Bortezomib and SAHA Synergistically Induce ROS-Driven Caspase-Dependent Apoptosis of Nasopharyngeal Carcinoma and Block Replication of Epstein–Barr Virus

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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 terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (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, whereas 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 patients with NPC.

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 approximately 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; ref. 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 (HDAC) inhibitors are 2 classes of promising therapeutic agents for cancer treatment. Bortezomib is a proteasome inhibitor approved by U.S. Food and Drug Administration (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 destabilization, accumulation of tumor suppressor p53, and inactivation of NF-κB (7, 8). Suberoylanilide hydroxamic acid (SAHA; also known as vorinostat) is a HDAC inhibitor approved by FDA for the treatment of cutaneous T-cell lymphoma (6). Its cytotoxicity is associated with the downstream effects of proteasome inhibition including cyclin destabilization, accumulation of tumor suppressor p53, and inactivation of NF-κB (7, 8). Suberoylanilide hydroxamic acid (SAHA; also known as vorinostat) is a HDAC 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 HDAC inhibitors in various cancer types include histone acetylation (20, 21), aggresome disruption (22), NF-kB inactivation (13, 14, 16, 17, 19), p53 and p21 upregulation (13, 15, 16, 18), c-Jun NH2-terminal kinase activation (13, 17), and mitochondrial membrane dysfunction (13, 16–18).

Proteasome and HDAC 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 [125I]2-fluoro-2’-deoxy-β-D-5-iodouracil-arabinofuranoside to selectively suppress the growth of Burkitt lymphoma xenografts in severe combined immunodeficient (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 mediates 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).

Because 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, and (v) evaluate the in vivo antitumor 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, Hong Kong SAR, China; ref. 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 nmol/L bortezomib (Selleck Chemicals), 5 μmol/L SAHA (Cayman Chemicals), or combination of them. To inhibit ROS generation and caspase activation, cells were treated with 12 nmol/L N-acetyl cysteine (NAC; Sigma-Aldrich), 50 μmol/L Z-VAD-FMK (Torcris Bioscience), 50 μmol/L Z-IETD-FMK, and 50 μmol/L Z-LEHD-FMK (R&D Systems) for 1 hour before treatment with bortezomib/SAHA. Cell lines were authenticated with an AmpF/STR Identifier PCR Amplication Kit (Applied Biosystems), 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.

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 nmol/L) and SAHA (0, 0.625, 1.25, 2.5, 5, and 10 μmol/L) for 48 hours except C666-1 for 72 hours. MTT (Invitrogen) assay was conducted as previously described (11). Absorbance at optical density (OD) 570 nm was measured with a multimode detector (DTX 880; Beckman Coulter). 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 and 72 hours, respectively. Supernatant and cells adhered to the plate were collected and washed with PBS. Cells were diluted to 106 cells/mL in Annexin V-binding buffer and stained with fluorescein isothiocyanate Annexin V and propidium iodide (PI; BD Pharmingen) according to the manufacturer’s protocol. The stained cells were detected by flow cytometry (LSRII; BD Biosciences) and data were analyzed by FlowJo software (Tree Star).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

HA and C666-1 cells were incubated with drugs for 48 and 72 hours, respectively. Following incubation, both floating and adherent cells were collected and washed twice with PBS. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was then conducted 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 assay

Dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) was used to analyze the intracellular ROS level. HA and C666-1 cells were treated with drugs for 24 and 48 hours, respectively. Adherent cells were then washed with PBS and stained with 2 μmol/L DCFH-DA.
diluted in PBS at 37°C in the dark for 30 minutes (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 and 48 hours, 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 and 48 hours, 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) 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 hours. 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 (1:200; Cell Signaling Technology) overnight at 4°C. Expression of the proteins was visualized with Alexa Fluor 488 Fab’2 fragment of goat anti-rabbit immunoglobulin G (IgG) antibody (1:500; Invitrogen) under fluorescence microscopy. Nuclei of cells were stained with 4’,6-diamidino-2-phenylindole (DAPI; Roche).

**Western blot analysis**

HA and C666-1 cells were treated with drugs for 24 and 48 hours, respectively. Protein from the cell cultures was extracted and Western blot analysis was conducted 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 (1:1,000; Cell Signaling Technology). Histone acetylation was detected with acetyl-histone H3 and H4 rabbit polyclonal antibodies (1:2,000; Millipore). Nk-x8 signaling was detected with p-p65, p65, and IKK-αβ rabbit polyclonal antibodies (1:1,000; Cell Signaling Technology). Tumor suppressor genes were detected with p-Rb, p-p53, and p-p53 rabbit polyclonal antibodies (1:1,000; Cell Signaling Technology). Expression of human β-actin was detected with β-actin antibody (1:5,000; Sigma-Aldrich) as a loading control.

**Quantitative PCR assay**

HA and C666-1 cells were treated with drugs for 48 and 72 hours, respectively. The viral load analysis by quantitative PCR assay was conducted as described previously (11). 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 GFP, were treated with drugs for 5 days. The supernatant was collected and EBV infection assay was conducted as previously described (11). Dauci cells infected with EBV with GFP 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 × 10⁶) were resuspended in medium containing 50 μL PBS plus 50 μL matrigel (BD Biosciences). HONE1 and HA (1 × 10⁵) cells were resuspended 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 to 6 weeks of age. When the tumors became palpable, 60 μg/kg bortezomib, 50 mg/kg SAHA, or combination of them dissolved in dimethyl sulfoxide (DMSO) at a volume of 10 μL was administered to nude mice of treatment group (n = 5) by intraperitoneal injection 5 days per week for 4 weeks. Equal volume of DMSO was administered by intraperitoneal 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: length × (width)² × 3.14/6. All mice were euthanized by intraperitoneal 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**

Experiments testing single drug versus combination drugs were done in triplicate and repeated at least 3 times. Differences in data were analyzed for statistical significance using unpaired Student t test. P value less than 0.05 was considered as significant. To evaluate the synergistic action of bortezomib and SAHA, isobolograms were generated from the different combinations of concentrations of each drug which inhibit 60% of NPC cell proliferation (IC₅₀, ref. 29). Isoboles for IC₅₀ that were located to the left of the additive isoboles (refer to Fig. 1B) indicated synergistic action. The combination index (CI) was calculated using the Chou and Talalay method (30). CI < 1, = 1, and >1 represent 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 nmol/L) and SAHA (0, 0.625, 1.25, 2.5, 5, and 10 μmol/L) for 48 hours (for 72 hours 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. Although each drug was able to reduce NPC cell proliferation in a dose-dependent manner, the combination of bortezomib and SAHA yielded much stronger antiproliferative effect. The isobolograms of IC₅₀s are shown in Fig. 1B. The isoboles for IC₅₀ were located to the left of the additive isoboles for all NPC cell lines, indicating synergistic action of bortezomib/SAHA. Combination of 30 nmol/L bortezomib and 5 μmol/L 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).

![Figure 1](image-url)
and C666-1 cells; Fig. 1C). The combination indices of NPC cells treated with the combination of 30 nmol/L bortezomib and 5 μmol/L SAHA or either drug alone are 0.3, 0.38, 0.33, and 0.04 for HK1-EBV, HONE1-EBV, HA, and C666-1 cells, respectively, when IC50s 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 (Supplementary Fig. S1).

**Bortezomib/SAHA induced caspase-dependent apoptosis of NPC cells**

HA and C666-1 NPC cells were treated with 30 nmol/L bortezomib, 5 μmol/L SAHA, or their combination for 48 and 72 hours, respectively, and assayed for apoptosis by Annexin V/PI 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 nmol/L bortezomib, 5 μmol/L SAHA, or their combination for different time duration (24, 48, and 72 hours) and assayed for proteolytic cleavage of 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 hours in HA cells and 48 hours 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 and -9 induced by bortezomib/SAHA (Fig. 2D).

**Bortezomib/SAHA’s cytotoxic effect was strongly associated with 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 showed enhanced ROS content in HA and C666-1 cells treated with bortezomib/SAHA at 24 hours, 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.
and 48 hours posttreatment, respectively. 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. Although 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 coincubation with either Z-VAD-FMK or NAC, indicating that the histone acetylation was caspase- and ROS-dependent (Fig. 3D and Supplementary 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 was 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 (Figs. 2D and 4C). These suggest ROS induction by bortezomib/SAHA led to subsequent caspase activation in NPC cells.

**Figure 4.** Effect of ROS on activation of caspases. HA and C666-1 cells were pretreated with either 1 μmol/L NAC or 50 μmol/L Z-VAD-FMK for 1 hour followed by treatment with combination of 30 nmol/L bortezomib and 5 μmol/L SAHA or either drug alone for 24 and 48 hours, 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.
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 and 48 hours, 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; ref. 12) at these time points (Fig. 5A and B). Addition of bortezomib effectively reduced the expression of SAHA-induced Zta in HA cells (Fig. 5A and B).

Time course experiment showed that bortezomib/SAHA induced proteolytic cleavage of PARP and caspase-9 at an earlier time point (12 hours posttreatment) when compared with either drug alone (24 hours posttreatment; Fig. 5C). Apoptosis was triggered at 12-hour posttreatment, whereas expression of Zta was observed later at approximately 24-hour posttreatment (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 hours posttreatment, the viral load was 1,258 genomes per cell in HA treated with SAHA and it dropped to 172 genomes per cell when HA was coincubated 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; ref. 12). Supernatants from
HONE1-EBV treated with SAHA could effectively infect approximately 29% Daudi cells with EBV. However, when bortezomib was added, only approximately 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 (Supplementary 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 \(\mu\)g/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 antitumor effect (Fig. 6 and Supplementary Fig. S4). For instance, on day 28, the tumor mass of C666-1 in the control group increased to 1,000 mg. The mass of tumors treated with bortezomib and SAHA increased to 760 and 580 mg respectively, whereas 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 antitumor effects of bortezomib/SAHA could also be observed in HONE1 and HA xenografts (Fig. 6D and Supplementary Fig. S4). The weight of mice was recorded throughout the experiments. Weight loss of approximately 5% was observed in mice treated with either the single drugs or their combination (Supplementary Fig. S4). No significant increase in weight loss was observed in the mice treated with the combined drug regimen. On the basis of the observations in all 3 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 showed that combination of bortezomib and SAHA (Fig. 7) could synergistically inhibit proliferation of all 4 EBV-positive NPC cell lines used 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, whereas 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 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 showed that ROS generation resulted in caspase activation and subsequent apoptosis of NPC cells because NAC effectively reduced caspase activation, whereas 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 as 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 upregulation of tumor suppressor genes (9). However, we did not find any upregulation of retinoblastoma (Rb) or p53 in the bortezomib/SAHA–treated NPC cells (refer to Supplementary Fig. S2A). The p53 expression was repressed by the combination treatment in HA cells, whereas such repression was not found in C666-1 cells. Because 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 nonmitochondrial production of ROS via the NADPH oxidase complex and endoplasmic reticulum system (32).

Our laboratory showed 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 showed 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 approximately 5%. Together, these in vivo data complemented our in vitro data on the augmented antitumor effects of bortezomib/SAHA in NPC over those of either bortezomib or SAHA alone. Indeed a clinical study showed possible efficacy of combined bortezomib and SAHA in the treatment of
relapsed and refractory multiple myeloma with acceptable toxicities (35).

In addition to synergistic antitumor 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 (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 patients with NPC and is thought to confer cytotoxicity through ROS generation (3). Because bortezomib/SAHA can enhance radiosensitivity in both gliomas and colorectal cancers (38, 39), coadministration 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 patients with NPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Profs. C.H. Tsai and J.Y. Chen for providing the HA cell line and C.C.F. Chan for the HK2 cell line. The authors also thank Profs. P.J. Farell, R.H.N. Chan, and J.D. Suthers for providing the EBV antibodies; Drs. A.K.L. Cheung and S.T. Cheung for their advice on nude mice experiments; and Profs. M.L. Lung and K.W. Lo for their help in authentication of cell lines.

Grant Support
This project is funded by NPC Area of Excellence (AoE/M06/88/Center for Nasopharyngeal Carcinoma Research), Committee on Research and Conference Grants (CRCG; #10401264), and Epstein–Barr virus research (# 20004525) grants (A.K.S. Chiang).

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Received August 7, 2012; revised February 20, 2013; accepted February 20, 2013; published OnlineFirst March 8, 2013.

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Mol Cancer Ther  Published OnlineFirst March 8, 2013.

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doi:10.1158/1535-7163.MCT-12-0811

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