Suberoylanilide hydroxamic acid (Zolinza/vorinostat) sensitizes TRAIL-resistant breast cancer cells orthotopically implanted in BALB/c nude mice

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Abstract
The purpose of this study was to examine whether histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA; Zolinza/vorinostat) could sensitize tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant breast carcinoma in vivo. BALB/c nude mice were orthotopically implanted with TRAIL-resistant MDA-MB-468 cells and treated i.v. with SAHA, TRAIL, or SAHA followed by TRAIL for four times during first 3 weeks. The effects of drugs on tumor growth and markers of apoptosis, metastasis, and angiogenesis were examined. SAHA sensitized TRAIL-resistant xenografts to undergo apoptosis through multiple mechanisms. Whereas TRAIL alone was ineffective, SAHA inhibited growth of MDA-MB-468 xenografts in nude mice by inhibiting markers of tumor cell proliferation, angiogenesis, and metastasis and inducing cell cycle arrest and apoptosis. The sequential treatment of nude mice with SAHA followed by TRAIL was more effective in inhibiting tumor growth, angiogenesis, and metastasis and inducing apoptosis than SAHA alone, without overt toxicity. Treatment of nude mice with SAHA resulted in down-regulation of nuclear factor-κB and its gene products (cycdin D1, Bcl-2, Bcl-XL, vascular endothelial growth factor, hypoxia-inducible factor-1α, interleukin-6, interleukin-8, matrix metalloproteinase-2, and matrix metalloproteinase-9) and up-regulation of DR4, DR5, Bak, Bax, Bim, Noxa, PUMA, p21^{CIP1}, tissue inhibitor of metalloproteinase-1, and tissue inhibitor of metalloproteinase-2 in tumor cells. Furthermore, control mice showing increased rate of tumor growth had increased numbers of CD31⁺ or von Willebrand factor-positive blood vessels and increased circulating vascular endothelial growth factor receptor 2-positive endothelial cells compared with SAHA-treated or SAHA plus TRAIL-treated mice. In conclusion, sequential treatment with SAHA followed by TRAIL may target multiple pathways in tumor progression, angiogenesis, and metastasis and represents a novel therapeutic approach to treat breast cancer. [Mol Cancer Ther 2009;8(6):1596–605]

Introduction
Acetylation and deacetylation of histones plays a role in the regulation of gene expression (1). Histone acetyltransferases and histone deacetylases (HDAC) have recently been shown to regulate cell proliferation, differentiation, and apoptosis in various hematologic and solid malignancies (2). Altered histone acetyltransferase or HDAC activity is associated with cancer by changing the expression pattern of selected genes. Hyperacetylation of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates euchromatic chromatin condensation and gene expression silencing (3). Five classes of HDAC inhibitors have been characterized and include benzamides (MS-275); hydroxamic acids [suberoylanilide hydroxamic acid (SAHA) and trichostatin A]; short-chain fatty acids (sodium butyrate and phenylbutyrate); cyclic tetrapeptide containing a 2-amino-8-oxo-9,10-epoxy-decanoyl moiety (trapoxin A); and cyclic peptides without the 2-amino-8-oxo-9,10-epoxy-decanoyl moiety (FK228). Recent studies have shown that HDAC inhibitors induce cell cycle arrest, differentiation, and apoptosis in vitro and in vivo (4, 5). HDAC inhibitors also inhibit endothelial cell proliferation and angiogenesis by down-regulating angiogenesis-related gene expression (6, 7). Phase I and II clinical trials on SAHA (Zolinza/vorinostat) have provided promising results (8, 9). Therefore, HDAC inhibitors are considered as candidate drugs in cancer therapy.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2L binds to TRAIL-R1 (DR4/Apo-2A), TRAIL-R2 (DR5/TRICK/Killer), TRAIL-R3 (TRID/DcR1/LIT), and TRAIL-R4 (TRUNDD/DcR2) cell surface receptors (10). DR4 and DR5 receptors contain a cytoplasmic death domain required for apoptosis, whereas DcR1 and
DcR2 lack a functional death domain and are termed as decoy receptors. Although many cancer cells undergo TRAIL-induced apoptosis, some cells are resistant to TRAIL, making it ineffective as an anticancer agent (10–14). Expression of certain apoptosis-related genes has been suggested to regulate sensitivity of cancer cells to TRAIL-mediated apoptosis, including nuclear factor-κB (NF-κB; refs. 15, 16), Akt/protein kinase B (12, 17), Bcl-2 (18), Bak and/or Bak (19, 20), and c-FLIP (21). Our recent work has shown that certain HDAC inhibitors can enhance the apoptosis-inducing potential of TRAIL in TRAIL-sensitive cells and sensitize TRAIL-resistant breast cancer cells in vitro (22) and thus can be considered as clinically viable candidates for combination therapy.

We have recently shown that HDAC inhibitors (SAHA, m-carboxycinnamic acid bis-hydroxamide, MS-275, and trichostatin A) can enhance the apoptosis-inducing potential of TRAIL in leukemia, multiple myeloma, and breast cancer cells in vitro (22–24). A synergism in apoptosis was observed in both TRAIL-sensitive and TRAIL-resistant cells on sequential treatments with HDAC inhibitors followed by TRAIL. HDAC inhibitors synergized with TRAIL through NF-κB and its gene products such as death receptors (DR4/TNF-R1 and DR5/TRAIL-R2), and some of the members of the Bcl-2 family, and engaging the mitochondrial pathway. However, the ability of SAHA to sensitize TRAIL-resistant breast cancer cells in a suitable xenograft model has not yet been shown. The objective of this study was to investigate the molecular mechanisms by which SAHA sensitizes TRAIL-resistant breast cancer cells in vivo. These in vivo data provide essential information about the effectiveness of anticancer agents for conducting clinical trials in future.

Materials and Methods

Reagents

Antibodies against DR4 and DR5 were purchased from Imgenex. Fluorescence-tagged anti-active caspase-3 antibody and untagged anti-active caspase-8 antibody were purchased from Cell Signaling. HDAC activity kit (fluorometric substrate) was purchased from Imgenex. Fluorescence-tagged anti-active caspase-3 antibody and untagged anti-active caspase-8 antibody were purchased from Upstate Cell Signaling Solutions. Antibodies against actin, p21CIP1, cyclin D1, Bcl-2, Bcl-XL, Bax, Bak, Bim, Noxa, PUMA, proliferating cell nuclear antigen (PCNA), Ki-67, hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), CD31, interleukin (IL)-6, IL-8, matrix metalloproteinase (MMP)-2, MMP-9, TIMP-1, TIMP-2, Il-1β, CD31, and von Willebrand factor were purchased from Santa Cruz Biotechnology. MMP2 (8B4) and MMP9 (2C3) antibodies recognize proforms and do not cross-react with other MMPs. TRAIL was synthesized as described earlier (25). Cell death detection kit (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) was purchased from Roche Applied Sciences. SAHA was provided by the National Cancer Institute.

Cells and Culture Conditions

MDA-MB-468 cells were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Western Blot Analysis

Western blot analysis was done as we described elsewhere (26). In brief, cell pellets were lysed in radioimmunoprecipitation assay buffer containing 1× protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad). Cell lysates were electrophoresed in 12.5% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. After blotting in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies at 1:1,000 dilution in TBS overnight at 4°C and then secondary antibodies conjugated with horseradish peroxidase at 1:5,000 dilution in TBS for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system.

Reverse Transcription Reaction, cDNA Synthesis, and PCR

The reverse transcription-PCR (RT-PCR) assay was done as per the manufacturer’s instructions (Access RT-PCR System; Promega). The primer sequences were published earlier (27).

IKK Assay

IKK assay was done as we described elsewhere (28). In brief, tumor lysates were incubated with 2 μg/mL anti-NEMO/IKKγ antibody for 2 h at 4°C. Immunocomplex was precipitated using protein G-Plus agarose beads overnight at 4°C. Beads were washed and then resuspended in 30 μL kinase buffer [50 mmol/L Tris-HCl (pH 8), 100 mmol/L NaCl, 2 mmol/L MgCl2, 1 mmol/L DTT, 1 mmol/L NaF, 1 mmol/L Na3VO4, 25 mmol/L β-glycerophosphate, 10 mmol/L NPP, and protease inhibitor cocktail (complete; Roche)] supplemented with ATP (1 mmol/L) in the presence of wild-type glutathione S-transferase-1:βgal55 and were incubated at 30°C for 30 min. Reactions were stopped by the addition of SDS loading buffer and subjected to SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membranes and blotted with a phosphospecific anti-IκBα (Ser32/Ser36) antibody.

HDAC Assay

HDAC activity in nuclear extract was measured as per manufacturer’s instructions (Upstate Cell Signaling Solutions). In brief, nuclear extracts were prepared from tumor samples derived from control and treatment groups and kept at -80°C. Samples (∼50 μg nuclear extract in 15 μL volume) were aliquoted in a 96-well plate. In each well, 10 μL HDAC assay buffer and 15 μL HDAC fluorometric substrate were added. Samples were mixed thoroughly and incubated at 37°C for 30 min. Following incubation, 20 μL diluted Activator solution was added to each well and the samples were mixed thoroughly. After 15 min of incubation at room temperature, the samples were read in a fluorescence plate reader with excitation of 350 to 380 nm and emission of 440 to 460 nm.
Antitumor Activity of SAHA and TRAIL

TRAIL-resistant MDA-MB-468 cells (2 × 10⁶ in Matrigel; Becton Dickinson) cells in a final volume of 0.1 mL were injected into the mammary fat pad of BALB/c nu/nu mice (4–6 weeks old). The mice were purchased from the National Cancer Institute. After tumor formation (100 mm³), mice (10 mice per group) were injected i.v. with vehicle (PBS), SAHA (35 mg/kg), TRAIL (15 mg/kg), or SAHA followed by TRAIL. In combination treatment, SAHA was administered 24 h before TRAIL treatment because our goal was to sensitize MDA-MB-468 cells, so that successive treatment with TRAIL would result in an enhanced apoptosis of tumor cells. Drugs were administered four times during the first 3 weeks after tumor formation. Tumor growth was followed by measurements of tumor diameters with a sliding caliper once a week. The tumor volume was calculated according to the formula: tumor volume = L × W² / 2, where L and W are the major and minor dimensions, respectively.

Immunohistochemistry

Immunohistochemistry of tumor tissues collected on day 23 was done as described elsewhere (10, 11). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays were done as per manufacturer’s instructions (Roche Applied Sciences).

Measurement of Circulating Endothelial Cells

Endothelial cells were collected and counted as we described elsewhere (29).

Statistical Analyses

The mean ± SE were calculated for each data point. Differences between groups were analyzed by one- or two-way ANOVA. Differences in the rates of tumor inhibitions were validated by χ² test. Significant differences among groups were calculated at P < 0.05.

Results

Effects of SAHA and/or TRAIL on Tumor Growth and Cell Proliferation in Xenografted Nude Mice

We recently showed that several HDAC inhibitors enhanced the apoptosis-inducing potential of TRAIL in breast cancer cell lines in vitro (22); we therefore sought to validate whether the combination SAHA and TRAIL is effective in xenograft model. After tumor formation, mice were injected with vehicle (PBS), TRAIL, SAHA, and SAHA followed by TRAIL four times during 3 weeks (Fig. 1). Whereas TRAIL was ineffective, the administration of SAHA alone resulted in inhibition of tumor growth (Fig. 1A). Interestingly, SAHA sensitized TRAIL-resistant tumor cells by inhibiting tumor growth. No toxicity was observed in the liver, spleen, and brain tissues of mice as measured by H&E staining (data not shown). These data suggest that the sequential treatment of mice with SAHA followed by TRAIL may sensitize TRAIL-resistant cells. Therefore, the combination of SAHA and TRAIL could be a viable option to treat breast cancer.

We next examined the effects of SAHA and/or TRAIL on tumor tissues derived from xenografted nude mice on day 23 by immunohistochemistry. Tumor cell proliferation was measured by staining tissues with anti-PCNA and anti-Ki-67 staining (Fig. 1B). Whereas TRAIL alone was ineffective, SAHA inhibited tumor cell proliferation as evident by less immunoreactivity with PCNA and Ki-67 antibody. PCNA- and Ki-67-positive tumor cells were quantified. Mean ± SE. *, #, P < 0.05, significantly different from respective control. C, HDAC activity in tumor tissues. HDAC activity in nuclear extract of tumor tissues was measured as per manufacturer’s instructions. Mean ± SE. *, P < 0.05, significantly different from respective control.

SAHA and TRAIL Inhibit Tumor Growth and Angiogenesis

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Figure 1. Interactive effects of SAHA and TRAIL on tumor growth and tumor cell proliferation in nude mice. A, interactive effects of SAHA with TRAIL on tumor regression in nude mice. Tumor-bearing nude mice were injected with vehicle, SAHA, TRAIL, or SAHA followed by TRAIL. Drugs were administered i.v. four times during first 3 wk. Tumor volume was measured weekly. Mean ± SE. *, #, P < 0.05, significantly different from respective control. B, interactive effects of SAHA with TRAIL on tumor cell proliferation. Tumor samples derived from xenografted nude mice were subjected to immunohistochemistry with anti-PCNA or anti-Ki-67 antibody. PCNA- and Ki-67-positive tumor cells were quantified. Mean ± SE. *, #, P < 0.05, significantly different from respective control. C, HDAC activity in tumor tissues. HDAC activity in nuclear extract of tumor tissues was measured as per manufacturer’s instructions. Mean ± SE. *, P < 0.05, significantly different from respective control.
inhibition of HDAC activity in tumor tissues than those derived from control mice (Fig. 1C). Treatment of mice with SAHA plus TRAIL resulted in similar HDAC activity compared with those received SAHA alone. These data suggest that SAHA induces its effect through acetylation of histones.

**Regulation of Apoptosis and Caspase-3 and Caspase-8 Activities by SAHA and TRAIL in Tumor Tissues Derived from Xenografted Mice**

Because SAHA sensitized TRAIL-resistant breast cancer xenografts in nude mice, we sought to examine the mechanism by which tumor cells underwent apoptosis. Activation of caspase-3 and caspase-8 and induction of apoptosis were measured in tumor tissues (Fig. 2A and B). MDA-MB-468 xenografts were resistant to TRAIL and showed no significant caspase-3 and caspase-8 activities and apoptosis. MDA-MB-468 xenografts treated with SAHA alone showed enhanced caspase-3 activity and apoptosis compared with control group. Sequential treatments of mice with SAHA followed by TRAIL sensitized TRAIL-resistant MDA-MB-468 tumor cells to undergo apoptosis and resulted in enhanced caspase-3 and caspase-8 activities compared with SAHA alone. These data clearly show that the increased activities of caspase-3 and caspase-8 and tumor cell apoptosis correlate with the ability of SAHA to sensitize TRAIL-resistant breast cancer cells.

**Regulation of Death Receptors, p21, and Cyclin D1 by SAHA and/or TRAIL in Tumor Tissues Derived from Xenografted Mice**

Our in vitro data showed that HDAC inhibitors induce apoptosis through regulation of death receptors and cell cycle regulatory genes (22). We therefore examined the expression of these genes in tumor tissues derived from xenografted nude mice treated with SAHA and/or TRAIL (Fig. 3). We first measured the expression of DR4 and DR5 by ELISA assay and immunohistochemistry. Whereas TRAIL alone was ineffective, SAHA enhanced the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 proteins and percent of DR4- or DR5-positive tumor cells (Fig. 3A and B). The combination of SAHA and TRAIL slightly had more effects on DR4 and DR5 expression than SAHA alone.
We have shown previously than SAHA causes growth arrest at G1-S stage of cell cycle in vitro (22). We therefore sought to examine the effects of SAHA and/or TRAIL on the expression of p21CIP1 and cyclin D1 in tumor tissues by immunohistochemistry (Fig. 3B). Whereas TRAIL was ineffective, SAHA enhanced the expression of p21CIP1 and inhibited the expression of cyclin D1 proteins. The combination of SAHA and TRAIL slightly had more effects on induction of p21CIP1 and down-regulation of cyclin D1. The relative expression of p21CIP1 and cyclin D1 proteins correlated with percent of tumor cells expressing these proteins. These data suggest that SAHA may cause growth arrest at G1-S stage of cell cycle by regulating p21CIP1 and cyclin D1.

Because SAHA or SAHA plus TRAIL regulated the expression of death receptors and cell cycle regulatory proteins, we confirmed the expression of DR4, DR5, p21, and cyclin D1 by Western blot analysis (Fig. 3C). Whereas TRAIL was ineffect, treatment of mice with SAHA enhanced the expression of DR4, DR5, and p21CIP1 and inhibited the expression of cyclin D1 in tumor tissues. The combination of SAHA plus TRAIL slightly had more effects on induction of p21CIP1 and completely inhibited cyclin D1 expression. These data suggest that SAHA may cause growth arrest at G1-S stage of cell cycle by regulating p21CIP1 and cyclin D1, and up-regulation of DR4 and DR5 may be one of the mechanisms of inducing sensitivity to TRAIL.

NF-κB is responsible for the expression by regulating many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis, and apoptosis. The function of NF-κB is inhibited by binding to NF-κB inhibitor (IκB), and imbalance of NF-κB and IκB has been associated with development of many diseases, including tumors. We confirmed the involvement of NF-κB pathway by measuring IKK kinase activity in tumor tissues derived from TRAIL and/or SAHA treated mice (Fig. 3D). Whereas treatment of mice with TRAIL had no effect on IKK activity, SAHA alone inhibited IKK activity. Furthermore, the combination of TRAIL plus SAHA was more effective in inhibiting IKK activity than single agent alone. These data show that SAHA or TRAIL plus SAHA can inhibit NF-κB and its gene products.

Regulation of Bcl-2 Family Members by SAHA and/or TRAIL in Tumor Tissues Derived from Xenographed Mice

Our in vitro data showed that HDAC inhibitors induce apoptosis through regulation of Bcl-2 family members in breast cancer cell lines (22); we therefore examined the...
expression of these genes by RT-PCR (Fig. 4A). Whereas TRAIL was ineffective, SAHA enhanced the expression of Bak, Bax, Bim, Noxa, and PUMA and inhibited the expression of Bcl-2 and Bcl-XL. The combination of SAHA plus TRAIL slightly had more effect on the regulation of these Bcl-2 family proteins.

We next confirmed the regulation of these genes by immunohistochemistry of tumor tissues derived from MDA-MB-468 xenografts (Fig. 4B). Tumor sections derived from control and treated mice were subjected to immunohistochemistry to examine the expression of Bcl-2 family members. SAHA induced the expression of Bak, Bax, Bim, Noxa, and PUMA and inhibited the expression of Bcl-2 and Bcl-XL. By comparison, TRAIL had no significant effect on the expression of these proteins. The combination of SAHA plus TRAIL slightly had more effect on the regulation of these Bcl-2 family proteins. The relative expression of Bcl-2, Bcl-XL, Bak, Bax, Bim, Noxa, and PUMA proteins correlated with percent of tumor cells expressing these proteins (Fig. 4B–D). Furthermore, the immunohistochemistry data on the regulation of Bcl-2 family members by SAHA are in agreement with gene expression data using RT-PCR.

**Interactive Effects of SAHA and/or TRAIL on Angiogenesis and Metastasis**

Angiogenesis is a critical step in solid tumor progression (30). To determine whether sensitization of TRAIL-resistant breast carcinoma by SAHA was due to regulation of angiogenesis, we first examined the number of blood vessels by staining tumor tissues with H&E, anti-CD31 antibody, and anti-von Willebrand factor antibody (Fig. 5A). Treatment of xenografted mice with SAHA resulted in significantly less blood vessel formation compared with control mice. TRAIL alone had no effect on the blood vessel formation. We observed significantly less blood vessels in mice treated with SAHA plus TRAIL compared with mice treated with SAHA alone or control. Control mice showing increased rate of tumor growth had increased numbers of CD31⁺ and von Willebrand factor-positive blood vessels compared with SAHA-treated or SAHA plus TRAIL-treated mice (Fig. 5A). We have shown that increases in the circulating VEGFR2-positive endothelial cells correlate directly with...
increase in tumor angiogenesis and can serve as in vivo indicators of tumor angiogenesis (29). The numbers of VEGFR2-positive endothelial cells in the blood derived from xenografted mice were also counted (Fig. 5B). As expected, control mice had increased circulating VEGFR2-positive endothelial cells compared with SAHA-treated or SAHA plus TRAIL-treated mice. By comparison, TRAIL had no effect on circulating VEGFR2-positive endothelial cells.

VEGF, HIF-1α, IL-6, and IL-8 have been shown to be associated with tumor progression including inhibition of cancer cell apoptosis and stimulation of angiogenesis (30). To further assess the inhibitory effect of SAHA on tumor microenvironment, immunohistochemistry and Western blotting of tumor samples were done. Treatment of mice with SAHA inhibited the expression of VEGF, HIF-1α, IL-6, and IL-8 in tumor tissues compared with untreated control group (Fig. 5C). TRAIL had no effect on the expression of these proteins. The combination of SAHA plus TRAIL slightly had more effect than SAHA alone. The relative expression of VEGF, HIF-1α, IL-6, and IL-8 proteins correlated with percent of tumor cells expressing these proteins (Fig. 5C). Our data show that SAHA can inhibit tumor angiogenesis and proinflammatory cytokines in breast cancer xenografts.

MMPs are a family of zinc-dependent endopeptidases (30). They are capable of digesting the different components of the extracellular matrix and basement membrane. The extracellular matrix gives structural support to cells and plays a central role in cell adhesion, differentiation, proliferation, and migration. TIMPs are endogenous regulators of MMPs.

Figure 5. Effects of SAHA and TRAIL on angiogenesis and metastasis. A, tumor tissue sections were stained with H&E, anti-CD31 antibody, and anti-von Willebrand factor antibody and the number of blood vessels were counted. Columns, mean; bars, SE. *, #, P < 0.05, significantly different from respective control. B, VEGFR2-positive circulating endothelial cells in mice on day 23. The blood cells from peripheral blood attached to the slide were stained with anti-VEGFR2 antibody and the number of positive cells was counted. C, top, expression of VEGF, HIF-1α, IL-6, and IL-8 in tumor tissues by immunohistochemistry. Tumor tissues were subjected to immunohistochemistry with anti-VEGF, anti-HIF-1α, anti-IL-6, and anti-IL-8 antibodies and photographed (magnification, ×20). Bottom left, protein expression of VEGF, HIF-1α, IL-6, and IL-8 in tumor tissues. Tumor cell lysates were prepared and subjected to the Western blot analysis for the measurement of VEGF, HIF-1α, IL-6, and IL-8. β-Actin was used as a loading control. Bottom right, percent of VEGF, HIF-1α, IL-6, and IL-8-positive tumor cells. Mean ± SE (n = 10). *, #, P < 0.05, significantly different from respective control. D, top, expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in tumor tissues by immunohistochemistry. Tumor tissues were subjected to immunohistochemistry with anti-MMP-2, anti-MMP-9, TIMP-1, and anti-TIMP-2 antibodies and photographed (magnification, ×20). Bottom left, protein expression of anti-MMP-2, anti-MMP-9, TIMP-1, and anti-TIMP-2 antibodies and photographed (magnification, ×20). Bottom right, protein expression of anti-MMP-2, anti-MMP-9, TIMP-1, and anti-TIMP-2 antibodies and photographed (magnification, ×20). Bottom left, protein expression of anti-MMP-2, anti-MMP-9, TIMP-1, and anti-TIMP-2 antibodies and photographed (magnification, ×20). Bottom right, percent of MMP-2, MMP-9, TIMP-1, and TIMP-2-positive tumor cells. Mean ± SE (n = 10). *, #, %, P < 0.05, significantly different from respective control.
the principal enzymes responsible for the degradation of extracellular matrix in metastasis, and reduce their proteolytic activity. Treatment of mice with SAHA down-regulated the expression of MMP-2 and MMP-9 and up-regulated the expression of TIMP-2 in tumor tissues compared with untreated control group as measured by immunohistochemistry and Western blot analysis (Fig. 5D). TRAIL had no effect on the expression of these proteins. The combination of SAHA plus TRAIL had effects similar to that of SAHA. The relative expression of MMP-2, MMP-9, and TIMP-2 proteins correlated with percent of tumor cells expressing these proteins (Fig. 5D). Our data show that SAHA can inhibit markers of metastasis in breast cancer xenografts.

Discussion

Our study sheds important new insight into the mechanisms of SAHA and its potential for combining with TRAIL for the treatment of breast cancer. Treatment of nude mice with SAHA caused a significant induction in apoptosis and inhibition in tumor growth, angiogenesis, and markers of metastasis. Treatment of nude mice with SAHA resulted in down-regulation of NF-κB and its gene products (cycillin D1, Bcl-2, Bcl-XL, VEGF, HIF-1α, IL-6, IL-8, MMP-2, and MMP-9) and up-regulation of DR4, DR5, Bak, Bax, Bin, Noxa, PUMA, p21Cip1, and TIMP-2 in tumor cells. Furthermore, control mice showing increased rate of tumor growth had increased numbers of CD31+ or von Willebrand factor-positive blood vessels and increased circulating vascular VEGFR2-positive endothelial cells compared with SAHA-treated or SAHA plus TRAIL-treated mice. Sequential treatments of athymic nude mice with SAHA followed by TRAIL caused a synergistic apoptotic response through activation of caspase-3, which was accompanied by regression of tumor growth and inhibition of markers of angiogenesis and metastasis. Together with previous studies showing that cancer chemotherapeutic drugs and irradiation up-regulate DR4 and/or DR5 expression (10, 11, 13, 31), our data also show that SAHA can up-regulate these death receptors in TRAIL-resistant breast cancer xenografts. Although several recent studies including ours have shown the additive or synergistic effects of HDAC inhibitors and TRAIL on apoptosis in vitro (22, 24), this is the first study showing the antitumor activity of SAHA plus TRAIL in breast carcinoma in vivo. Here, we show that SAHA can sensitize TRAIL-resistant breast cancer xenografts in nude mice through induction of growth arrest and apoptosis and inhibition of markers of cell proliferation, angiogenesis, and metastasis.

Activation of death receptor pathway is a new strategy for targeted therapy of cancer. HDAC inhibitors have been shown to improve the efficacy of chemotherapeutics, ionizing radiation, bortezomib, and cytotoxic cytokine (22, 24, 32, 33). TRAIL is a particularly promising candidate for cancer therapy as it provokes cell death in tumor cells while sparing most normal cells (34). In cancer patients, phase I and II clinical trials using agonistic monoclonal antibodies that engage the human TRAIL receptors DR4 and DR5 have shown limited/no toxicity (35). Nevertheless, some cancer cells were refractory to TRAIL, suggesting that treatment with TRAIL alone may be insufficient for cancer therapy. The up-regulation of DR4 and/or DR5 may give an advantage for enhancing the apoptosis-inducing potential of TRAIL in cancer cells and sensitizing those cancer cells that are resistant to TRAIL alone. In the present study, administration of SAHA in nude mice up-regulated the expression of DR4 and DR5 in tumor tissues; this could be one of the mechanisms of inducing sensitivity in TRAIL-resistant xenografts. Our data suggest that SAHA can enhance the therapeutic potential of TRAIL in cancer patients.

In addition to the induction of death receptors by HDAC inhibitors, the regulation of Bcl-2 family members will also play a significant role in sensitization of TRAIL-resistant cancer cells (22). Based on our data, it appears that SAHA selectively induces proapoptotic members such as Bax, Bak, Noxa, Bim, and PUMA and inhibits antiapoptotic Bcl-2 and Bcl-Xl expression in vivo. Bcl-2 family members mainly exert their apoptotic effects by acting at the level of mitochondria and play crucial role in cancer growth and progression (36). Inactivation of both Bax and Bak was required for tumor growth and was selected for in vivo during tumorigenesis (37). HDAC inhibitors can also induce TRAIL (38), suggesting the activation of death receptor pathway without the requirement of exogenous TRAIL. Thus, HDAC inhibitors can induce apoptosis by linking both death receptor and mitochondrial pathways of apoptosis.

Tumor growth and metastasis depend on the development of a neovasculature in and around the tumor (39). Angiogenesis is regulated by the balance between stimulatory (e.g., basic fibroblast growth factor, IL-6, IL-8, MMP-2, MMP-9, transforming growth factor-β1, and VEGF) and inhibitory (e.g., angiostatin, IL-10, and IFN) factors released by the tumor and its environment (39). Overexpression of basic fibroblast growth factor (40) and VEGF (41) has been found in the tissue, serum, and urine of patients with bladder cancer and also has been associated with disease progression. HDAC inhibitors have been recently shown to inhibit endothelial cell migration, invasion, vascular sprouting in vitro, and vasculature formation in animal models of cancer (42-44). Our data show that SAHA inhibited tumor growth by inhibiting angiogenesis and its marker such as VEGF and its effects on angiogenesis were further enhanced in the presence of TRAIL. Similar to our previous studies with chemotherapeutic drugs and TRAIL on angiogenesis (29), we observed a positive correlation between inhibition of angiogenesis and tumor regression.

Hypoxia, frequently found in the center of solid tumor, is associated with resistance to chemotherapy by activation of signaling pathways that regulate cell proliferation, angiogenesis, and apoptosis (45). Hypoxia can increase the resistance of cancer cells to drug-induced apoptosis by activation of phosphatidylinositol 3′-kinase/Akt, MEK/mitogen-activated protein kinase (extracellular signal-regulated kinase), and NF-κB signaling pathways (46). Expression of HDACs is often up-regulated under angiogenic stimuli such as hypoxia in cancer cells. HDAC inhibitors FK228 and TSA
down-regulates hypoxia-responsive genes and hypoxia-induced angiogenesis by the suppression of HIF-1α activity (47). In the present study, SAHA inhibited the expression of HIF-1α and angiogenesis, and its effects were further enhanced in the presence of TRAIL, suggestive of a positive relationship between HIF-1α expression and angiogenesis.

MMPs and their tissue inhibitors (TIMPs) represent a regulatory system playing a crucial role in extracellular matrix metabolism (48, 49). High tumor grade and lymph node positivity are associated with poor prognosis in breast carcinoma. MMPs degrade the extracellular matrix, and type IV collagenases MMP-2 and MMP-9 have been linked to invasive behavior of several malignancies (48). TIMP-1 and TIMP-2 inhibit their activity and are therefore considered to have an inhibitory effect on tumor progression (50). The lack of TIMP-1 protein expression is associated with a favorable prognosis in patients with node-positive high-grade breast carcinoma (51). In the present study, SAHA down-regulated the expression of MMP-1 and MMP-2 and up-regulated the expression of TIMP-1 and TIMP-2 in xenografted tumors. Our data suggest that SAHA, in addition to tumor growth and angiogenesis, can also inhibit tumor metastasis.

We have shown that NF-κB is constitutively active in breast cancer, and down-regulation of this transcription factor enhances therapeutic response of anticancer drugs and TRAIL (15, 22). NF-κB regulates the expression of genes involved in cancer cell invasion, metastasis, and resistance to chemotherapy (52). In normal unstimulated cells, NF-κB is maintained in the cytoplasm with its inhibitor protein IκBα, whereas, in cancer cells, NF-κB is in the nucleus and constitutively activates target genes. Overexpression of a transdominant-negative mutant of the inhibitory protein IκBα results in down-regulation of constitutively active NF-κB, induction of DR5, and tumor necrosis factor receptor 1-associated death domain expression and enhancement of TRAIL sensitivity (15). NF-κB may promote cell growth and proliferation by regulating expression of genes such as c-myc, cyclin D1, and IL-6 and inhibit apoptosis through activation of expression of antiapoptotic genes, such as Bcl-2 and Bcl-Xi (52). NF-κB-mediated expression of genes involved in angiogenesis, invasion, and metastasis, may further contribute to the progression of breast cancer (52). Constitutive NF-κB activity has also been shown in primary breast cancer tissue samples and suggested to have prognostic importance for a subset of primary tumors. In the present study, SAHA inhibited the activation of NF-κB and its gene products such as VEGF, IL-6, HIF-1α, Bcl-2, Bcl-Xi, MMP-2, MMP-7, MMP-9, and IL-6 in tumor tissues. These findings suggest that NF-κB may play a role in human breast cancer development and/or progression, and SAHA can inhibit these processes through regulation of NF-κB-regulated gene products.

In summary, we have developed a novel strategy of combining SAHA with TRAIL for the treatment of human breast cancer. Given that SAHA up-regulates proapoptotic members DR4, DR5, Bax, Bak, Bim, PUMA, and NOXA and down-regulates antiapoptotic Bcl-2 and Bcl-Xi, it is possible that sensitization of TRAIL-induced apoptosis may occur at various stages of apoptotic pathways involving both cell-extrinsic and cell-intrinsic pathways. Furthermore, the ability of SAHA to inhibit NF-κB and its gene products (VEGF, HIF-1α, IL-6, IL-8, MMP-2, and MMP-9) and induce the expression of TIMP-2 will further enhance its antitumor activity through regulation of markers of angiogenesis and metastasis. Thus, our results show the efficacy of a novel therapy that combines SAHA and TRAIL for the treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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