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The HDAC1 Inhibitor CBUD-1001 Enhances TRAIL-induced Apoptosis in Colorectal Cancer Cells

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Abstract. *Background/Aim: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential anti-tumor agent. However, resistance to TRAIL has been reported in a number of clinical trials. In this study, we investigated the molecular mechanisms by which a novel histone deacetylase (HDAC) inhibitor, CBUD-1001, sensitizes colorectal cancer (CRC) cells to TRAIL-induced apoptosis. Materials and Methods: Apoptotic cell death induced by CBUD-1001 and/or TRAIL was assessed on human CRC cells using the MTT assay, FACS analysis and nuclei staining. The involved molecular mechanisms were explored through western blotting analysis. Results: We demonstrated that combined with CBUD-1001, TRAIL significantly enhanced TRAIL-induced apoptosis in CRC cells via mitochondria-mediated pathways. We also found that hyper-acetylation of histone by CBUD-1001 treatment leads to up-regulation of death receptor (DR) 5 in a dose- and time-dependent manner. Furthermore, we identified that enhanced sensitivity to TRAIL by combination with CBUD-1001 depends on the MAPK/CHOP axis, being a key mediator of DR5. Conclusion: A novel HDAC inhibitor CBUD-1001 sensitizes TRAIL-induced apoptosis via up-regulation of DR5, and that CBUD-1001 and TRAIL combination treatment offers an effective strategy to overcome TRAIL resistance in CRC cells.*

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Colorectal cancer (CRC) is a common life-threatening and malignant tumor in humans (1, 2). CRC is a heterogeneous disease, the cause of which appears to be related to lifestyle patterns, genetic factors, and epigenetic modifications (3). Among the risk factors, epigenetic alterations of tumor suppressor genes are closely related to CRC progression and metastasis (4, 5). Given their dynamic and reversible characteristics, epigenetic changes are potential targets in CRC treatment.

Tumor necrosis factor-related (TNF) apoptosis-inducing ligand (TRAIL), which belongs to the TNF cytokine super-family, induces cancer therapy *via* death receptor (DR) 4 (TRAIL-R1) or DR5 (TRAIL-R2) in various cancer cells while showing only negligible effects on normal cells (6, 7). After binding to DRs, TRAIL induces cell death through extrinsic and intrinsic apoptosis pathways (8). As a result of selectivity to cancer cells, TRAIL is currently the subject of clinical trials as cancer therapy (9). However, several tumor types have demonstrated profound resistance to TRAIL treatment, primarily due to constitutive expression of intracellular TRAIL resistance–mediating factors, such as FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein (FLIP) and inhibitor of apoptosis protein family members, in cancer cells (10, 11). The effectiveness of TRAIL may, therefore, be limited by development of resistance, and an agent that enhances TRAIL-induced apoptosis and sensitizes cancer cells to TRAIL is urgently needed.

Histone deacetylases (HDACs) are epigenetic enzymes that play critical roles in the regulation of gene expression and transcription. HDACs catalyze deacetylation of histones and epigenetically regulate chromatin architecture and gene expression, particularly during oncogenesis. Inhibition of HDACs reverses an aberrant epigenetic status and displays potent antitumor activity by inducing cell-cycle arrest,

differentiation, and/or apoptosis in diverse cancer cells (12). Numerous reports indicate HDAC overexpression in many cancers, with HDAC1, HDAC2, and HDAC8 being overexpressed in colon cancer in particular, and inhibit specific tumor suppressor genes, resulting in an aberrant epigenetic status compared to adjacent normal cells (13). Several studies have found that HDAC inhibitors can increase the level of DRs in various cancer cells, enhancing the apoptosis-inducing potential of TRAIL. Moreover, combined treatment with HDAC inhibitors reduces the dose of each agent and strengthens the effect of TRAIL-based therapy (14-17).

In our previous study, we synthesized a novel fluorinated aminophenyl-benzamide–based HDAC1 inhibitor, CBUD-1001, and demonstrated that it triggers apoptotic cell death and epithelial-mesenchymal transition progression in CRC cells, indicating it as a potent therapeutic agent for CRC therapy (18). Therefore, a deeper understanding of the molecular mechanism involved in use of CBUD-1001 as a therapeutic agent for CRC is needed. A new perspective of CBUD-1001 as a target for TRAIL resistance may provide insights into the role of HDAC inhibitors in cancer therapy.

This study evaluated the effect of treatment with a combination of CBUD-1001 and TRAIL on human CRC cells and TRAIL-resistant CRC in particular. We also investigated the molecular mechanism responsible for enhanced sensitivity of TRAIL by HDAC inhibition.

Materials and Methods

Reagents. TRAIL was purchased from PeproTech (Rocky Hill, NJ, USA) and dissolved in 0.1% bovine serum albumin (BSA) to 100 ng/μl. Annexin V FITC Apoptosis Detection Kit II Part A was purchased from BD Biosciences. MEK inhibitor (U0126), p38 inhibitor (SB202190), and JNK inhibitor (SP600125) were purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture. The human colorectal cancer cell lines DLD-1 and HCT116 were purchased from the American Type Culture Collection (Manassas, VA, USA), and DLD-1 and HCT116 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cell viability assays were based on an MTT assay, with 1×10^4 cells seeded in 96-well plates and pre-treated with U0126, SB202190, and SP600125. TRAIL (5, 10, 30, 50, 100, and 200 ng/ml) and CBUD-1001 (0.1, 1, and 3 μM) were applied for 24 h. Next, 30 μl of thiazolyl blue (MTT solution, 5 mg/ml; Duchefa Biochemie) was added to each well and incubated at 37˚C for 3 h. Cultured medium containing MTT solution was removed, 500 μl of dimethyl sulfoxide was added, and the mixture was shaken until the crystals dissolved. The cells were detected by measuring the absorbance at 590 nm using a microplate reader.

Flow cytometry analysis. Apoptotic cell death was determined by staining the cells with Annexin V-FITC (Ex/Em, 488/519nm). 1×10^5 cells in a 60 mm culture dish were treated with CBUD-1001 (1 μM) and/or TRAIL (50 ng/ml). The cells were washed twice with cold phosphate-buffered saline (PBS) and then resuspended in 500 μl of binding buffer (10 μM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Annexin V-FITC (1 μl) and PI (1 μg/ml) were added, and the cells were analyzed with a BD Accuri C6 flow cytometer (BD Biosciences).

Hoechst 33258 staining. The cells (1×10^3) were seeded in 24-well plates and treated with CBUD-1001 (1 μM) and/or TRAIL (50 ng/ml) for 24 h. The cells were washed twice with 1× PBS, and 300 μl of cell FIX solution was added for 10 min. After washing twice with 1× PBS and addition of 300 μl of Hoechst 33258 stain, the cells were left in the dark for 15 min at room temperature. The Hoechst 33258 was removed, and the cells were washed with 1× PBS and observed using a fluorescence microscope (Lionheart FX, BioTek).

Protein extraction. DLD-1 cells were harvested by resolving them in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 1.0 mM EDTA, 0.1% SDS, 0.01% sodium azide at pH 7.4, and protease inhibitors) and were centrifuged at 18,000 × *g* at 4˚C for 20 min. Mitochondrial and cytosolic fractions from DLD-1 cells were collected using a mitochondria isolation kit for cultured cells (Thermo Fisher Scientific, Waltham, MA, USA). DLD-1 cells were centrifuged in a harvested cell suspension in a 2 ml microcentrifuge tube at 850 × g for 2 min. Pellet-added Reagent A and mitochondria were extracted in Reagent B and Reagent C in a mitochondrial buffer, followed by centrifugation at 700 × *g* for 10 min at 4˚C. The supernatant was further centrifuged at 12,000 × *g* for 15 min at 4˚C to pellet the mitochondria. The crude mitochondrial fraction was resuspended for washing and centrifuged at 12,000 × *g* for 15 min at 4 ˚C. The pellets were collected as the mitochondrial fraction.

Western blot analysis. The same 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-caspase 3 (1:1,000, sc-7148, Santa Cruz Biotechnology, Dallas, TX, USA), anti-PARP-1 (1:1,000, sc-7150, Santa Cruz Biotechnology), anti-DR5 (1:1,000, sc-166624, Santa Cruz Biotechnology), anti-caspase 8 (1:1,000, sc-73526, Santa Cruz Biotechnology), anti-BID (1:1,000, sc-11423, Santa Cruz Biotechnology), anti-Bax (1:1,000, sc-7480, Santa Cruz Biotechnology), cytochrome c (1:1,000, sc-13156, Santa Cruz Biotechnology), anti-Bcl-xL (1:1,000, sc-8392, Santa Cruz Biotechnology), anti-ac-histone H3 (1:1,000, sc-56616, Santa Cruz Biotechnology), anti-ac-histone H4 (1:1,000, sc-515319, Santa Cruz Biotechnology), anti-caspase9 (1:3,000, #9502, Cell Signaling Technology, Danvers, MA, USA), anti-FLIP (1:3,000, #8510, Cell Signaling Technology), anti-XIAP (1:3,000, #14334, Cell Signaling Technology), anti-p-p38 (1:3,000, #4511, Cell Signaling Technology,), anti-p-ERK (1:3,000, #9106, Cell Signaling Technology), anti-p-JNK(1:3,000, #4668, Cell Signaling Technology), anti-CHOP (1:3,000, #2895, Cell Signaling Technology), anti-DR4 (1:1,000, 1167, ProSci Inc, USA), and anti-actin (1:3,000, A2066, Sigma-Aldrich, St. Louis, MO, USA) at 4 ˚C for overnight. Membranes were probed with goat anti-mouse and goat anti-rabbit antibodies (Santa Cruz Biotechnology). Horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (sc-2005,

Santa Cruz Biotechnology), goat anti-mouse (sc-2005, Santa Cruz Biotechnology), and mouse anti-goat (sc-2354, Santa Cruz Biotechnology) secondary antibodies were used at concentrations of 1:3,000 for 1 h at room temperature. Protein expression was detected using the ECL prime (Millipore, Billerica, MA, USA) and chemiluminescent image system (Fusion Solo system, Villber Lourmat, Collégien, France).

Statistical analysis. All experiments were repeated three times. The data are presented as the mean±standard and analyzed using Graphpad Prism 5.0. Differences between multiple groups were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. p-Value<0.05 was considered statistically significant.

Results

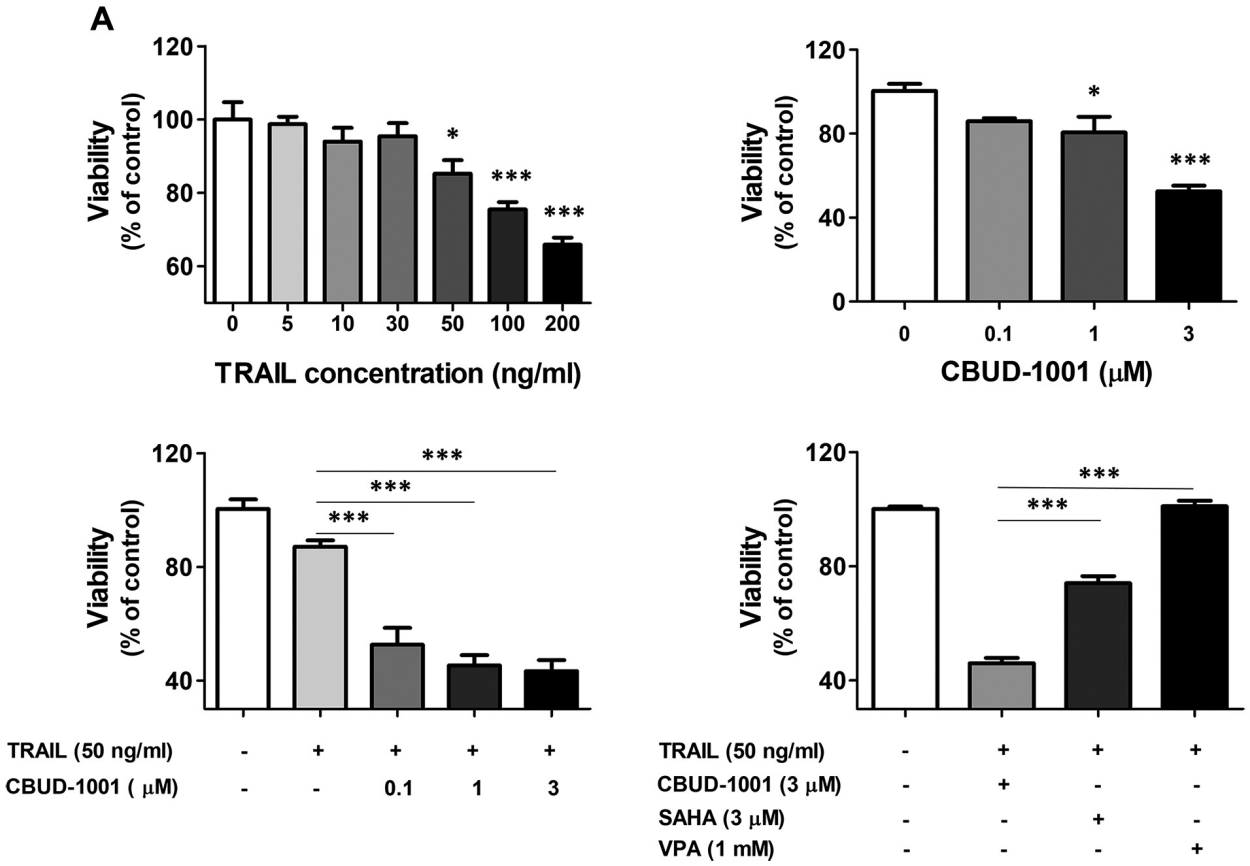
CBUD-1001 sensitizes human CRC cells to TRAIL-induced apoptosis. To investigate interactions between TRAIL and CBUD-1001 combination on cells regarding induction of apoptosis, we treated DLD-1 cells with CBUD-1001 or/and TRAIL at varying concentrations for 24 h. When TRAIL was dose-dependently applied, cell growth was inhibited at a TRAIL dose of 50 ng/ml (Figure 1A upper left). Figure 1A (upper right) shows that, CBUD-1001 decreased cell viability by 50% at a concentration of 3 μM in the DLD-1 cell line. DLD-1 cells responded to CBUD-1001 (1 μM) with TRAIL (50 ng/ml), showing approximately 50% growth inhibition (Figure 1A, lower left). In addition, co-treatment of CBUD-1001 and TRAIL produced a better effect compared to combining SAHA (3 μM) or VPA (1 mM) with TRAIL (Figure 1A, lower right).

We measured the rate of apoptotic cell flow through flow cytometric analysis with Annexin V-FITC/PI staining. As shown in Figure 1B, the cells treated with CBUD-1001 alone experienced greater apoptotic cell death when treated with a 50 ng/ml dose of TRAIL compared to cells that were given a combined treatment of CBUD-1001 and TRAIL. Apoptotic cell death was quantified by counting the cells in the lower right and upper right quadrants in the Figure. Treatment of DLD-1 cells with either CBUD-1001 or TRAIL alone induced 19.53% and 10.25% apoptosis in DLD-1 cells, respectively. In agreement with cell growth inhibition, combination treatment of CBUD-1001 and TRAIL dramatically increased Annexin V/PI–positive cells (38.54%) by 4-fold compared to treatment with TRAIL alone, indicating that cell viability decreased due to apoptosis caused by co-treatment of CBUD-1001 with TRAIL (Figure 1B).

To confirm apoptosis, a cell morphology assessment was conducted by treating DLD-1 with CBUD-1001 or/and TRAIL. When apoptosis occurs, changes in cell morphology, such as cell shrinkage, nuclear condensation, and fragmentation, can be observed through fluorescence microscopy after straining with Hoechst 33258. As shown in Figure 1C, the nuclei of the cells in the control were round and displayed blue fluorescence, whereas treated cells exhibited cell shrinkage, nuclear condensation, fragmentation, and strong blue-white fluorescence when co-treated with CBUD-1001 and TRAIL. Unlike intact, round, and large nuclei, apoptotic bodies of irregular shapes and sizes appeared in apoptotic cells (white arrow in the Figure). These data indicate that co-treatment by CBUD-1001 and TRAIL synergistically suppressed DLD-1 cell proliferation.

Combined treatment with CBUD-1001 and TRAIL accelerates apoptosis induction via a mitochondria-mediated apoptotic pathway. To evaluate the mechanisms responsible for apoptosis caused by a combination of CBUD-1001 and TRAIL, we examined the expression levels of several pro-apoptosis and anti-apoptosis proteins in DLD-1 cells. Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme (FLICE)-inhibitory proteins (FLIPs) are anti-apoptotic proteins that can inhibit death receptor-mediated apoptosis by binding to FADD or caspase 8. Western blot analysis showed that the level of FLIP was significantly decreased by treatment with CBDU-1001 (1 μM) plus TRAIL (50 ng/ml) compared with to levels in cells treated with either TRAIL or CBUD-1001 alone. In contrast, expression of the active form of caspase 8 was significantly increased by treatment with CBUD-1001 plus TRAIL compared to levels in DLD-1 cells treated with either TRAIL or CBUD-1001 alone. In addition, the specific substrates for caspase 8, BID, and induction of cytochrome c release and cleaved caspase 9 are important events in the signaling of apoptosis for cells that follow mitochondrial-dependent pathways. The pro-forms of BID, cytochrome c, and caspase 9 showed change in protein amount after co-treatment of CBUD-1001 and TRAIL. The expression of caspase inhibitor protein X-linked inhibitor-of-apoptosis (XIAP) was also reduced after treatment with CBDU-1001 and TRAIL compared to treatments with single agents alone. In addition, higher levels of cleaved caspase 3 and PARP following treatment with TRAIL were observed in DLD-1 cells treated with TRAIL or CBUD-1001 alone compared with the control (Figure 2). Collectively, these data indicate that co-treatment of CBUD-1001 and TRAIL enhances TRAIL-induced apoptosis through extrinsic and intrinsic apoptotic pathways.

CBUD-1001 potentiates TRAIL-induced apoptosis through DR5 up-regulation by hyper-acetylation of histone. To determine the mechanism through which CBUD-1001 sensitizes TRAIL-induced apoptosis in CRC cells, we tested whether CBUD-1001 affected protein levels of DRs and acetylated histone in time- and dose-dependent manners. As shown in Figure 3A, treatment with CBUD-1001 markedly increased the levels of acetyl-histone H3 and H4 proteins for up to 16 h in CRC cells. The level of DR5 protein was up-regulated by CBUD-1001 treatment for up to 24 h, while the level of DR4 was not changed. These observations suggest



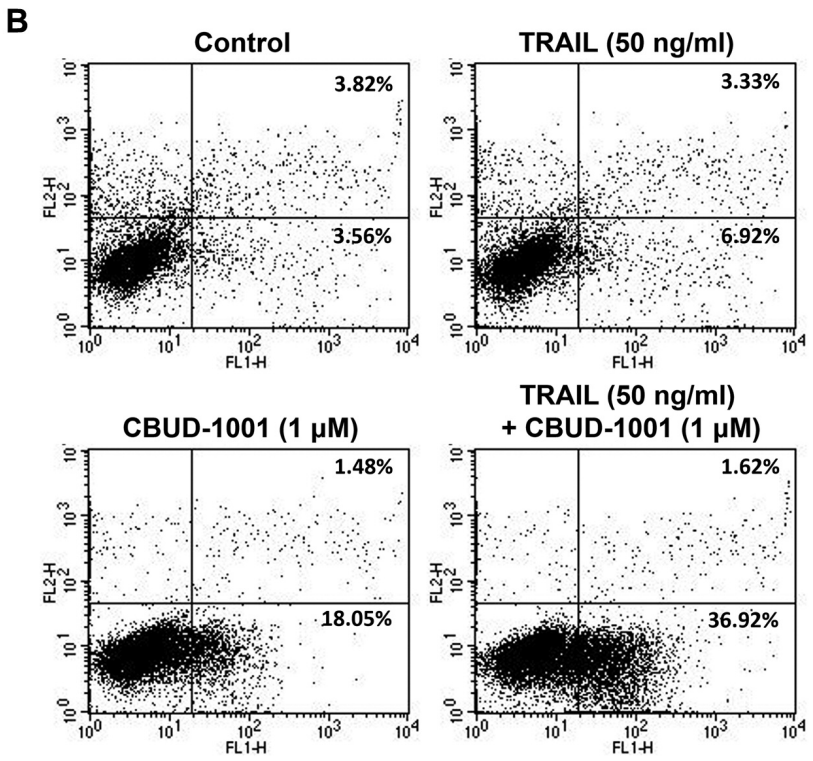


Figure 1. *Continued*

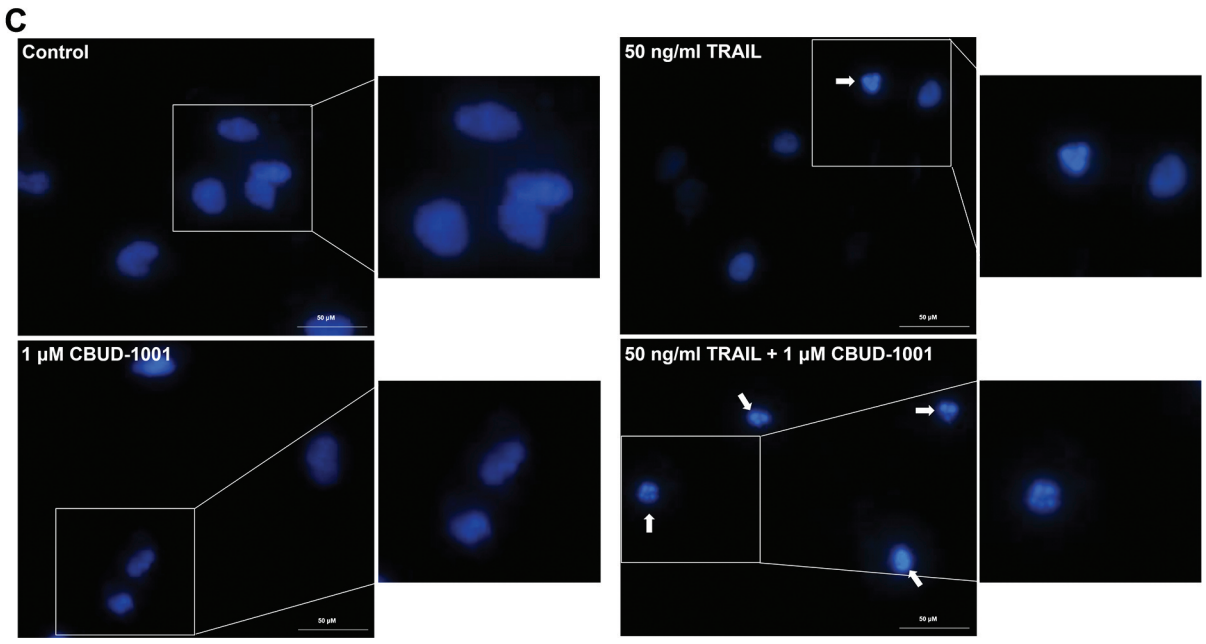


Figure 1. *CBUD-1001 and TRAIL combination treatment induces apoptosis in DLD-1 cells. DLD-1 cells were treated with TRAIL and CBUD-1001 at various concentrations for 24 h. (A) DLD-1 cells using TRAIL (50 ng/ml) and/or CBUD-1001 (0.1, 1, and 3 μM) treatment were compared with SAHA (3 μM)- or VPA (1 mM)-treated cells by an MTT assay. (B) DLD-1 cells treated with TRAIL (50 ng/ml) and/or CBUD-1001 (1 μM) were stained with Annexin V-FITC/PI and analyzed using flow cytometry. (C) DLD-1 cells were analyzed for apoptosis through Hoechst staining. $p<0.05$ and $p<0.001$.*

that DR5 expression is influenced by CBUD-1001 following histone acetylation.

After exposure to various concentrations of CBUD-1001 for 24 h, the levels of acetyl-histone H3 and H4 increased dramatically in a dose-dependent manner (Figure 3B). The level of DR5 also was up-regulated in a dose-dependent manner, and the level of DR4 did not show any alteration after CBUD-1001 treatment. These results imply that CBUD-1001 sensitizes CRC cells to TRAIL through up-regulation of DR5 following hyper-acetylation of histones.

CBUD-1001 effectively activates the MAPK/CHOP pathway involved in up-regulation of DR5. Activation of mitogen-activated protein kinase (MAPK) subfamilies may play an important role in TRAIL receptor induction (19, 20). We, therefore, examined whether CBUD-1001 could regulate MAPKs using western blot analysis with antibodies that recognize the phosphorylated forms of these kinases in CRC cells. As shown in the first and second panels of Figure 4A, the protein levels of phospho-p38, phospho-ERK, and phospho-JNK were up-regulated by CBUD-1001 treatment in a time-dependent manner. Treatment with various concentrations of CBUD-1001 for 24 h also induced up-regulation of phospho-p38, phospho-ERK, and phospho-JNK in a dose-dependent manner (Figure 4B, first and second panels). CHOP, a member of the C/EBP family of transcription factors, is a substrate for p38 MAPK and a key regulator of DR5 (21, 22), we conducted an experiment to determine whether the protein level of CHOP is regulated by CBUD-1001 treatment in CRC cells. Like the MAPK protein, expression of CHOP significantly increased in a time- and dose-dependent manner (Figure 4A and 4B, fourth panel).

To confirm that MAPK signaling is involved with CBUD-1001-induced TRAIL sensitivity, the cells were pretreated with U0126 (an MEK inhibitor), SB202190 (a p38 inhibitor), and SP600125 (a JNK inhibitor). Cell viability was evaluated using an MTT assay following incubation with CBUD-1001 for 24 h. As shown in Figure 4C, treatment with CBUD-1001 alone reduced cell viability in a dose-dependent manner, with the percentage of viable cells decreased by approximately 50% at the maximum concentration. However, following treatment with MAPK inhibitors U0126, SB202190, and SP600125 and CBUD-1001, the percentage of viable cells recovered significantly to 76.92±1.544%, 78.36±1.505%, and 79.31±2.554%, respectively. These results suggest that CBUD-1001 is responsible for TRAIL sensitivity through mitogen-activated protein kinases (MAPKs) and the CCAAT/enhancer-binding protein homologous protein (CHOP). Overall, it was demonstrated that CBUD-1001 treatment enhances TRAIL sensitivity by regulating the

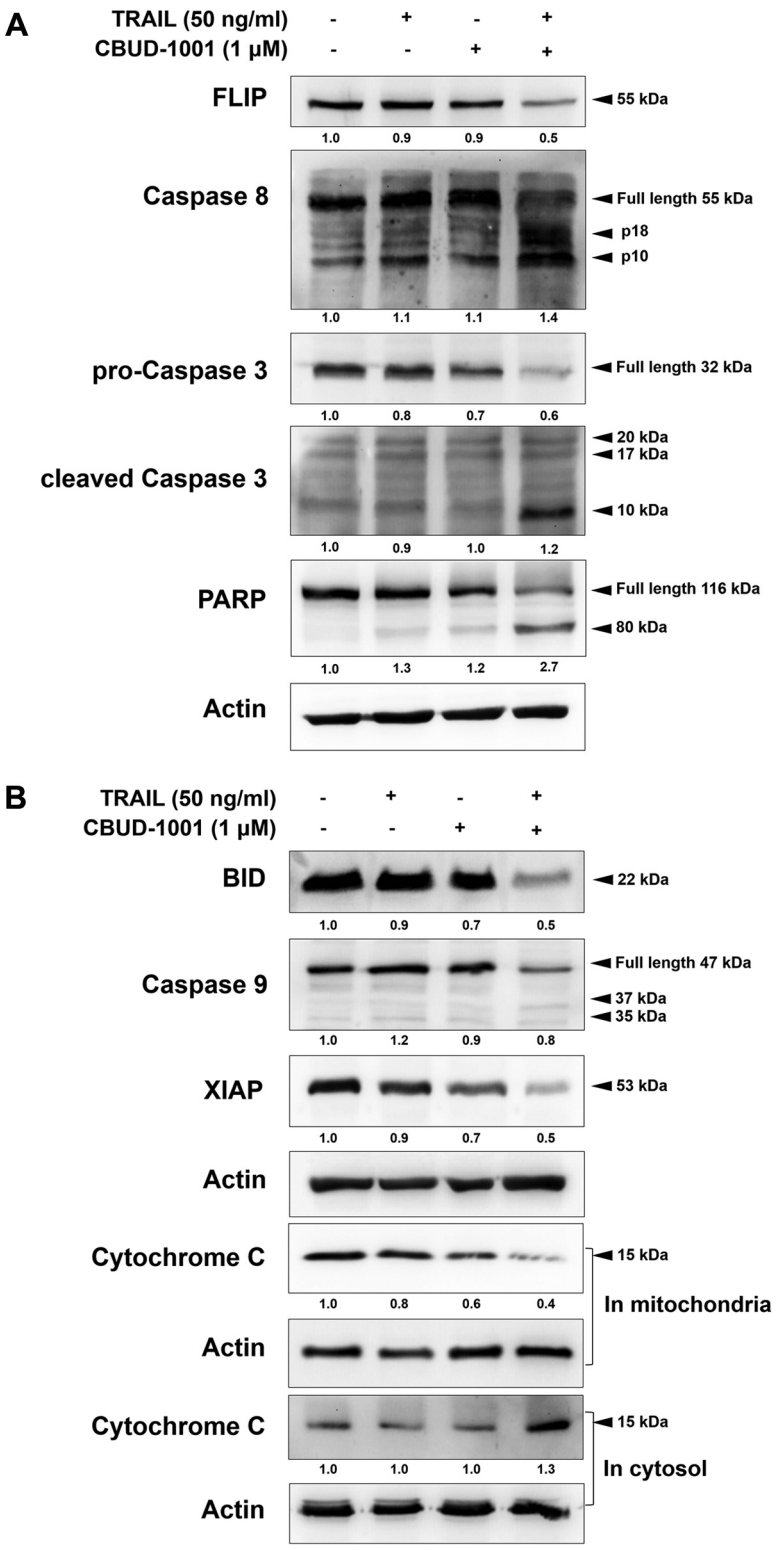


Figure 2. *CBUD-1001 and TRAIL combination treatment alters expression of apoptosis in DLD-1 cells. DLD-1 cells were treated with TRAIL (50 ng/ml) and/or CBUD-1001 (1 μM) for 24 h. (A) The cells and total cell lysates were prepared for western blot analysis of pro-apoptotic proteins (caspase 8, 9, and 3, and PARP). (B) Total cell lysates were prepared for western blot analysis of anti-apoptotic proteins (FLIP and XIAP) and pro-apoptotic protein BID. Mitochondria and cytosol lysates were prepared for western blot analysis of cytochrome c.*

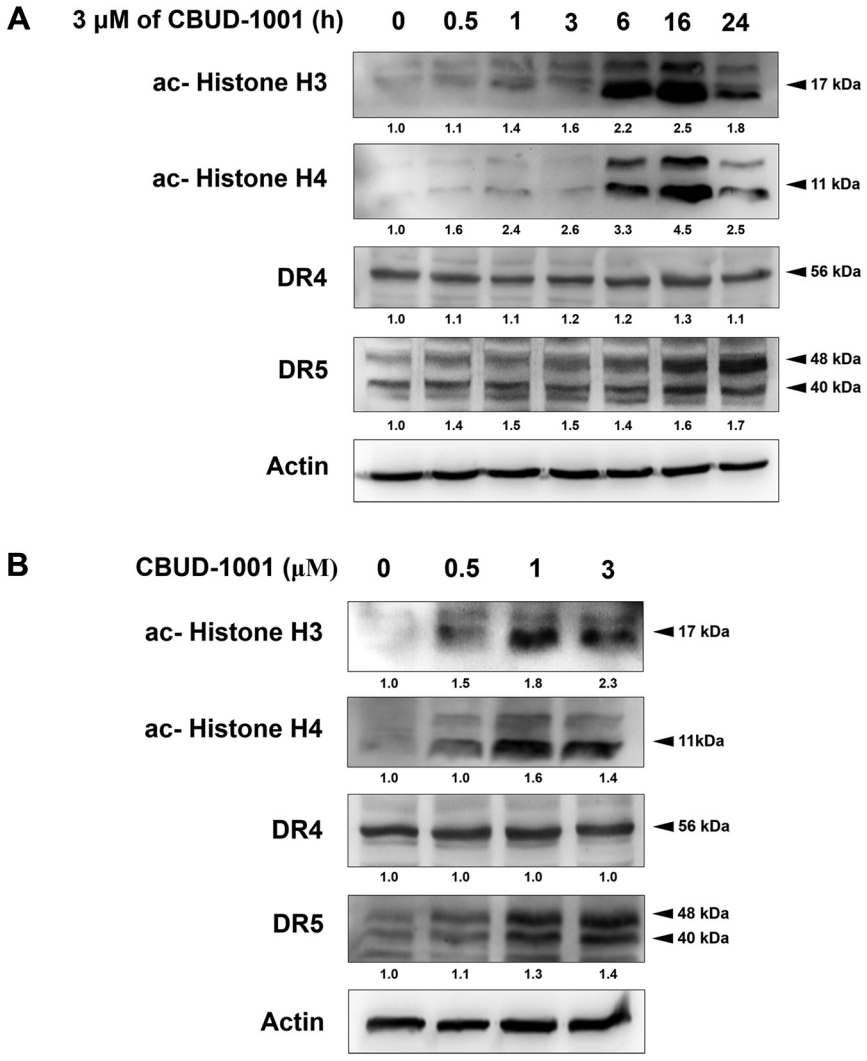


Figure 3. *CBUD-1001 levels affect acetyl-histone H3 and H4 proteins acetylation, in a time- and dose-dependent manner in DLD-1 cells. (A, B) Cells were incubated with CBUD-1001 at the indicated times and concentrations for 24 h. Total cell lysates were subjected to western blotting and then investigated for histone acetylation and death receptors 4 and 5 (DR4, DR5).*

MAPK-CHOP-DR5 pathway. The proposed molecular mechanisms by which CBUD-1001 treatment induced TRAIL resistance are illustrated in Figure 5.

Discussion

Resistance to current treatment strategies using programmed cell death presents a major obstacle in oncology (23). Epigenetic alterations trigger defective apoptotic signaling, which promotes programmed cell death (13). TRAIL offers potential benefits in cancer therapy because of its selective toxicity in cancer cells (9). In this study, the effect of combination therapy of CBUD-1001 (a novel HDAC inhibitor that induces epigenetic alteration) and TRAIL on CRC cells was evaluated.

We first evaluated the effects of treatment with TRAIL alone, CBUD-1001 alone, and TRAIL plus CBUD-1001 on cell viability in DLD-1 and HCT116 cell lines. DLD-1 cells were resistant to TRAIL-induced apoptosis over a broad range of doses compared to HCT116 cells (24, 25). In our previous study, we measured the HDAC-inhibitor effect of CBUD-1001 using HCT116 cells (18). In this study, the synergistic effect of CBUD-1001 and TRAIL was investigated using TRAIL-resistant DLD-1 cells. Our results show that TRAIL's effects decreased in a dose-dependent manner, and CBUD-1001 showed a half-

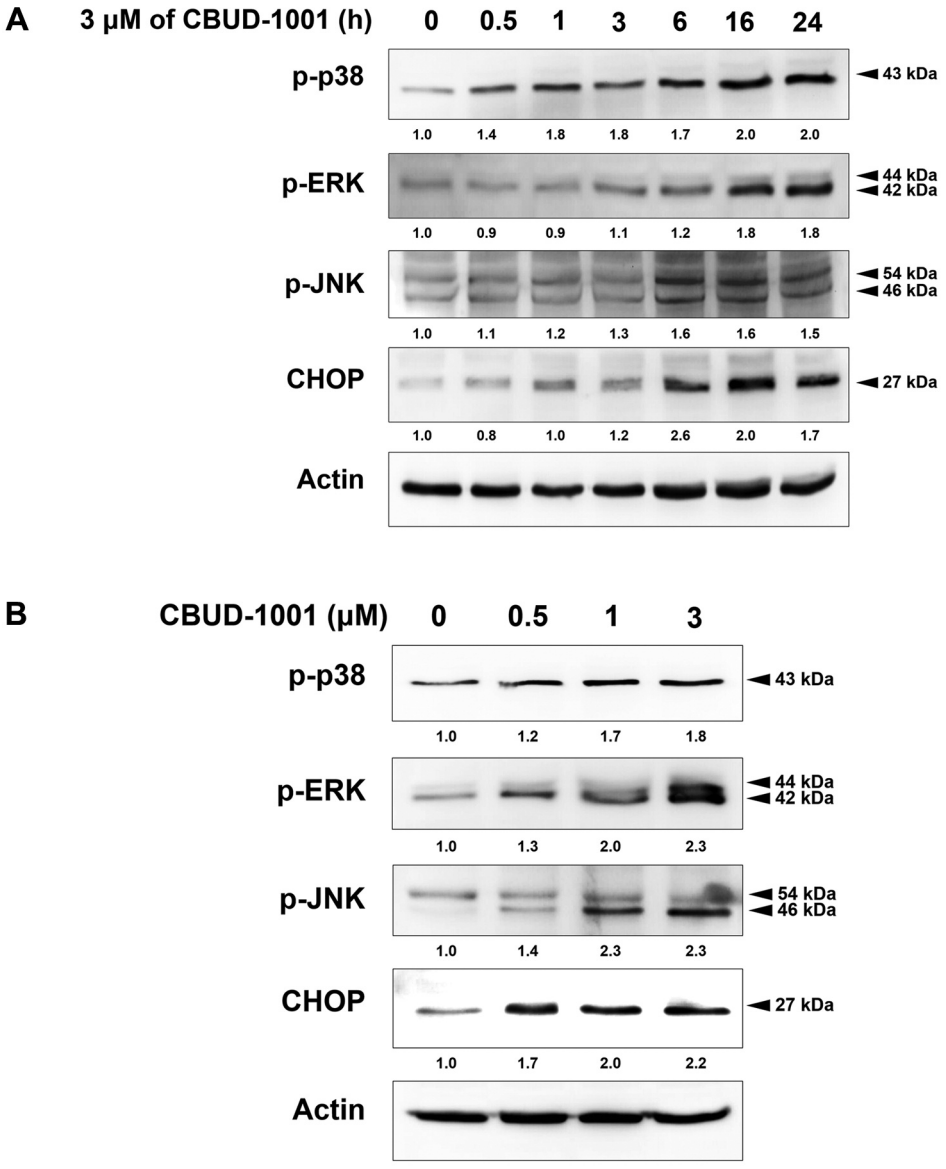


Figure 4. *Continued*

maximal inhibitory concentration (IC50) similar to previous results (18). Also, co-treatment with CBUD-1001 and TRAIL further reduced cell viability in both cell lines. In addition, combined treatment with SAHA or VPA and TRAIL was associated with greater cell viability than the CBUD-1001 and TRAIL combination. This result indicates that combined treatment with CBUD-1001 and TRAIL has a better synergistic effect compared to a treatment that combines TRAIL and approved HDAC inhibitors in CRC cells. Previous studies have revealed that HDAC inhibitors plus TRAIL significantly induced apoptosis in various types of cancer cells (14-17). We also showed that treatment with TRAIL plus CBUD-1001 significantly increased the number of Annexin V-positive cells, suggesting that the observed reduction in cell viability associated with combined CBUD-1001 and TRAIL treatment was caused by apoptotic cell death.

According to previous studies, combined treatment with other agents and TRAIL have shown sensitize cancer cells towards several mechanisms, like up-regulation of TRAIL receptors, caspase cascade, mitochondria-mediated pathway and down-regulation of anti-apoptotic proteins. (7, 14-16).

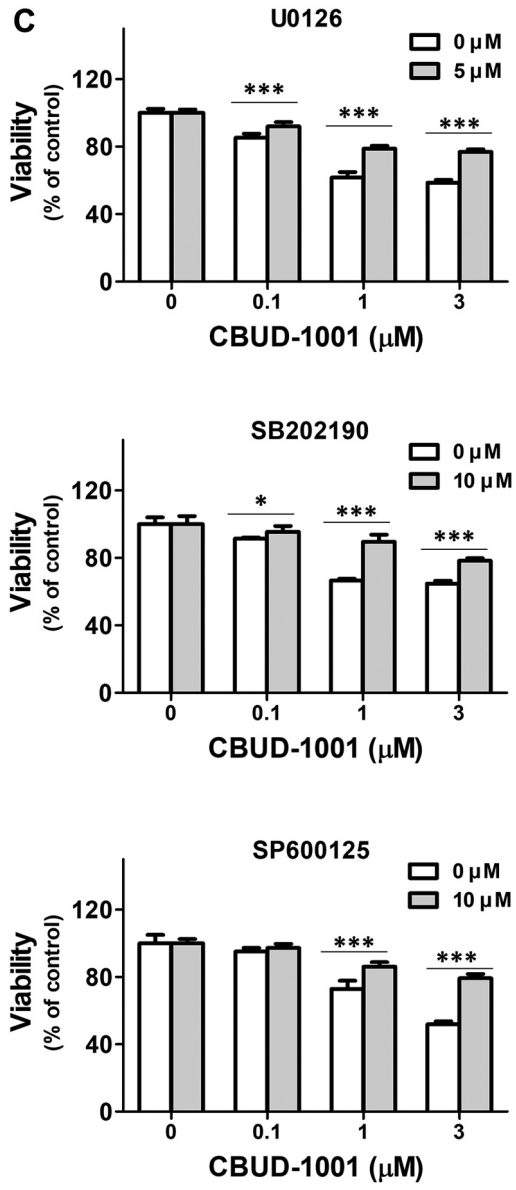


Figure 4. *MAPK pathway facilitation by CBUD-1001 is required for cell apoptosis. (A, B) The cells were incubated with TRAIL and/or CBUD-1001 at the indicated times and concentrations at 24 h. Total cell lysates were subjected to western blotting, and p-ERK, p-p38, p-JNK, and CHOP were measured. (C) DLD-1 cells were pre-treated with MEK inhibitor (U0126), p38 inhibitor (SB202190), or JNK inhibitor (SP600125) for 1 h and then treated with CBUD-1001 for 24 h. $p<0.05$ and $p<0.001$.*

In our study, we identified the alteration of apoptosis-related molecules, including FLIP, BID, XIAP, and caspase cascades, produced by treatment with a combination of CBUD-1001 and TRAIL. FLIP is highly expressed in CRC cells and can inhibit apoptotic signals by preventing recruitment of caspase 8 at the death-inducing signaling complex and suppressing the subsequent activation of the caspase cascade (26). Recent studies reported that XIAP can bind and potently inhibit caspase 3, 7, and 9 directly (27, 28). In our study, we observed that combined treatment with CBUD-1001 and TRAIL decreased the levels of FLIP and XIAP in DLD-1 cells. We also found that levels of the cleaved forms of caspase 8 and 3 increased after treatment with a combination of CBUD-1001 and TRAIL, decreasing the levels of pro-forms of caspase 8, 3, and 9. The cleaved form of caspase 8 amplifies the intrinsic pathway by BID cleavage, causing mitochondrial dysfunction, extracellular release of cytochrome c, and activation of caspase 9 (29). Similarly, we showed that the levels of BID, cytochrome c, and caspase 9 were regulated by co-treatment with CBUD-1001 and TRAIL, indicating their involvement of both extrinsic and intrinsic pathways in overcoming TRAIL resistance caused by CBUD-1001.

TRAIL binds to DR4 and DR5 on the cell surface and induces apoptosis. Up-regulation of DR4 or DR5 may provide an advantage in enhancing the apoptosis-inducing potential of TRAIL in cancer cells and sensitizing those cancer cells resistant to TRAIL alone (8). Epigenetic alterations are involved in inhibiting or inducing cancer by a balance of histone acetyltransferase and HDAC. HDACs display a variety of functions such as apoptosis, cell motility, and DNA repair by chromatin structure remodeling (30). Abnormally high HDAC activity regulates the expression of apoptosis-related proteins and induces tumorigenesis through epigenetic mechanisms (31). A number of studies has demonstrated that HDAC inhibitors can up-regulate DR4 or/and DR5 (32, 33). Our observations revealed that CBUD-1001 treatment enhances level of DR5 in a time- and dose-dependent manner, with CBUD-1001 inducing an elevation of DR5 *via* epigenetic regulation and increasing TRAIL sensitization in cancer cells.

Various mechanisms have been proposed to explain the expression of DR5, including induction of p53, YY1, SP1, and Foxo3; regeneration of reactive oxygen species; and MAPK pathway activation (34). Several recent studies have suggested that activation of ERK1/2 signaling, c-Jun NH2-terminal kinase (JNK), and the p38 MAPK pathway plays an important role in DR5 induction (19, 20, 34). We, therefore, investigated whether MAPK played a role in DR5 regulation affected by CBUD-1001 treatment. Our results indicate that MAPK (p-p38, p-ERK, and p-JNK) activation was involved in DR5 up-regulation after CBUD-1001 treatment in DLD-1 cells. In addition, we provided evidence that inhibition of MAPK activation with U0126, SB202190, or SP600125 can counteract increased cell viability, suggesting that CBUD-1001 triggers MAPK activation to induce DR5.

CHOP belongs to the family of CCAAT/enhancer-binding proteins (C/EBPs), and transcription factors are known to be

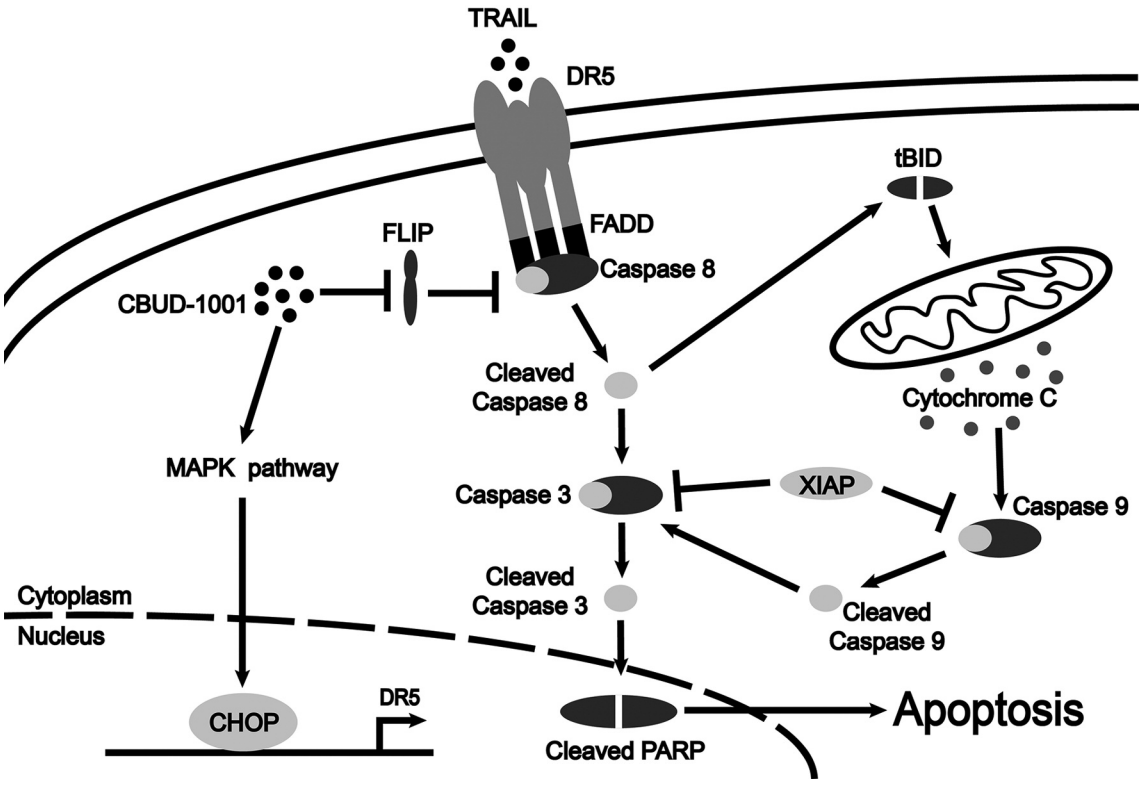


Figure 5. *Schematic summary of the proposed mechanism of CBUD-1001- and TRAIL-induced apoptosis in DLD-1 cells.*

involved in endoplasmic reticulum stress, including the unfolded protein response. CHOP can be combined with other C/EBP family members to regulate transcriptional activity (35). Results of our previous study suggest that transactivation of CHOP through MAPK activation is an important regulator of DR5. Various stimuli promote CHOP binding to the DR5 promoter and up-regulation DR5 (36-39). We determined that activation of MAPK proteins by CBUD-1001 treatment increased protein levels of CHOP. Our results are supported by results from previous studies demonstrating the function of CHOP in up-regulating DR5 and enhancing the effect of TRAIL by MAPK activation. Therefore, these data demonstrated that histone acetylation by CBUD-1001 increases TRAIL sensitivity through regulation of MAPK/CHOP/DR5 signaling and the proposed molecular mechanisms are illustrated in Figure 5.

In conclusion, we investigated the effects of CBUD-1001 and TRIAL on the viability, apoptosis, and morphological changes of DLD-1 cells. We observed various effects of CBUD-1001 or TRAIL separately and in combination on the expression of several apoptosis-related molecules, MAPK proteins, and receptors in cancer cells. Our results showed that combination treatment of CBUD-1001 and TRAIL may target TRAIL signaling and could serve as an effective therapeutic strategy against CRC cells. Improved understanding of molecular mechanisms may facilitate the use of CBUD-1001 or TRAIL targets and the selection of appropriate combinations.

Conflicts of Interest

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

Authors' Contributions

SWK and SLK participated in design of the study. HKK synthesized HDAC inhibitor, CBUD-1001. MWS, SLK, HCY, SKY, SYS, and STL performed all *in vitro* experiments and wrote the manuscript. SWK edited the draft and supervised all experimental procedures. All Authors have read and approved the final manuscript.

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