation.

3.2. Sangivamycin induces apoptosis by activating caspase-9 and -7 in PEL cells

We next investigated whether the cytotoxic effects of sangivamycin were attributable to apoptotic cell death. Apoptosis is induced by executioner caspases, including caspase-3 and -7, which have been previously activated via an intrinsic pathway (caspase-9) or an extrinsic pathway (caspase-8). We monitored the cleavage (i.e., activation) of caspase-7 by Western blotting of lysates prepared from cells pretreated with 0.1 μM sangivamycin (Fig. 2A). Active caspase-9 and -7 was detected in both BC3 and BC2 cells. In contrast, cleavage of these caspases was not evident in sangivamycin-treated DG75 cells. Further, compared to sangivamycin-treated DG75 cells, the level of cleaved PARP increased in sangivamycin-treated BC3 and BC2 cells. IF assays using antibodies detecting active caspase-7 and cleaved PARP showed that caspase-7 activation was induced in BC3 cells after 3 h of incubation in 0.1 μM sangivamycin (Fig. 2B). Thus, sangivamycin suppressed the growth of PEL cells by triggering apoptosis mediated by caspase-7, via activation of caspase-9. In addition, Western blotting showed that caspase-8 was not appreciably cleaved in sangivamycin-treated cells (data not shown). However, the activation of caspase-8 and -9 was detected colorimetrically (Fig. 2C). Thus,

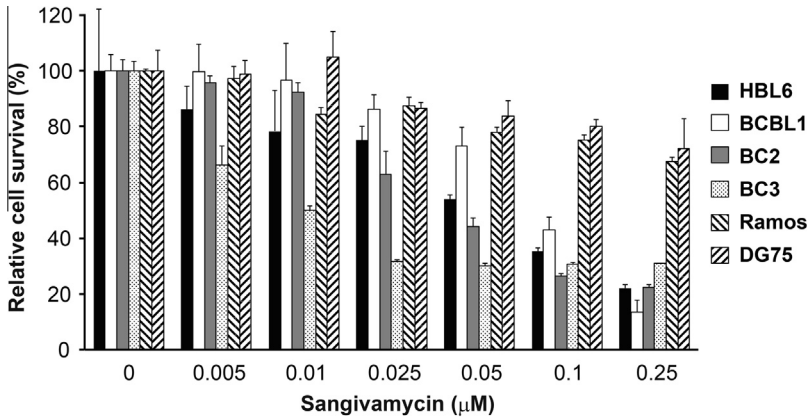

Fig. 1. The cytotoxic effects of sangivamycin on PEL cells and KSHV-uninfected lymphoma cells. KSHV-infected PEL cells (i.e., HBL6, BCBL1, BC2 and BC3 cells) and KSHV-uninfected lymphoma cells (i.e., DG75 and Ramos cells) were incubated with various concentrations of sangivamycin for 24 h and then subjected to cell viability assays. For each cell type, viability was assessed in six replicate wells. The optical density was measured at 450 nm; the values yielded by untreated cells were considered to be 100%. Standard deviations were determined by analysis of the data from three independent experiments, and are indicated by the bars.

Table 1
Cytotoxic effects of sangivamycin on B lymphoma cells.

	HBL6	BCBL1	BC2	BC3	Ramos	DG75
CC_{50} (nM)	60.6	88.4	42.2	10.0	>500	>500

CC_{50}, cytotoxic concentration of sangivamycin that reduces cell viability by 50%.

sangivamycin may induce the activation of not only caspase-9 but also caspase-8. These results thus indicate that sangivamycin suppressed the growth of PEL cells by triggering apoptosis via the activation of caspase-7.

3.3. Sangivamycin suppresses Erk signaling in PEL cells

It is known that KSHV activates Erk [15], NF-κB [17,20], and Akt signaling [15] in PEL cells and that these actions mitigate apoptosis and promote cell growth. We therefore explored whether sangivamycin influenced Akt, NF-κB, and Erk signaling. When BCBL1 and BC3 cells were treated with 0.1 μM sangivamycin for 9 h, the

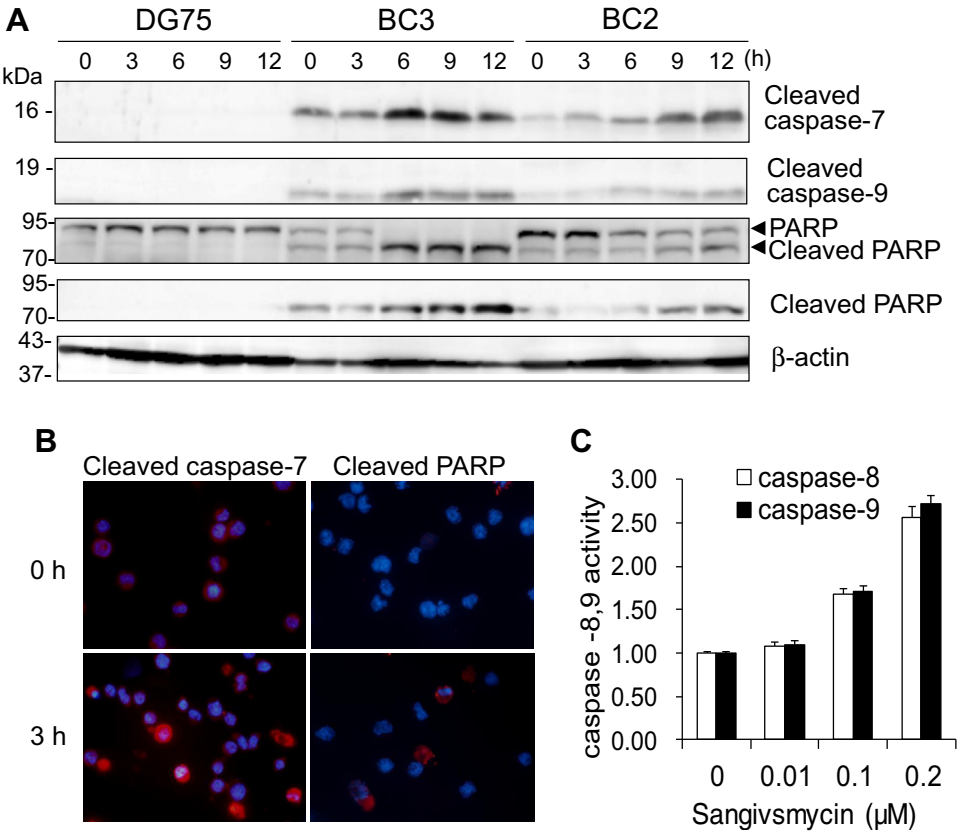

Fig. 2. Apoptosis-inducing effects of sangivamycin on PEL and KSHV-uninfected cells. (A) Immunoblotting using antibodies against caspase-7 and -9, PARP, and cleaved PARP. PEL (BC3 and BC2) and KSHV-uninfected lymphoma DG75 cells were cultured with 100 nM sangivamycin for 0, 3, 6, 9, or 12 h. (B) Immunofluorescence analysis using anti-active caspase-7 and anti-cleaved PARP antibodies. BC3 cells were cultured with 100 nM sangivamycin for 3 h and fixed in ice-cold methanol. The fixed cells were incubated with primary antibodies and then with Alexa Fluor 594 donkey anti-rabbit IgG and DAPI (to stain the nucleus). (C) Changes in the activity of caspase-8 and -9 in BC3 cells. Cells were cultured with sangivamycin for 3 h prior to harvesting. Caspase activity was measured using the Caspase-Glo Assay. Caspase activity in sangivamycin-untreated cells was defined as relative light units (RLUs) of unity. The error bars show the standard deviations.

extent of phosphorylation of Ser473 of Akt (to form p-Akt) was decreased in BCBL1 and BC3 PEL cells (Fig. 3A). The level of IκBα protein (an inhibitor of NF-κB) was unaffected by sangivamycin treatment. Expression of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ was induced in BCBL1 and BC3 cells treated with sangivamycin, but the level of the K-bZIP did not change. K-bZIP, encoded by the early gene K8, is a viral transcription factor initiating the lytic replication of KSHV. Thus, sangivamycin did not kill PEL cells in a manner involving lytic induction. We measured the levels of proteins acting upstream of Erk and downstream of Akt, but the levels of Ser259-phosphorylated Raf and Ser9-phosphorylated GSK-3β did not change upon addition of sangivamycin (data not shown). Apart from enhancing the phosphorylation of Akt, sangivamycin treatment for 6 and 9 h suppressed the phosphorylation of Thr202 and Tyr204 in Erk1/2 (to yield p-Erk1/2) in HBL6 PEL cells (Fig. 3B). We next investigated the effect of sangivamycin on phosphorylation of the p38 MAPK and JNK and Erk. When BC3 cells were treated with 0.1 μM sangivamycin for 12 h, the extent of Erk1/2 phosphorylation was significantly decreased, whereas the phosphorylation status of p38 MAPK and JNK did not change (Fig. 3C). We confirmed the inhibition of Erk phosphorylation by sangivamycin using IF with anti-phosphorylated Erk1/2 antibodies. The level of phosphorylated Erk was reduced 12 h after the start of sangivamycin treatment in BC3 cells (Fig. 3D). In addition, we measured the cytotoxic effects of an Akt inhibitor and a MEK inhibitor (U0126) on PEL cells to confirm that Akt and Erk signaling contribute to the survival and growth of such cells. The Akt inhibitor, which inhibits Akt phosphorylation, is an irreversible and specific inhibitor of Akt signaling. U0126 inhibits MEK1 and MEK2, which can phosphorylate and activate Erk. When BC3 and KSHV-uninfected Ramos cells were treated with 100 μM Akt inhibitor or 100 μM U0126 for 24 h, treatment with both the Akt inhibitor and U0126 resulted in a remarkable decrease in BC3 cell viability, compared to that of KSHV-uninfected Ramos cells (Fig. 3E). These data indicate that Akt and MEK-Erk signaling contribute strongly to the survival and proliferation of PEL cells.

We thus showed that sangivamycin suppressed Akt and Erk phosphorylation, which is necessary to activate Akt and Erk signaling. It has been reported that PKC is a target molecule of sangivamycin [6]. PKC phosphorylates MEK and Erk1/2, and these signaling proteins become activated only after such phosphorylation [21]. Together, these data suggest that sangivamycin suppresses PKC activity, causing dephosphorylation of Erk. Sangivamycin affects not only Erk but also Akt signaling in PEL cells, and this contributes to the killing of PEL cells. Akt signaling is important for the survival and growth of many types of cancer cells, including KSHV-infected lymphoma cells. The KSHV-encoded viral G-protein-coupled receptor promotes endothelial cell survival via stimulation of the phosphatidylinositol-3-kinase-Akt pathway [22], upregulating the synthesis of vascular endothelial growth factor (VEGF) receptor 2, which in turn triggers VEGF release. Sangivamycin treatment elevated the levels of $p21^{Cip1}$, suggesting that sangivamycin induced a G1-S cell cycle arrest, leading to the apoptosis of PEL cells. Additionally, it was reported that the

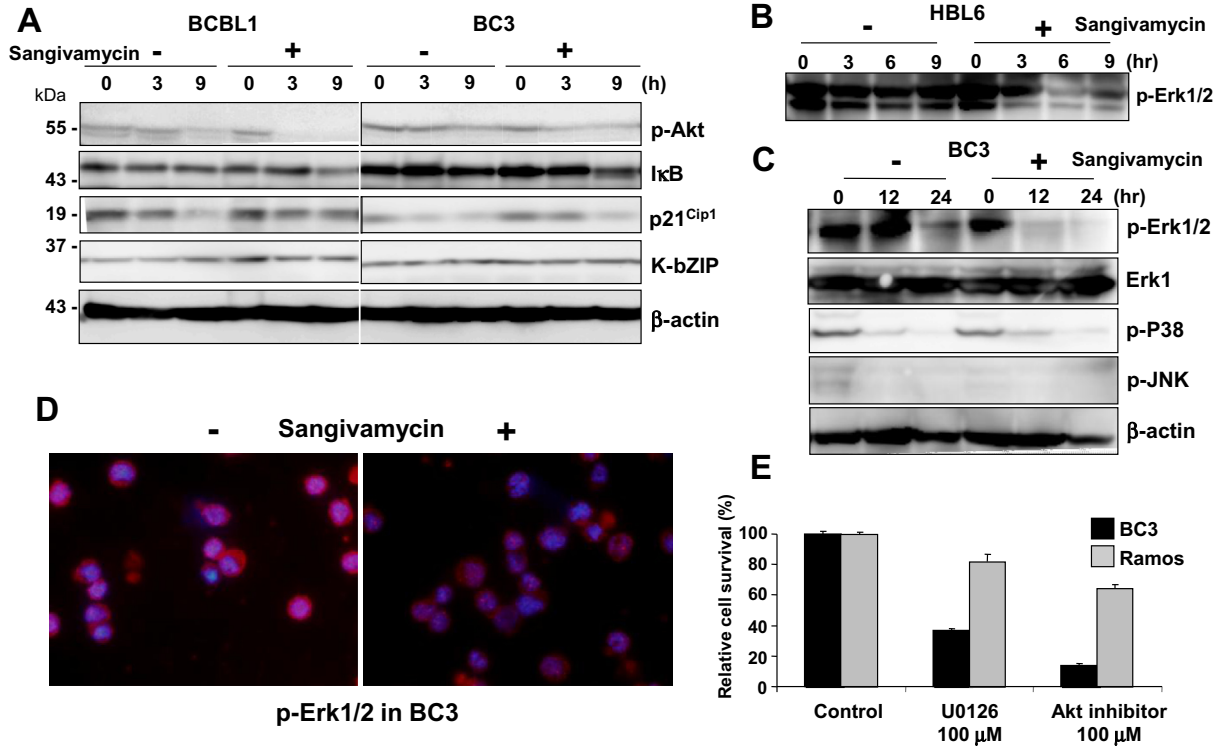

Fig. 3. Suppression of Erk signaling in PEL cells by sangivamycin. (A) Immunoblotting using antibodies against phospho-Akt, IκBα, $p21^{Cip1}$, and K-bZIP. BCBL1 and BC3 cells were cultured with or without 0.1 μM sangivamycin for 0, 3, or 9 h and then harvested. To monitor Akt and NF-κB activity, cell cycle arrests, and induction of the lytic cycle, the levels of phosphorylated Akt, IκBα, $p21^{Cip1}$, and K-bZIP were determined. (B) Inhibition of Erk phosphorylation by sangivamycin. HBL6 PEL cells were cultured with 0.1 μM sangivamycin, and whole-cell lysates were subjected to immunoblotting using anti-Thr202/Tyr204-phospho-Erk rabbit polyclonal antibodies. (C) The effects of sangivamycin treatment on phosphorylation of Erk, p38, and JNK in BC3 PEL cells. Cells were cultured with 0.1 μM sangivamycin and whole-cell lysates were subjected to immunoblotting with anti-Thr202/Tyr204-phospho-Erk1/2, -Erk1, -Thr180/Tyr182-phospho-p38 MAPK, and -Thr183/Tyr185-phospho-JNK antibodies. (D) IF assay of sangivamycin-treated BC3 cells using anti-phospho-Erk antibodies. BC3 cells were cultured with or without 0.1 μM sangivamycin for 12 h then stained with anti-Thr202/Tyr204-phospho-Erk1/2 rabbit antibodies and incubated with Alexa Fluor 594 donkey anti-rabbit IgG containing DAPI. (E) The cytotoxic effects of an Akt inhibitor and a MEK inhibitor, U0126, on PEL cells. KSHV-infected BC3 and uninfected Ramos cells were incubated with 100 μM Akt inhibitor and 100 μM MEK inhibitor for 24 h, and then assessed for viability. The viabilities of untreated cells were defined to be 100%.

knockdown of PKC reduced Akt phosphorylation at Ser473 and increased the level of $p21^{Cip1}$ [23]; these observations are consistent with our data. We found that sangivamycin suppressed the phosphorylation of Erk and Akt, thus inhibiting Erk and Akt signaling, which is necessary for the survival of PEL cells. Such inhibition by sangivamycin may cause the apoptosis of PEL cells.

3.4. Low levels of sangivamycin with GA or valproate suppress the proliferation of PEL cells

Previously, we reported that the HSP90 inhibitor geldanamycin (GA) induced apoptosis in PEL cells by inhibiting NF-κB signaling [17,19]. Valproate (valproic acid) also induces the apoptosis of PEL cells, accompanied by KSHV re-activation [24]. It is well known that combined therapies for lymphoma are more effective than monotherapies. Therefore, we investigated whether treatment with a low concentration of sangivamycin in combination with GA or valproate (also at low levels) inhibited the proliferation of BCBL1 cells. Such cells were treated with 25 nM sangivamycin alone, 10 nM GA alone (Fig. 4A), 0.5 mM valprolate alone (Fig. 4B), a combination of 25 nM sangivamycin and 10 nM GA (Fig. 4A), or a combination of 25 nM sangivamycin and 0.5 mM valprolate (Fig. 4B). Cell viability was measured after 1, 2, or 3 days. Sangivamycin, GA, or valproate alone did not inhibit the growth of BCBL1 cells. However, the combination of 25 nM sangivamycin with 10 nM GA or 25 nM sangivamycin with 0.5 mM valproate suppressed the proliferation of BCBL1 cells in a synergistic manner. Such drug mixtures are thus novel strategies for the treatment of PEL. The HSP90 inhibitor GA induces apoptosis in PEL cells by stabilizing IκBα, which in turn suppresses NF-κB signaling [19]. Constitutive activation of NF-κB signaling is essential for the survival of PEL cells. HSP90 functions as a scaffold for the IKK complex, and thus contributes to upregulation of NF-κB activity by destabilizing IκB. We found that the inhibition of NF-κB and Erk signaling by GA and sangivamycin strongly compromised the survival of PEL cells, indicating that co-activation of NF-κB and Erk may be important in this context. Valproate is clinically used as an anti-epileptic drug and a mood stabilizer. Additionally, valproate functions as an inhibitor of histone deacetylases and induces the apoptosis of transformed cells, including PEL and EBV-infected lymphoma cells [24]. These findings, and our present data, suggest that histone deacetylation as well as signaling by NF-κB and Erk enhance the survival of PEL cells.

Finally, we examined the effects of sangivamycin on lytic replication in PEL cells. The addition of *n*-butyrate induces viral replication in KSHV-infected BCBL1 cells. As treatment with 25 nM sangivamycin remarkably reduced the viability of BCBL1 cells, such cells were treated with 0.5–10 nM sangivamycin in the presence or absence of 1.5 mM *n*-butyrate for 24 h. Sangivamycin did not induce KSHV lytic replication in BCBL1 cells that were not treated with *n*-butyrate (Fig. 4C). Further, sangivamycin did not affect the level of virus production in *n*-butyrate-treated BCBL1

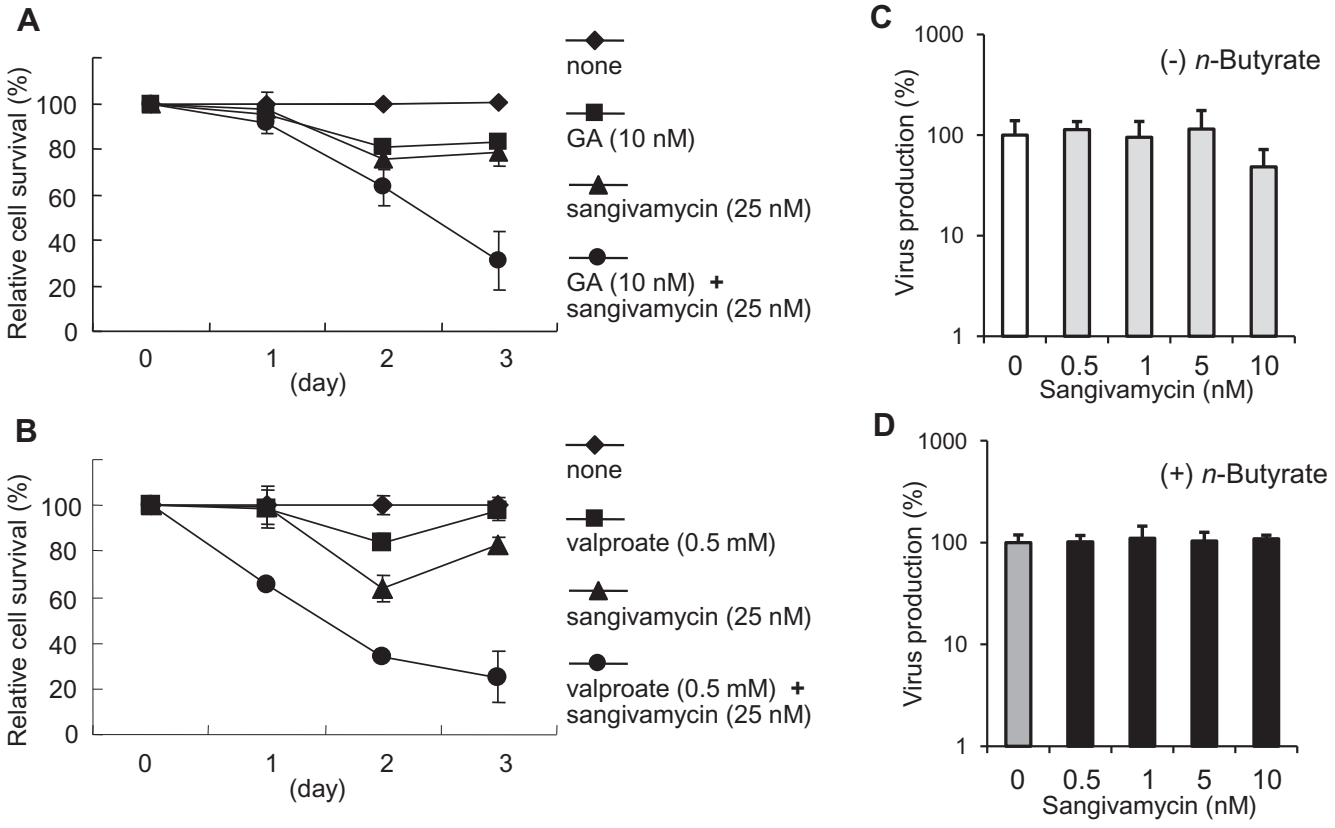

Fig. 4. Enhancement of the cytotoxic effects of geldanamycin (GA) or valproate by sangivamycin. (A) The cytotoxic effects of GA alone, sangivamycin alone, or a combination of sangivamycin and GA, on BCBL1 cells. (B) The cytotoxic effects of valproate alone, sangivamycin alone, or a combination of sangivamycin and valproate, on BCBL1 cells. Cells were treated with 25 nM sangivamycin, 10 nM GA, 0.5 mM valprolate, a combination of 25 nM sangivamycin and 10 nM GA, or a combination of 25 nM sangivamycin and 0.5 mM valproate, and cell viability was measured after 1, 2, and 3 days. The viability of untreated cells was defined to be 100%. (C) The effect of sangivamycin on lytic induction in BCBL1 cells not treated with *n*-butyrate. The cells were treated with sangivamycin for 24 h, and culture medium containing virus particles was harvested. The KSHV genome levels were quantified via real-time PCR. The KSHV genome copy number in sangivamycin-untreated BCBL1 cells was defined to be 100%. (D) Effects of sangivamycin on lytic replication in BCBL1 cells treated with *n*-butyrate. BCBL1 cells were treated with sangivamycin in the presence of 1.5 mM *n*-butyrate for 24 h to induce lytic replication. KSHV genomes were purified from the culture medium and quantified via real-time PCR. The KSHV genome copy number in medium from sangivamycin-untreated cells was defined to be 100%.

cells, in which lytic replication was induced (Fig. 4D). Fig. 3A shows that sangivamycin did not induce lytic replication in either BCBL1 or BC3 cells. Sangivamycin did not induce K-bZIP expression, which is induced during lytic replication. Together, our data show that sangivamycin induces apoptosis in PEL cells without production of progeny virus; thus, sangivamycin may serve as a useful treatment for PEL without any risk of *de novo* KSHV infection.

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