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Combination of SAHA and bortezomib up-regulates $p16^{INK4A}$ and $p21^{WAF1}$ and induces apoptosis of Epstein-Barr virus-positive Wp-restricted Burkitt's lymphoma and lymphoblastoid cell lines

Running title: SAHA/bortezomib on Wp-restricted BL and LCLs

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Summary:

Epstein-Barr virus (EBV) latent proteins exert anti-apoptotic effects on EBV-transformed lymphoid cells by down-regulating Bim, $p16^{INK4A}$ and $p21^{WAF1}$. However, the potential therapeutic effects of targeting these anti-apoptotic mechanisms remain unexplored. Here, we tested both *in vitro* and *in vivo* effects of the combination of histone deacetylase (HDAC) and proteasome inhibitors on the apoptosis of six endemic Burkitt's lymphoma (BL) lines of different latency patterns (types I and III and Wp-restricted) and three lymphoblastoid cell lines (LCLs). We found that the combination of HDAC and proteasome inhibitors (e.g. SAHA/bortezomib) synergistically induced the killing of Wp-restricted and latency III BL and LCLs but not latency I BL cells. The synergistic killing was due to apoptosis, as evidenced by the high percentage of annexin V positivity and strong cleavage of PARP and caspase-3. Concomitantly, SAHA/bortezomib up-regulated the expression of $p16^{INK4A}$ and $p21^{WAF1}$ but did not affect the level of Bim or BHRF1 (viral homologue of Bcl2). The apoptotic effects were dependent on reactive oxygen species generation. Furthermore, SAHA/bortezomib suppressed the growth of Wp-restricted BL xenografts in nude mice. This study provides the rationale to test the novel application of SAHA/bortezomib on the treatment of EBV-associated Wp-restricted BL and post-transplant lymphoproliferative disorder.

Introduction

Epstein-Barr virus (EBV) establishes life-long latent infections in normal human B cells and is closely associated with post-transplant lymphoproliferative disorder (PTLD) and endemic Burkitt's lymphoma (BL) (Rickinson and Kieff 2007). PTLD and its *in vitro* experimental model, lymphoblastoid cell line (LCL), are associated with the most immunogenic form of latency (latency III) in which all EBV nuclear antigens (EBNA-1, -2, -LP, -3A, -3B and -3C) and latent membrane proteins (LMP-1, -2A and -2B) are expressed. Endemic BL can display either type I or Wp-restricted (~15% of the endemic BL) latency (Kelly, *et al* 2013). In type I latency, only EBNA-1 is expressed. In Wp-restricted latency, EBNA-1, EBNA-LP, EBNA-3A, EBNA-3B and EBNA-3C are expressed (Rowe, *et al* 2009). BL cell lines can also switch to express latency III infection after long period of *in vitro* culture (Gregory, *et al* 1990).

Most of the EBV latent proteins expressed in Wp-restricted or type III latency were shown to be oncogenic and **might** contribute to the resistance of EBV-associated lymphomas to standard chemotherapy. Leao *et al.* showed that EBV-positive BL cells of type III latency were more resistant to the killing by nocodazole or taxol in comparison to EBV-negative or latency I BL cells (Leao, *et al* 2007). Kelly *et al.* showed that Wp-restricted BL cells were also more resistant to the treatment with ionomycin or anti-IgM when compared with latency I BL cells (Kelly, *et al* 2005). Vereide *et al.* found that EBV conferred essential anti-apoptotic effects on both Wp-restricted BL and PTLD cells but not on latency I BL cells (Vereide and Sugden 2011). EBNA-LP, -3A, -3B and -3C are expressed in both Wp-restricted and type III latency but not in type I latency (Rickinson and Kieff 2007). Among these viral latent proteins, EBNA-3A and EBNA-3C **were** shown to have anti-apoptotic functions in different B-cell malignancies. Tumour suppressor genes such as Bim, $p16^{INK4A}$ and $p21^{WAF1}$ are down-regulated by EBNA-3A or -3C through epigenetic modification of

the host cell genomes (Anderton, *et al* 2008, Cooper, *et al* 2003, Hertle, *et al* 2009, Maruo, *et al* 2006, Maruo, *et al* 2011, Paschos, *et al* 2009, Saha, *et al* 2011, Skalska, *et al* 2010). The epigenetic modification by the virus latent proteins could be achieved either through interaction with C-terminal binding protein or direct recruitment of histone deacetylases (HDAC) such as HDAC-1 and HDAC-2 (Chinnadurai 2002, Knight, *et al* 2003, Radkov, *et al* 1999, White, *et al* 2010).

HDAC inhibitors can inhibit various types of HDAC enzymes and mediate potent anti-cancer effect in a wide range of malignancies. We have reported that an FDA-approved HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), can induce growth arrest and apoptosis of EBV-positive gastric carcinoma and nasopharyngeal carcinoma cells by disrupting the EBV latency (Hui and Chiang 2010, Hui, *et al* 2012). HDAC inhibitors can also induce the expression of Bim, $p16^{INK4A}$ and $p21^{WAF1}$ (Gui, *et al* 2004, Zhou, *et al* 2009). The induction of apoptosis and up-regulation of tumour suppressor genes by HDAC inhibitors can be greatly enhanced when co-administered with proteasome inhibitors such as bortezomib (Emanuele, *et al* 2007, Heider, *et al* 2009, Hui and Chiang 2014, Pei, *et al* 2004, Yu, *et al* 2003). However, it remains unknown whether combination of HDAC and proteasome inhibitors can affect the expression of tumour suppressor genes and induce apoptosis of EBV-associated B-cell malignancies.

In this study, we tested the hypothesis that combination of HDAC inhibitors (e.g. SAHA) and proteasome inhibitors (e.g. bortezomib) can synergistically induce apoptosis of Wp-restricted BL and LCLs. Our results showed that SAHA/bortezomib can preferentially induce the killing of Wp-restricted and latency III BL cells but not latency I BL cells. The synergistic killing can also be achieved in EBV-transformed LCLs which express type III latency. The synergism of SAHA/bortezomib is probably mediated through **reversal** of the suppressive effects of EBNA-3 proteins on $p16^{INK4A}$

and $p21^{WAF1}$ **with** subsequent induction of apoptosis in the Wp-restricted BL and LCLs. Furthermore, the drug combination can suppress the growth of Wp-restricted BL xenografts *in vivo*. Our findings provide the rationale to test the efficacy of SAHA/bortezomib **in** the treatment of Wp-restricted BL and primary EBV-driven lymphoproliferative diseases such as PTLD.

Materials and Methods:

Cell culture

Akata 31 (Ak31) is an EBV-negative Burkitt's lymphoma (BL) cell line. Akata 2003 (Ak2003), Mutu-I, Daudi, P3HR1-c16, Raji and Mutu-III are EBV-positive BL cell lines (obtained from Prof. M. Allday and Prof. P. Farrell, Imperial College, UK). LCL330, LCL337 and LCL354 were established by immortalization of peripheral blood mononuclear cells (PBMCs) of paediatric post-transplant lymphoproliferative disorder (PTLD) patients with B95-8 strain of EBV. All the cell lines were cultured in RPMI medium1640 and supplemented with fetal bovine serum (FBS; 10% and 15% for BL cell lines and LCLs, respectively), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) and were grown in a humidified incubator at 37°C with 5% CO_2.

Chemicals

MS-275, apicidin, panobinostat, sodium butyrate and bortezomib were purchased from Selleck (Selleck Chemicals, Houston, TX). SAHA was purchased from Cayman (Cayman Chemicals, Ann Arbor, MI). N-acetyl-cysteine (NAC) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

BL and LCLs (5 X 10^5 cells/ml) were seeded in triplicates in 96-well plates and

treated with various concentrations of drugs for 24, 48 and 72 hr. MTT (Invitrogen, Carlsbad, CA) assay was performed and percentage of cell proliferation was calculated as previously described (Hui and Chiang 2010, Hui, *et al* 2012). **Three independent experiments were performed for each MTT assay.**

Annexin V/propidium iodide (AV/PI) assay

BL and LCLs (5 X 10^5 cells/ml) were treated with combination of SAHA and bortezomib for 48 hr. The treated cells were stained with FITC-AV and propidium iodide (PI; BD Pharmingen™, Heidelberg, Germany) and the percentage of AV/PI-positive cells was calculated as previously described (Hui, *et al* 2012, Hui, *et al* 2013). **Two independent experiments were performed in each cell line.**

Cell cycle analysis

BL cells or LCLs (5 X 10^5 cells/ml) were treated with combination of SAHA and bortezomib for 12, 24 and 48 hr. The treated cells were subjected to cellular DNA content analysis by flow cytometry as previously described (Hui and Chiang 2010). **Three independent experiments were performed in each cell line.**

Western blot analysis

BL cells or LCLs (5 X 10^5 cells/ml) were treated with various concentrations of drugs for 24, 48 and 72 hr. Protein from the cell cultures was extracted and western blot analysis was performed as previously described (Hui and Chiang 2010). EBV lytic proteins (Zta), acetylated histone (acetyl-H3), apoptotic proteins (PAPR and cleaved caspase-3) and α-tubulin were detected with the antibodies described previously (Hui, *et al* 2012). EBV latent proteins were detected with mouse anti-LMP1 monoclonal, rabbit anti-EBNA-1 polyclonal (Prof. Jaap Middeldorp, VU

University, Netherlands), rat anti-EBNA-2 monoclonal (Prof. Elisabeth Kremmer, Institute of Molecular Immunology, Germany) and sheep anti-EBNA-3A polyclonal antibodies (Exalpha Biologicals, Maynard, MA). EBV Bcl2 homologue BHRF1 was detected with mouse anti-BHRF1 monoclonal antibody (Millipore, Temecula, CA). Expression of tumour suppressor genes was detected with mouse anti-p16 monoclonal (BD Biosciences, San Jose, CA), rabbit anti-p21 and anti-Bim polyclonal antibodies (Cell Signaling Technology, Beverly, MA), respectively. **At least 2 independent experiments were performed in each western blotting.**

Nude mice experiment

Female BALB/c nude (nu/nu) mice were purchased at 5-6 weeks of age from the Laboratory Animal Unit (LAU), The University of Hong Kong. The mice were housed in LAU under a pathogen-free condition. All experiments were performed in accordance with the LAU guidelines and approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of The University of Hong Kong. P3HR1-c16 (1 X 10^7) cells were re-suspended in 200 µl serum-free culture medium. The cells were subcutaneously injected at the right flanks of female BALB/c nude (nu/nu) mice at 6-7 weeks of age. When the tumours became palpable, 60 µg/kg bortezomib, 50 mg/kg SAHA or their combination dissolved in DMSO in 10 µl was administered to nude mice of the treatment group (n=5) by intraperitoneal (IP) injection 5 days per week for 4 weeks. Equal volume of DMSO was administered by IP injection to nude mice of the control group (n=5). The size and the weight of the tumours were measured as described previously (Hui*, et al* 2012, Hui*, et al* 2013).

Statistical analysis

Synergism of SAHA and bortezomib was analyzed with isobologram analysis and combination index (CI) calculation as described previously (Hui and Chiang 2014, Hui, *et al* 2013). Curves that lie under the additive isobole in isobologram suggest synergism and vice versa. CI<1, =1 and >1 represents synergy, additivity and antagonism, respectively. All statistical analyses were performed with GraphPad Prism Version 5.0 software.

Results

BL cells of Wp-restricted or type III latency were more resistant to the treatment with HDAC inhibitors than those of type I latency, independent of lytic cycle induction.

We tested the effects of different HDAC inhibitors on proliferation of EBV-positive BL cells which express different forms of viral latency. BL cells of type I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) and type III (Raji and Mutu-III) latencies were treated with five different HDAC inhibitors, including MS-275, apicidin, panobinostat, suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (NaB) for 24, 48 and 72 hr. The treated cells were analyzed for cell proliferation by MTT assay (only the results on SAHA were shown because similar anti-proliferation effects by the HDAC inhibitors were observed; Fig. 1a). According to the data, all these HDAC inhibitors could suppress the proliferation of BL cells in a dose- and time-dependent manner. Half maximal inhibitory concentration (IC50) values of the five HDAC inhibitors on the proliferation of BL cells at 48-hr time point were determined (Table 1). The IC50 values of HDAC inhibitors on BL cells of Wp-restricted (Daudi and P3HR1-c16) and type III (Raji and Mutu-III) latencies were higher than those of latency I (AK2003 and Mutu-I), suggesting that BL cells of Wp-restricted and type III latencies were more resistant to the treatment with HDAC inhibitors. Since HDAC inhibitors were shown to induce EBV lytic cycle in BL cells

(Ghosh, *et al* 2012), we also investigated whether the enhanced resistance to the treatment with HDAC inhibitors was related to the refractoriness of EBV lytic cycle induction in the Wp-restricted and latency III BL cells (only the results on SAHA were shown because similar ability of lytic cycle induction by the HDAC inhibitors were observed; Fig. 1b). We found that HDAC inhibitors could not induce the expression of Zta in almost all these BL cell lines (except weak induction was observed in P3HR1-c16) regardless of the forms of viral latency, indicating that the differential responses of the BL cell lines to killing by HDAC inhibitors were unrelated to EBV lytic cycle induction. Collectively, our data showed that Wp-restricted and latency III BL cells were more resistant to the treatment with HDAC inhibitors when compared with latency I BL cells and the drug resistance was independent of EBV lytic cycle induction.

Combination of SAHA/bortezomib induced synergistic killing of Wp-restricted and latency III BL cells.

Since combination of SAHA and bortezomib, a proteasome inhibitor, can synergistically induce cell death in various types of cancers (Emanuele, *et al* 2007, Hui, *et al* 2013, Pitts, *et al* 2009, Zhang, *et al* 2009), we also investigated the effect of combining SAHA/bortezomib on the killing of BL cell lines expressing various types of EBV latency. We treated latency I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) or latency III (Raji and Mutu-III) BL cells with combinations of various concentrations of SAHA (0, 0.125, 0.25, 0.5, 1 and 2 μM) and bortezomib (0, 1, 2, 4, 8, 16, 32 and 64 nM). The treated cells were analyzed for cell proliferation by MTT assay (Fig. 2a). When compared with either drug alone, SAHA/bortezomib yielded a much stronger anti-proliferative effect in the Wp-restricted and latency III BL cells. However, such enhanced killing could not be observed in the latency I BL cells.

Isobologram analysis was performed to determine the synergism between SAHA/bortezomib (Fig. 2b). Whilst the isoboles lied to the left of the additive isoboles for the Wp-restricted and latency III BL cells, they lied to the right for the latency I BL cells, indicating synergism of SAHA/bortezomib in the BL cells of Wp-restricted or type III latency. The synergistic anti-proliferative effect of bortezomib and other HDAC inhibitors (including MS-275, apicidin, panobinostat and NaB) could also be observed in the Wp-restricted P3HR1-c16 cells (supplementary Fig. 1).

Synergistic killing by SAHA/bortezomib could also be achieved in LCLs.

To test whether the synergistic killing **could** also be observed in other EBV-associated B cell lines, we established a panel of EBV-transformed lymphoblastoid cell lines (LCLs), including LCL330, LCL337 and LCL354, from PBMCs of **patients with** post-transplant lymphoproliferative disorder (PTLD). According to the MTT assay and isobologram analysis, SAHA/bortezomib could also induce killing of all three LCLs **synergistically** (Fig. 3a). To confirm the EBV latency in the LCLs and BL cells, we examined the expression pattern of EBV latent proteins in **the** cell lines by western blot analysis. BL cells of type I latency, including **AK2003** and Mutu-I only expressed **EBNA-1** protein. The Wp-restricted BL cell lines, Daudi and P3HR1-c16, expressed **EBNA-1** and the **EBNA-3** proteins (here shown by EBNA-3A). BL cells and LCLs of type III latency, including Raji, Mutu-III, LCL330, LCL337 and LCL354, expressed EBNA-1, EBNA-2, EBNA-3A and LMP-1 (Fig. 3b).

SAHA/bortezomib induced apoptosis of Wp-restricted BL cells and latency III LCLs.

We postulated that SAHA/bortezomib could antagonize the function of EBNA-3 proteins and induce cell cycle arrest and apoptosis of Wp-restricted BL

cells and LCLs. P3HR1-c16 and LCL337 cells were treated with SAHA/bortezomib for 12, 24, 48 and 72 hr, stained with propidium iodide and subjected to flow cytometric analysis of the cell cycle (Fig. 4a and c). The data at 72 hr were not suitable for cell cycle analysis because the majority of the cells underwent apoptosis following the drug treatment. Following treatment of P3HR1-c16 cells with the drugs for 24 hr, SAHA clearly arrested cell cycle at G1 phase whereas both bortezomib and SAHA/bortezomib arrested similar percentages of cells at G2/M phase. In LCL337, SAHA, bortezomib and SAHA/bortezomib arrested similar percentages of cells at G1 phase. These data indicated no apparent additive or synergistic effect between SAHA and bortezomib on cell cycle arrest in both P3HR1-c16 and LCL337. We also analyzed the percentages of the sub-G1 populations, which represent the cells undergoing apoptosis (Fig. 4b & 4d). The percentages of sub-G1 populations increased over time from 12 hr to 72 hr in the cells treated with SAHA/bortezomib or either drug alone. At 48-hr time point, significantly higher percentages of sub-G1 populations was observed in the cells treated with SAHA/bortezomib (~37% and ~53% in P3HR1-c16 and LCL337, respectively) when compared with those treated with either drug alone. We confirmed the enhanced apoptosis by annexinV/propidium iodide (AV/PI) staining **(Fig. 4e)**. Following 48 hr treatment with SAHA, bortezomib or SAHA/bortezomib, the percentages of apoptotic cells (AV+) increased to 17.1%, 20.0% and 58.5% in P3HR1-c16 and to 13.7%, 39.7% and 86.6% in LCL337, respectively. **To further analyze the relationship between the cell cycle arrest and apoptosis, we performed time course experiments to investigate the kinetics of expression of several cell cycle and apoptotic markers following treatment with SAHA/bortezomib (Fig. 4f). Expression of apoptotic markers (cleaved PARP and**

caspase-3) appeared at 4 hr and 8 hr in LCL337 and P3HR1-c16, respectively. However, there was no significant alternation of the protein levels of the cell cycle regulators (cyclin-D1, CDK4, cyclin-B1 and p-cdc2) in both cell lines until 24 hr when down-regulation of CDK4, cyclin-B1 and p-cdc2 was observed. The early activation of apoptotic markers in the cell lines suggested the existence of two cellular subpopulations in response to the treatment with SAHA/bortezomib, one sensitive to apoptosis and the other arresting in the cell cycle.

SAHA/bortezomib induced the expression of p16^{INK4A} and p21^{WAF1}, concomitant with the up-regulation of apoptotic markers.

Tumour suppressor **proteins**, including Bim, p16^{INK4A} and p21^{WAF1}, were shown to be down-regulated by EBNA-3 proteins**, hence facilitating** the growth of EBV-associated malignancies (Anderton*, et al* 2008, Cooper*, et al* 2003, Hertle*, et al* 2009, Maruo*, et al* 2006, Maruo*, et al* 2011, Paschos*, et al* 2009, Saha*, et al* 2011). We investigated whether SAHA/bortezomib could **increase the levels of these tumour suppressor proteins** in Wp-restricted BL and LCLs. We treated P3HR1-c16 cells and LCL337 with combination of SAHA/bortezomib for 24 hr. The cell lysates of the treated cells were analyzed for expression of tumour suppressor **proteins** (p16^{INK4A}, p21^{WAF1}, p27 and Bim), apoptotic markers (PARP, cleaved PARP and cleaved caspase-3) and cell cycle regulatory proteins (cyclin-D1, CDK4, cyclin-B1 and p-cdc2) by western blotting (Fig. 5). When compared with either drug alone, SAHA/bortezomib significantly induced the expression of p16^{INK4A} and p21^{WAF1} but not p27 (Fig. 5a) **nor** Bim (Fig. 5b) in both P3HR1-c16 and LCL337 cells. Concomitantly, SAHA/bortezomib increased the expression of cleaved PARP and cleaved caspase-3 and decreased the expression of **PARP**, CDK4, cyclin-B1 and p-cdc2. **The up-regulation of the apoptotic marks and down-regulation of cell**

cycle markers were consistent with the data presented in Fig. 4f. Together, the data indicated that induction of apoptosis and down-regulation of cell cycle regulatory proteins mediated by SAHA/bortezomib might be related to the up-regulation of $p16^{INK4A}$ and $p21^{WAF1}$.

Up-regulation of $p16^{INK4A}$ and $p21^{WAF1}$ and induction of apoptosis were dependent on the generation of reactive oxygen species (ROS).

We and others have reported that SAHA/bortezomib could synergistically induce apoptosis of various types of cancer cells through an ROS-dependent mechanism (Denlinger, *et al* 2004, Heider, *et al* 2009, Heider, *et al* 2008, Hui, *et al* 2013, Pei, *et al* 2004, Yu, *et al* 2003). We postulated that SAHA/bortezomib could mediate a similar apoptotic mechanism in both Wp-restricted BL and LCLs. We pre-treated P3HR1-c16 and LCL337 with 12 mM N-acetyl cysteine (NAC), an ROS scavenger, for 1 hr before treatment with SAHA/bortezomib. Addition of NAC could significantly reduce the cleavage of PARP and caspase-3 in both P3HR1-c16 and LCL337 (Fig. 5c). **Interestingly, NAC also significantly reduced the protein levels of $p16^{INK4A}$ and $p21^{WAF1}$ (Fig. 5d and supplementary Fig. 2). Taken together, the data showed that SAHA/bortezomib could induce the up-regulation of $p16^{INK4A}$ and $p21^{WAF1}$ and apoptosis in Wp-restricted BL and LCLs via an ROS-dependent mechanism (Fig. 5e).**

SAHA/bortezomib significantly suppressed the growth of Wp-restricted BL xenografts in nude mice.

Finally, we attempted to examine the *in vivo* effect of SAHA/bortezomib on growth suppression of Wp-restricted BL and LCL xenografts. However, only the xenografts of P3HR1-c16, but not LCLs, could grow in nude mice. The mice bearing

the P3HR1-c16 xenografts were treated with either DMSO (vehicle control) (n=5), 50 mg/kg SAHA (n=5), 60 μg/kg bortezomib (n=5) or combination of 50 mg/kg SAHA and 60 μg/kg bortezomib (n=5) for 5 days per week over 15 days by intraperitoneal injection (Fig. 6a). Toxicity of either SAHA, bortezomib or their combination on nude mice was assessed by monitoring their weight (Fig. 6b). Despite weight loss of approximately 5%, no other noticeable toxicity was observed in the mice treated with SAHA/bortezomib or either drug alone. The growth of tumours was measured during the experimental period (Fig. 6c). On day 6 post-treatment, mean tumour volumes of mice treated with vehicle control, SAHA or bortezomib started to exceed that of the group treated by SAHA/bortezomib. At the end of experiment (day 15 post-treatment), the mean tumour volumes of mice treated with vehicle control, SAHA, bortezomib and SAHA/bortezomib were 4093 mm^3, 2950 mm^3, 2948 mm^3, and 1048 mm^3, respectively. Whilst either SAHA or bortezomib alone suppressed the growth of tumours, their combination mediated much stronger suppressive effect ($P < 0.01$) (Fig. 6d).

Discussion

Epstein-Barr virus (EBV) latent proteins exert anti-apoptotic effects on Wp-restricted Burkitt's lymphoma (BL) and lymphoblastoid cell line (LCL) through down-regulation of Bim, $p16^{INK4A}$ and $p21^{WAF1}$ (Anderton, *et al* 2008, Cooper, *et al* 2003, Hertle, *et al* 2009, Maruo, *et al* 2006, Maruo, *et al* 2011, Paschos, *et al* 2009, Saha, *et al* 2011, White, *et al* 2010). Histone deacetylase (HDAC) inhibitors can synergize with proteasome inhibitors to up-regulate these tumour suppressor genes (Emanuele, *et al* 2007, Heider, *et al* 2009, Hui and Chiang 2014, Pei, *et al* 2004, Yu, *et al* 2003). In this study, we hypothesized that combination of HDAC and proteasome inhibitors **could synergistically** induce apoptosis of Wp-restricted BL and

LCL.

We first tested the responses of six endemic BL cell lines, which expressed either type I, Wp-restricted or type III latency, to a panel of HDAC inhibitors. **We found that both** Wp-restricted and latency III BL cell lines displayed higher resistance to killing by HDAC inhibitors when compared to latency I BL cell lines (refer to Fig. 1 & Table 1). Despite identical cellular background **of Mutu-I (latency I) and Mutu-III (latency III) cell lines (Gregory, *et al* 1990),** Mutu-III cells were more resistant to HDAC inhibitor-mediated killing when compared with Mutu-I cells, **strongly implicating** the involvement of EBV latent proteins in the mechanism of drug resistance. **Among the latent genes,** EBNA-LP, -3A, -3B and -3C are expressed in both Wp-restricted and type III EBV latency. However, both EBNA-LP and EBNA-3B are not essential for the growth transformation of LCLs (Rickinson and Kieff 2007). **We speculated that EBNA-3A and -3C might contribute to** the resistance of Wp-restricted BL cells to the killing effect of HDAC inhibitors (Anderton, *et al* 2008, Skalska, *et al* 2010). Since HDAC inhibitors were reported to induce EBV lytic cycle in BL cell lines, we also analyzed whether the expression of EBV lytic proteins might be involved in the drug resistance of Wp-restricted or latency III BL cells (Iwamoto, *et al* 2011, Jung, *et al* 2007, Rodriguez, *et al* 1999). We found that HDAC inhibitors could only induce low expression levels of EBV lytic proteins in P3HR1-c16 cells, but not in other BL cell lines (refer to Fig. 1). Indeed, we had shown that HDAC inhibitors preferentially induce EBV lytic cycle in EBV-positive epithelial rather than B-cell malignancies (Hui and Chiang 2010, Hui, *et al* 2012). The data implied that the mechanism of drug resistance of Wp-restricted or latency III BL cells was independent of EBV lytic cycle induction.

Our laboratory and others had previously reported that proteasome inhibitors such as bortezomib could potentiate the anti-tumour effect of HDAC inhibitors such

as SAHA in various types of cancers (Emanuele, *et al* 2007, Hui, *et al* 2013, Miller, *et al* 2009, Zhang, *et al* 2009). Moreover, combination of HDAC and proteasome inhibitors can up-regulate tumour suppressor genes in different cancer cell lines (Emanuele, *et al* 2007, Heider, *et al* 2009, Hui and Chiang 2014, Pei, *et al* 2004, Yu, *et al* 2003). Here, we demonstrated that SAHA/bortezomib could synergistically induce killing of Wp-restricted (Daudi and P3HR1-c16) or latency III (Raji and Mutu-III) BL cells but not latency I BL lines (Ak2003 and Mutu-I). Interestingly, the drug combination could also induce synergistic killing of EBV-transformed LCLs which also express type III latency. The synergistic killing was due to apoptosis as evidenced by the higher percentage of annexin V-positive and sub-G1 populations and stronger cleavage of PARP and caspase-3.

EBNA-3 proteins could provide anti-apoptotic effect through down-regulation of tumour suppressor genes, including Bim, $p16^{INK4A}$ and $p21^{WAF1}$ (Anderton, *et al* 2008, Cooper, *et al* 2003, Hertle, *et al* 2009, Maruo, *et al* 2006, Maruo, *et al* 2011, Paschos, *et al* 2009, Saha, *et al* 2011, White, *et al* 2010). We analyzed the expression level of Bim, $p16^{INK4A}$ and $p21^{WAF1}$ and the viral encoded Bcl2 homologue, BHRF1, upon treatment with SAHA/bortezomib **and found that the drug combination** could significantly up-regulate the expression of $p16^{INK4A}$ and $p21^{WAF1}$, but not Bim nor BHRF1. **One potential pro-apoptotic effect of $p16^{INK4A}$ and $p21^{WAF1}$ was mediated by arresting cells at G1 or G2/M phases (Davis, *et al* 1998, Mandl-Weber, *et al* 2010, Park, *et al* 2002, Wang, *et al* 2010). We did not observe such casual relationship between cell cycle arrest and enhanced apoptosis in both Wp-restricted BL and LCLs upon treatment with SAHA/bortezomib which seemed to briskly induce apoptosis of Wp-restricted BL and LCLs without arrest of the cell cycle. Indeed, $p16^{INK4A}$ has been shown to preferentially induce apoptosis in proliferating cells rather than G1-arrested cells (Al-Mohanna, *et al***

2004, Minami, *et al* 2003). p21^{WAF1} can also directly promote apoptosis in different cancer cell lines through mechanisms involving the activation of TNF receptors or induction of pro-apoptotic protein, Bax (Abbas and Dutta 2009). In addition, the induction of apoptosis and up-regulation of p16^{INK4A} and p21^{WAF1} by SAHA/bortezomib were reactive oxygen species (ROS)-dependent, which is consistent with previous findings that ROS generation induced the expression of p16^{INK4A} and p21^{WAF1} and triggered apoptosis of different cancer cell lines (Donadelli, *et al* 2006, Hui and Chiang 2014, Hui, *et al* 2013, Kim and Wong 2009, Luo, *et al* 2011). We further evaluated the effect of SAHA/bortezomib on Wp-restricted BL xenografts in nude mice and found that the drug combination significantly suppressed the growth of Wp-restricted BL xenografts in nude mice, indicating that the *in vitro* anti-tumour effect of SAHA/bortezomib could also be achieved *in vivo*.

Collectively, our results proposed a novel synergistic action of SAHA/bortezomib in Wp-restricted BL and LCLs through the up-regulation of p16^{INK4A} and p21^{WAF1}, in a ROS-dependent pathway and possibly reversing the suppressive effect of EBNA-3 proteins on these tumour suppressor proteins. The potential application of this drug regimen as an alternative clinical regimen for the treatment of Wp-restricted BL and PTLD should be further investigated.

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Authorship

KFH and YYL designed the work, performed experiments, analyzed the data and wrote the manuscript; PLY performed experiments and analyzed the data; JMM interpreted the data and revised the manuscript; AKC conceived the work, interpreted the data and wrote the manuscript.

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Figure legends:

Fig 1. Effects of SAHA on proliferation of EBV-positive BL cells and induction of EBV lytic cycle. (A) EBV-positive BL cells of type I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) or type III latency (Raji and Mutu-III) were treated with increasing concentrations of SAHA for 24, 48 and 72 hr. Cell proliferation was measured by MTT reduction assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. **Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments.** (B) The cellular and viral proteins of BL cells treated with increasing concentrations of SAHA for 48 hr were extracted and analyzed for expression of an acetylated cellular histone H3 protein, acetyl-H3, and a viral lytic protein, Zta, by western blotting. α-tubulin was detected as a loading control. Anti-human IgG antibody (IgG) was used to induce the lytic cycle of AK2003 cells (AK03+IgG) and the protein extracted was loaded as a positive control of Zta expression.

Fig 2. Effects of SAHA/bortezomib on proliferation of EBV-positive BL cells. (A) EBV-positive BL cells of type I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) or type III latency (Raji and Mutu-III) were treated with various combinations of SAHA (0, 0.125, 0.25, 0.5, 1, and 2 μM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. **Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (B) Synergisms of proliferation inhibition of different BL cell lines were analyzed by isobologram analysis.**

Fig 3. Effects of SAHA/bortezomib on proliferation of EBV-transformed LCLs. (A) Three EBV-transformed LCLs, including LCL330, LCL337 and LCL354, were treated with various combinations of SAHA (0, 0.125, 0.25, 0.5, 1, and 2 μM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay. **Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments.** (B) Isobologram analysis was performed to analyze the synergism of SAHA and bortezomib on the LCLs. (C) Cellular proteins of six BL cell lines and three LCLs were extracted and analyzed for expression of EBV latent proteins including EBNA-1, EBNA-2, EBNA-3A and LMP-1 by western blotting. α-tubulin was detected as a loading control. EBV-negative sub-clone of Akata cells, AK31, was used as a negative control.

Fig 4. Effects of SAHA/bortezomib on cell cycle arrest and apoptosis of Wp-restricted BL and LCLs. P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) or either drug alone **for 12, 24, 48 and 72 hr. Cell cycle status was analyzed by flow cytometry and representative figures for (A) P3HR1-c16 and (C) LCL337 at 12, 24 and 48 hr are shown. The percentages of sub-G1 population of (B) P3HR1-c16 and (D) LCL337 at 12, 24, 48 and 72 hr are shown. The results were analyzed for statistical significance using One-way ANOVA Dunnett's Multiple Comparison Test. P value less than 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with SAHA/bortezomib.** Error bars represent the standard error of mean (SEM) of data

obtained in at least three independent experiments. **(E)** Annexin V/propidium iodide (AV/PI) staining was performed to analyze for the percentages of apoptotic cells upon treatment with SAHA/bortezomib or either drug alone for 48 hr. AV+/PI- population represents early apoptotic cells whilst AV+/PI+ population represents late apoptotic/ necrotic cells. **(F) P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) for 0, 2, 4, 8, 12 and 24 hr. The cellular proteins were extracted and analyzed for expression of PARP, cleaved PARP, cleaved caspase-3, cyclin-D1, CDK4, cyclin-B1 and p-cdc2 by western blot analysis. α-tubulin was detected as a loading control.**

Fig 5. The roles of $p16^{INK4A}$, $p21^{WAF1}$ and ROS in the apoptosis of Wp-restricted BL and LCLs. P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) or either drug alone for 24 hr. (A) The cellular proteins were extracted and analyzed for expression of $p16^{INK4A}$, $p21^{WAF1}$, p27, PARP, cleaved PARP, cleaved caspase-3, cyclin-D1, CDK4, cyclin-B1 and p-cdc2 by western blot analysis. α-tubulin was detected as a loading control. (B) Expression of the cellular pro-apoptotic protein Bim, viral latent protein EBNA-3A and viral encoded Bcl2 homologue BHRF1 was analyzed. (C) P3HR1-c16 cells and LCL337 were pretreated with N-acetyl cysteine (NAC) for 1 hr followed by treatment with SAHA, bortezomib or SAHA/bortzomib for 24 hr. Expression of PAPR, cleaved PARP and cleaved caspase-3 proteins was analyzed. (D) Expression of $p16^{INK4A}$ and $p21^{WAF1}$ proteins was analyzed. (E) Schematic diagram illustrating the roles of $p16^{INK4A}$, $p21^{WAF1}$ and ROS in the mechanism of apoptosis induced by SAHA/bortezomib.

Fig 6. Effects of SAHA/bortezomib on growth of Wp-restricted BL xenografts established in nude mice. P3HR1-c16 cells were inoculated into the right flanks of nude mice. The mice were treated with either DMSO (vehicle control) (n=5), 50 mg/kg SAHA (n=5), 60 μg/kg bortezomib (n=5) or combination of 50 mg/kg SAHA and 60 μg/kg bortezomib (n=5) for 5 days per week over 15 days by intraperitoneal injection. (A) The tumours were resected from the nude mice at the end of the experiment. (B) The mice were weighed at 0, 1, 6, 8, 12 and 15 days post-treatment. (C) Tumour size measurements were performed at 0, 1, 6, 8, 12 and 15 days post-treatment by using a caliper. (D) Average tumour masses of mice of control and treated groups were shown. The results were analyzed for statistical significance using One-way ANOVA Dunnett's Multiple Comparison Test. P value less than 0.05 was considered statistically significant; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with SAHA/bortezomib. Error bars represent the standard error of mean (SEM) of data obtained from the nude mice (n=5).

Supplementary Fig 1. Effects of combination of either MS-275, apicidin, SAHA or sodium butyrate (NaB) with bortezomib on proliferation of Wp-restricted BL cells. P3HR1-c16 cells were treated with combination of either MS-275 (0, 0.5, 1, 2, 4 and 8 μM), apicidin (0, 31.25, 62.5, 125, 250 and 500 nM), panobinstat (0, 3.125, 6.25, 12.5, 25 and 50 nM), or NaB (0, 0.6, 1.2, 2.4, 4.8 and 9.6 mM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. Synergisms of proliferation inhibition of the cells following treatment with different drug combinations were analyzed by isobologram analysis.

Supplementary Fig 2. Western blot results in Fig. 4d were quantitated by densitometry. The bars represent the relative protein levels compared to the loading control.

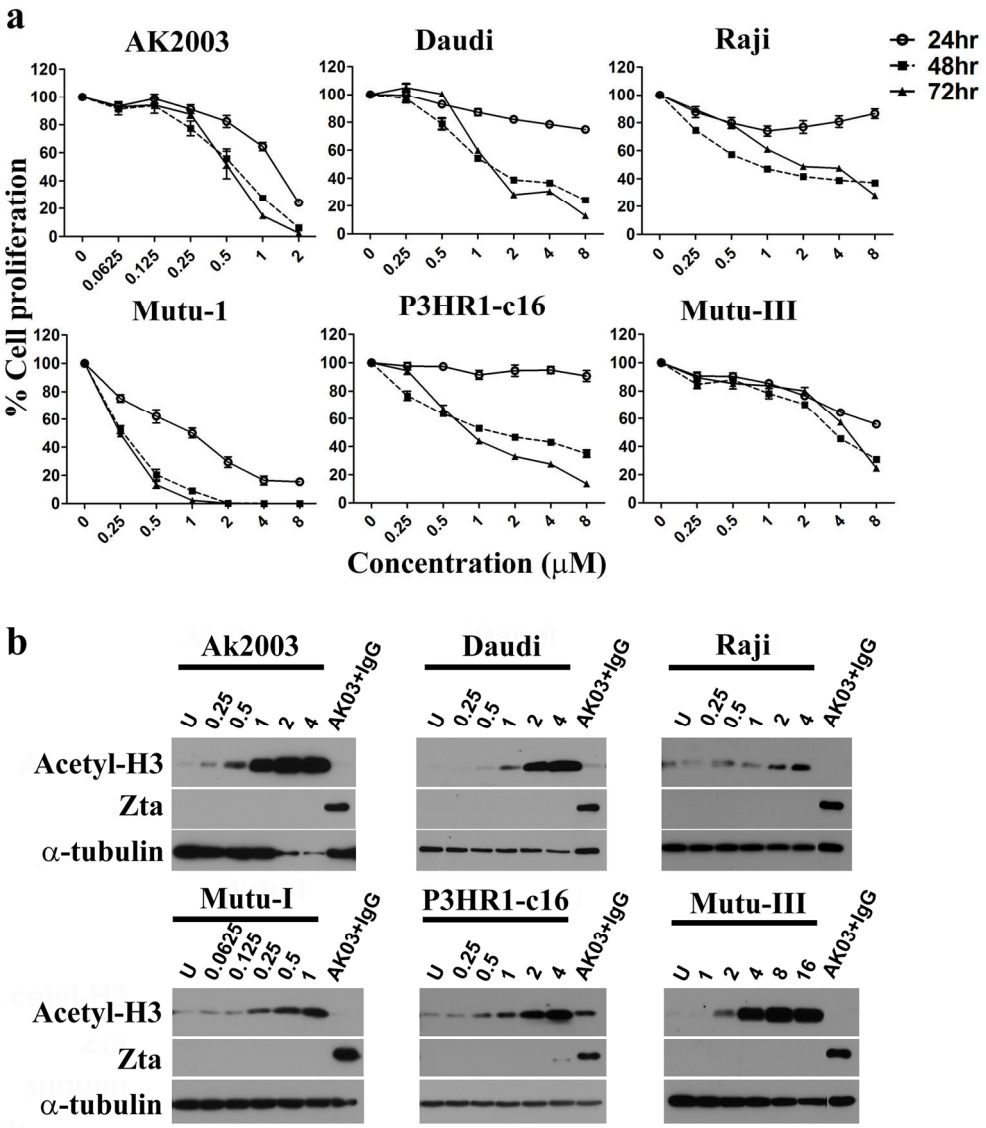

Fig 1. Effects of SAHA on proliferation of EBV-positive BL cells and induction of EBV lytic cycle. (A) EBV-positive BL cells of type I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) or type III latency (Raji and Mutu-III) were treated with increasing concentrations of SAHA for 24, 48 and 72 hr. Cell proliferation was measured by MTT reduction assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (B) The cellular and viral proteins of BL cells treated with increasing concentrations of SAHA for 48 hr were extracted and analyzed for expression of an acetylated cellular histone H3 protein, acetyl-H3, and a viral lytic protein, Zta, by western blotting. α-tubulin was detected as a loading control. Anti-human IgG antibody (IgG) was used to induce the lytic cycle of AK2003 cells (AK03+IgG) and the protein extracted was loaded as a positive control of Zta expression.
206x237mm (300 x 300 DPI)

HDAC inhibitor		MS-275 (μM)	Apicidin (nM)	Panobinostat (nM)	SAHA (μM)	NaB (mM)
Cell line	Latency					
AK2003	I	0.37	90.79	4.51	0.55	0.49
Mutu-I	I	0.70	78.48	4.65	0.26	0.40
Daudi	Wp	0.80	501.70	17.28	1.72	1.56
P3HR1-c16	Wp	11.29	684.3	33.47	1.84	5.57
Raji	III	5.61	1220.00	33.28	1.31	10.57
Mutu-III	III	3.90	971.70	75.71	3.73	7.74

Table 1. Values of half maximal inhibitory concentration (IC50) of HDAC inhibitors on proliferation of BL cells

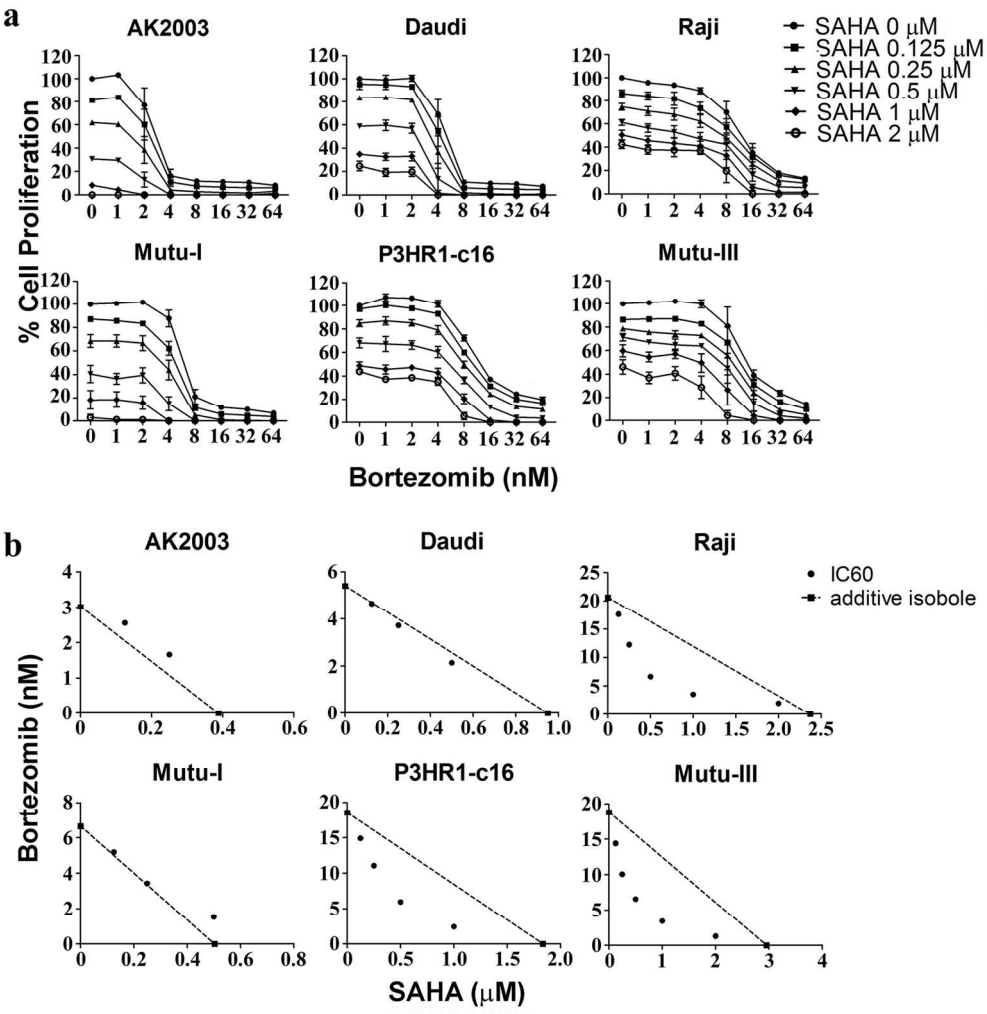

Fig 2. Effects of SAHA/bortezomib on proliferation of EBV-positive BL cells. (A) EBV-positive BL cells of type I (AK2003 and Mutu-I), Wp-restricted (Daudi and P3HR1-c16) or type III latency (Raji and Mutu-III) were treated with various combinations of SAHA (0, 0.125, 0.25, 0.5, 1, and 2 μM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (B) Synergisms of proliferation inhibition of different BL cell lines were analyzed by isobologram analysis.
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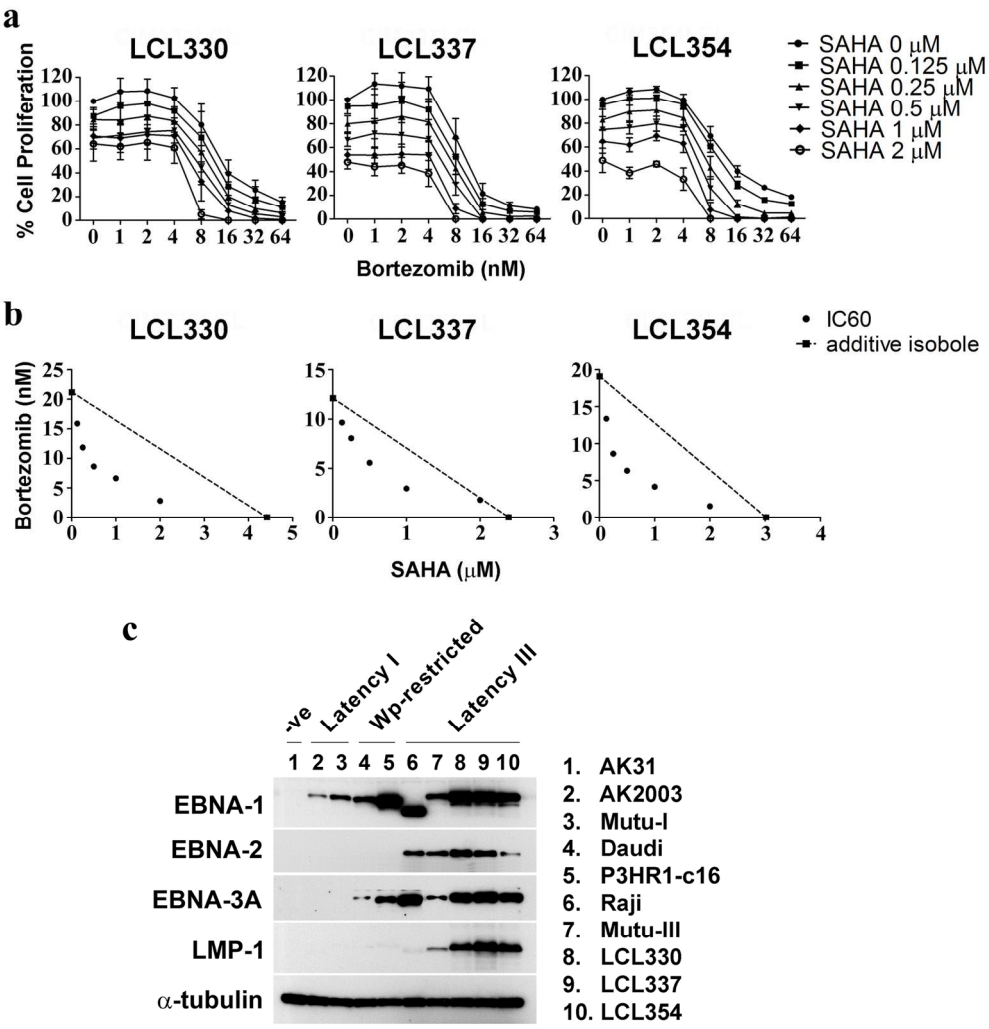

Fig 3. Effects of SAHA/bortezomib on proliferation of EBV-transformed LCLs. (A) Three EBV-transformed LCLs, including LCL330, LCL337 and LCL354, were treated with various combinations of SAHA (0, 0.125, 0.25, 0.5, 1, and 2 μM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (B) Isobologram analysis was performed to analyze the synergism of SAHA and bortezomib on the LCLs. (C) Cellular proteins of six BL cell lines and three LCLs were extracted and analyzed for expression of EBV latent proteins including EBNA-1, EBNA-2, EBNA-3A and LMP-1 by western blotting. α-tubulin was detected as a loading control. EBV-negative sub-clone of Akata cells, AK31, was used as a negative control.
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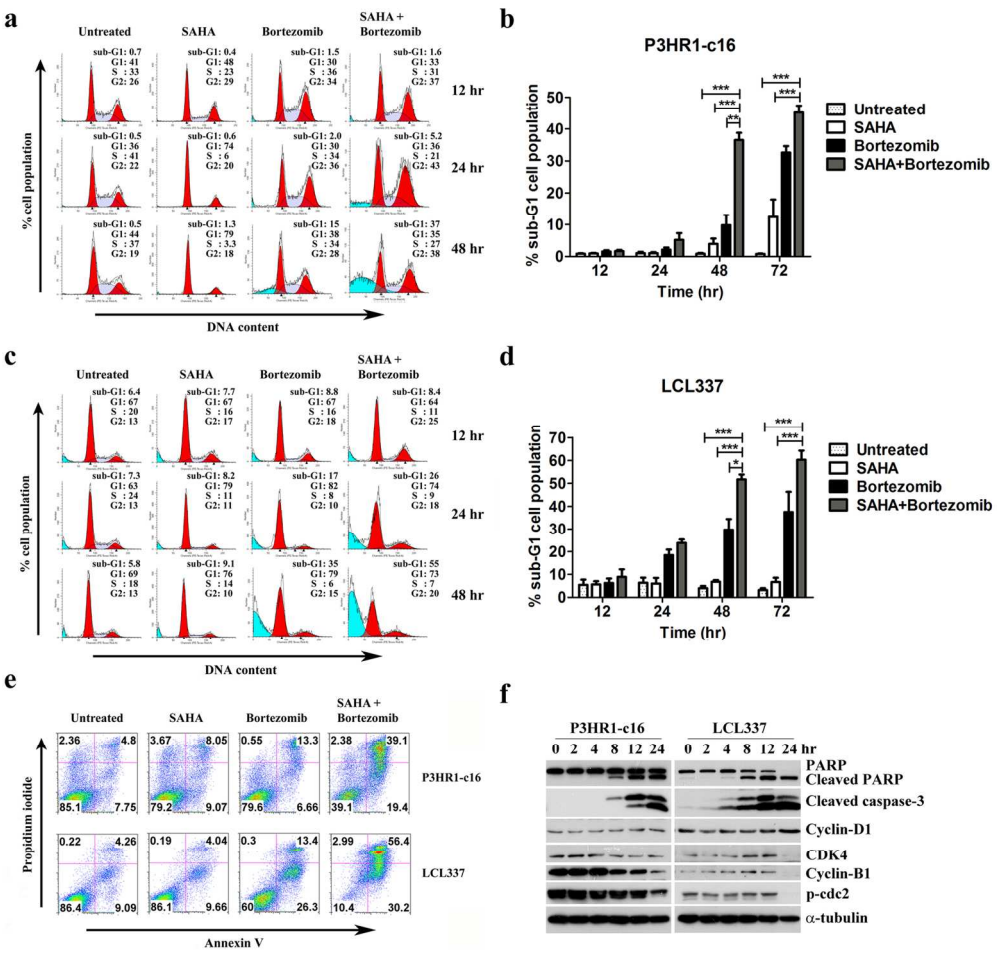

Fig 4. Effects of SAHA/bortezomib on cell cycle arrest and apoptosis of Wp-restricted BL and LCLs. P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) or either drug alone for 12, 24, 48 and 72 hr. Cell cycle status was analyzed by flow cytometry and representative figures for (A) P3HR1-c16 and (C) LCL337 at 12, 24 and 48 hr are shown. The percentages of sub-G1 population of (B) P3HR1-c16 and (D) LCL337 at 12, 24, 48 and 72 hr are shown. The results were analyzed for statistical significance using One-way ANOVA Dunnett's Multiple Comparison Test. P value less than 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with SAHA/bortezomib. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments. (E) Annexin V/propidium iodide (AV/PI) staining was performed to analyze for the percentages of apoptotic cells upon treatment with SAHA/bortezomib or either drug alone for 48 hr. AV+/PI- population represents early apoptotic cells whilst AV+/PI+ population represents late apoptotic/ necrotic cells. (F) P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) for 0, 2, 4, 8, 12 and 24 hr. The cellular proteins were extracted and analyzed for expression of PARP, cleaved PARP, cleaved caspase-3, cyclin-D1, CDK4, cyclin-B1 and p-cdc2 by western blot analysis. α-tubulin was detected as a loading control.
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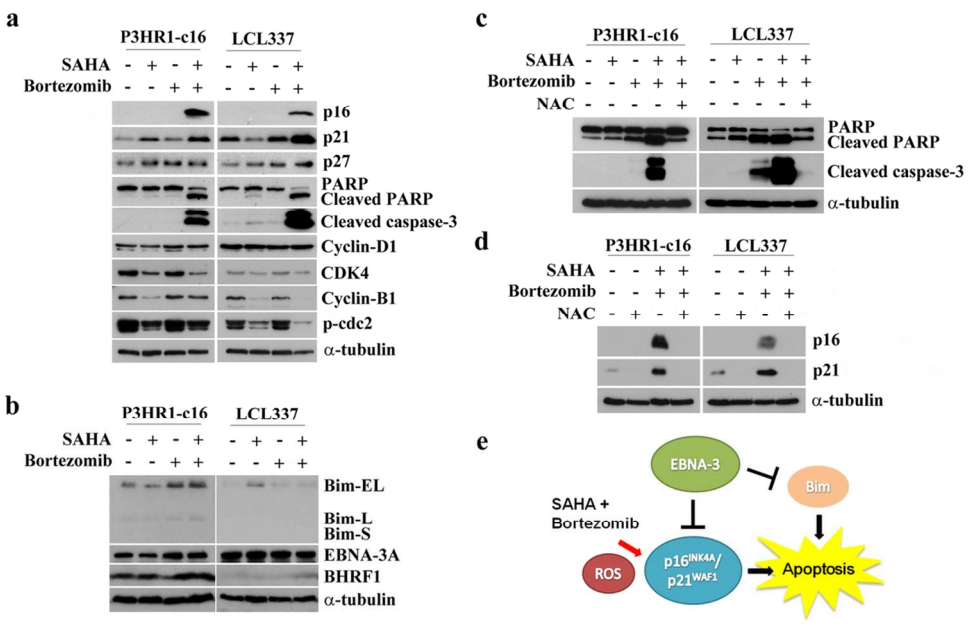

Fig 5. The roles of p16INK4A, p21WAF1 and ROS in the apoptosis of Wp-restricted BL and LCLs. P3HR1-c16 and LCL337 were treated with combination of SAHA (2 μM and 1 μM for P3HR1-c16 and LCL337, respectively) and bortezomib (8 nM) or either drug alone for 24 hr. (A) The cellular proteins were extracted and analyzed for expression of p16INK4A, p21WAF1, p27, PARP, cleaved PARP, cleaved caspase-3, cyclin-D1, CDK4, cyclin-B1 and p-cdc2 by western blot analysis. α-tubulin was detected as a loading control. (B) Expression of the cellular pro-apoptotic protein Bim, viral latent protein EBNA-3A and viral encoded Bcl2 homologue BHRF1 was analyzed. (C) P3HR1-c16 cells and LCL337 were pretreated with N-acetyl cysteine (NAC) for 1 hr followed by treatment with SAHA, bortezomib or SAHA/bortzomib for 24 hr. Expression of PAPR, cleaved PARP and cleaved caspase-3 proteins was analyzed. (D) Expression of p16INK4A and p21WAF1 proteins was analyzed. (E) Schematic diagram illustrating the roles of p16INK4A, p21WAF1 and ROS in the mechanism of apoptosis induced by SAHA/bortezomib.
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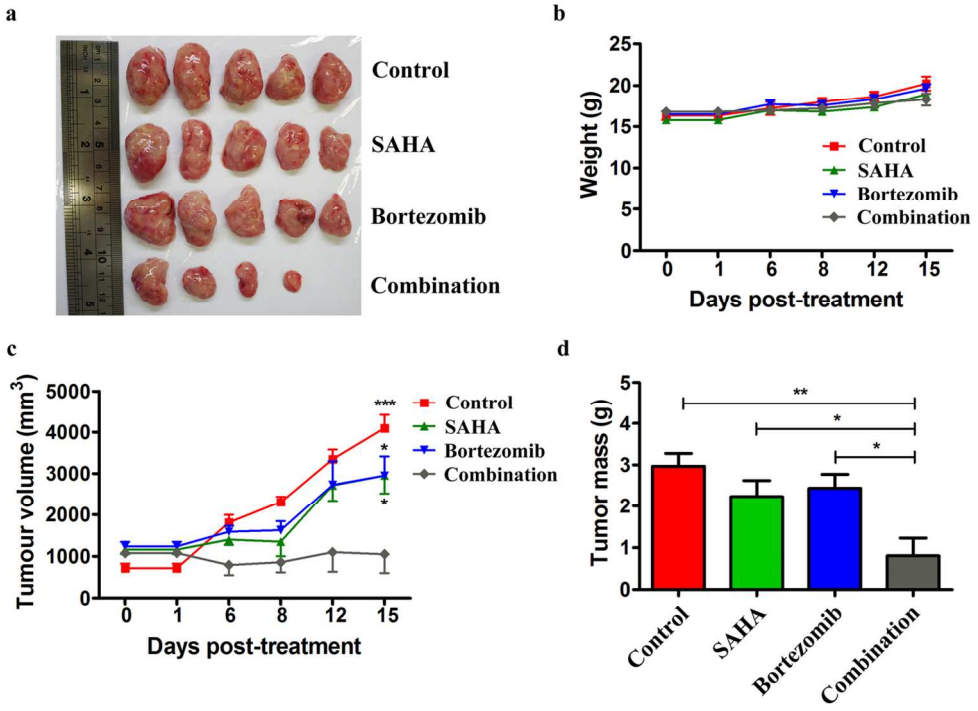

Fig 6. Effects of SAHA/bortezomib on growth of Wp-restricted BL xenografts established in nude mice. P3HR1-c16 cells were inoculated into the right flanks of nude mice. The mice were treated with either DMSO (vehicle control) (n=5), 50 mg/kg SAHA (n=5), 60 μg/kg bortezomib (n=5) or combination of 50 mg/kg SAHA and 60 μg/kg bortezomib (n=5) for 5 days per week over 15 days by intraperitoneal injection. (A) The tumours were resected from the nude mice at the end of the experiment. (B) The mice were weighed at 0, 1, 6, 8, 12 and 15 days post-treatment. (C) Tumour size measurements were performed at 0, 1, 6, 8, 12 and 15 days post-treatment by using a caliper. (D) Average tumour masses of mice of control and treated groups were shown. The results were analyzed for statistical significance using One-way ANOVA Dunnett's Multiple Comparison Test. P value less than 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with SAHA/bortezomib. Error bars represent the standard error of mean (SEM) of data obtained from the nude mice (n=5).
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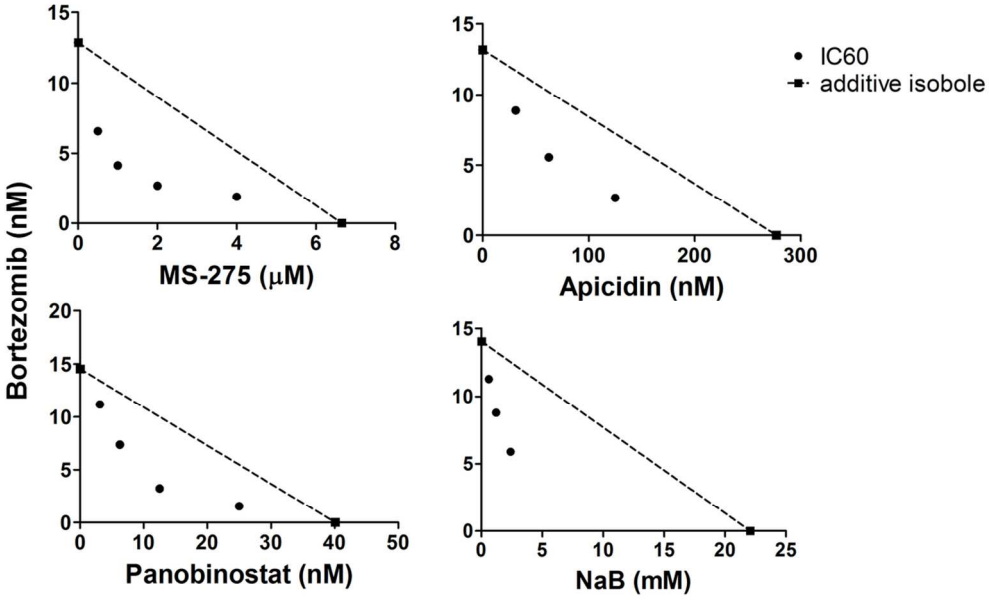

Supplementary Fig 1. Effects of combination of either MS-275, apicidin, SAHA or sodium butyrate (NaB) with bortezomib on proliferation of Wp-restricted BL cells. P3HR1-c16 cells were treated with combination of either MS-275 (0, 0.5, 1, 2, 4 and 8 μM), apicidin (0, 31.25, 62.5, 125, 250 and 500 nM), panobinstat (0, 3.125, 6.25, 12.5, 25 and 50 nM), or NaB (0, 0.6, 1.2, 2.4, 4.8 and 9.6 mM) and bortezomib (0, 1, 2, 4, 8, 16, 32, and 64 nM) for 48 hr. Cell proliferation was determined by MTT assay and presented as percentages of cell proliferation of treated cells compared with those of untreated cells. Synergisms of proliferation inhibition of the cells following treatment with different drug combinations were analyzed by isobologram analysis.
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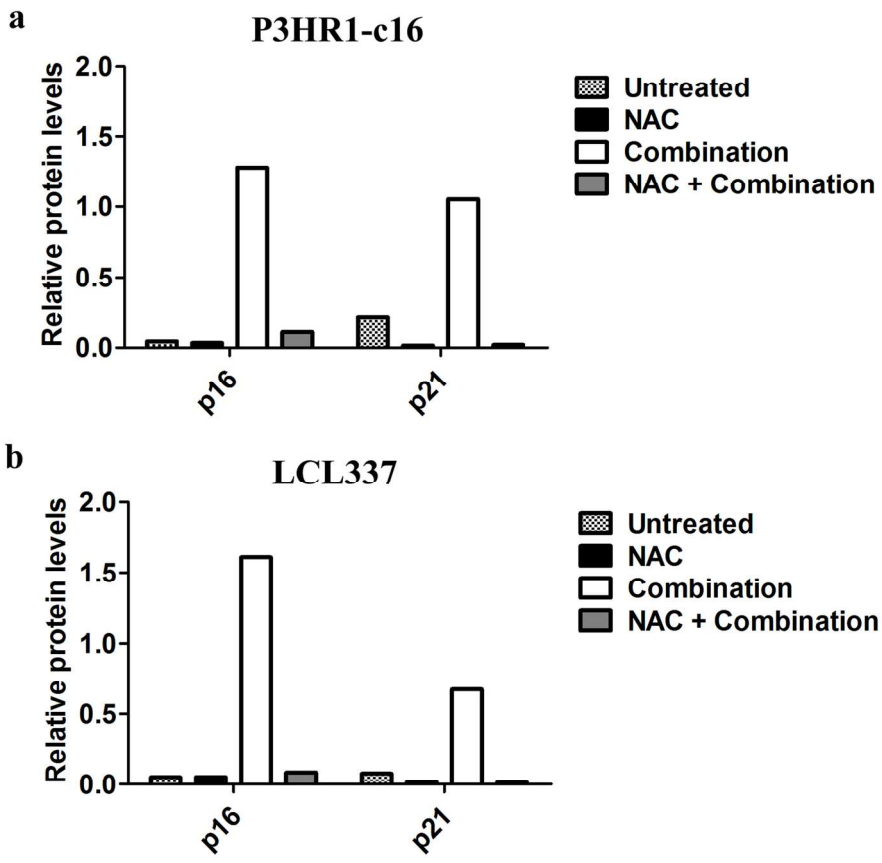

Supplementary Fig 2. Western blot results in Fig. 4d were quantitated by densitometry. The bars represent the relative protein levels compared to the loading control.
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