MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo.

Rakesh K. Srivastava¹, Razelle Kurzrock² and Sharmila Shankar³,*

¹Department of Pharmacology, Toxicology and Therapeutics, and Medicine, The University of Kansas Cancer Center, The University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. ²Department of Investigational Cancer Therapeutics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030. ³Department of Pathology and Laboratory Medicine, The University of Kansas Cancer Center, The University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160.

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Abbreviations: TRAIL, TNFα-related apoptosis-inducing ligand; EMT, epithelial-mesenchymal transition; HDAC, histone deacetylase; HAT, histone acetyltransferases

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*Correspondence: Dr. Sharmila Shankar, Department of Pathology and Laboratory Medicine, The University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. Email: sshankan@kumc.edu

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Abstract

Histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) show promise for the treatment of cancers. The purpose of this study was to examine the molecular mechanisms by which HDAC inhibitor MS-275 sensitizes TRAIL-resistant breast cancer cells in vivo, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition (EMT). Balb c nude mice were orthotopically implanted with TRAIL-resistant invasive breast cancer MDA-MB-468 cells, and treated intravenously with MS-275, TRAIL or MS-275 followed by TRAIL, four times during first three weeks. Treatment of mice with TRAIL alone had no effect on tumor growth, metastasis, angiogenesis and EMT. By comparison, MS-275 sensitized TRAIL-resistant xenografts by inducing apoptosis, inhibiting tumor cell proliferation, angiogenesis and metastasis, and reversing EMT. Treatment of nude mice with MS-275 resulted in down-regulation of NFκB and its gene products (cyclin D1, Bcl-2, Bcl-XL, VEGF, HIF-1α, IL-6, IL-8, MMP-2 and MMP-9), and up-regulation of DR4, DR5, Bax, Bak, and p21/CIP1 in tumor cells. Furthermore, MS-275 treated mice demonstrated significantly reduced tumor growth and decreased circulating vascular VEGFR2-positive endothelial cells, CD31-positive or von Willebrand Factor-positive blood vessels and lung metastasis compared to control mice. Interestingly, MS-275 caused “cadherin switch” and reversed EMT as demonstrated by up-regulation of E-cadherin and down-regulation N-cadherin and transcription factors Snail, Slug, and ZEB1. In conclusion, sequential treatments of mice with MS-275 followed by TRAIL may target multiple pathways to reverse EMT, and inhibit tumor progression, angiogenesis and metastasis, and represent a novel therapeutic approach to treat cancer.
Introduction

Human HDACs comprise a family of 18 different members, which have been grouped into four classes (1). Class I (HDAC1-3, 8), class II (HDAC4-7, 9, 10 of which HDAC4, 5, 7, and 9 form the class IIa subgroup due to a common structural organization, while HDAC6 and HDAC10 are members of class IIb), class III (also referred to as sirtuins (SIRT1-7)), and class IV (HDAC11). Classes I, II, and IV HDACs are zinc-dependent enzymes and show some sequence similarities, while class III HDACs are NAD1-dependent enzymes without homology with the other HDACs. Class I HDACs are mainly localized inside the nucleus, where their predominant substrates are found. Class II HDACs and class IV HDACs are localized both in the nucleus and in the cytoplasm, and are able to shuttle in and out of the nucleus in response to specific cellular signals. Many potent non-selective HDAC inhibitors have been developed, but their toxicity often constitutes a limitation in therapy. Therefore, there is a need to develop selective inhibitors for cancer therapy. Selective HDAC inhibitors should in principle be able to avoid such undesirable effects, thus proving to be less toxic.

Acetylation and deacetylation of histones plays a role in the regulation of gene expression (2). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) have recently been shown to regulate cell proliferation, differentiation and apoptosis in various hematologic and solid malignancies (3). Altered HAT 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 deacylation mediates eukaryotic chromatin condensation and gene expression silencing (4). HDAC inhibitors induce cell cycle arrest, differentiation, and apoptosis in vitro and in vivo (5-6). 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 MS-275 have provided promising results (8-9). MS-275 is currently in phase II trials, while SAHA has recently been approved by FDA for treating cutaneous T cell lymphoma. Therefore, HDAC inhibitors are considered as candidate drugs in cancer therapy.

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). Although many cancer cells undergo TRAIL-induced apoptosis, some cells are resistant to TRAIL, making it ineffective as an anticancer agent (10-12). Expression of certain apoptosis-related genes has been suggested to regulate sensitivity of cancer cells to TRAIL-mediated apoptosis, including NFκB (13), Akt/PKB (14), Bcl-2 (15), Bax and/or Bak (16), and c-FLIP (17). 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 (5), and thus can be considered as clinically viable candidates for combination therapy.

Epithelial-Mesenchymal Transition (EMT) is an extreme form of cellular plasticity defined by loss of epithelial cell morphology, dissociation of cell-cell contacts, reduction in proteins mediating cell-cell contacts, remodeling of the actin cytoskeleton, and acquisition of mesenchymal cell shape (18). During EMT, cells diminish epithelial gene expression and acquire mesenchymal gene expression (19-20). Cortical actins, the actin filament bundles below the plasma membrane, reorganize or are lost, while stress fibers comprising F-actin are gained. EMT is considered a key process driving tumor cell invasiveness and metastasis. It is associated with a downregulation of epithelial proteins, including E-cadherin, γ-catenin/plakoglobin, α-catenin, and β-catenin (21), and with an induction of mesenchymal proteins, including α-smooth muscle actin (α-SMA), fibronectin, N-cadherin, or vimentin (21-22). This is mediated by
transcription factors like Twist, E12/E47, and members of the Snail and ZEB protein families (19-20). The zinc finger transcriptional repressor, ZEB1, inhibits E-cadherin expression by recruiting HDAC. Loss of E-cadherin is associated with tumor invasiveness, metastatic dissemination, and poor prognosis in several solid tumors (23). Recent evidence indicates, however, that in addition to the loss of E-cadherin, another adhesion molecule, N-cadherin, is upregulated in invasive tumor cell lines (24). N-cadherin is present in the most invasive and dedifferentiated breast cancer cell lines and its exogenous expression in tumor cells induces a scattered morphology and increased motility, invasion, and metastasis (25). Down-regulation of E-cadherin and up-regulation of N-cadherin, cadherin switch, are believed to induce EMT. However, the molecular mechanisms by which HDAC inhibitors regulate EMT is not well understood.

We have recently demonstrated that HDAC inhibitors (MS-275, m-carboxycinnamic acid bis-hydroxamide and trichostatin A) can enhance the apoptosis-inducing potential of TRAIL in leukemia, multiple myeloma and breast cancer cells in vitro (5, 26). HDAC inhibitors synergized with TRAIL by engaging the mitochondrial pathway. In this paper, we have extended our previous studies to examine the mechanisms by which class I selective HDAC inhibitor MS-275 reverses EMT and sensitizes TRAIL-resistant breast cancer MDA-MB-468 xenografts in vivo.

**Materials and Methods**

**Reagents.** Antibodies against DR4 and DR5 were purchased from IMGENEX Inc. (San Diego, CA). Fluorescence tagged-antibody against active caspase-3, anti-E-cadherin, anti-ZEB1 and
anti-Slug antibodies and HDAC activity kit (fluorometric detection) were purchased from Cell Signaling (Beverly, MA). Antibodies against actin, P21[^CIP1], cyclin D1, Bcl-2, Bcl-X\(_L\), Bax, Bak, PCNA, Ki67, HIF-1\(\alpha\), VEGF, VEGFR2, CD31, IL-6, IL-8, ZEB1, MMP-2, MMP-9, I\(\kappa\)B\(\alpha\), CD31, and vWF were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against Snail and N-Cadherin were purchased from R &D Systems. TRAIL was synthesized as described earlier (27). Cell death detection kit (TUNEL) was purchased from Roche Applied Sciences (Indianapolis, IN). MS-275 was provided by the National Cancer Institute, Bethesda, MD.

**Cells and Culture Conditions.** MDA-MB-468 cell line was purchased from the American Type Culture Collection (Manassas, VA) and passaged in the laboratory for fewer than 6 months after receipt. No authentication for the cell line was done by the authors. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

**Western Blot Analysis.** Western blot analysis was performed as we described elsewhere (28).

**IKK Assay.** IKK assay was performed as we described elsewhere (29). In brief, tumor lysates were incubated with 2 \(\mu\)g/ml of anti-NEMO/IKK\(\gamma\) antibody for 2 h at 4\(^0\)C. Immunocomplex was precipitated using protein G-PLUS-Agarose beads overnight at 4\(^0\)C. Beads were washed and then resuspended in 30 \(\mu\)l of kinase buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 2mM MgCl\(_2\), 1 mM DTT, 1 mM NaF, 1mM Na\(_3\)VO\(_4\), 25 mM \(\beta\)-glycerophosphate, 10 mM NPP, and protease inhibitor cocktail (complete, Roche) supplemented with ATP (1 mM) in the presence of wild-type glutathione S-transferase (GST)-I\(\kappa\)B\(\alpha\)\(_{1-55}\) and were incubated at 30\(^0\)C for 30 min. Reactions were stopped by the addition of SDS loading buffer and were subjected to SDS-PAGE. Proteins were electrotransferred to PVDF membranes and blotted with a phospho-specific anti-I\(\kappa\)B\(\alpha\) (Ser\(^{32}\)-Ser\(^{36}\)) antibody.
**HDAC Assay.** HDAC activity in nuclear extract was measured as per manufacturer’s instructions (Upstate Cell Signaling).

**Antitumor activity of MS-275 and TRAIL.** TRAIL-resistant MDA-MB-468 (2 X 10^6 cells in Matrigel, Becton Dickinson, Bedford, MA) cells in a final volume of 0.1 ml were injected into the mammary fatpad of Balb/c nu/nu mice (4-6 weeks old). After tumor formation (100 mm^3), mice (10 mice per group) were injected i.v. with vehicle (PBS), MS-275 (35 mg/kg), TRAIL (15 mg/kg), or MS-275 followed by TRAIL. In combination treatment, MS-275 was administered 24 h prior to TRAIL treatment because our goal was to sensitize MDA-MB-468 tumors so that successive treatment with TRAIL would result in an enhanced apoptosis. Drugs were administered four times during the first three weeks after tumor formation. Tumor volume (TV) was calculated according to the formula: TV = L X W^2/2, where L and W are the major and minor dimensions, respectively. In vivo experiments were performed at the University of Texas Health Science Center at Tyler under protocol approved by IACUC.

**Immunohistochemistry.** Immunohistochemistry of tumor tissues collected on day-23 was performed as described elsewhere (30). TUNEL assays were performed as per manufacturer’s instructions (Roche Applied Sciences).

**Measurement of circulating endothelial cells.** Endothelial cells were collected and counted as we described elsewhere (31).

**Statistical Analyses.** The mean and SEM 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 \( \chi^2 \) test. Significant differences among groups were calculated at P < 0.05.
Results

Effects of MS-275 and/or TRAIL on tumor growth, HADC activity, cell proliferation, caspase-3 activity and apoptosis in xenografted nude mice. We recently showed that several HDAC inhibitors enhanced the apoptosis-inducing potential of TRAIL in several cancer cell lines in vitro (5), we therefore sought to validate whether the combination MS-275 and TRAIL is effective in xenograft model. After tumor formation, mice were injected with vehicle (PBS), TRAIL, MS-275, and MS-275 followed by TRAIL four times during three weeks (Fig. 1). While TRAIL was ineffective, the administration of MS-275 alone resulted in inhibition of tumor growth (Fig. 1 A). Interestingly, MS-275 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 MS-275 followed by TRAIL may sensitize TRAIL-resistant cells. Therefore, the combination of MS-275 and TRAIL could be an attractive strategy to treat TRAIL-resistant breast cancer patients.

We next measured HDAC activity in tumor samples by fluorometric assay. While TRAIL alone was ineffective, treatment of mice with MS-275 resulted in a significant inhibition of HDAC activity in tumor tissues than those derived from control mice (Fig. 1B). Treatment of mice with MS-275 plus TRAIL resulted in similar HDAC activity compared to those received MS-275 alone. These data suggest that MS-275 exerts its biological effects by inhibiting HDAC activity.

We next examined the effects of MS-275 and/or TRAIL on tumor-cell proliferation on day-23 by immunohistochemistry using anti-Ki67 and anti-PCNA antibodies (Fig. 1C). While
TRAIL alone was ineffective, MS-275 inhibited tumor-cell proliferation as evident by less immunoreactivity with PCNA and Ki67 antibodies. Furthermore, the combination of MS-275 plus TRAIL had more effect on inhibition of tumor cell proliferation than MS-275 alone. These data suggest that MS-275 or MS-275 plus TRAIL inhibited tumor growth by inhibiting tumor-cell proliferation.

Since the combination of MS-275 plus TRAIL was effective in regressing tumor growth in xenografted nude mice, we sought to examine the mechanism by which tumor cells underwent apoptosis. Activation of caspase-3 and induction of apoptosis were measured in tumor tissues (Fig. 1D). MDA-MB-468 xenografts treated with MS-275 alone showed enhanced caspase-3 activity and apoptosis compared to control group. TRAIL had no effect on caspase-3 activity and apoptosis in MDA-MB-468 xenografts. Sequential treatments of mice with MS-275 followed by TRAIL sensitized TRAIL-resistant MDA-MB-468 tumor cells to undergo apoptosis, and resulted in enhanced caspase-3 activity compared to MS-275 alone. These data clearly demonstrate that the increased activity of caspase-3 and tumor-cell apoptosis correlate with regression in tumor volume.

**Regulation of death receptors, p21, cyclin D1 and NFκB by MS-275 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 (5). We therefore examined the expression of these genes in tumor tissues derived from xenografted nude mice treated with MS-275 and/or TRAIL (Fig. 2). The expressions of death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) were measured by ELISA and immunohistochemistry (Fig 2A and B). While TRAIL alone was ineffective, MS-275 enhanced the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 proteins and percent of DR4 or DR5 positive tumor cells (Fig. 2A
and 2B). The combination of MS-275 and TRAIL slightly had more effects on DR4 and DR5 expression than MS-275 alone.

We have previously shown than MS-275 causes growth arrest at G1/S stage of cell cycle in vitro (5). We therefore sought to examine the effects of MS-275 and/or TRAIL on the expression of p21\(^{CIP1}\) and cyclin D1 in tumor tissues by immunohistochemistry (Fig. 2B). While TRAIL was ineffective, MS-275 enhanced the expression of p21\(^{CIP1}\) and inhibited the expression of cyclin D1 proteins. The combination of MS-275 and TRAIL slightly had more effects on induction of P21\(^{CIP1}\) and downregulation of cyclin D1. The expression levels of P21\(^{CIP1}\) and cyclin D1 proteins correlated with number of tumor cells. These data suggest that MS-275 may cause growth arrest at G1/S stage of cell cycle by inducing P21\(^{CIP1}\) and inhibiting cyclin D1.

Since MS-275 or MS-275 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. 2C). While TRAIL was ineffective, treatment of mice with MS-275 enhanced the expression of DR4, DR5 and p21\(^{CIP1}\) and inhibited the expression of cyclin D1 in tumor tissues. The combination of MS-275 plus TRAIL had no additional effect on induction of P21\(^{CIP1}\) and completely inhibited cyclin D1 expression. These data suggest that MS-275 may cause growth arrest at G1/S stage of cell cycle by regulating P21\(^{CIP1}\) and cyclin D1, and the upregulation of death receptors may be one of the mechanisms of inducing sensitivity to TRAIL.

NF\(_\text{K}\)B is a transcription factor, which regulates many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis (32). The function of NF\(_\text{K}\)B is inhibited by binding to NF\(_\text{K}\)B inhibitor (I\(_\text{K}\)B), and imbalance of NF\(_\text{K}\)B and I\(_\text{K}\)B has been associated with development of many diseases, including tumors. We therefore confirmed the involvement of NF\(_\text{K}\)B pathway by measuring IKK kinase activity in tumor tissues derived from
TRAIL and/or MS-275 treated mice (Fig. 2D). While treatment of mice with TRAIL had no effect on IKK activity, MS-275 alone inhibited IKK activity. Furthermore, the combination of TRAIL plus MS-275 was more effective in inhibiting IKK activity than single agent alone. We next confirmed the involvement of NFκB by immunohistochemistry using P65-NFκB antibody. Although TRAIL was ineffective, MS-275 inhibited the activation of NFκB (Fig. 2D). By comparison, the combination of MS-275 plus TRAIL was more effective in inhibiting NFκB activation than MS-275 alone. These data demonstrate that MS-275 or TRAIL plus MS-275 can inhibit NFκB and its gene products.

**Regulation of Bcl-2 family members by MS-275 and/or TRAIL in tumor tissues derived from xenografted mice.** Our *in vitro* data showed that HDAC inhibitors induce apoptosis through regulation of Bcl-2 family members in cancer cells (5), we therefore examined the expression of these genes by immunohistochemistry and western blot analysis (Fig. 3). Treatment of mice with MS-275 resulted in the induction of Bax and Bak, and inhibition of Bcl-2 and Bcl-XL expression. By comparison, TRAIL had no significant effect on the expression of these proteins. The combination of MS-275 plus TRAIL slightly had more effect on the regulation of these Bcl-2 family proteins. The relative expression of Bcl-2, Bcl-XL, Bax and Bak proteins correlated with percent of tumor cells expressing these proteins (Fig. 3B-C). These data suggest that MS-275 may regulate apoptosis through mitochondrial pathway by changing the expression patterns of Bcl-2 family members.

**Effects of MS-275 and/or TRAIL on angiogenesis.** Angiogenesis is a critical step in solid tumor progression (33). VEGF, HIF-1α, IL-6 and IL-8 have been shown to be associated with tumor progression through stimulation of angiogenesis (33). We first assess the effects of MS-275 and/or TRAIL on tumor microenvironment by measuring the markers of angiogenesis by
immunohistochemistry. Treatment of mice with MS-275 inhibited the expression of VEGF, HIF-1α, IL-6 and IL-8 in tumor tissues compared to untreated control group (Fig. 4A). TRAIL had no effect on the expression of these proteins. The combination of MS-275 plus TRAIL slightly had more effect than MS-275 alone. The relative expression of VEGF, HIF-1α, IL-6 and IL-8 proteins correlated with percent of tumor cells expressing these proteins (Fig. 4A). We next confirmed the effects of MS-275 and/or TRAIL on the expression of these proteins by Western blot analysis. Our data demonstrate that MS-275 can inhibit tumor angiogenesis and pro-inflammatory cytokines in breast cancer xenografts. Treatment of mice with MS-275 inhibited the expression of VEGF, HIF-1α, IL-6 and IL-8 in tumor tissues compared to untreated control group (Fig. 4B). As expected, TRAIL had no effect on the expression of these proteins. These data suggest that MS-275 can regulate angiogenesis in tumor tissues by regulating inflammatory cytokines and HIF-1α.

We have shown that increases in the circulating vascular endothelial growth factor receptor 2 (VEGFR2)-positive endothelial cells correlate directly with increase in tumor angiogenesis and can serve as in vivo indicators of tumor angiogenesis (31). We therefore decided to count the numbers of VEGFR2-positive endothelial cells in the blood derived from mice of different treatment groups (Fig. 4C). As expected, control mice had increased circulating VEGFR2-positive endothelial cells compared to MS-275 or MS-275 plus TRAIL treated mice. By comparison, TRAIL had no effect on circulating VEGFR2-positive endothelial cells.

In order to determine whether regression in tumor growth by MS-275 or MS-275 plus TRAIL was due to inhibition of angiogenesis, we next examined the number of blood vessels by staining tumor tissues with H&E, anti-CD31 antibody and anti-vWF antibody (Fig. 4D).
Treatment of xenografted mice with MS-275 resulted in significantly less blood vessel formation compared to control mice. TRAIL alone had no effect on the blood vessel formation. We observed significantly less blood vessels in mice treated with MS-275 plus TRAIL compared to mice treated with MS-275 alone or control. Control mice demonstrating increased rate of tumor growth had increased numbers of CD31- and vWF-positive blood vessels compared to MS-275 or MS-275 plus TRAIL treated mice. Overall, these data suggest that MS-275 can inhibit angiogenesis by regulating VEGF, HIF-1α, and inflammatory cytokines (IL-6 and IL-8).

**Effects of MS-275 and/or TRAIL on metastasis.** Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases (33). They are capable of digesting the different components of the extracellular matrix (ECM) and basement membrane. The ECM gives structural support to cells and plays a central role in cell adhesion, differentiation, proliferation and migration. We therefore sought to measure the expression of MMP-2 and MMP-9, and number of tumor nodules in the lungs (Fig. 5). Treatment of mice with MS-275 down-regulated the expression of MMP-2 and MMP-9 in tumor tissues compared to untreated control group as measured by immunohistochemistry and western blot analysis (Fig. 5A and C). TRAIL had no effect on the expression of MMP-2 and MMP-9. The combination of MS-275 plus TRAIL had slightly more effects on these proteins than MS-275 alone. The relative expression of MMP-2 and MMP-9 proteins correlated with percent of tumor cells expressing these proteins. Treatment of mice with MS-275 inhibited tumor metastasis in the lungs (as demonstrated by counting number of tumor nodules) than untreated control or TRAIL-treated mice (Fig. 5D). Interestingly, lung metastasis was not observed in MS-275 plus TRAIL treated mice. Our data demonstrate that MS-275 can inhibit tumor-cell metastasis by inhibiting MMP-2 and MMP-9 in breast cancer xenografts.
Interactive effects of MS-275 and/or TRAIL on reversal of Epithelial-mesenchymal transitions. EMT are vital for morphogenesis during embryonic development and are also implicated in the conversion of early stage tumors into invasive malignancies (19-20). Several key inducers of EMT are transcription factors (Twist, Snail, Slug, ZEB-1 and ZEB-2) that repress E-cadherin expression (19-20). During EMT, epithelial proteins (e.g. E-cadherin) are inhibited and mesenchymal proteins (e.g. N-cadherin) are induced. We therefore measured the expression of E-cadherin and N-cadherin in tumor tissues derived from MS-275 and/or TRAIL treated mice (Fig. 6). MS-275 induced the expression of E-cadherin and inhibited the expression of N-cadherin in tumor tissues, a situation for reversal of EMT. Treatment of mice with TRAIL had no effect on the expression of these cadherins. The combination of MS-275 plus TRAIL had effects similar to that of MS-275 on the expression of E-cadherin and N-cadherin. We next examined the effects of MS-275 and/or TRAIL on the expression of transcription factors ZEB1, Snail and Slug. Treatment of mice with MS-275 resulted in inhibition of ZEB1, Snail and Slug. By comparison, TRAIL had no effect on the expression of these transcription factors. The interactive effects of MS-275 plus TRAIL on the inhibition of ZEB1, Snail and Slug were either similar or slightly higher compared to that of MS-275 alone. These data suggest that MS-275 can reverse the EMT by changing the expression of E-cadherin, N-cadherin and the transcription factors ZEB1, Snail and Slug, thus inhibit metastasis.

Discussion

Our study sheds important new insight into the mechanisms of MS-275 and its potential for combining with TRAIL for the treatment of breast cancer. Treatment of nude mice with
MS-275 inhibited tumor growth, angiogenesis and metastasis. MS-275 also reversed epithelial-mesenchymal transition by inducing the expression of E-cadherin and inhibiting the expression of mesenchymal protein N-cadherin and transcription factors ZIB-1, Snail, and Slug compared to control group. Treatment of nude mice with MS-275 resulted in down-regulation of NFκB and its gene products (cyclin D1, Bcl-2, Bcl-XL, VEGF, HIF-1α, IL-6, IL-8, MMP-2 and MMP-9), and up-regulation of DR4, DR5, Bax, Bak, and p21/CIP1 in tumor cells. Furthermore, control mice demonstrating increased rate of tumor growth had increased numbers of CD31-positive or von Willebrand Factor (vWF)-positive blood vessels, increased circulating vascular VEGFR2-positive endothelial cells and elevated lung metastasis compared to MS-275 or MS-275 plus TRAIL treated mice. Sequential treatments of nude mice with MS-275 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, irradiation and histone deacetylase inhibitor SAHA upregulate DR4 and/or DR5 expression (6, 10-11, 34), our data also demonstrate that MS-275 can up-regulate these death receptors in TRAIL-resistant breast cancer xenografts. Although we have recently demonstrated the additive or synergistic effects of HDAC inhibitors and TRAIL on apoptosis in several cancers (5-6, 26), this is the first study showing that the MS-275 can reverse EMT transition in breast tumor tissues and thus may be responsible for reduced metastasis. Here, we demonstrate that MS-275 can sensitize TRAIL-resistant breast cancer xenografts in nude mice through induction of growth arrest and apoptosis, and inhibition of tumor-cell proliferation, angiogenesis and metastasis.

Activation of death receptor pathway is a new strategy for targeted therapy of cancer. We and others have shown that HDAC inhibitors can improve the efficacy of chemotherapeutics,
ionizing radiation, bortezomib and cytotoxic cytokine (5, 26, 35). TRAIL is a particularly promising candidate for cancer therapy as it provokes cell death in tumor cells while sparing most normal cells. In cancer patients, phase 1 and 2 clinical trials using agonistic mAbs that engage the human TRAIL receptors DR4 and DR5 have demonstrated limited / no toxicity (36). Nevertheless, some cancer cells refractory to TRAIL, suggesting that treatment with TRAIL alone may be insufficient for cancer therapy. In the present study, administration of MS-275 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.

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 (5). In the present study, MS-275 selectively induces proapoptotic members such as Bax and Bak and inhibits antiapoptotic Bcl-2 and Bcl-X$_L$ expression in tumor tissues. 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 (37). The inactivation of both Bax and Bak during tumor growth and development in vivo has been demonstrated, and SAHA has been shown to induce the expression of Bax, Bak, PUMA and Noxa in tumor tissues (6). 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 upon the development of a neovasculature in and around the tumor (39). Angiogenesis is regulated by the balance between stimulatory (e.g. bFGF, IL-6, IL-8, MMP-2, MMP-9, TGFβ1, VEGF) and inhibitory (e.g. angiostatin, IL-10, interferon) factors released by the tumor and its environment (39). HDAC inhibitors have been
shown to inhibit endothelial cell migration, invasion, vascular sprouting in vitro, and vasculature formation in animal models of cancer (1). Our data demonstrate that MS-275 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.

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 (40). Hypoxia can increase the resistance of cancer cells to drug-induced apoptosis by activation of PI3K/Akt, MEK/ERK, and NFκB signaling pathways (41). Expression of HDACs is often up-regulated under angiogenic stimuli such as hypoxia in cancer cells. HDAC inhibitors downregulate hypoxia-response genes and hypoxia-induced angiogenesis by the suppression of HIF-1α activity (42). Similarly, SAHA inhibited the expression of HIF-1α in breast cancer xenograft. In the present study, MS-275 inhibited the expression of HIF-1α and angiogenesis, and its effects were further enhanced in the presence of TRAIL. Increased level of HIF-1α expression has been positively correlated with angiogenesis (1). Our studies have demonstrated that HDAC inhibitors can inhibit angiogenesis.

Matrix metalloproteinases (MMPs) are a superfamily of Zn(2+)-dependent proteases that are capable of cleaving the proteinaceous component of the extracellular matrix (ECM) (43). ECM remodeling and/or degradation by MMPs are expected to affect cell fate and behavior during many developmental and pathological processes. Recent studies have shown that the expression of MMP mRNAs and proteins associates tightly with diverse developmental and pathological processes, such as tumor metastasis and mammary gland involution. In the present study, MS-275 down-regulated the expression of MMP-2 and MMP-9 in tumor tissues, and also inhibited the lung metastasis. We have recently demonstrated that SAHA inhibited the
expression of MMP-2 and MMP-9 and upregulated the expression of TIMP-1 and TIMP-2 in MDA-MB-468 breast cancer xenograft (6), suggesting a role of HDAC inhibitor in inhibition of tumor metastasis.

Malignant transformation is characterized by a phenotype "switch" from E- to N-cadherin, which is associated with increased motility and invasiveness of the tumor and altered signaling, leading to decreased apoptosis. At the molecular level, EMT is characterized by loss of E-cadherin and increased expression of several transcriptional repressors of E-cadherin expression (ZEB-1, ZEB-2, Twist, Snail, and Slug) (19-20). E-cadherin plays an essential role in EMT, tumor progression, and metastasis (19-20). In human tumors, loss or reduction of E-cadherin expression can be caused by somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the CDH1 promoter (44). Such silencing can occur either by DNA hypermethylation or through the action of transcription factors such as Slug, Snail, and Twist (45). Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. Furthermore, E-cadherin is a part of a complex that regulates β-catenin signaling and the activity of Rho GTPase (46), further impinging on cell migration and invasion. Aberrant expression of N-cadherin in breast cancer cells can contribute to invasiveness and metastasis by making the cells more motile (47). We show that the treatment of mice with MS-275 resulted in the induction of E-cadherin and inhibition of N-cadherin compared to that of control or TRAIL treated mice. This phenomenon is commonly observed in reversal of EMT.

The ectopic expression of Snail, Slug, ZEB1 and ZEB2 in epithelial cells induces dramatic phenotypic changes accompanied by increased cellular motility and invasiveness (19). In addition, these transcription factors have been reported to correlate negatively in transformed cell lines with epithelial marker expression levels such as E-cadherin, desmoplakin, cytokeratin...
18, and MUC-1 (48). These transcription factors activate EMT by binding to E-box elements present in the E-cadherin promoter, suppressing synthesis of this cell-cell adhesion protein. ZEB1 also promotes EMT by repressing expression of basement membrane components and cell polarity proteins. In the present study, MS-275 inhibited the expression of Snail, Slug and ZEB1 transcription factors. The inhibition of these transcription factors correlated with the induction of E-cadherin and inhibition of N-cadherin, suggesting a reversal of EMT.

We have shown that NFκB is constitutively active in breast cancer, and down-regulation of this transcription factor enhances therapeutic response of anticancer drugs and TRAIL (5-6, 13). NFκB regulates the expression of genes involved in cancer cell invasion, metastasis, and resistance to chemotherapy (32, 49). NFκB promotes cell growth and proliferation by regulating expression of genes such as c-myc, cyclin D1, and IL-6 and inhibits apoptosis through expression of anti-apoptotic genes, such as Bcl-2 and Bcl-XL (32). NFκB-mediated expression of genes, involved in angiogenesis, invasion and metastasis, may further contribute to its role on tumor progression. Constitutive NFκB activity has also been demonstrated in primary breast cancer tissue samples and suggested to have prognostic importance for a subset of primary tumors. In the present study, MS-275 inhibited the activation of NFκB and its gene products such as VEGF, IL-6, HIF-1α, Bcl-2, Bcl-XL, MMP-2, MMP-7, MMP-9 and IL-6 in tumor tissues. These findings suggest that NFκB may play a role in human breast cancer progression, metastasis and angiogenesis, and MS-275 can inhibit these processes through regulation of NFκB-regulated gene products.

In summary, we have demonstrated that histone deacetylase inhibitor MS-275 inhibits breast cancer tumor growth, angiogenesis and metastasis and also reverses EMT. The ability of MS-275 to sensitize TRAIL resistant breast cancer xenografts suggest a beneficial role of histone
modification in enhancing the therapeutic potential of TRAIL. Our data clearly demonstrated that MS-275 can sensitize TRAIL-resistant breast cancer xenografts through the involvement of both cell-extrinsic and cell-intrinsic pathways of apoptosis. Furthermore, the ability of MS-275 to inhibit NFκB and its gene products, and induce the expression of DR4, DR5 and p21/CIP1 will further enhance its antitumor activity of TRAIL through regulation apoptosis, angiogenesis and metastasis. In addition, MS-275 drives invasive breast cancer cells to undergo the reversal of EMT (by inducing epithelial cell markers, inhibiting mesenchymal cell marker and regulating the expression of transcription factors Snail, Slug and ZEB-1), leading to the suppression of cancer metastasis. Thus, our data suggest that MS-275 can be combined with TRAIL to treat invasive breast cancer.

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References


Figure Legends

Fig. 1. Interactive effects of MS-275 and TRAIL on tumor growth, tumor-cell proliferation and apoptosis in nude mice. (A), Structure of MS-275, and interactive effects of MS-275 and TRAIL on tumor regression in nude mice. Top panel, the structure of MS-275. Bottom panel, effects of MS-275 and TRAIL on tumor regression. Tumor-bearing nude mice were injected with vehicle, MS-275, TRAIL, or MS-275 followed by TRAIL. Drugs were administered i.v. four times during first three weeks. Tumor volume was measured weekly. Data represent mean ± SEM. *, or @ = significantly different from respective control, P < 0.05. (B), HDAC activity in tumor tissues. HDAC activity in nuclear extract of tumor tissues was measured as per manufacturer’s instructions. Data represent mean ± SEM. * = significantly different from respective control, P < 0.05. (C), Interactive effects of MS-275 and TRAIL on tumor cell proliferation. Tumor samples derived from xenografted nude mice were subjected to immunohistochemistry with anti-Ki67 or anti-PCNA antibody. Ki67 and PCNA positive tumor cells were quantified. Data represent mean ± SEM. *, or @ = significantly different from respective control, P < 0.05. (D), Interactive effects of MS-275 and TRAIL on caspase-3 activity, and apoptosis. (Upper panel), Immunohistochemical analyses of active caspase-3 (green = activate caspase-3; red = nucleus). (Middle and lower panels), Quantification of caspase-3 positive, and TUNEL positive cells. Data represent mean ± SEM. *, or # = significantly different from respective control, P < 0.05.

Fig. 2. Expression of death receptors, p21/CIP1 and cyclin D1 in tumor tissues. (A) Expression of death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 in tumor tissues. DR4 and DR5 expression in tumor tissues derived from xenografted nude mice was measured by ELISA. *, #, or & = significantly different from respective control, P < 0.05. (B), Left panel: expression of
DR4, DR5, P21\textsuperscript{CIP1} and cyclin D1 in tumor tissues by immunohistochemistry. Tumor tissues were subjected to immunohistochemistry with anti-DR4, anti-DR5, anti-p21\textsuperscript{CIP1} and anti-cyclin D1 antibodies, and photographed under a microscope (20X magnification). Right panel: percent of DR4, DR5, p21 and cyclin D1 positive tumor cells. Data represent mean (n = 10) ± SEM. *, @, &, or % = significantly different from respective control, P < 0.05. (C), Protein expression of DR4, DR5, P21\textsuperscript{CIP1} and cyclin D1 in tumor tissues. Tumor-cell lysates were prepared and subjected to the Western blot analysis for the measurement of DR4, DR5, P21\textsuperscript{CIP1} and cyclin D1. β-actin was used as a loading control. Band intensities were quantified and are shown below each blot. (D), Top panel, IKK kinase assay. After immunoprecipitation of the IKK complex with an anti-IKK\textgamma antibody, an \textit{in vitro} IKK kinase assay was carried out by incubation of the immunoprecipitated proteins with a purified GST-IκB\textalpha1-55 fusion protein as substrate. A Western blot analysis was then performed using an antibody specific for Ser32-36 phosphorylated IκB\textalpha and IKK\textgamma. Band intensities were quantified and are shown below each blot. Bottom Panel, expression of p65-NFκB in tumor tissues by immunohistochemistry.

\textbf{Fig. 3.} Expression of Bcl-2 family members in tumor tissues derived from xenografted nude mice. (A), Expression of Bcl-2, Bcl-X\textsubscript{L}, Bax, and Bak in tumor tissues. Tumor tissues were subjected to immunohistochemistry with anti-Bcl-2, anti-Bcl-X\textsubscript{L}, anti-Bax, and anti-Bak antibodies, and photographed under a microscope. (B), Percent of Bcl-2 and Bcl-X\textsubscript{L} positive tumor cells. Data represent mean (n = 10) ± SEM. *, or # = significantly different from respective control, P < 0.05. (D), Percent of Bax, and Bak positive tumor cells. Data represent mean (n = 10) ± SEM. *, or # = significantly different from respective control, P < 0.05. (D), Expression of Bcl-2, Bcl-X\textsubscript{L}, Bax, and Bak in tumor tissues. Tumor-cell lysates were prepared and subjected to the Western blot analysis for the measurement of Bcl-2, Bcl-X\textsubscript{L}, Bax, and Bak.
β-actin was used as a loading control. Band intensities were quantified and are shown below each blot.

**Fig. 4.** Effects of MS-275 and/or TRAIL on angiogenesis. (A), Expression of VEGF, HIF-1α, IL-6 and IL-8. Upper Panel, Tumor tissues were subjected to immunohistochemistry with anti-VEGF, anti-HIF-1α, anti-IL-6 and anti-IL-8 antibodies, and photographed (magnification 20X). Lower panel: percent of VEGF, HIF-1α, IL-6 and IL-8 positive tumor cells. Data represent mean (n = 10) ± SEM. *, or # = significantly different from respective control, P < 0.05. (B), 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. Band intensities were quantified and are shown below each blot. (C), VEGF receptor 2 (VEGFR2)-positive circulating endothelial cells. The blood cells from peripheral blood attached to the slide were stained with anti-VEGFR2 antibody, and the number of positive cells was counted. (D), Tumor tissue sections were stained with H & E, anti-CD31 antibody and anti-von Willebrand Factor (vWF) antibody and the number of blood vessels were counted. Each column represents the mean ± SEM. *, or # = significantly different from respective control, P < 0.05.

**Fig. 5.** Effects of MS-275 and/or TRAIL on metastasis. (A), Expression of MMP-2 and MMP-9 in tumor tissues by immunohistochemistry. Tumor tissues were subjected to immunohistochemistry with anti-MMP-2, anti-MMP-9 antibodies, and photographed (magnification 20X). (B), Quantification of MMP-2 and MMP-9 positive tumor cells. Data represent mean (n = 10) ± SEM. #, or % = significantly different from respective control, P < 0.05. (C), Protein expression of MMP-2 and MMP-9 in tumor tissues. Tumor-cell lysates were prepared and subjected to the Western blot analysis for the measurement of MMP-2, and MMP-
9. β-actin was used as a loading control. Band intensities were quantified and are shown below each blot. (D), Number of tumor nodules in lungs. Number of tumor nodules in the lungs were counted. Data represent mean ± SEM. #, or % = significantly different from respective control, P < 0.05.

**Fig. 6.** Effects of MS-275 and/or TRAIL on epithelial-mesenchymal transition. (A), Expression of E-cadherin, N-cadherin, ZEB-1, Snail, and Slug in tumor tissues by immunohistochemistry. Tumor tissues were subjected to immunohistochemistry with anti-E-cadherin, anti-N-cadherin, anti-ZEB-1, anti-Snail, and anti-Slug antibodies, and photographed (magnification 20X). (B), Protein expression of E-Cadherin, N-cadherin, ZEB-1, Snail, and Slug in tumor tissues. Tumor-cell lysates were prepared and subjected to the Western blot analysis for the measurement of E-cadherin, N-cadherin, ZEB-1, Snail, and Slug. Band intensities were quantified and are shown below each blot. β-actin was used as a loading control.
FIG. 5

A B C D

Control MS-275 TRAIL MS-275 + TRAIL

% Positive Cells

MMP-2 MMP-9

Control MS-275 TRAIL MS-275 + TRAIL

No of tumor nodules

Control MS-275 TRAIL MS-275 + TRAIL

10 kDa MMP-2

100 kDa MMP-9

40 kDa β-Actin
Molecular Cancer Therapeutics

MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo

Rakesh K Srivastava, Razelle Kurzrock and Sharmila Shankar

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