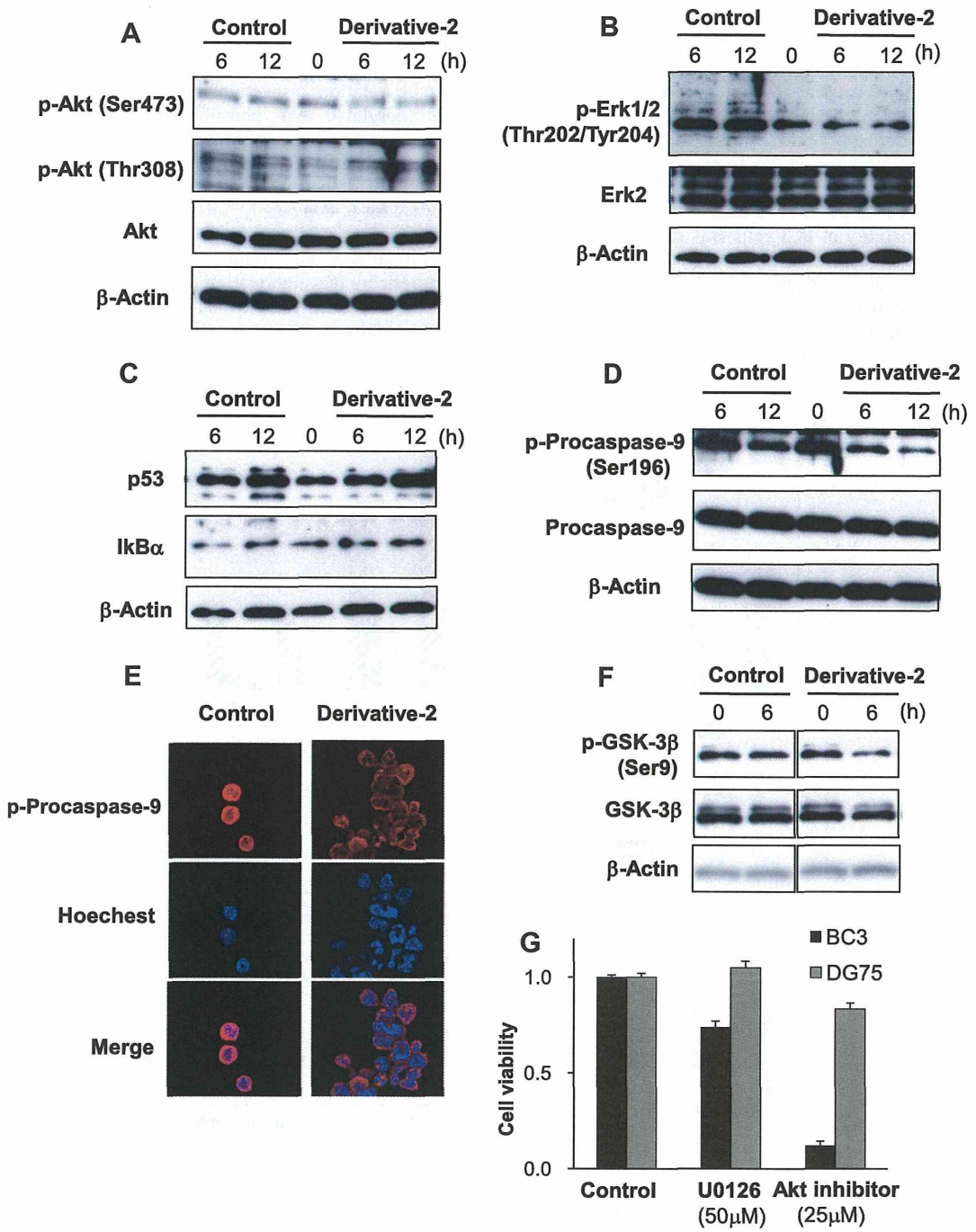

Fig. 3. Derivative-2 suppresses phosphorylation of Akt, Erk and procaspase-9 in PEL cells. (A and B) Suppression of Ser473 phosphorylation at Akt and Thr202/Tyr204 at Erk1/2 in PEL cells caused by derivative-2. BC3 cells were cultured with 25 μM derivative-2 or control vehicle (DMSO) for 0, 6, or 12 h and then harvested. To elucidate Akt and Erk activities, whole-cell lysates were analyzed by Western blotting with anti-Ser473-phospho-Akt, anti-Thr308-phospho-Akt and anti-Thr202/Tyr204-phospho-Erk1/2 antibodies. (C and D) Derivative-2 effects on the amount of p53, IκBα and Ser196-phosphorylated procaspase-9 in BC3 cells. Cells were cultured with 25 μM derivative-2 and cell lysates were subjected to immunoblotting with anti-p53, -IκBα and -Ser196-phosho-procaspase-9 antibodies. (E) Immunofluorescence (IF) assay of Ser196 procaspase-9 phosphorylation in BC3 cells treated with derivative-2. BC3 cells treated with 25 μM derivative-2 for 12 h were stained with anti-Ser196-phosho-procaspase-9 and then incubated with Alexa594-conjugated anti-rabbit IgG and Hoechest33342 (to stain the nucleus). (F) Suppression of GSK-3β Ser9 phosphorylation in BC3 cells caused by derivative-2. BC3 cells were cultured with 25 μM derivative-2 for 6 h and cell lysates were subjected to blotting with anti-Ser9-phospho-GSK-3β antibody. (G) The cytotoxic effects of a MEK inhibitor (U0126) or an Akt inhibitor on PEL cells. BC3 and KSHV-uninfected Ramos cells were incubated with U0126 or Akt inhibitor for 24 h and then assessed for viability.

of NF-κB) were unaffected by derivative-2 (Fig. 3C). Ser473-phosphorylated Akt (activated Akt) phosphorylates its substrates, such as procaspase-9 and GSK-3β [8–10]. Because derivative-2 reduced Ser473-phosphorylated Akt, we examined whether derivative-2 affected the phosphorylation of procaspase-9 and GSK-3β. When BC3 cells were treated with derivative-2 for 6 and 12 h, Ser196 phosphorylation of procaspase-9 was decreased as compared with the control vehicle (Fig. 3D). We confirmed the suppression of

procaspase-9 phosphorylation by derivative-2 using an IF assay. The level of Ser196-phosphorylated procaspase-9 was reduced 12 h after the start of derivative-2 treatment in BC3 cells (Fig. 3E). Furthermore, treatment with derivative-2 resulted in decreased Ser9 phosphorylation in GSK-3β (Fig. 3F). In addition, we measured the cytotoxic effects of a U0126, a MEK (Erk kinases) inhibitor and an Akt inhibitor on PEL to confirm that Erk and Akt signaling contribute to the growth and survival of PEL cells (Fig. 3G). When BC3 and DG75 cells were treated with U0126 or Akt inhibitor, the viability of BC3 cells was reduced significantly by the Akt inhibitor compared to DG75 cells. The viability of BC3 cells treated with U0126 was decreased to 75% of the vehicle control, whereas the viability of DG75 cells treated with U0126 was unchanged. These data indicate that Akt signaling contributes significantly to the survival and proliferation of PEL compared with MEK-Erk signaling.

PDK1-PI3K and mTORC2 induce Ser308-Akt and Ser473-Akt phosphorylation, respectively, causing Akt activation [8]. Ser473-phosphorylated Akt phosphorylates Ser196 in procaspase-9, resulting in suppression of activation of procaspase-9 by proteolytic cleavage [9,10]. Thus, Akt contributes to suppression of the cascade of caspase-9-mediated apoptosis by phosphorylation of procaspase-9. Conversely, Ser473-phosphorylated Akt phosphorylates Ser9 at GSK-3β, causing GSK-3β inactivation [8]. GSK-3β downregulates cell proliferation, glycogen synthesis and protein synthesis by phosphorylation of cyclin D1, glycogen synthase and eIF2B, respectively. Akt-mediated phosphorylation of Ser9 at GSK-3β induces GSK-3β inactivation by inhibiting the active site

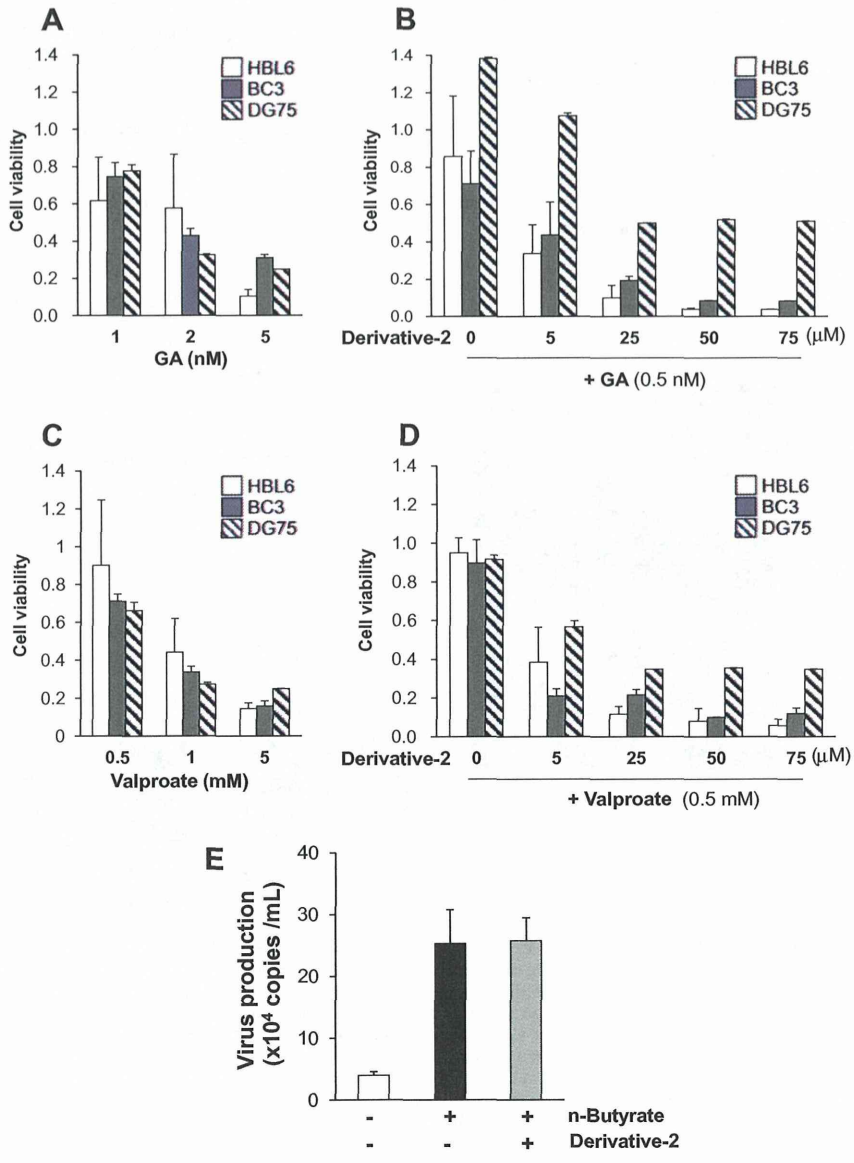

Fig. 4. Facilitation of derivative-2 cytotoxic activities with a low concentration of geldanamycin (GA) or valproate against PEL cells. (A and B) The cytotoxic effect of GA alone or in combination with derivative-2 on PEL cells. PEL cells (HBL6 and BC3), or KSHV-uninfected DG75 cells were cultured with 1, 2, or 5 nM GA (A) or a combination of 0.5 nM GA and 0, 5, 25, 50, or 75 μM derivative-2 (B) for 24 h and cell viability was measured. The viability of untreated cells was defined as 1.0. (C and D) The cytotoxic effect of valproate alone or in combination with derivative-2 on PEL cells. HBL6, BC3 or DG75 cells were treated with 0.5, 1 or 5 mM valproate or a combination of 0.5 mM valproate and derivative-2 and cultured for 24 h. (E) Derivative-2 effects on KSHV replication in BCBL1 cells. BCBL1 cells were cultured for 24 h with or without 25 μM derivative-2 in the presence of 1.5 mM *n*-butyrate. Culture medium containing virus particles was harvested and KSHV genome copy numbers were quantified by real-time PCR.

availability of GSK-3β [8]; thus, Akt promotes cell proliferation and glycogen synthesis *via* GSK-3β-phosphorylation. We showed that derivative-2 induced activation of procaspase-9 (Fig. 2B and C) and reduced Akt phosphorylation at Ser473 and procaspase-9 at Ser196 in PEL cells. In addition, derivative-2 resulted in decreased Ser9 phosphorylation in GSK-3β. Collectively, these data suggest that derivative-2 induced activation of caspase-9 and inhibition of cell proliferation in PEL cells by suppressing Akt, which plays an important role in inactivation of caspase-9 and GSK-3β.

Because derivative-2 suppressed Erk1/2 phosphorylation (Fig. 3B), we examined the effects of derivative-2 on other MAPKs; however, unlike Erk1/2, derivative-2 did not change the phosphorylation of p38 MAPK and JNK (data not shown). Akt [4,7] and Erk [4,7,22,24] signaling are activated in PEL to maintain the malignant phenotype and to ensure PEL cell survival. In particular, KSHV activates Raf-MEK-Erk signaling, allowing the establishment of a KSHV infection [24] and survival of KSHV-infected cells [7]. We found that derivative-2 suppressed the phosphorylation of Erk1/2 as well as Akt; thus inhibition of Erk signaling also contributed to PEL cell apoptosis.

In this report, four groups of water-soluble fullerene derivatives were evaluated in terms of their effect on cell proliferation. Our data demonstrated that only the pyrrolidinium-type fullerene (derivative-2), which induces apoptosis of HL-60 cells by ROS generation [16], decreased the viability of KSHV-infected PEL cells but not KSHV-uninfected B-lymphoma cells (Fig. 1B). We examined whether derivative-2 induced the mitochondrial abnormalities caused by ROS; release of cytochrome c from mitochondria was observed in both PEL cells and KSHV-uninfected B-lymphoma cells (data not shown), indicating that derivative-2 induces apoptosis only in PEL cells by an ROS generation-independent mechanism, which is closely related to Akt suppression and caspase-9 activation.

3.4. Derivative-2 in combination with geldanamycin (GA) or valproate enhances the cytotoxic effects on PEL cells

Generally, combined therapies for treatment of cancer, including lymphoma, are more effective than monotherapies. We previously found that the HSP90 inhibitor, geldanamycin (GA), induced apoptosis in PEL [23]. Furthermore, valproate (sodium valproate) reportedly also induces apoptosis in PEL cells, accompanied by KSHV reactivation [25]. Therefore, we investigated whether derivative-2 treatment in combination with low concentrations of GA or valproate enhanced the cytotoxic effects of derivative-2 on PEL cells. When PEL (HBL6 and BC3 cells) were treated with GA alone (Fig. 4A) or valproate alone (Fig. 4C) for 24 h, treatment with 1 nM GA and 0.5 nM valproate slightly affected PEL cell proliferation. Next, PEL cells were treated with a combination of various concentrations of derivative-2 and 0.5 nM GA (Fig. 4B) or 0.5 nM valproate (Fig. 4D). The combination treatment with GA or valproate resulted in significantly decreased cell viability of HBL6 and BC3 cells compared with derivative-2 alone (Fig. 1B). As shown in Fig. 1B, 25 μM derivative-2 alone decreased cell viability to approximately 50% of the vehicle control, whereas the viability of HBL6 and BC3 cells treated with the combination of 25 μM derivative-2 with 0.5 nM GA (or 0.5 mM valproate) decreased to less than 20%. Thus, the combination of derivative-2 with a low concentration of GA or valproate enhanced the suppression of PEL cell proliferation in a synergistic manner. Combination treatment with such drugs is a novel chemotherapeutic strategy for PEL. Finally, we examined the effect of derivative-2 on lytic replication in PEL cells. Treatment with 1.5 mM *n*-butyrate induced viral replication in KSHV-infected BCBL1 cells, and co-treatment with 1.5 mM *n*-butyrate and 25 μM derivative-2 did not affect KSHV lytic replication in BCBL1 cells (Fig. 4E). These data show that derivative-2 induces apoptosis in PEL cells without production of progeny virus. Thus, the present study provided a rationale indicating derivative-2 as a potential molecular candidate agent for PEL treatment without *de novo* KSHV production and infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.068.

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