Colorectal cancer (CRC) displays noticeable resistance to chemotherapeutic drugs or innovative tumor cell apoptosis-inducing agents such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Thus, sensitizers are needed to enhance the effects of TRAIL-based cancer therapies. Elevated tumor cell death has been reported when various HDAC inhibitors are administered with TRAIL in various human cancers; however, SB939–TRAIL combined treatment has not been reported. In this study, we determined the ability of SB939 and TRAIL, as single agents or in combination, to inhibit the growth and survival of colorectal cancer cells. Our results demonstrated the effects of SB939 and TRAIL on cell viability, apoptosis, and morphological changes in HT-29 cells. SB939 treatment induces hyper-acetylation of histones and death receptors (DR) by activating MAPK proteins in a dose- and time-dependent manner. The ability of SB939 to sensitize HT-29 cells suggests that SB939 can induce essential changes in cell signaling pathways. Thus, the pan-HDAC inhibitor SB939 sensitizes TRAIL-induced apoptosis via up-regulation of DR5, and SB939–TRAIL combined treatment may target the MAPK pathways and serve as an effective therapeutic strategy against CRC. 

#These authors contributed equally to this work

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**Abstraction**

Colorectal cancer (CRC) displays noticeable resistance to chemotherapeutic drugs or innovative tumor cell apoptosis-inducing agents such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Thus, sensitizers are needed to enhance the effects of TRAIL-based cancer therapies. Elevated tumor cell death has been reported when various HDAC inhibitors are administered with TRAIL in various human cancers; however, SB939–TRAIL combined treatment has not been reported. In this study, we determined the ability of SB939 and TRAIL, as single agents or in combination, to inhibit the growth and survival of colorectal cancer cells. Our results demonstrated the effects of SB939 and TRAIL on cell viability, apoptosis, and morphological changes in HT-29 cells. SB939 treatment induces hyper-acetylation of histones and death receptors (DR) by activating MAPK proteins in a dose- and time-dependent manner. The ability of SB939 to sensitize HT-29 cells suggests that SB939 can induce essential changes in cell signaling pathways. Thus, the pan-HDAC inhibitor SB939 sensitizes TRAIL-induced apoptosis via up-regulation of DR5, and SB939–TRAIL combined treatment may target the MAPK pathways and serve as an effective therapeutic strategy against CRC. 

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**Introduction**

Colorectal cancer is the third most common cause of cancer mortality worldwide, with more than 1.9 million new cases and 935,000 deaths in 2020 (1). The development of CRC is caused by several risk factors, including age, environment, genetic factors, and epigenetic modifications. Epigenetic modifications enhance or promote the expression of tumor suppressor genes and are involved in CRC progression and metastasis(2, 3). Therefore, epigenetic modifications may represent a valuable therapeutic strategy against cancer.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a variety of tumor cells through the stimulation of death receptors but has an insignificant effect on normal cells. The death receptors DR4 and DR5 combine with TRAIL to induce apoptosis, and then recruit pro-caspase 8, which forms a death-inducing signaling complex (DISC). DISC cleaves caspase 8 and activation of extrinsic or intrinsic pathways (4, 5). Therefore, TRAIL is an attractive therapeutic agent for cancer. However, some tumor types are resistant to TRAIL through the expression of anti-aptotic proteins, such as FADD-like interleukin-1beta-converting enzyme (FLICE)-inhibitory protein (FLIP), X-linked inhibitor of apoptosis protein (XIAP), and Bel-2 family proteins (6, 7).

Histone modification is associated with the regulation of gene transcription and expression. Histone acetyltransferases (HATs) increase transcriptional activity by catalyzing the conversion of lysine residues of ε –NH2 groups to acetyl groups, whereas histone deacetylases (HDACs) decrease the expression of tumor suppressor genes that bind by deacetylating histone 3 at lysine 9 and 14 in target promoters. HDACs are classified into four classes according to sequence homology to the original yeast enzyme and domain organization. Class I (HDAC 1, 2, 3, and 8), class II (HDAC 4, 5, 6, 7, 9, and 10), and class IV (HDAC 11) are subdivided based on sequence similarity to yeast deacetylases, which are zinc-dependent amidohydrolases. Class III is the sirtuin protein, which requires NAD as a cofactor for its catalytic function (8, 9). Abnormal expression of class I HDAC is associated with CRC (10). In numerous cancers, HDACs are promising targets for therapeutic intervention, as they are involved in the regulation of proliferation, migration, differentiation, angiogenesis, and apoptosis (11). In CRC, it has been shown to contribute to the control of proliferation, apoptosis, and metastasis.

HDAC inhibitors are chemical compounds that have attracted interest as novel targeted therapies for various malignant tumors. HDAC inhibitor treatment compensates for histone methylation changes and changes in histone modulator expression. Furthermore, inhibition of HDAC using HDAC inhibitors causes changes in chromatin structure and alters gene expression at various levels, including miRNA expression and transcription factor activity (12). The most commonly reported effect of HDAC inhibitors on CRC cells is the induction of apoptosis (8, 13).

The hydroxamic acid-based HDAC inhibitor SB939 potently inhibited class I, II, and IV HDACs. In a previous study, it prevented the proliferation of CRC cell lines...
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(COLO 205 and HCT116) at values of 0.56 and 0.48 µM. SB939 had better pharmacokinetic, physicochemical, and pharmaceutical properties than SAHA, an HDAC inhibitor. In vivo evaluation of SB939 demonstrated better pharmacokinetic properties and high tumor concentrations of SB939 lead to superior antitumor efficacy (14). However, the mechanism of action of SB939 is not well understood. In this study, we investigated the anticancer efficacy of SB939 and TRAIL alone or in combination using cell viability, apoptosis detection assay, and cell shrinking. Furthermore, we analyzed protein levels to explore the molecular mechanisms of SB939 treatment in HT-29 cells. Our findings provide compelling evidence that SB939 binds to TRAIL to kill CRC cells.

Materials and Methods

Reagents
TRAIL (solvent, 0.1% bovine serum albumin [BSA] to a concentration of 100 ng/µL ) was acquired from Pro-Tech (Rocky Hill, NJ, USA). SB939 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The Annexin V FITC Apoptosis Detection Kit II Part A was acquired from BD Biosciences (San Jose, CA, USA).

Cell culture
Human colorectal cancer cells (RKO, SW620, and HT-29) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RKO cells were maintained in minimum essential medium Eagle (MEM), whereas SW620 and HT-29 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell viability assay
Cell viability assays were performed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. CRC cells were plated in 24-well plates and treated with TRAIL (5, 10, 30, 50, 100, or 200 ng/mL) and/or SB939 (0, 0.1, 0.5, 1, 10, 20, or 50 µM) for 24 h. Thiazolyl blue (5 mg/mL; Duchefa Biochemie, Haarlem, NH, Netherlands) was added to each well and the plate was incubated at 37 °C for 3 h. The culture medium containing the MTT solution was removed, and 300 µL of DMSO was added. Next, the crystals were shaken until they dissolved, and the cells were detected by measuring the absorbance at 590 nm using a microplate reader.

Flow cytometry analysis
For apoptosis analysis, HT-29 cells were plated at a density of 5 × 10⁶. Apoptotic cells were identified by flow cytometry using the BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

Hoechst 33258 staining
HT-29 cells (1 × 10⁵) were seeded in 24-well plates and treated with TRAIL (100 ng/mL) and/or SB939 (0.5 µM) for 24 h. The cells were washed with 1X PBS and the cell FIX solution was added for 10 min. The cells were washed with 1X PBS and stained with 300 µL Hoechst33258 (10 μg/mL) in the dark for 15 min at room temperature (15–25 °C).

Protein extraction and western blot analysis
HT-29 cells were harvested by resolving them in radioimmunoprecipitation assay (RIPA) buffer and were centrifuged at 14,000 RPM at 4 °C for 30 min. Cytosolic and nuclear fractions HT-29 cells were collected using Buffer A (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol [DTT], 0.5 mmol/L phenylmethylsulphonylfluoride [PMSF], 0.4% v/v Nonidet P-40 [NP-40] and protease inhibitor [PI] cocktail) and Buffer B (20 mmol/L HEPES [pH 7.9], 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, and PI cocktail).

Equal amounts of cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8–15%) and transferred to polyvinylidene fluoride membranes blocked with 3% BSA. Membranes were probed with various antibodies, comprising anti-FLIP (1:2,500, #8510, Cell Signaling Technology, Danvers, MA, USA), anti-caspase 8 (1:2,500, #9746, Cell Signaling Technology), anti-PARP-1 (1:1,000, sc-7150, Santa Cruz Biotechnology, Dallas, TX, USA), anti-Bid (1:1,000, sc-11423, Santa Cruz Biotechnology), anti-Mcl-1 (1:2,500, #4572, Cell Signaling Technology), anti-XIAP (1:2,500, #14334, Cell Signaling Technology), anti-Cytochrome C, anti-caspase 3 (1:1,000, sc-7148, Santa Cruz Biotechnology), anti-active caspase 3 (1:2,500, 559565, BD Pharmingen, San Jose, CA, USA), anti-DR4 (1:2,500, 1167, ProSci Inc, Poway, CA, USA), anti-DR5 (1:2,500, 2019, ProSci Inc), anti-act-histone H3 (1:1,000, sc-56616, Santa Cruz Biotechnology), anti-ac-histone H4 (1:1,000, sc-515319, Santa Cruz Biotechnology), anti-p-p38 (1:2,500, #14334, Cell Signaling Technology), anti-CHOP (1:2,500, #4511, Cell Signaling Technology), anti-caspase 8 (1:2,500, #8510, Cell Signaling Technology, Danvers, MA, USA), anti-caspase 9 (1:500, sc-8510, Cell Signaling Technology, Danvers, MA, USA), and a chemiluminescent imaging system (Fusion Solo system, Vilber Lourmat, Collegien, France).

Statistical analysis
Data are presented as mean ±SE of at least three independent experiments. All the data were entered into GraphPad Prism5.0 software was used to perform one-way ANOVA followed by Bonferroni’s post hoc test. Statistical significance was set at P < 0.05.

Results
TRAIL-SB939 combined treatment induced apoptosis in CRC cells
To assess the effects of TRAL and/or SB939 on the viability of the CRC cell lines (RKO, SW620, HT-29) and its variants, MTT assays were performed on cells treated with various concentrations of TRAIL or/and SB939 for 24 h. RKO cells demonstrated a dose-dependent decrease in cell viability upon TRAIL or SB939 treatment for 24 h, whe-
reas SW620 and HT-29 were unchanged (Supplementary Figure 1). Figure 1A shows that RKO cells responded to SB939 (0.5 µM) with TRAIL (10 ng/mL), showing approximately 20% growth inhibition. TRAIL-resistant cells (e.g., SW620 and HT-29) also showed decreased growth following TRAIL-SB939 combination treatment.

We detected apoptosis by measuring Annexin V-PI-positive HT-29 cells exposed to TRAIL alone, SB939 alone, or their combination. Apoptotic cell death was quantified by counting the number of cells on a graph. Treatment of HT-29 cells with SB939 or TRAIL alone induced 10.72% and 11.79% apoptosis, respectively. In agreement with cell viability, the combination treatment of SB939 and TRAIL dramatically increased 25.82% of Annexin V/PI-positive cells by 2-fold compared to treatment with TRAIL alone, indicating that cell viability decreased due to apoptosis caused by co-treatment SB939 with TRAIL (Figure 1B).

Hoechst 33258 staining was performed to detect cell shrinkage, nuclear condensation, and fragmentation, and we investigated nuclear fragmentation following the co-treatment of TRAIL and SB939 in HT-29 cells. As shown in Figure 1C, TRAIL-SB939 combined treatment induced morphological changes in HT-29 cell shrinkage, whereas TRAIL or SB939 treatment alone did not change. After co-treatment with TRAIL and SB939, cell apoptosis was significantly increased in CRC cells.

TRAIL-SB939 combined treatment accelerates apoptosis induction via extrinsic and intrinsic apoptotic pathway

Next examined whether TRAIL-SB939 combined treatment has the potential to modulate the expression of pro- and anti-apoptotic proteins in HT-29 cells. FLIP is a major apoptosis-regulatory protein that inhibits death receptor-mediated apoptosis by interrupting caspase8 activity. Western blot analysis showed that the level of FLIP was dramatically decreased in cells treated with SB939 and TRAIL compared to that in cells treated with either SB939 or TRAIL alone. As expected, the active form of caspase8 was much higher after SB939 plus TRAIL treatment. To identify the next extrinsic apoptosis pathway, the active form of caspase3 and PARP was increased, whereas the levels of full-length caspase3 and PARP were decreased in combination therapy. In addition, the combination of SB939 and TRAIL was dependent on the initiation of intrinsic apoptotic pathways, and molecules were examined by western blot analysis. The pro-forms of Bid, which link the extrinsic and intrinsic apoptotic pathways, were altered by combined treatment. The cytochrome C, and caspase 9 also, changed the amount of protein after co-treatment with SB939 and TRAIL. The expression of the anti-apoptotic protein myeloid cell leukemia-1 (Mcl-1) and X-linked inhibitor of apoptosis (XIAP) decreased after treatment with SB939 and TRAIL, compared to treatment with single agents alone (Figure 2). These results suggest that TRAIL-SB939 combined treatment sensitizes TRAIL-resistant CRC cells to TRAIL and induces mitochondria-mediated apoptosis.

SB939 treatment enhances DR5 expression by hyperacetylation of histone in CRC cells

To define the underlying mechanisms by which SB939 enhances TRAIL-induced apoptosis, we examined alterations in protein expression by treating HT-29 cells with SB939 in a time- or dose-dependent manner. First, we performed a western blot analysis for histone acetylation, acetyl-H3, and H4. Treatment with SB939 significantly
up-regulated the expression of hyper-acetyl-H4 for up to 30 min, and the expression of acetyl-H3 changed for up to 16 h. The expression of the DR5 protein was increased by SB939 treatment for up to 3 h, whereas the expression of DR4 did not change (Figure 3). Moreover, the levels of acetyl-histones H3 and H4 changed, and the level of DR5 increased in a dose-dependent manner. However, the levels of DR4 did not change after the SB939 treatment. These results imply that DR5 up-regulation was induced by histone hyper-acetylation.

SB939 treatment induces the MAPK/CHOP pathway involved in the up-regulation of DR5

To determine whether the MAPK pathway is involved in TRAIL-induced apoptosis in CRC cells, we assessed the expression of MAPK proteins using western blot analysis. As shown in Figure 4, in HT-29 cells, the levels of phospho-p38, phospho-ERK, and phospho-JNK increased in SB939 treatment in a time-dependent manner. Moreover, treatment with various concentrations of SB939 for 24 h up-regulated the expression of MAPK proteins in a
dose-dependent manner. CHOP and C/EBP homologous proteins are transcription factors that are elevated through the MAPK pathway induction and play an important role in increasing the protein level of DR5. The level of CHOP protein following SB939 induction increased markedly in the nucleus (Figure 4). The results showed that phosphorylation of MAPK proteins and CHOP was induced in a time- and dose-dependent manner.

Discussion

TRAIL-induced apoptosis of cancer cells is a promising therapy in oncology; however, toxicity and resistance to TRAIL are limiting factors. TRAIL resistance in various cancer cells is significantly affected by anti-apoptotic signaling molecules (15). Several reports have demonstrated that inhibition of HDAC1, HDAC2, and HDAC8 could enhance sensitivity to TRAIL in CRC cells with a TRAIL-resistant phenotype, resulting in an aberrant epigenetic status compared to adjacent normal cells (13, 16). The mechanism responsible for the potentiation effect of class I HDAC inhibition on TRAIL-induced apoptosis involves the up-regulation of DR5 and down-regulation of FLIP, IAP, and Bcl-2 (17-19). In this study, we demonstrated that SB939 and TRAIL combination therapy overcame TRAIL-resistant cell death in CRC cells.

The identification of drugs or agents that can overcome TRAIL resistance and render cancer cells sensitive to TRAIL-induced apoptosis is critical for enhancing the effectiveness of TRAIL-based cancer treatment. Several TRAIL-based combinations have been developed to date (20-22). This can be applied to the design of novel cancer-selective therapeutic drugs (23, 24). The present study demonstrated that sub-clinically achievable doses of SB939, 0.1 or 0.5 uM, significantly and synergistically increased TRAIL toxicity in vitro. Previous studies have shown that a combination of HDAC inhibitor and TRAIL significantly increased the number of apoptotic cells in various types of cancer. We showed that SB939-TRAIL treatment increased the number of annexin V- and PI-positive cells. Moreover, we observed that SB939-TRAIL combined treatment resulted in greater cell shrinkage than treatment with a single agent. These results indicate that the combined treatment with SB939 and TRAIL has a synergistic effect on CRC cells.

To date, the synergistic induction of apoptosis by HDAC inhibitor and TRAIL in CRC cells has been assessed as a potential therapeutic, because combined treatment with HDAC inhibitor and TRAIL sensitizes cancer cells to several mechanisms, such as cleaving caspase8 and activating pro-caspase or by cleavage of BID, which causes mitochondrial dysfunction and decreases anti-apoptotic proteins (25-27). In this study, we demonstrated that anti-apoptotic down-regulation, caspase cascade, and mitochondria-mediated apoptotic pathway were induced after SB939 and TRAIL combination for 24h. Previous studies have shown that FLIP is highly expressed in CRC cells and can inhibit apoptotic signals by preventing the recruitment of caspase8 at the death-inducing signaling complex (DISC) and suppressing the subsequent activation of the caspase cascade (28). In addition, X-linked inhibitors of apoptosis protein, XIAP, are important anti-apoptotic proteins for caspase suppression (29), and we studied the effects of TRAIL and HDAC inhibitor combined therapy on these proteins. We found that TRAIL-SB939 combination therapy decreased the protein levels of FLIP and XIAP, anti-apoptotic proteins that have been linked to TRAIL resistance in tumor cells.

TRAIL binds to DR4 and DR5 on the cell surface and promotes apoptosis (4). Inhibitors of HDAC classes induce death receptor-mediated apoptosis via histone acetylation (21, 26). Therefore, we evaluated the protein levels of histone acetylation and death receptor in SB939-treated HT29 cells. We found that SB939 dramatically increased the levels of DR5, but not DR4, in a time- and dose-dependent manner. We then increased histone acetylation by treating SB939 cells. Therefore, we speculated that one of the mechanisms of SB939-mediated TRAIL sensitization in CRC cells is the up-regulation of histone acetylation and DR5.

Several studies have reported the involvement of p53-dependent and -independent pathways, SP1, Foxo3, ROS, and the activation of MAPK signaling pathways in the sensitization of cancer cells to TRAIL-induced apopto-
sis (6, 30, 31). The expression of MAPK proteins induces TRAIL-sensitized apoptosis by HDAC inhibitors and the up-regulation of DR5, leading to apoptosis. To examine whether the MAPK pathways are responsible for SB939-induced up-regulation of DR5, we examined the effect of SB939 on the phosphorylation of MAPK family members in CRC cells. SB939 increased the phosphorylation of p38, ERK, and JNK. Therefore, we suggested that SB939 triggers MAPK activation to induce DR5 expression.

CHOP is up-regulated by the MAPK pathway and is involved in MAPK-mediated apoptosis (32). CHOP, which belongs to the C/EBP family, is a transcription factor (33). CHOP also enhances DR5 expression by binding to the DR5 promoter in the MAPK signaling pathway (34, 35). If CHOP expression is blocked, DR5 expression is down-regulated (36). Our results showed that CHOP was decreased when the level of MAPK proteins was suppressed in HT-29 cells by treatment with SB939. Therefore, we considered that the phospho-p38, ERK, and JNK-mediated CHOP-DR5 pathway is involved in SB939-induced apoptosis in HT-29 cells, although direct evidence was obtained by knocking out the key regulator.

In conclusion, our data showed that SB939 induces the death receptor 5 via the MAPK/CHOP pathway by histone acetylation, and SB939 combined with TRAIL treatment overcomes TRAIL resistance by activating the caspase cascade and inducing mitochondria-mediated apoptosis in CRC cells. The use of TRAIL-based combination therapy with pan-HDAC inhibitors SB939 may be a potentially effective therapeutic strategy for human colorectal cancer patients.

Authors’ Contributions
SWK and SLK designed the study. MWS evaluated all experiments and wrote the manuscript. SWK and SYS edited the draft and supervised all the experimental procedures. All the authors have read and approved the final manuscript.

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Conflicts of Interest
The Authors declare that there are no conflicts of interest concerning this article.

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