Bortezomib and SAHA synergistically induce ROS-driven caspase-dependent apoptosis of nasopharyngeal carcinoma and block replication of Epstein-Barr virus

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Running title: Bortezomib and SAHA on NPC apoptosis

Keywords: Bortezomib; SAHA; nasopharyngeal carcinoma; Epstein-Barr virus; apoptosis

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Author contributions
KFH and BHWL conceived and designed the study, carried out the experimental work and wrote the manuscript. DNH carried out the experimental work. GSWT generated NP and NPC cell lines for the study. AKSC conceived and designed the study, interpreted the data and wrote the manuscript.

Grant support
This project is funded by NPC Area of Excellence (AoE/M 06/08 Center for...
Nasopharyngeal Carcinoma Research), CRCG (#10401264) and Epstein-Barr virus research (# 20004525) grants of A.K.S. Chiang.

**Disclosure of potential conflicts of interest**

The authors disclosed no potential conflicts of interest.
Abstract

A novel drug combination of a proteasome inhibitor, bortezomib and a histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA) was tested in nasopharyngeal carcinoma (NPC) both in vitro and in vivo. Dose-response of different concentrations of bortezomib and SAHA on inhibition of cell proliferation of NPC was determined. Mechanisms of apoptosis and effects on lytic cycle activation of Epstein-Barr virus (EBV) were investigated. Combination of bortezomib and SAHA (bortezomib/SAHA) synergistically induced killing of a panel of NPC cell lines. Pronounced increase in sub-G1, annexin V-positive and TUNEL-positive cell populations were detected after treatment with bortezomib/SAHA when compared with either drug alone. Concomitantly, markedly augmented proteolytic cleavage of PARP, caspase-3, -7, -8 and -9, reactive oxygen species (ROS) generation and caspase-8-dependent histone acetylation were observed. ROS scavenger, N-acetyl cysteine, diminished the apoptotic effects of bortezomib/SAHA while caspase inhibitor, Z-VAD-FMK, significantly suppressed the apoptosis without decreasing the generation of ROS. Bortezomib inhibited SAHA’s induction of EBV replication and abrogated production of infectious viral particles in NPC cells. Furthermore, bortezomib/SAHA potently induced apoptosis and suppressed the growth of NPC xenografts in nude mice. In conclusion, the novel drug combination of bortezomib and SAHA is highly synergistic in the killing of NPC cells in vitro and in vivo. The major mechanism of cell death is ROS-driven caspase-dependent apoptosis. Bortezomib antagonizes SAHA’s activation of EBV lytic cycle in NPC cells. This study provides a strong basis for clinical testing of the combination drug regimen in NPC patients.
Introduction:

Nasopharyngeal carcinoma (NPC) is concentrated in Southern Chinese populations in Southeast Asia, North Africans, Greenlanders and Alaskan Eskimos but is rare in other populations of the world. It has a predilection to affect young adult males. The incidence rate reaches ~25 per 100,000 males in Southern Chinese (1). The disease has uniquely strong association with Epstein-Barr virus (EBV) when compared with other head and neck malignancies (2). Radiotherapy is the mainstay of NPC treatment and confers cytotoxicity through generation of reactive oxygen species (ROS) (3). Chemotherapy is required in locally advanced or metastatic cases and commonly used chemotherapeutic drugs include cisplatin and docetaxel (4). Although NPC is both radiosensitive and chemosensitive, the standard treatment regimen results in significant long term sequelae. Moreover, the disease relapse rate is relatively high with poor survival chance for recurrent or metastatic disease (5). Development of novel therapeutic strategies against the disease is clearly needed.

Proteasome and histone deacetylase inhibitors are two classes of promising therapeutic agents for cancer treatment. Bortezomib is a proteasome inhibitor approved by FDA for the treatment of multiple myeloma and relapsed mantle cell lymphoma (6). Its cytotoxicity is associated with the downstream effects of proteasome inhibition including cyclin destabilisation, accumulation of tumor suppressor p53 and inactivation of nuclear factor-κB (NF-κB) (7, 8). Suberoylanilide hydroxamic acid (SAHA; also known as vorinostat) is a histone deacetylase inhibitor approved by FDA for the treatment of cutaneous T-cell lymphoma. It alters gene expression by histone acetylation and mediates various cellular effects, including cell differentiation, cell cycle arrest and apoptosis, in different cancer cell types (9-12).
Combination of bortezomib and SAHA (bortezomib/SAHA) was shown to be effective in the treatment of hematologic malignant cells such as multiple myeloma (13), mantle cell lymphoma (14), cutaneous T-cell lymphoma (15) and leukemia (16, 17). Bortezomib/SAHA triggers apoptosis through caspase activation (14, 16-18) and ROS generation (13-16, 19) in various types of cancers. Other cellular effects of combined proteasome and histone deacetylase inhibitors in various cancer types include histone acetylation (20, 21), aggresome disruption (22), NF-κB inactivation (13, 14, 16, 17, 19), p53 and p21 up-regulation (13, 15, 16, 18), c-Jun NH2-terminal kinase activation (13, 17) and mitochondrial membrane dysfunction (13, 16-18).

Proteasome and histone deacetylase inhibitors were also reported to induce EBV lytic cycle in different EBV-associated malignancies and lead to specific therapeutic effects against the cancer cells (11, 12, 23, 24). Bortezomib was reported to induce EBV lytic cycle in EBV-positive Burkitt lymphoma and gastric carcinoma cells (25). Induction of EBV lytic cycle by bortezomib could activate the radioisotope $[^{125}\text{I}]$2′-fluoro-2′-deoxy-β-D-5-iodouracil-arabinofuranoside to selectively suppress the growth of Burkitt lymphoma xenografts in SCID mice (25). Our laboratory has previously shown that SAHA could significantly induce viral lytic cycle in EBV-positive gastric carcinoma and NPC cells and mediate enhanced apoptosis (11, 12). The lytic cycle induction and tumor growth suppression mediated by SAHA could also be observed in NPC xenografts established in nude mice (12).

Since bortezomib and SAHA have synergistic action on various malignant cell types and both drugs can induce viral lytic cycle in EBV-associated malignancies, we set out to investigate the effects of combining bortezomib and SAHA in the treatment of
EBV-positive NPC. Specifically, we aimed to (i) determine the dose-response of different concentrations of bortezomib/SAHA on inhibition of NPC cell proliferation (ii) examine the effect of bortezomib/SAHA on apoptosis of NPC cells (iii) delineate the mechanisms of apoptosis (iv) analyze the effect of bortezomib/SAHA on EBV lytic cycle induction and its relationship with apoptosis (v) evaluate the \textit{in vivo} anti-tumor effect of bortezomib/SAHA on NPC xenografts in nude mice.
Materials and Methods:

Cell lines and drug treatment

HONE1 is an EBV-negative NPC cell line. HK1-EBV, HONE1-EBV, HA and C666-1 are EBV-positive NPC cell lines (12). NP460 is a normal nasopharyngeal epithelial cell line immortalized with human telomerase reverse transcriptase gene (26). Human kidney 2 (HK2) is a normal kidney cell line immortalized with human papillomavirus 16 E6/E7 genes (Gift from Prof. G.C.F. Chan, The University of Hong Kong) (27). All the cell lines were cultured as previously described (12, 26, 27). In most of the experiments in this study, NPC cells grown to 70% confluence were treated with either 30 nM bortezomib (Selleck Chemicals, Houston, TX), 5 μM SAHA (Cayman Chemicals, Ann Arbor, MI) or combination of them. To inhibit ROS generation and caspase activation, cells were treated with 12 mM N-acetyl cysteine (NAC; Sigma-Aldrich, St. Louis, MO), 50 μM Z-VAD-FMK (Torcris Bioscience, Bristol, UK), 50 μM Z-IETD-FMK and 50 μM Z-LEHD-FMK (R&D Systems, Minneapolis, MN) for 1 hr before treatment with bortezomib/SAHA. Cell lines were authenticated with an AmpF/STR Identifiler PCR Amplication Kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. The data were analyzed by GeneScan and GeneMapper™ ID Software (Applied Biosystems). The STR profiles were compared with DSMZ database. Cells were tested on August 2011.

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

NPC cells, including HK1-EBV, HONE1-EBV, HA and C666-1, were seeded in triplicates in 96-well plates and treated with various combinations of bortezomib (0, 7.5, 15, 30, 60 and 120 nM) and SAHA (0, 0.625, 1.25, 2.5, 5 and 10 μM) for 48 hr except C666-1 for 72 hr. MTT (Invitrogen) assay was performed as previously
described (11). Absorbance at optical density (OD) 570 nm was measured with a multimode detector (DTX 880; Beckman Coulter, Fullerton, CA). The percentage of cell proliferation was calculated as (OD of treated cells)/(OD of untreated cells) × 100%.

**Annexin V/propidium iodide assay**

HA and C666-1 cells were incubated with drugs for 48 hr and 72 hr, respectively. Supernatant and cells adhered to the plate were collected and washed with phosphate buffered saline (PBS). Cells were diluted to 10^6 cells/ml in annexin V binding buffer and stained with FITC annexin V and propidium iodide (BD Pharmingen™, Heidelberg, Germany) according to the manufacturer’s protocol. The stained cells were detected by flow cytometry (LSRII; BD Biosciences, San Jose, CA) and data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

HA and C666-1 cells were incubated with drugs for 48 hr and 72 hr, respectively. Following incubation, both floating and adherent cells were collected and washed twice with PBS. TUNEL staining was then performed with APO-BrdU™ TUNEL Assay Kit (Invitrogen) following manufacturer’s instructions. The stained cells were detected by flow cytometry (LSRII, BD Biosciences) and data were analyzed by FlowJo software (Tree Star).

**Dichlorofluorescein diacetate (DCFH-DA) assay**

Dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) was used to analyze the intracellular reactive oxygen species level. HA and C666-1 cells were treated with
drugs for 24 hr and 48 hr, respectively. Adherent cells were then washed with PBS and stained with 2 μM DCFH-DA diluted in PBS at 37°C in the dark for 30 min (28). The DCFH-DA dye oxidized by ROS can be excited by a 488-nm laser. The cells were washed twice with PBS before flow cytometric analysis (LSRII, BD Biosciences) and data were analyzed by FlowJo software (Tree Star).

**JC-1 assay**

HA and C666-1 cells were treated with drugs for 24 hr and 48 hr, respectively. One million cells were collected and washed once with PBS. The cells with loss of mitochondrial membrane potential, which was reflected by decreased JC-1 red signal, were detected with Flow Cytometry Mitochondrial Membrane Potential Detection Kit (BD Biosciences) following the manufacturer’s instructions. The stained cells were detected by flow cytometry (LSRII, BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**Caspase-3, -7 in situ assay**

HA and C666-1 cells were treated with drugs for 24 hr and 48 hr, respectively. One million cells were collected and washed once with PBS. The cells with active caspase were then detected with CaspaTag Caspase-3, -7 In Situ Assay Kit, Fluorescein (Millipore, Billerica, MA) following the manufacturer’s instructions. The stained cells were detected by flow cytometry (LSRII, BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**Immunocytochemistry**

HA cells grown on cover slips coated with 0.1% gelatin were treated with drugs for 48
hr. Cells were fixed with acetone for 10 minutes at room temperature. The fixed cells were then stained with cleaved caspase-3 or cleaved PARP rabbit polyclonal antibodies antibodies (1:200; Cell Signaling Technology) overnight at 4°C. Expression of the proteins was visualized with Alexa Fluor 488 F(ab’)2 fragment of goat anti-rabbit IgG antibody (1:500; Invitrogen, Carlsbad, CA) under fluorescence microscopy. Nuclei of cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Roche, Mannheim, Germany).

**Western blot analysis**

HA and C666-1 cells were treated with drugs for 24 hr and 48 hr, respectively. Protein from the cell cultures was extracted and western blot analysis was performed as described previously (11). EBV proteins and apoptotic proteins were detected with the antibodies reported previously (12). Cleavage of caspase-8 was detected with cleaved caspase-8 antibody (1:1000; Cell Signaling Technology, Beverly, MA). Histone acetylation was detected with acetyl-histone H3 and H4 rabbit polyclonal antibodies (1:2000; Millipore, Temecula, CA). NK-κB signaling was detected with p-p65, p65 and IKK-αβ rabbit polyclonal antibodies (1:1000; Cell Signaling Technology, Beverly, MA). Tumor suppressor genes were detected with p-Rb, p-p53 and p53 rabbit polyclonal antibodies (1:1000; Cell Signaling Technology). Expression of human β-actin was detected with β-actin antibody (1:5000; Sigma-Aldrich) as a loading control.

**Quantitative PCR assay**

HA and C666-1 cells were treated with drugs for 48 hr and 72 hr, respectively. The viral load analysis by quantitative PCR assay was performed as described previously.
EBV viral load was presented as number of viral genomes per cell. Data were determined by triplicate wells in a 96-well plate format.

**Infection assay**

HONE1-EBV cells, which contain EBV with green fluorescent protein, were treated with drugs for 5 days. The supernatant was collected and EBV infection assay was performed as previously described (11). Daudi cells infected with EBV with green fluorescent protein would give fluorescence under flow cytometric analysis (LSRII, BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**Nude mice experiment**

C666-1 cells ($5 \times 10^6$) were re-suspended in medium containing 50 µl PBS plus 50 µl matrigel (BD Biosciences). HONE1 and HA ($1 \times 10^7$) 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 5-6 weeks of age. When the tumors became palpable, 60 µg/kg bortezomib, 50 mg/kg SAHA or combination of them dissolved in DMSO at a volume of 10 µl was administered to nude mice of 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 control group (n=5). The size of the tumors were measured twice weekly using a caliper and the tumor volume was estimated by the following formula: $\text{length} \times (\text{width})^2 \times 3.14 / 6$. All mice were euthanized by IP injection of 200 mg/kg pentobarbital at the end of the experiment (when mice of control group had tumors reaching 2 cm in diameter). Tumors were dissected and weighed after euthanasia, followed by extraction of protein for western blot analysis.
Statistical analysis

*In vitro* experiments were performed in triplicate and repeated at least 3 times. Data were analyzed for statistical significance using unpaired Student *t* test. *P* value < 0.05 was considered statistically significant. To evaluate the synergism of bortezomib and SAHA, isobolograms were generated from inhibitory concentrations plotted as a function of the concentration of bortezomib versus the concentration of SAHA. Here, the IC60 curves were plotted on the isobolograms and were compared with the additive isoboles comparing the two IC60s of each drug. Curves that lie under the additive isobole suggest synergism and vice versa (29). The combination index (CI) was calculated using the Chou and Talalay method using Microsoft Excel software (30). CI<1, =1 and >1 represents synergy, additivity and antagonism, respectively. All statistical analyses were determined using GraphPad Prism Version 5.0 software.
Results

Bortezomib and SAHA inhibited NPC cell proliferation in a synergistic manner

HK1-EBV, HONE1-EBV, HA and C666-1 cells were treated with various combinations of bortezomib (0, 7.5, 15, 30, 60 and 120 nM) and SAHA (0, 0.625, 1.25, 2.5, 5 and 10 μM) for 48 hr (for 72 hr in the case of C666-1 due to much slower proliferation rate). The relative cell proliferation was determined by MTT assay and the dose-response curves are shown in Fig. 1a. While each drug was able to reduce NPC cell proliferation in a dose-dependent manner, the combination of bortezomib and SAHA yielded much stronger anti-proliferative effect. The isobolograms of IC60s are shown in Fig. 1b. The isoboles for IC60 lie below the additive isoboles for all NPC cell lines, suggesting synergism of bortezomib/SAHA in their anti-proliferative effects. Combination of 30 nM bortezomib and 5 μM SAHA induced a significantly stronger inhibition of NPC cell proliferation when compared with either drug alone (P<0.05 for HK1-EBV, P<0.01 for HONE1-EBV, P<0.001 for HA and C666-1 cells; Fig. 1c). The combination indices of NPC cells treated with the combination of 30 nM bortezomib and 5 μM SAHA are 0.3, 0.38, 0.33 and 0.04 for HK1-EBV, HONE1-EBV, HA and C666-1 cells, respectively, when IC60s are compared. As the combination indices are all <1, it suggests synergistic anti-proliferative effect. The synergistic killing of NPC cells could also be observed upon treatment with combination of bortezomib and other HDAC inhibitors, including LBH-589, sodium butyrate and valproic acid (Fig. 1d). No synergistic killing was observed in NP460 normal nasopharyngeal cells and HK2 normal kidney cells (Fig. S1).

Bortezomib/SAHA induced caspase-dependent apoptosis of NPC cells

HA and C666-1 NPC cells were treated with 30 nM bortezomib, 5 μM SAHA or their combination, for 48 and 72 hr, respectively, and assayed for apoptosis by annexin V/
propidium iodide staining. Bortezomib/SAHA induced a higher percentage of apoptotic cells, when compared with either drug alone, in both NPC cell lines. The percentages of annexin V-positive populations upon treatment with bortezomib, SAHA and bortezomib/SAHA increased to 29%, 7% and 76%, respectively in HA cells and 42%, 53% and 79%, respectively in C666-1 cells (Fig. 2a). To study the kinetics of apoptosis, HA and C666-1 cells were treated with 30 nM bortezomib, 5 μM SAHA or their combination for different time duration (24, 48 and 72 hr) and assayed for proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and caspase-3 by western blot analysis. Cleavage of PARP and caspase-3 was observed at an earlier time point upon treatment with both drugs (24 hr in HA cells and 48 hr in C666-1 cells) when compared with either drug alone (Fig. 2b). Upon treatment with bortezomib/SAHA, increased number of NPC cells expressing cleaved PARP and cleaved caspase-3 were also detected by immunofluorescent staining (Fig. 2c). The apoptosis was caspase-dependent because a pan-caspase inhibitor, Z-VAD-FMK, significantly suppressed the cleavage of PARP and caspase-3, -7 and -9 induced by bortezomib/SAHA (Fig. 2d).

Bortezomib/SAHA’s cytotoxic effect was strongly associated with reactive oxygen species (ROS)

Bortezomib/SAHA induced ROS generation in a wide variety of cancer cell lines (13-16, 19). We, therefore, hypothesized that ROS generation were also involved in bortezomib/SAHA-induced apoptosis in NPC cells. DCFH-DA assay demonstrated enhanced ROS content in HA and C666-1 cells treated with bortezomib/SAHA at 24 hr and 48 hr post-treatment, respectively. N-acetyl cysteine (NAC), a ROS scavenger, significantly reduced the generation of ROS (Fig. 3a). NAC also effectively
suppressed apoptosis, as indicated by the reduction of TUNEL-positive and sub-G1 populations in NPC cells upon treatment with bortezomib/SAHA. The TUNEL-positive NPC cells dropped from 52.4% to 1.74% for HA and from 57.8% to 41.9% for C666-1 (Fig. 3b). The corresponding sub-G1 population dropped from 38.9% to 6.9% for HA and from 40.7% to 17.9% for C666-1 (Fig. 3c). We also examined other possible mechanisms of cell death that would be induced by bortezomib/SAHA. While SAHA mildly induced acetylation of histone H3 and H4, addition of bortezomib resulted in a more significant increase in histone acetylation (Fig. 3d). Meanwhile, the histone acetylation induced by bortezomib/SAHA reduced upon co-incubation with either Z-V AD-FMK or NAC, indicating that the histone acetylation was caspase- and ROS-dependent (Fig. 3d and Fig. S2). Specific caspase-8 inhibitor Z-IETD-FMK, but not caspase-9 inhibitor Z-LEHD-FMK, completely reduced the level of acetylated histone and partially reduced the cleavage of PARP, showing that bortezomib/SAHA-induced cell death was partially related to caspase-8-dependent histone acetylation (Fig. 3e). NK-κB activation and mitochondrial membrane potential were shown to be closely associated with ROS generation (31, 32). However, according to our results, no significant change in expression of NF-κB-related proteins, including p-p65, p65 and IKK-α/β, was observed after treatment with combined bortezomib/SAHA when compared with either drug alone (Fig. 3d). The drug combination also did not result in significant loss of mitochondrial membrane potential in the bortezomib/SAHA-treated cells (Fig. 3f).

Caspase activation were regulated by ROS signaling in NPC cells
To further investigate the roles of caspases and ROS in NPC apoptosis, we analyzed the effects of NAC and Z-VAD-FMK on caspase activation and ROS generation
mediated by bortezomib/SAHA. NAC effectively suppressed the cleavage of PARP and caspase-3, -7 and -9 in both HA and C666-1 cells (Fig. 4a). Moreover, NAC significantly decreased the percentage of cells containing active caspase-3/-7 following treatment with bortezomib/SAHA (Fig. 4b). The percentages decreased from 39.3% to 5.75% in HA cells and from 58% to 10.5% in C666-1 cells. On the other hand, Z-VAD-FMK, which effectively suppressed caspase activation, did not reduce ROS generation induced by bortezomib/SAHA (Fig. 2d & 4c). These suggest ROS induction by bortezomib/SAHA led to subsequent caspase activation in NPC cells.

**Bortezomib inhibited SAHA’s induction of EBV lytic protein expression and abrogated production of infectious viral particles in NPC cells**

Induction of EBV lytic cycle could lead to apoptosis of EBV-positive NPC cells (12). We, therefore, investigated the expression of EBV immediate-early lytic (Zta) and latent (EBNA1) proteins in HA and C666-1 cells after treatment with bortezomib/SAHA for 24 hr and 48 hr, respectively. As expected, SAHA significantly induced Zta expression in HA cells but not in C666-1 cells (in which Zta could only be detected after treatment with SAHA for 3 days) (12) at these time points (Fig. 5a & b). Addition of bortezomib effectively reduced the expression of SAHA-induced Zta in HA cells (Fig. 5a & b). Time course experiment showed that bortezomib/SAHA induced proteolytic cleavage of PARP and caspase-9 at an earlier time point (12 hr post-treatment) when compared with either drug alone (24 hr post-treatment) (Fig. 5c). Apoptosis was triggered at 12 hr post-treatment while expression of Zta was observed later at ~24 hr post-treatment (Fig. 5c). Expression of EBV early lytic protein, BMRF1, was suppressed to a greater extent than that of Zta protein and expression of
EBV late lytic protein, gp350/220, was totally abrogated after treatment with bortezomib/SAHA when compared with SAHA alone (Fig. 5c). Bortezomib could also abrogate SAHA-induced EBV DNA replication and infectious virus production. At 48 hr post-treatment, the viral load was 1258 genomes per cell in HA treated with SAHA and it dropped to 172 genomes per cell when HA was co-incubated with bortezomib (Fig. 5d). The effect cannot be observed in C666-1 as SAHA was not capable of activating EBV DNA replication in C666-1 as reported previously (data not shown) (12). Supernatants from HONE1-EBV treated with SAHA could effectively infect ~29% Daudi cells with EBV. However, when bortezomib was added, only ~3% Daudi cells were infected with EBV (Fig. 5e). Moreover, the synergistic killing by bortezomib/SAHA could be observed similarly in both EBV-negative and EBV-positive NPC cells (Fig. S3). These data suggest that the synergism of bortezomib/SAHA on apoptosis of NPC cells was independent of the presence of EBV. However, bortezomib effectively abrogated SAHA-induced viral replication while potentiating SAHA’s induction of apoptosis of NPC cells.

**Bortezomib/SAHA significantly suppressed NPC tumor growth in vivo**

We evaluated the in vivo effect of bortezomib/SAHA on growth suppression of NPC xenografts established in nude mice. C666-1, HONE1 and HA cells were inoculated subcutaneously at the right flanks of nude mice. The mice (n=5) were either treated with DMSO (vehicle control), 60 μg/ml bortezomib, 50 mg/kg SAHA or combination of them for 5 days per week over 4 weeks by intraperitoneal injection. Growth of tumors was measured twice weekly during the experimental period. While either bortezomib or SAHA alone suppressed the growth of NPC tumors, their combination mediated a much stronger anti-tumor effect (Fig. 6 and Fig. S4). For instance, on day
28, the tumor mass of C666-1 in the control group increased to 1000 mg. The mass of tumors treated with bortezomib and SAHA increased to 760 mg and 580 mg respectively, while that treated with drug combination gave a much smaller increase in tumor mass to 270 mg (Fig. 6b). Western blot analysis showed that bortezomib/SAHA could induce a stronger proteolytic cleavage of PARP and caspases in the tumors when compared with either drug alone (Fig. 6c). Similar anti-tumor effects of bortezomib/SAHA could also be observed in HONE1 and HA xenografts (Fig. 6d and S4). The weight of mice was recorded throughout the experiments. Weight loss of ~5% was observed in mice treated with either the single drugs or their combination (Fig. S4). No significant increase in weight loss was observed in the mice treated with the combined drug regimen. Based on the observations in all three mouse tumor models (C666-1, HONE1 and HA), the toxicity (generally mild) seems to be similar for either the single drugs or their combination.
Discussion

We demonstrated that combination of bortezomib and SAHA could synergistically inhibit proliferation of all four EBV-positive NPC cell lines employed in this study. The synergistic killing was specific to NPC when compared with normal nasopharyngeal and kidney cells and was mediated by increased apoptosis. We showed that bortezomib/SAHA could significantly induce activation of caspase-3, -7 and -9 while addition of caspase inhibitor, Z-VAD-FMK, could effectively reduce the cleavage of the caspases and PARP, suggesting a caspase-dependent apoptosis of the NPC cells. We also observed a significant increase in ROS generation in NPC cells after treatment with bortezomib/SAHA. The ROS generation likely played a critical role in the induction of apoptosis of NPC cells because N-acetyl cysteine (NAC), a ROS scavenger, could markedly reduce the sub-G1 cell populations, in line with previously reported function of ROS generation in various cancer cell types upon treatment with bortezomib/SAHA (13-16, 19). We sought to clarify the relationship between ROS generation and caspase-dependent apoptosis in the NPC cells as conflicting data on the link between ROS generation and caspase activation have been reported in the literature (33, 34). Our data demonstrated that ROS generation resulted in caspase activation and subsequent apoptosis of NPC cells because NAC effectively reduced caspase activation while Z-VAD-FMK failed to reduce ROS production.

We have also investigated the potential roles of several signaling pathways which could mediate apoptosis in the NPC cells upon treatment by bortezomib/SAHA. Bortezomib was found to potentiate SAHA’s acetylation of histones H3 and H4 in the NPC cells. Furthermore, the histone acetylation was ROS- and caspase-8-dependent since both NAC and caspase-8 specific inhibitor, Z-IETD-FMK could markedly...
reduce the acetylation of the histones. The results were similar to the induction of caspase-8-dependent histone acetylation by combination of HDAC and proteasome inhibitors in leukemic cells (21). One of the major effects mediated by histone hyperacetylation is up-regulation of tumor suppressor genes (9). However, we did not find any up-regulation of Rb or p53 in the bortezomib/SAHA-treated NPC cells (refer to Fig. S2a). p53 expression was repressed by the combination treatment in HA cells whereas such repression was not found in C666-1 cells. Since enhanced apoptosis was observed in both HA and C666-1 cells, the cell death was unlikely related to the p53 pathway. It is unexpected that NF-κB inactivation, which was seen in vitro in various cancer cell lines after treatment with bortezomib/SAHA, was not observed in the NPC cells. However, this finding is in line with that in a clinical study on patients with multiple myeloma where the clinical response to bortezomib/SAHA did not correlate to the expression pattern of NF-κB p65 subunit protein in the bone marrow (35). Loss of mitochondrial membrane potential was not significantly detected in the bortezomib/SAHA-treated NPC cells, which was consistent with the finding that caspase-9 specific inhibitor, Z-LEHD-FMK, could not suppress the apoptosis of NPC cells. We postulated that the apoptosis was not mediated through the intrinsic mitochondrial pathway but through the non-mitochondrial production of ROS via the NADPH oxidase complex and endoplasmic reticulum system (32).

Our laboratory demonstrated that NPC cells proceeded to apoptosis following EBV lytic cycle induction by SAHA (12). In this study, we critically investigated the expression kinetics of EBV lytic proteins (Zta, Rta, BMRF1 and gp350/220) and apoptotic markers (PARP and cleaved caspase-9) after treatment with either bortezomib or SAHA alone or bortezomib/SAHA in the NPC cells. We found that
bortezomib/SAHA induced apoptosis of NPC cells at an earlier time point than either drug alone and bortezomib reduced SAHA’s induction of EBV lytic cycle. Moreover, cleavage of caspase-9 and PARP was detected much earlier than the expression of EBV lytic proteins, indicating that bortezomib/SAHA potently and briskly induced cell death through apoptotic pathways independent of EBV lytic cycle activation. Interestingly, this drug combination will also serve to minimize the production of this oncogenic virus from the NPC cells.

We examined the *in vivo* effect of bortezomib/SAHA in NPC xenografts in nude mice. Our results demonstrated that combination of 60 μg/kg bortezomib and 50 mg/kg SAHA could suppress the growth of NPC tumors, including C666-1, HONE1 and HA, in a more-than-additive manner, when compared with either drug alone. Take C666-1 as an example, while bortezomib or SAHA resulted in reduction of tumor mass by 24% (P<0.05) and 42% (P<0.05), respectively, bortezomib/SAHA resulted in reduction of tumor mass by 72% (P<0.01) when compared with the vehicle treated group. Increased proteolytic cleavage of PARP and caspases was also observed in the NPC xenografts. Of note, no apparent toxicity was observed in the mice treated with the drug combination except weight loss of ~5%. Together, these *in vivo* data complemented our *in vitro* data on the augmented anti-tumor effects of bortezomib/SAHA in NPC over those of either bortezomib or SAHA alone. Indeed a clinical study demonstrated possible efficacy of combined bortezomib and SAHA in the treatment of relapsed and refractory multiple myeloma with acceptable toxicities (35).

In addition to synergistic anti-tumor effect, combination of bortezomib and SAHA
could potentially reduce the side effects of either drug alone. Despite its efficacy in multiple myeloma, 64% of patients receiving bortezomib experienced peripheral neuropathy which is a dose-limiting factor of this drug in the treatment of this disease (29, 36). The peripheral neuropathy is due to accumulation of ubiquitinated proteins in neuron cells after inhibition of proteasome by bortezomib. Recent studies have shown that SAHA can reduce bortezomib-induced neuropathy by increasing Schwann cell autophagy (37). Radiotherapy is the mainstay of treatment for NPC patients and is thought to confers cytotoxicity through ROS generation (3). Since bortezomib/SAHA can enhance radiosensitivity in both gliomas and colorectal cancers (38, 39), co-administration of the drug combination with radiotherapy could potentially result in enhanced therapeutic effect.

In summary, combination of bortezomib and SAHA synergistically induced killing of NPC cells. The major mechanism of cell death is ROS-driven caspase-dependent apoptosis. Furthermore, bortezomib could effectively abrogate SAHA-induced EBV replication in NPC cells. In vivo, bortezomib/SAHA potently induced apoptosis and suppressed the growth of NPC xenografts in nude mice. Taken together, our study provides a strong basis to progress to clinical testing of this drug combination regimen in NPC patients.

Acknowledgements
We thank Prof. CH Tsai and Prof. JY Chen for providing the HA cell line and Prof. GCF Chan for the HK2 cell line. We thank Prof. PJ Farrell, Prof. KH Chan and Prof. JM Middeldorp for providing the EBV antibodies. We also thank Dr. AKL Cheung and Dr. ST Cheung for their advice on nude mice experiments. We are also grateful to
Prof. ML Lung and Prof. KW Lo for their help in authentication of cell lines.
References:

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Figure legends:

Figure 1. Effects of bortezomib/SAHA on cell proliferation of EBV-positive NPC cells. HK1-EBV, HONE1-EBV and HA cells were treated with various combinations of BTZ and SAHA for 48 hr. C666-1 cells were treated for 72 hr. (A) Data are presented as percentages of cell proliferation as determined by MTT assays. (B) Synergisms of proliferation inhibition of different cell lines were analyzed by isobologram analysis. (C) Percentages of cell proliferation of NPC cells upon treatment with combination of 30 nM BTZ and 5 μM SAHA were compared to those treated with either drug alone. (D) Percentages of cell proliferation of HA upon treatment with combination of 30 nM BTZ and HDAC inhibitors including 100 nM LBH-689, 3 mM sodium butyrate and 5 mM valproic acid for 48 hr were determined. The results were analyzed for statistical significance using unpaired student’s t-test. P value < 0.05 was considered statistically significant; ***p < 0.001, **p < 0.01 and *p < 0.05. Error bars represent the standard error of mean (SEM) of data obtained in at least three independent experiments.

Figure 2. Effects of bortezomib/SAHA on apoptosis of EBV-positive NPC cells. (A) HA cells and C666-1 cells were treated with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 48 hr and 72 hr, respectively. The treated cells were assayed for apoptosis by annexin V/propidium iodide (AV/PI) staining. AV+/PI- population represents early apoptotic cells while AV+/PI+ population represents late apoptotic/ necrotic cells. (B) NPC cells were treated with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 24, 48 or 72 hr followed by detection of expression of PARP, cleaved PARP and cleaved caspase-3 by western blot analysis. (C) After treatment for 24 hr, expression of cleaved caspase-3 and cleaved PARP (green
signals) in HA cells was detected by immunofluorescent staining. DAPI (blue signals) stained the cell nuclei. (D) HA cells and C666-1 cells were pre-treated with 50 μM Z-VAD-FMK for 1 hr followed by treatment with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 24 hr and 48 hr, respectively. Expression of PARP, cleaved PARP and cleaved caspase-3, -7 and -9 was detected by western blot analysis. β-actin served as loading control.

**Figure 3.** Role of reactive oxygen species (ROS) in bortezomib/SAHA-induced apoptosis. (A) HA cells and C666-1 cells were pre-treated with 1 μM N-acetyl-cysteine (NAC) for 1 hr followed by treatment with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 24 hr and 48 hr, respectively. Percentages of cells with increased ROS level were detected by DCFH-DA assay. (B) Percentages of apoptotic cells upon drug treatments were detected by TUNEL staining. (C) Cell cycle distributions of cells upon drug treatments were analyzed by propidium iodide staining. (D) Expression of acetyl-H3 and H4, p-p65, p65 and IKK-αβ proteins was detected by western blotting. (E) Expression of PARP, cleaved caspase-8, acetyl-H3 and acetyl-H4 in HA cells after pre-treatment with either Z-VAD-FMK, Z-IETD-FMK or Z-LEHD-FMK for 1 hr and treatment with BTZ and SAHA was detected by western blotting. (F) Decreases in mitochondrial membrane potential in HA cells were analyzed by JC-1 assay.

**Figure 4.** Effect of reactive oxygen species (ROS) on activation of caspases. HA and C666-1 cells were pre-treated with either 1 μM N-acetyl-cysteine (NAC) or 50 μM Z-VAD-FMK for 1 hr followed by treatment with combination of 30 nM bortezomib and 5 μM SAHA or either drug alone for 24 hr and 48 hr, respectively. (A) Expression
of PARP, cleaved PARP and cleaved caspase-3, -7 and -9 was detected by western blot analysis. β-actin served as loading control. (B) Percentages of cells with active caspase-3/-7 were detected by FLICA-Z-DEVD assay. (C) Percentages of cells with increased ROS level were detected by DCFH-DA assay.

Figure 5. Effects of bortezomib/SAHA on EBV lytic cycle induction in EBV-positive NPC cells. HA cells and C666-1 cells were pre-treated with either 1 μM N-acetyl-cysteine (NAC) or 50 μM Z-VAD-FMK for 1 hr followed by treatment with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 24 hr and 48 hr, respectively. (A & B) Expression of EBV Zta and EBNA1 proteins was detected by western blot analysis. (C) HA cells were treated with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for increasing duration of time. Expression kinetics of cellular proteins (PARP, cleaved PARP and cleaved caspase-9) and EBV proteins (Zta, Rta, BMRF1, Gp350/220 and EBNA1) was analyzed by western blotting. β-actin served as loading control. (D) HA cells treated with combination of 30 nM BTZ and 5 μM SAHA or either drug alone for 48 hr were analyzed for induction of EBV DNA replication by quantitative PCR. Data are presented as number of viral genomes per cell. Error bars show standard errors of triplicate wells. (E) HONE1-EBV cells were treated with 30 nM BTZ and 5 μM SAHA or either drug alone for 5 days. Culture supernatants, which would contain GFP-expressing recombinant EBV particles released by NPC cells, were used to infect Daudi cells for 48 hr. The amount of infectious EBV particles was estimated by the level of GFP expression detected by flow cytometry. Data are presented as the percentages of EBV infection (GFP-expressing Daudi cells).
**Figure 6.** Effects of bortezomib/SAHA on tumor growth suppression of NPC xenografts in nude mice. C666-1 cells were subcutaneously injected into the right flanks of nude mice. When the tumors were palpable, the mice were either treated with combination of 60 μg/kg bortezomib and 50 mg/kg SAHA or either drug alone for 5 days/week over 4 weeks by intraperitoneal injection. (A) The size of tumors during the period of experiment was measured twice weekly using a caliper. Data are presented as the mean tumor volumes of mice in both treatment and control groups on the days post-treatment. (B) Average tumor masses of mice of control and treated groups were shown. Error bars represent the standard error of mean (SEM) of tumor masses. (C) Protein samples extracted from the tumors were tested for expression of PARP, cleaved PARP and cleaved caspase-3,-7 and -9 proteins by western blot analysis. β-actin served as loading control. (D) The in vivo effects of bortezomib and SAHA on the percentage of tumor growth suppression of C666-1, HONE1 and HA xenografts were shown.

**Figure 7.** Chemical structures of (A) bortezomib and (B) SAHA.
Figure 4

\(a\)

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[Images of Western blotting for PARP, Cleaved PARP, Cleaved caspase-3, Cleaved caspase-7, Cleaved caspase-9, and \(\beta\)-actin]

\(b\)

[Images of flow cytometry for untreated, BTZ, SAHA, BTZ + SAHA, BTZ + SAHA + NAC, and histograms for HA and C666-1]

\(c\)

[Images of flow cytometry for untreated, BTZ + SAHA, BTZ + SAHA + NAC, and BTZ + SAHA + Z-VAD, and histograms for HA and C666-1]
**Figure 6**

(a) Graph showing the number of cells over days post treatment for Control, BTZ, SAHA, and BTZ+SAHA conditions.

(b) Bar graph comparing cell counts for Control, BTZ, SAHA, and BTZ+SAHA conditions.

(c) Western blot images for PARP, Cleaved PARP, Cleaved caspase 3, and β-actin.

(d) Bar graph showing % Tumor growth suppression for C666-1, HONE1, and HA conditions.
Molecular Cancer Therapeutics

Bortezomib and SAHA synergistically induce ROS-driven caspase-dependent apoptosis of nasopharyngeal carcinoma and block replication of Epstein-Barr virus

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Mol Cancer Ther Published OnlineFirst March 8, 2013.

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0811

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