Synergistic combination of histone deacetylase inhibitor suberoylanilide hydroxamic acid and oncolytic adenovirus ZD55-TRAIL as a therapy against cervical cancer

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Received March 20, 2014; Accepted January 21, 2015

DOI: 10.3892/mmr.2015.3355

Abstract. Oncolytic adenoviruses (OA) have been investigated as virotherapeutic agents for the treatment of cervical cancer and thus far results are promising. However, the cytotoxicity of the viruses requires improvement. The present study demonstrated that this can be achieved by combining ZD55-TRAIL, an OA containing the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene, with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). It was demonstrated that these agents act synergistically to kill HeLa cells by inducing G2 growth arrest and apoptosis. Notably, in a mouse xenograft model, ZD55-TRAIL/SAHA combination inhibited tumor growth. At the molecular level, it was found that upregulation of IκBα and the p50 and p65 subunits of nuclear factor-κB induced by ZD55-TRAIL, can be abrogated by SAHA treatment. These data strongly suggested that ZD55-TRAIL/SAHA co-treatment may serve as an effective therapeutic strategy against cervical cancer.

Introduction

Cervical cancer remains one of the leading causes of cancer-associated mortality in females, accounting for 14% of all mortality due to gynecological cancer worldwide (1). The most common symptom of cervical cancer is abnormal vaginal bleeding, however, in the majority of cases there may be few clear manifestations until it progresses into the advanced stage. The treatment of cervical cancer may include surgery, chemotherapy, radiotherapy, immunotherapy and vaccine therapy or a combination of these. Surgery, including local excision is suitable for this type of cancer in the early stages. Radiotherapy combined with cisplatin-based chemotherapy can markedly improve the survival rate of patients with cervical cancer at advanced stages (2), however, these strategies may fail if tumors have metastasized. Furthermore, chemotherapy resistance has created a major challenge for the treatment of cervical cancer. Thus, the identification of new strategies and agents for the treatment of cervical cancer is urgently required (3).

Cancer targeting Gene-Viro-Therapy represents a promising approach for cancer therapy, which is designed through inserting an antitumor gene into an oncolytic viral vector that has the ability to selectively replicate in tumor cells but not in normal cells (4-6). More than 12 different oncolytic viruses are currently undergoing phase I-III clinical trials against various types of cancer (7). ZD55-TRAIL is an oncolytic adenovirus (OA) previously constructed in the laboratory of Xinyuan Institute of Medicine and Biotechnology at Zhejiang Sci-Tech University (Hangzhou, China), with the antitumor gene tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) inserted into the oncolytic vector ZD55 with E1B55-kDa deletion (8). Several *in vitro* and *in vivo* experiments have demonstrated the antitumor effects of ZD55-TRAIL in models of cervical, lung, breast, colorectal and hepatocellular cancer (8-10). However, due to their genetic heterogeneity, it is likely that certain cancer cells within a tumor have different sensitivities to oncolytic viruses. Therefore, it is necessary to reinforce the antineoplastic activity of the virus to obtain the highest therapeutic intervention.

Suberoylanilide hydroxamic acid (SAHA) is a potent inhibitor of class I and II histone deacetylases (HDACs). These enzymes are responsible for deacetylation of histones and certain other proteins, thus maintaining chromatin in a more relaxed state, and thereby allowing transcription of different regulatory genes that are involved in tumor development and growth (11-13). In xenograft and *in vitro* models,

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Key words: suberoylanilide hydroxamic acid, ZD55-TRAIL, cervical cancer, apoptosis, nuclear factor-κB

certain HDAC inhibitors, including SAHA have demonstrated antitumor activity against cervical cancer (14-16). The present study investigated whether SAHA is able to enhance the anti-cancer activity of ZD55-TRAIL against cervical cancer, and the mechanism underlying the synergistic therapeutic effects of ZD55-TRAIL and SAHA.

Materials and methods

Reagents. The OA ZD55-TRAIL was constructed as described previously (8) and preserved in the laboratory of Xinyuan Institute of Medicine and Biotechnology at Zhejiang Sci-Tech University. The HDAC inhibitor SAHA was purchased from Calbiochem (San Diego, CA, USA). All the compounds used in the present study were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium (0.1% v/v DMSO). Caspase-8, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Caspase-3, poly adenosine diphosphate (ADP)-ribose (PARP), IκBα, p65 and p50 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell lines and culture conditions. The human cervical cancer cell line HeLa was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HeLa-luc cell line, a light producing cell line from HeLa cells stably transfected with the luciferase gene, was purchased from Perkin Elmer (Waltham, MA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum (FBS; Gibco-BRL) at 37˚C in a humidified air atmosphere with 5% CO_2.

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was determined using an MTT assay. Briefly, HeLa cells were plated in 96-well plates at a density of $1x10^4$ in 100 μl culture medium. Cells were treated with SAHA (0.1, 0.2, 0.4 or 0.8 μM) and ZD55-TRAIL (2, 4, 8 or 16 MOI) independently or in combination for 48 h. After 48 h, MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (10 μl, 5 mg/ml) was added to the cells, which were then cultivated for a further 3 h. Absorbance was measured as 490 nm using a fluorescence reader (Finstruments Multiskan EX; MTX LAB Systems Inc., Männedorf, Switzerland).

Hoechst 33342 staining. Morphological characteristics of cell apoptosis were detected using an Hoechst staining assay. HeLa cells were treated with SAHA (0.4 μM), ZD55-TRAIL (8 MOI) or SAHA in combination with ZD55-TRAIL for 24 h. Subsequently, 5 μl of Hoechst 33342 (Sigma-Aldrich) was added to the cells for 30 min and results were observed under an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan). Untreated cells served as the control.

Western blot analysis. HeLa cells were cultured and treated as mentioned above. Following the indicated treatments, cells were washed twice with phosphate-buffered saline (PBS) and lysed in RIPA buffer (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40 and 10 mM EDTA). Proteins were separated by electrophoresis on 12% SDS polyacrylamide gel, transferred onto a polyvinylidene fluoride membrane, immunostained with the appropriate antibody and visualized. The primary antibodies and dilutions used were as follows: Rabbit monoclonal anti-caspase-8 (1:1,000), rabbit monoclonal anti-caspase-3 (1:200), rabbit monoclonal anti-PARP (1:200), rabbit monoclonal anti-IκBα (1:200), mouse monoclonal anti-p65 (1:200), mouse monoclonal anti-p50 (1:200), rabbit monoclonal anti-GAPDH (1:1,000) and rabbit monoclonal anti-β-actin (1:1,000). The secondary antibodies used were IR Dye 700-conjugated anti-rabbit (1:10,000) and IR Dye 800-conjugated anti-mouse (1:5,000) immunoglobulin G antibodies (Lorne Laboratories, Ltd., Lower Early, UK).

Flow cytometric analysis. Apoptosis was determined using an annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, HeLa cells were treated with SAHA, ZD55-TRAIL or SAHA in combination with ZD55-TRAIL for 24 h, and then harvested by centrifugation at 300 x g for 5 min. Following this, the cells were washed twice with 1X annexin V binding buffer, resuspended in binding buffer and co-stained with annexin V and propidium iodide (PI). The prepared cells were analyzed using a FACScan flow cytometer and CELLQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

To analyze the DNA distribution, cells were harvested by trypsinization followed by washing twice with cold PBS and then fixed in 70% ethanol at -20˚C for at least 12 h. Subsequently, cells were stained with phosphatidylinositol in the dark at room temperature for 30 min and subsequently analyzed using a FACScan flow cytometer (BD FACSAria I; Becton Dickinson). The cell cycle profile was analyzed using FlowJo software (version 7.6.5; Treestar, Inc., Ashland, OR, USA).

Animal experiments. Female BALB/c nude mice (4-weeks old) were purchased from the Shanghai Experimental Animal Center (Shanghai, China). The study was approved by the Ethics Committee of the Women's Hospital, College of Medicine, Zhejiang University (Hanzhou, China). The National Institutes of Health (Bethesda, MD, USA) guidelines on the ethical use of animals were strictly followed in all the experiments. For establishment of xenograft tumors, HeLa-luc cells were subcutaneously injected into the right flank of each mouse at a density of $5x10^6$ cells in 100 μl DMEM. Once tumors had reached 100-150 mm^3 in size, mice were divided randomly into three groups (n=6). The ZD55-TRAIL group was treated with $1x10^9$ plaque-forming units of ZD55-TRAIL daily for 5 days by intratumoral injection and the combination group was treated with the virus plus SAHA (25 mg/kg, intraperitoneal injection). The control group received PBS. The tumor burdens were monitored using the Xenogen IVIS Kinetic imaging system (Caliper Life Sciences, Hopkinton, MA, USA).

Immunohistochemistry. At day 12, additional mice were humanely sacrificed and tumors were excised. Following fixing in 4% paraformaldehyde and embedding in

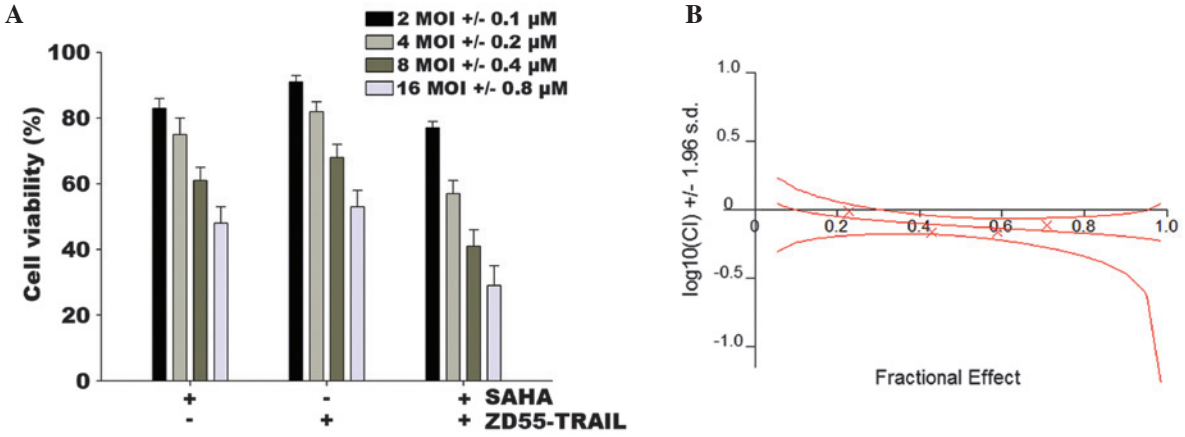

Figure 1. SAHA enhances ZD-55-TRAIL-induced cytotoxicity in HeLa cells. (A) HeLa cells were treated with SAHA (0.1-0.8 μM), ZD55-TRAIL (2-16 MOI) alone or ZD55-TRAIL-SAHA combination for 48 h. Cell viability was measured using an MTT assay. Data are expressed as the mean ± standard deviation (n=3). (B) Combination index (CI) at the different concentrations of ZD55-TRAIL and SAHA in HeLa cells was determined using a CalcuSyn software program. CI values <0 correspond to a synergistic interaction. SAHA, suberoylanilide hydroxamic acid; MOI, multiplicity of infection; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

paraffin, the tumors were cut into 4 μm-thick sections. Immunohistochemical staining with anti-TRAIL primary antibody at 1:500 dilutions was conducted. For expression detection, an avidin-biotin-peroxidase complex reagent (Vector Laboratories, Burlingame, CA, USA) was used. Slides were immersed in 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.006% hydrogen peroxide. Sections were counterstained with hematoxylin and eosin (H&E).

Apoptosis was measured by TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay using an *in situ* Cell Apoptosis Detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions.

Statistical analysis. All data are expressed as the mean ± standard deviation and analyzed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). Data analysis was performed using the Student's t-test and $P<0.05$ was considered to indicate a statistically significant difference.

Results

SAHA enhances ZD55-TRAIL-mediated growth inhibition in HeLa cells. To assess whether the HDAC inhibitor SAHA enhances ZD55-TRAIL-induced cell death in HeLa cells, an MTT assay was performed as mentioned in Materials and methods. Fig. 1A shows that the synergistic combination of ZD55-TRAIL and SAHA induced cell death. HeLa cells infected with ZD55-TRAIL at a MOI of 16 retained 53% cell viability, whereas SAHA (0.8 μM) eliminated 51% of the cells. However, when ZD55-TRAIL (16 MOI) and SAHA (0.8 μM) were used together, 71% of the cells were killed. Combination index (CI) analysis revealed that the CI for different concentrations of the agents was 0.685-0.975, demonstrating that co-treatment of SAHA and ZD55-TRAIL has a synergistic suppressive effect on HeLa cell survival (Fig. 1B).

SAHA enhances ZD55-TRAIL-induced apoptosis in HeLa cells. Subsequently, Hoechst 33342 staining was designed to investigate the morphological alterations of HeLa cells treated with a combination of SAHA and ZD55-TRAIL or SAHA and ZD55-TRAIL alone. The results demonstrated that compared with treatment of ZD55-TRAIL alone, co-treatment with SAHA led to marked apoptosis characterized by chromatin condensation, nuclear fragmentation and apoptotic bodies (Fig. 2A). To quantify the effects of SAHA on TRAIL-induced apoptosis in HeLa cells, Annexin-V-fluorescein isothiocyanate (FITC)/PI double staining was used to analyze cell apoptosis (Fig. 2B). The results demonstrated that the apoptotic rate of HeLa cells co-treated with ZD55-TRAIL and SAHA was 15.7%, which is nearly three times more than that of ZD55-TRAIL treatment alone (5.8%). Furthermore, western blot analysis demonstrated that ZD55-TRAIL was able to induce activation of caspase-8, caspase-3 and cleavage of PARP. The activation of this caspase pathway was further increased by co-treatment of SAHA and ZD55-TRAIL (Fig. 2C). Since caspase-8, caspase-3 and PARP are the main apoptotic proteins involved in the extrinsic apoptotic signaling pathway (17), our results suggested that SAHA promotes ZD55-TRAIL-induced activation of the extrinsic apoptotic signaling pathway, which may synergistically enhance ZD55-TRAIL-induced HeLa cell apoptosis.

Effects of SAHA and ZD55-TRAIL treatment on the cell cycle. To determine whether the anti-proliferative effects of ZD55-TRAIL and SAHA may also result from cell cycle arrest, cell cycle analyses were performed on HeLa cells treated for 24 h. As shown in Fig. 3, when used individually, treatment with SAHA had only a partial effect on the cell cycle. ZD55-TRAIL arrested a greater number of cells in the G2 phase (8.9%). However, treatment with a combination of ZD55-TRAIL and SAHA caused 22.8% cells to enter the G2 cell cycle phase.

SAHA inhibits ZD55-TRAIL-induced upregulation of IκBα, p50 and p65. To elucidate the mechanism of the enhanced cytotoxicity of combined ZD55-TRAIL and SAHA, the protein expression of IκBα, p65 and p50 was then determined by western blotting. As shown in Fig. 4, ZD55-TRAIL alone induced a partial increase in the levels of IκBα, p65 and

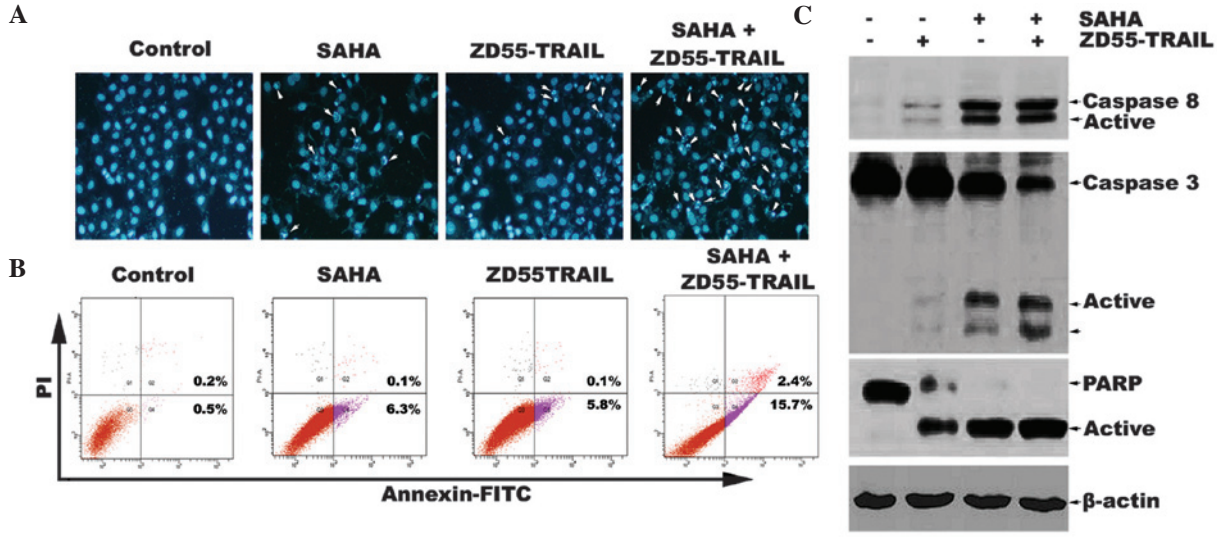

Figure 2. SAHA enhances ZD-55-TRAIL-induced apoptosis and activation of the extrinsic apoptotic signaling pathway. (A) HeLa cells were treated with ZD55-TRAIL (8 MOI), SAHA (0.4 μM) or ZD55-TRAIL plus SAHA for 24 h. Nuclei were stained with Hoechst 33342 to visualize chromatin condensation, nuclear shrinkage or fragmentation by immunofluorescence microscopy (magnification, x200). (B) Annexin V staining caused by phosphatidylserine externalization was examined by flow cytometry. (C) Immunoblotting analysis for activation of caspase-8, caspase-3 and cleavage of PARP in cell lysates of HeLa cells treated for 48 h. β-actin was used as a loading control. SAHA, suberoylanilide hydroxamic acid; PARP, poly adenosine diphosphate (ADP)-ribose; FITC, fluorescein isothiocyanate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Active, activated/phosphorylated form of the respective protein.

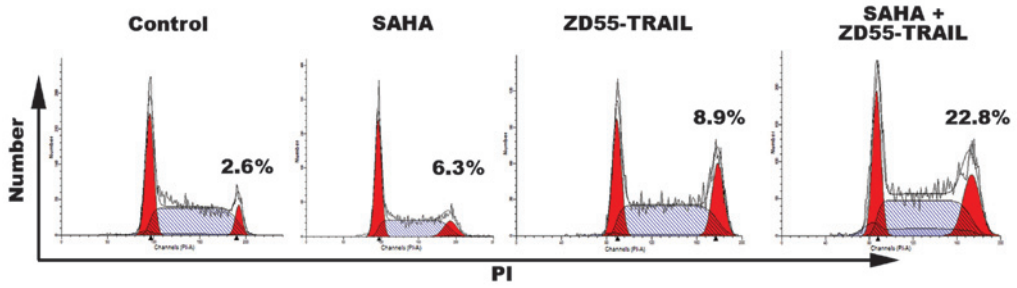

Figure 3. Effect of ZD-55-TRAIL/SAHA combination on the cell cycle. HeLa cells were treated with ZD55-TRAIL at a multiplicity of infection of 8, SAHA (0.4 μM) or ZD55-TRAIL combined with SAHA for 24 h and then stained with phosphatidylinositol as described in Materials and methods. The DNA content was analyzed by flow cytometry. The representative data are shown. SAHA, suberoylanilide hydroxamic acid; PI, propidium iodide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

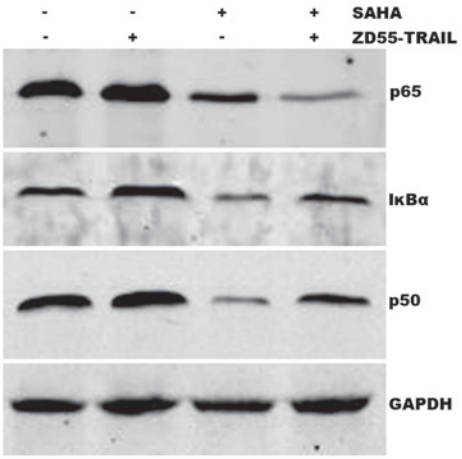

Figure 4. SAHA inhibits ZD-55-TRAIL-induced upregulation of IκBα and the p50 and p65 subunits of nuclear factor-κB. HeLa cells were cultured for 48 h with ZD55-TRAIL at a multiplicity of infection of 8, SAHA (0.4 μM) or the virus plus SAHA. Cell lysates were fractionated on 12% SDS-polyacrylamide gels and analyzed by western blotting with antibodies against IκBα, p65 and p50. GAPDH was used as a loading control. SAHA, suberoylanilide hydroxamic acid; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

p50 protein, consistent with the hypothesis that activation of the NF-κB pathway by ZD55-TRAIL leading to induced drug resistance is responsible for the insensitivity of cancer cells to this anticancer therapy agent (18-21). By contrast, SAHA inhibited expression of IκBα, p65 and p50. Notably, ZD55-TRAIL-induced upregulation of IκBα, p65 and p50 was significantly inhibited by SAHA.

SAHA enhances ZD55-TRAIL-mediated cervical tumor growth suppression in vivo. To determine whether SAHA plus ZD55-TRAIL treatment enhances tumor growth suppression, *in vivo* experiments were performed using a cervical tumor xenograft model established by HeLa-luc cells. Compared with mice treated with PBS or ZD55-TRAIL, mice treated with SAHA plus ZD55-TRAIL demonstrated significant growth suppression (P=0.026 vs. ZD55-TRAIL; Fig. 5A). The average luciferase activity in the mice receiving combination therapy was ~$4.6x10^7$ photons/sec (p/s) at the end of the experiment. Whereas, the average luciferase activity of mice injected with ZD55-TRAIL or PBS was $3x10^8$ p/s and

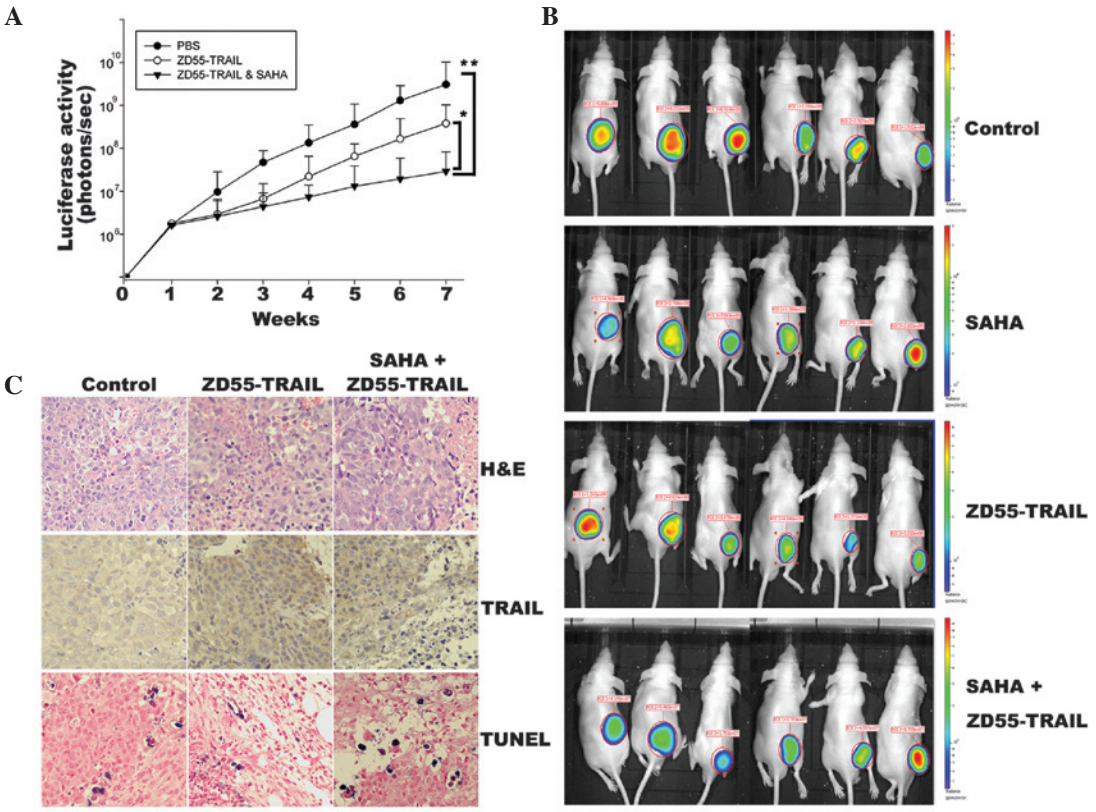

Figure 5. Synergistic effects of ZD55-TRAIL and SAHA *in vivo*. (A) Luciferase-expressing HeLa cells were injected into the right flank of BALB/c mice. The control group (n=6) received vehicle, the ZD55-TRAIL group (n=6) was injected with the ZD55-TRAIL virus and the combination group was treated with ZD55-TRAIL plus SAHA at the indicated doses. Data are presented as the mean ± standard error of the mean of six mice. *P=0.007 vs. control; **P=0.026 vs. the ZD55-TRAIL group. (B) Bioluminescence imaging was used to monitor tumor growth of HeLa-Luc cells and the intensity of the bioluminescent images were detected once a week. Bioluminescent images were acquired for each group of mice at week 7. (C) Representative immunohistochemical staining of paraffin-embedded tumor sections for H&E, TRAIL and TUNEL (magnification, x200). SAHA, suberoylanilide hydroxamic acid; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

$1.5x10^9$ p/s, respectively (Fig. 5B). Furthermore, analysis of tumors by immunohistochemical staining using anti-TRAIL antibody revealed that there was a strong expression of TRAIL in xenografts treated with ZD55-TRAIL and that the highest expression of TRAIL was observed in tumor sections that received combined injections of SAHA with ZD55-TRAIL (Fig. 5C). To investigate the possible mechanisms underlying tumor growth inhibition induced by ZD55-TRAIL combined with SAHA, a TUNEL assay was used to verify whether the action of the combination therapy was due to their proapoptotic effect. As shown in Fig. 5C, ZD55-TRAIL plus SAHA caused profound cell death in the tumor mass via apoptosis. These results demonstrated that the treatment of cervical tumors with ZD55-TRAIL plus SAHA enhances growth suppression in parallel to enhanced apoptosis, a finding consistent with our *in vitro* results.

Discussion

Monotherapy of cancer often has limited success, as the tumor cells usually develop resistance to the agents used and tumors are usually genetically diverse (7,22). Therefore, it is important to identify combinations of two or more therapeutic agents, acting through different mechanisms with synergistic effects without increasing adverse effects. Preclinical studies revealed that the efficacy of oncolytic viruses can be improved by combination with chemotherapeutic agents (23-25). ONYX-015, a E1B-55 kDa gene-deleted OA, in combination with chemotherapy has been assessed in phase I-II trials for patients with advanced cancer (26,27). The present study investigated the possibility of combining ZD55-TRAIL with SAHA, an HDAC inhibitor that may be a novel therapeutic agent for patients with cervical squamous cell carcinoma (14-16,28,29), in order to reinforce their antitumor activities. The present study demonstrated that subclinically achievable doses of SAHA (30), namely, 0.1-0.8 μM, significantly and synergistically increased ZD55-TRAIL oncotoxicity *in vitro*. SAHA also has been demonstrated to reinforce the cytotoxicity of the virus *in vivo*.

TRAIL is a promising anticancer agent due to its selective induction of apoptosis in various types of cancer cells, without toxic effects on normal cells (17,31). ZD55-TRAIL has been demonstrated to exert strong cytopathic effects on various cancer cell lines via expression of the TRAIL protein, thereby inducing enhanced apoptosis (8-10). However, TRAIL resistance has been observed in ~50% of assessed cancer cells, including cervical cancer cells (32). Susceptibility to TRAIL-induced apoptosis could be regulated at multiple levels in the apoptotic signaling cascade. Previous studies have suggested that activation of the NF-κB pathway contributes to resistance of cancer cells to TRAIL-induced apoptosis (33,34). It was reported that SAHA enhanced the cytotoxic and apoptotic effects of TRAIL and upregulated the expression of TRAIL

death receptors (35-37). In the present study, the expression of IκBα and the p50 and p65 subunits of NF-κB was upregulated in HeLa cells infected with ZD55-TRAIL. By contrast, HeLa cells treated with SAHA demonstrated a significant inhibition of IκBα, p50 and p65 expression. Notably, addition of SAHA abrogated upregulation of IκBα, p50 and p65, which may contribute to enhanced cell death induced by combination therapy. These results are consistent with previous observations that artesunate, an effective and safe anti-malarial drug, effectively enhances TRAIL-mediated cytotoxicity in human cervical carcinoma cells by suppressing pro-survival proteins and the transcriptional activity of NF-κB (32).

Apoptosis of cancer cells in response to TRAIL is initiated by binding of the ligand to death receptors (DR4 and DR5), followed by recruitment of caspase-8 via Fas-associated death domain protein and activation of caspase-3 leading to PARP cleavage (17). By contrast, it has been demonstrated that extrinsic and intrinsic apoptotic pathways were activated by treatment with SAHA, as evidenced by processing of initiator caspase-8 and caspase-9 (38,39). In our experiments activation of caspase-8 was observed in HeLa cells treated with SAHA. In addition, there were increased levels of caspase-8, caspase-3 and PARP cleavage in HeLa cells treated with ZD55-TRAIL/SAHA combination compared with that of cells treated with ZD55-TRAIL or SAHA alone. This suggested that the enhanced activation of the extrinsic apoptotic pathway also accounts for the observed enhanced therapeutic effects of combined ZD55-TRAIL and SAHA.

In clinical settings, the therapeutic approach to advanced cervical cancer is chemotherapy, however, severe adverse effects and resistance are major problems. OAs provide a new platform to treat cervical cancer as biotherapeutic agents that lack cross-resistance with chemotherapy (40-42). The present study demonstrated for the first time, to the best of our knowledge, that SAHA sensitizes human cervical cancer cells to ZD55-TRAIL-induced cell death *in vitro* and *in vivo*. This synergistic effect is associated with inhibition of the NF-κB signaling pathway and enhanced activation of the extrinsic apoptosis pathway. These results suggest that ZD55-TRAIL, used in combination with the HDAC inhibitor SAHA, represents a promising novel targeted approach for treating cervical cancer.

Acknowledgements

This study was supported by the Natural Science Foundation of Zhejiang Province (grant no. Y2090455), the Foundation of Zhejiang Municipal Commission of Population and Family Planning (grant no. 2009-74) and the Foundation of Health and Family Planning Commission of Zhejiang Province (grant no. 2012RCB013).

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