Low-Dose Combinations of LBH589 and TRAIL Can Overcome TRAIL-resistance in Colon Cancer Cell Lines

SANG-CHEOL LEE[1,2*], HEE-JEONG CHEONG[1,2*], SOOK-JA KIM[1,2], JINA YOON[3], HAN JO KIM[4], KYOUNG HA KIM[1,2], SE HYOUNG KIM[3], HYUN JUNG KIM[3], SANG BYUNG BAE[4], CHAN-KYU KIM[3], NAMSU LEE[1,2], KYU TAEG LEE[4], SUNG KYU PARK[3], DAE SIK HONG[3], HEE SOOK PARK[1,2] and JONG-HO WON[1,2]

[1]*Division of Hematology-Oncology, Department of Internal Medicine, and*
[2]*Institute for Clinical Molecular Biology Research, Soonchunhang University College of Medicine, Soonchunhang University Hospital Seoul, Seoul, Republic of Korea;*
[3]*Divison of Hematology-Oncology, Department of Internal Medicine, Soonchunhang University College of Medicine, Soonchunhyang University Hospital Bucheon, Bucheon, Gyeonggi-Do, Republic of Korea;*
[4]*Divison of Hematology-Oncology, Department of Internal Medicine, Soonchunhang University College of Medicine, Soonchunhyang University Hospital Cheonan, Cheonan, Chungchungnam-Do, Republic of Korea*

Abstract. *Background: Despite the considerable advances in the treatment of colorectal cancer, substantial changes in treatment strategies are required to overcome the problems of drug resistance and toxicity. Materials and Methods: Combinations of Pan-deacetylase inhibitor LBH589 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were studied in three colon cancer cell lines, HCT116, colo205, and HT29 (HCT116 and colo205 are TRAIL sensitive, whereas HT29 is TRAIL resistant). Results: It was found that TRAIL-induced cytotoxicity was enhanced by LBH589 cotreatment in the TRAIL-sensitive cell lines, and in the TRAIL-resistant HT29 cell line. The cytotoxicity of low-dose TRAIL plus LBH589 was found to be comparable to that of high-dose TRAIL plus LBH589. Additionally, TRAIL and LBH589 were significantly less toxic to normal UCB mononuclear cells than to the three colon cancer cell lines examined. Conclusion: LBH589 enhances TRAIL-induced apoptosis in human colon cancer cell lines, especially those resistant to TRAIL-induced apoptosis.*

More than 1 million new cases of colon and rectum cancer develop every year (1), and its disease-specific mortality rate has reached almost 33% in the developed world. This situation persists despite the considerable advances made in our understanding of the molecular pathogenesis, diagnosis, and the treatment of colorectal cancer, and the use of active targeting drugs for the treatment of metastatic colorectal cancer during the past decade. Furthermore, although overall survival for non-resectable disease has been improved to almost 2 years, cure rates remain low (2). Drug resistance and treatment toxicity are obvious limitations, and because continuous chemotherapy combined with or without targeting drug inevitably induces toxicity, patients often cannot continue with chemotherapy despite practical evidence of cancer control.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein that was initially identified because of the homology its extracellular domain shares with CD95L, tumor necrosis factor (TNF), and lymphotoxin α (3-5). TRAIL is a potent inducer of apoptosis in a variety of transformed or cancer cells of human and mouse origin, but it does not appear to be toxic to normal cells (5-8). Furthermore, a relatively high proportion of the tumor cell lines tested to date has been found to be sensitive to the cytotoxic effects of TRAIL (5, 7), and evidence for the safety and potential efficacy of TRAIL therapy against breast and colon cancer was recently obtained using a severe combined immunodeficiency mouse model (9, 10). In addition, some authors have shown that combinations of chemotherapeutic agents and TRAIL can augment TRAIL-induced apoptosis in several cancer cell types, and suggested that TRAIL may have potential use as a cancer treatment (11-14). However, although TRAIL has been shown to be a promising cancer drug, the possibility of TRAIL-induced hepatotoxicity has yet be resolved (15).

*These Authors contributed equally to this study.

Correspondence to: Jong-Ho Won, MD, Ph.D., Professor, Division of Hematology-Oncology, Soonchunhyang University Hospital Seoul, 22 Daesagwan-gil, Yongsan-gu, Seoul 140-743, Republic of Korea. Tel: +82 27099203, Fax: +82 27099200, e-mail: jhwon@schmc.ac.kr

Key Words: TRAIL, histone deacetylase inhibitor, LBH589, colon cancer, apoptosis.

On the other hand, the histone deacetylase inhibitors (HDACIs) are a new class of structurally diverse, neoplastic targeting agents. LBH589 (Panobinostat) is a true pan-deacetylase inhibitor (DACi), as demonstrated by its potent inhibition of all class I, II, and IV purified recombinant HDAC enzymes at nanomolar concentrations (16). LBH589 alters gene expression to promote the up-regulation of pro-apoptotic genes and the down-regulation of anti-apoptotic genes (17), and mediates acetylation-dependent changes in nonhistone proteins involved in cell-cycle regulation, to regulate multiple oncogenic pathways (18-20). Furthermore, LBH589 has potent *in vitro* anti-tumor activity against a wide range of cancer cell lines representing hematologic malignancies and solid tumors, including cutaneous T-cell lymphoma, acute myeloid leukemia, chronic myeloid leukemia, multiple myeloma, Hodgkin's lymphoma, and breast, colon, prostate and pancreatic cancer (21, 22). Moreover, in contrast to its effect on cancer cell lines, LBH589 is relatively sparing of normal cell lines (16, 21).

In earlier reports, combinations of HDACIs and TRAIL were found to demonstrate synergistic activity against leukemia and prostate cancer cell lines (23, 24). In this study, we tested combinations of TRAIL and the novel pan-DACi LBH589 in three colon cancer cell lines.

Materials and Methods

Reagents. Recombinant human TRAIL was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). LBH589 was provided by Novartis (Basel, Switzerland), and dissolved in dimethylsulfoxide (DMSO) at 20 mM as a stock solution, which was stored in ampules at –20˚C and thawed immediately prior to experiments. All chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

Antibodies. Antibodies for caspase-3, cleaved caspase-3, X-linked inhibitor of apoptosis protein (XIAP), and acetyl-histone H3 (Ac-H3) were obtained from Cell Signal Technology (Beverly, MA, USA), antibodies for BH3 interacting domain death agonist (BID), B-cell lymphoma 2 (BCL-2), BCL-2-associated X protein (BAX) and death receptor 4 (DR4) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DR5 antibody was purchased from ProSci (Poway, CA, USA), caspase-8 antibody from Stressgen (Ann Arbor, MI, USA), caspase-9, cytochrome c, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 antibodies were obtained from BD Pharmingen (Mountain View, CA, USA). β-actin antibody was purchased from Sigma.

Cell culture and mononuclear cell (MNC) isolation from umbilical cord blood (UCB). The HT29 and HCT116 human colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The colo205 human colon cancer cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). HT29 and colo205 cells were cultured in RPMI-1640 (Gibco, Rockville, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco) in an incubator with humidified 5% carbon dioxide/95% at 37˚C. HCT116 cells were cultured in McCoy's 5A medium (Gibco) supplemented with 10% FBS (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco) also in an incubator with humidified 5% carbon dioxide/95% at 37˚C. Cell media were changed twice weekly.

UCB was obtained at the time of full-term delivery, after the study protocol had been approved by our hospital's Ethics Committee. UCB was collected after clamping and cutting cords by draining blood into sterile collection tubes containing the anticoagulant citrate-phosphate dextrose. MNCs were isolated from UCB by Ficoll-Hypaque (1.077 g/cm^3; Sigma) density centrifugation (400 × g for 25 min). Interfacial UCB MNCs were collected and washed with phosphate-buffered saline (PBS; Gibco).

Cell viability assays. Cell viabilities were assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay kits (Roche, Mannheim, Germany), which measured numbers of viable cells. Briefly, equal numbers of cells were cultured in 96-well plates. UCB MNCs, HT29, HCT116, and colo205 cells were treated with TRAIL (10, 100, 500, 1000 ng/ml), LBH589 (10, 50, 100, 500 nM), or TRAIL (10, 100 ng/ml) plus LBH589 (50 nM). After 24 hours of treatment, 10 μl of the yellow MTT labeling reagent was added to culture media. Cells were incubated for a further 4 hours at 37˚C and then 100 μl of the solubilization solution was added to dissolve the purple formazan crystals formed. After standing overnight, absorbance was measured at 562 nm using an ELISA reader.

Annexin-V-fluorescein staining assay. HT29 cells were treated with TRAIL (10 or 100 ng/ml), LBH589 (50 nM), or TRAIL plus LBH589 for 24 hours, whereas HCT116 cells were treated with TRAIL (10 ng/ml), LBH589 (50 nM), or TRAIL plus LBH589 for 24 hours. The following procedure was conducted using Annexin-V-fluorescein isothiocyanate (FITC) kits (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, cells (1×10^6) were washed twice with PBS and incubated with FITC-conjugated annexin-V at room temperature for 15 minutes in the dark, resuspended in 400 μl of annexin-V binding buffer and analyzed by flow cytometry (Cytomics FC500; Beckman Coulter, Fullerton, CA, USA).

Preparation of nuclear and cytosol extracts. Cells were treated with TRAIL (10 ng/ml), LBH589 (50 nM), or TRAIL plus LBH589 at these concentrations for 24 hours. Cells were then harvested by trypsinization, washed with PBS, and lysed by incubation at 4˚C for 10 minutes in 100 μl of buffer A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin, and 0.1 μM aprotinin at pH 7.9]. Cell lysates were centrifuged and supernatants (cytosol extracts) were stored at –70˚C. Pellets were resuspended in 50 μl ice-cold buffer B [20 mM HEPES, 1.5 mM MgCl2, 420 mM KCl, 0.2 mM ethylenediaminetetra-acetic acid (EDTA), 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 0.1 μM aprotinin, and 25% glycerol at pH 7.9]. After incubation at 4˚C for 30 minutes, 50 μl of ice-cold buffer C [20 mM HEPES, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin and 0.1 μM aprotinin, 20% glycerol at pH 7.9] was added. Mixtures were then centrifuged at 12,000 xg at 4˚C for 15 minutes, and supernatants (nuclear extracts) were stored at -70˚C. The protein concentrations of extracts were determined using a colorimetric bicinchoninic acid method (Pierce, Rockford, IL, USA).

Total cellular protein extraction. Cells were treated with TRAIL (10 ng/ml), LBH589 (50 nM), or TRAIL plus LBH589 at these concentrations for 24 hours, harvested by trypsinization, and washed with PBS. Total cellular proteins were isolated using ice-cold PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2.0 μg/mL aprotinin, and protease inhibitor cocktail (Roche) by passing them through a 21-gauge needle, and then centrifuging them at 12,000 × g to remove cellular debris. The protein concentrations in extracts were determined using the colorimetric bicinchoninic acid method (Pierce).

Western blotting. Equal amounts of protein were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad, Hercules, CA, USA). The membranes were then blocked with 5% nonfat dried milk (Bio-Rad) in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween 20 (TTBS) for 1 hour at room temperature and then incubated in primary antibodies diluted to 1:1000; 1:3000 in 5% nonfat dried milk/TTBS overnight at 4˚C with gentle shaking. The membranes were then washed with TTBS (3×15 minutes) and subsequently incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark) or HRP-conjugated goat anti-mouse IgG (Dako) diluted to 1:5000 in 5% nonfat dried milk/TTBS for 2 hours at room temperature. The membranes were then washed as described above and developed using the ECL detection system (Amersham, Buckinghamshire, UK).

Results

Sensitivities of human colon cancer cell lines and normal UCB MNCs to TRAIL. We first determined the sensitivities of human colon cancer cell lines (HT29, HCT116, and colo205) and normal UCB MNCs to TRAIL by using MTT assays. Cells were exposed to TRAIL at different concentrations (0-1000 ng/ml) for 24 hours. HCT116 and colo205 exhibited dose-dependent decreases in cell survival after TRAIL treatment, whereas HT29 showed minimal response to TRAIL in terms of decreases in cell survival. Normal UCB MNCs were affected minimally by TRAIL (Figure 1A).

Effects of LBH589 on the growth of human colon cancer cell lines and normal UCB MNCs. To investigate the effects of LBH589 on the growth and survival of human colon cancer cell lines and normal UCB MNCs, we used MTT assays after exposing cells to LBH589 at different concentrations (0-500 nM) for 24 hours. As shown in Figure 1B, the three colon cancer cell whereas normal UCB MNCs showed no LBH589-induced cell reduction in survival.

Acetylation of histone by LBH589. To determine whether LBH589 induces the hyperacetylation of histone in human colon cancer cell lines, we treated cells with 50 nM of LBH589 for 24 hours and analyzed the expression of acetyl-histone H3 (Ac-H3) by Western blotting. As shown in Figure 1C, all three colon cancer cell lines exhibited a strong increase in histone H3 acetylation.

Synergistic effects of LBH589 on TRAIL-induced cytotoxicity and apoptosis. To investigate the synergistic effect of LBH589 on TRAIL-induced cytotoxicity, we used MTT assays after exposing cells to TRAIL (10 and 100 ng/ml), LBH589 (50 nM), or both for 24 hours. As illustrated in Figure 2A, combined treatment enhanced TRAIL-induced cytotoxicity in HCT116 and colo205 cells (TRAIL-sensitive) and in HT29 cells (TRAIL-resistant). In HT29 cells, 10 ng/ml TRAIL plus 50 nM LBH589 significantly reduced cell viability to 62%, whereas 10 ng/mL TRAIL alone only reduced viability to 93%. At 100 ng/ml, TRAIL reduced cell viability to 85%, whereas 100 ng/ml TRAIL plus 50 nM LBH589 reduced viability to 54%. This result means that the cytotoxic effect of low dose TRAIL plus LBH589 is comparable with that of high-dose TRAIL plus LBH589. Both TRAIL and LBH589 were significantly less toxic to normal UCB MNCs, and their treatment in combination did not even have an additive cytotoxic effect.

To determine whether reductions in viable cell numbers after treatment with TRAIL plus LBH589 are mediated by the induction of apoptosis, cells were stained with annexin V-FITC and analyzed by flow cytometry. In line with our cell viability data, TRAIL plus LBH589 was much more potent than the single agents alone in terms of inducing apoptosis by Annexin V-staining. TRAIL 10 ng/ml and 50 nM LBH589 caused 51.4% and 16% apoptosis, respectively, of TRAIL-sensitive HCT116 cells, whereas the combination of TRAIL and LBH589 at these concentrations caused 58.1% apoptosis (Figure 2B). In TRAIL-resistant HT29 cells, 10 or 100 ng/ml of TRAIL alone caused 9.8% and 15.9% apoptosis, respectively, whereas the combination of 10 or 100 ng/ml TRAIL plus 50 nM of LBH589 caused 42.9% and 43.7% apoptosis, respectively (Figure 2B). Again, the overall effect of the TRAIL at 10 ng/ml plus LBH589 at 50 nM combination was similar to that of TRAIL at 100 ng/ml plus LBH589 at 50 nM, which suggests that TRAIL at 10 ng/ml is sufficient to obtain the synergistic effect. Accordingly, in subsequent experiments, we used low-dose (10 ng/ml) TRAIL plus LBH589 (50 nM) as the combined treatment. These results suggest that low-dose TRAIL plus LBH589 benefits from the synergistic enhancement of apoptosis and that this extends to TRAIL-resistant cell lines.

The activations of caspases by treatments. To determine whether the increased apoptosis caused by TRAIL (10 ng/ml) plus LBH589 (50 nM) was mediated by the activation of caspases, we assessed the activation of caspase-3, -8 and -9 by Western blotting. In HCT116 and colo205 cells (TRAIL-sensitive), treatment with TRAIL alone enhanced the activation of caspase-3, -8 and -9, and when these cells were treated with TRAIL plus LBH589, activations were similar to those observed for treatment with TRAIL alone (Figure 3A). However, in HT29 cells (the TRAIL-resistant cell line),

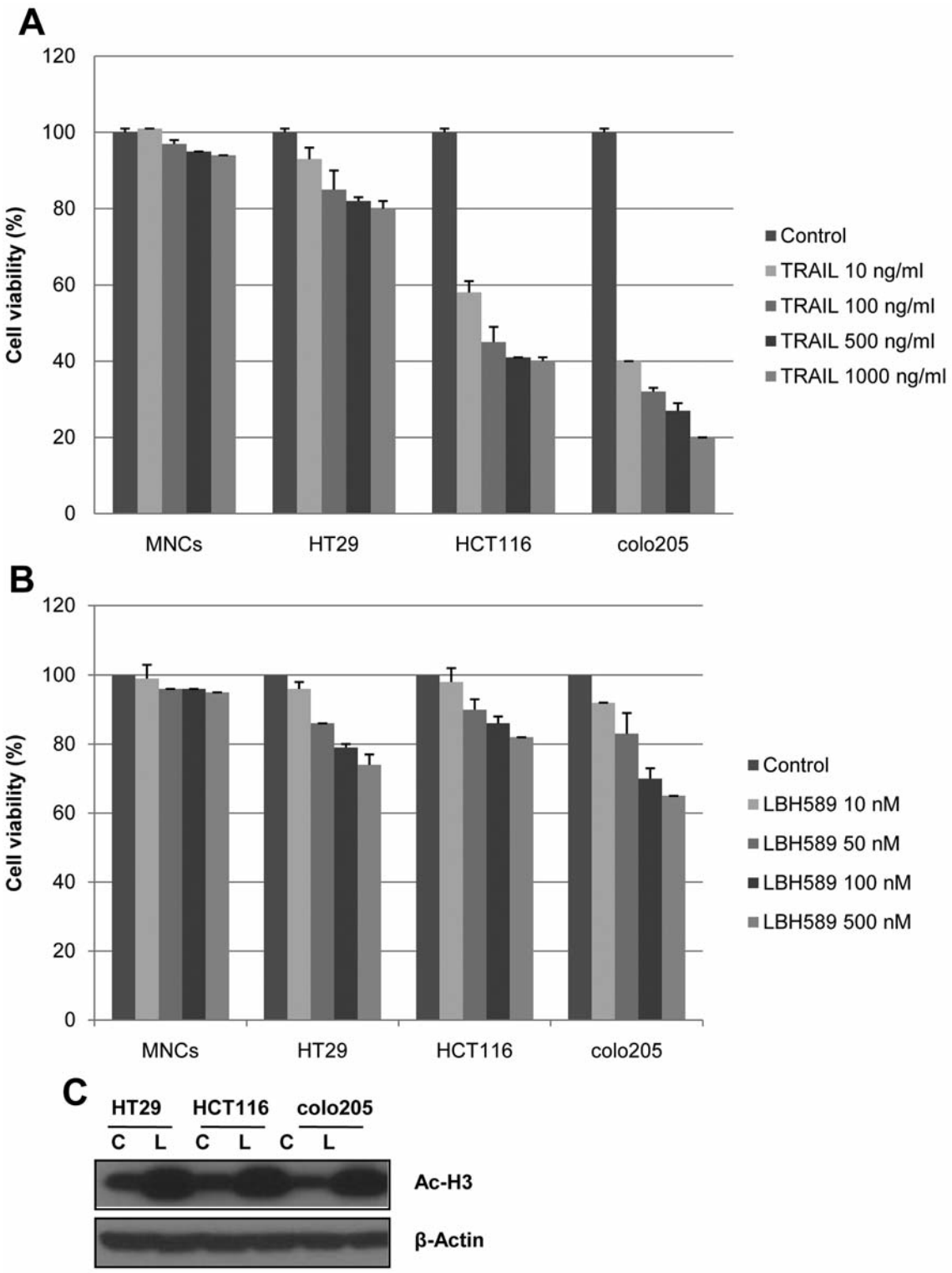

Figure 1. *Effects of TRAIL and LBH589. A: Tumor cell sensitivity to TRAIL. B: Cytotoxicity of LBH589. TRAIL-sensitive (HCT116 and colo205) and TRAIL resistant (HT29) colon cancer cells, and normal mononuclear cells from umbilical cord blood MNCs were treated for 24 hours with recombinant human TRAIL or LBH589. Cell viabilities were assessed using MTT assays. Results are given as percentages of the controls (untreated cells). Data points show the averages of triplicate results from representative experiments. C: Effect of LBH589 on the acetylation of histone. Cells were treated with 50 nM of LBH589 for 24 hours. Whole-cell lysates were analyzed by Western blotting. β-Actin was used as an internal control. C, Control (untreated cells); L, 50 nM of LBH589.*

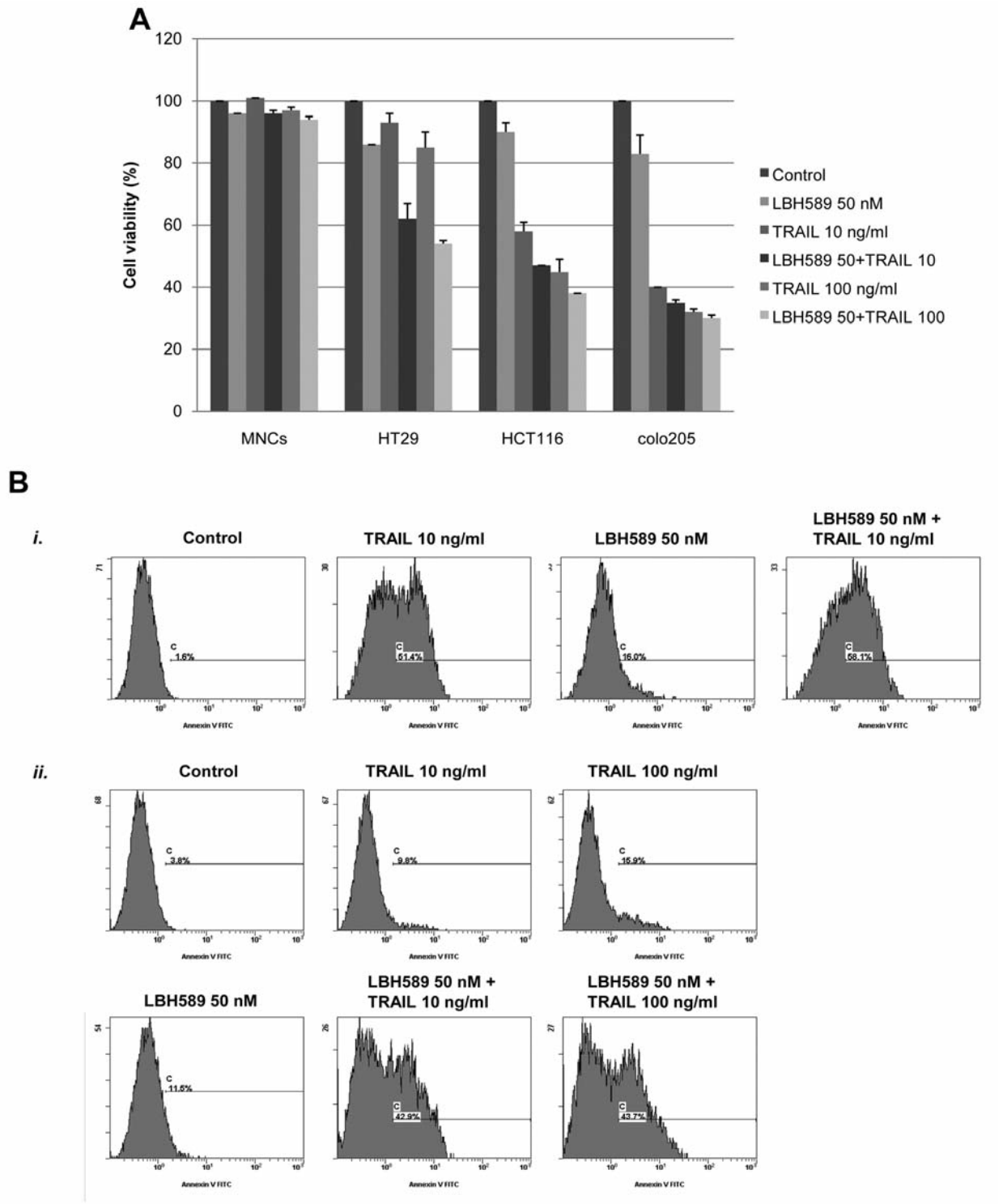

Figure 2. *Responses of the three human colon cancer cell lines to TRAIL, LBH589, or TRAIL plus LBH589. A: Synergism of LBH589 on TRAIL-induced cytotoxicity. The three human colon cancer cells and normal UCB MNCs were treated with 10 or 100 ng/ml of TRAIL, 50 nM of LBH589, or TRAIL plus LBH589 at these concentrations for 24 hours. Cell viabilities were assessed using MTT assays. The controls were untreated cells. Results are the means of three independent experiments. B: Synergistic effects of LBH589 on TRAIL-induced apoptosis. i. HCT116 cells were treated with 10 ng/ml of TRAIL, 50 nM of LBH589, or TRAIL plus LBH589 at these concentrations for 24 hours. ii. HT29 cells were treated with 10 or 100 ng/ml of TRAIL, 50 nM of LBH589, or TRAIL plus LBH589 at these concentrations for 24 hours. Treated cells were stained with annexin-V FITC and analyzed by flow cytometry. Untreated cells were used as controls.*

treatment with TRAIL or LBH589 alone did not activate caspase-3, -8 and -9, but combined treatment enhanced the activation of all three caspases (Figure 3A). These results suggest that the simultaneous activations of intrinsic and extrinsic apoptotic pathways are probably responsible for the synergism shown by TRAIL and LBH589.

To confirm the caspase dependence of the increased levels of apoptosis observed, we investigated, using MTT assays, whether the pan-caspase inhibitor Z-VAD could prevent the cell death caused by TRAIL, LBH589, or TRAIL plus LBH589. HT29 cells were pretreated with Z-VAD for 1 hour before addition the reagents, and Z-VAD was found to strongly inhibited the cell death induced by TRAIL or by the combination of TRAIL and LBH589 (Figure 3B).

The expression of TRAIL death receptors (DR4 and DR5). Since the expression of death receptors is critical for TRAIL-induced apoptosis, we examined whether TRAIL, LBH589, or TRAIL plus LBH589 affected the expressions of DR4 and DR5 by Western blotting. In HCT116 and colo205 cells (TRAIL-sensitive), treatment with TRAIL alone slightly increased DR4 expression and markedly increased DR5 expression, but treatment with LBH589 alone had little effect on the expression of DR4 and DR5 in the HCT116 cells and had moderate effect in the colo205 cells. When these cells were treated with TRAIL plus LBH589, the expression of DR4 and DR5 was affected in the same way as treatment with TRAIL alone (Figure 4A). On the other hand, in TRAIL-resistant HT29 cells, neither TRAIL nor LBH589 increased the expression of DR4 and DR5, whereas treatment with TRAIL plus LBH589 clearly increased their expression, although the expression of DR5 was more increased (Figure 4A).

The expressions of pro-apoptotic and anti-apoptotic proteins. To evaluate the mechanisms responsible for the synergism shown by TRAIL and LBH589, we investigated the expression patterns of several pro-apoptotic and anti-apoptotic proteins by Western blotting. In HCT116 and colo205 cells (TRAIL-sensitive), TRAIL markedly reduced the expression of BID, and LBH589 alone slightly reduced the expression of BID. In HT29 cells, neither TRAIL nor LBH589 alone changed BID expression. Treatment with TRAIL and LBH589 markedly reduced the expression levels of BID in both TRAIL-sensitive and -resistant cells (Figure 4B).

BAX and BCL-2 are major regulators of cytochrome *c* release from mitochondria to the cytosol. In HCT116 and colo205 cells, BCL-2 expression was reduced after treatment with TRAIL or LBH589, and treatment with TRAIL plus LBH589 markedly reduced BCL-2 expression in colo205 cells. However, in HT29 cells, neither TRAIL nor LBH589 altered BCL-2 expression, whereas TRAIL plus LBH589 clearly reduced its expression (Figure 4B). However, TRAIL plus LBH589 did not induce an expressional change in BAX neither in TRAIL-sensitive nor in TRAIL-resistant cells (Figure 4B). When HT29 cells were treated with TRAIL plus LBH589, cytochrome *c* was found to be synergistically released from mitochondria to the cytosol (Figure 4B). XIAP (a member of the IAP protein family) can bind to and directly inhibit caspase-3 and -9, and thus, act as an important modulator of TRAIL activity. In HT29 and HCT116 cells, TRAIL and LBH589 alone had little or no effect on the expression of XIAP, whereas TRAIL plus LBH589 markedly suppressed XIAP expression (Figure 4B). To determine whether the addition of LBH589 to TRAIL inhibits NF-κB activation, we measured NF-κB levels in nuclear fractions. In HT29 and HCT116 cells, TRAIL and LBH589 alone failed to suppress NF-κB activation, whereas TRAIL plus LBH589 markedly suppressed NF-κB activation (Figure 4B). In case of colo205 cells, TRAIL alone markedly suppressed the activation of XIAP and NF-κB. therefore synergistic effect of TRAIL and LBH589 is not showed because they are highly sensitive to TRAIL.

Discussion

Palliative chemotherapy can improve survival, lessen symptoms, and improve quality of life for patients with metastatic colorectal cancer. Survival increases from 12 months with fluorouracil monotherapy to roughly 2 years after adding irinotecan, oxaliplatin, or targeting drugs. In fact, the main advance made in the management of metastatic colorectal cancer during the past 5 years has been the addition of targeted treatments. However, the only licensed targeting drugs, cetuximab, bevacizumab, and panitumumab, have had only a relatively small effect on survival outcomes (2), and thus, substantive changes in treatment strategies are urgently required to overcome the continuing problems of drug resistance and toxicity.

The death ligand TRAIL has recently emerged as potential cancer chemotherapeutic agent because it preferentially induces apoptosis in transformed or malignant cells without inducing hepatotoxicity (25, 26). Currently, recombinant human TRAIL is being tested in phase I clinical trials. Furthermore, agonistic antibodies against DR4 and DR5, which directly activate the extrinsic apoptotic pathway, have also undergone phase I and II trials (27). Colon carcinomas express both DR4 and DR5 receptors, but respond variously to TRAIL, and some tumors are resistant to TRAIL-induced apoptosis (28, 29). In the present study, we found that the HDAC inhibitor LBH589 effectively and synergistically increases TRAIL-induced apoptosis in human colon cancer cell lines. Although synergistic activity has been reported for HDACIs and TRAIL in leukemia, multiple myeloma, prostate cancer, pancreatic cancer, and mesothelioma cell lines, the present study provides first evidence of synergistic activity against colon cancer cell lines (23, 24, 30-32). In HT29 human

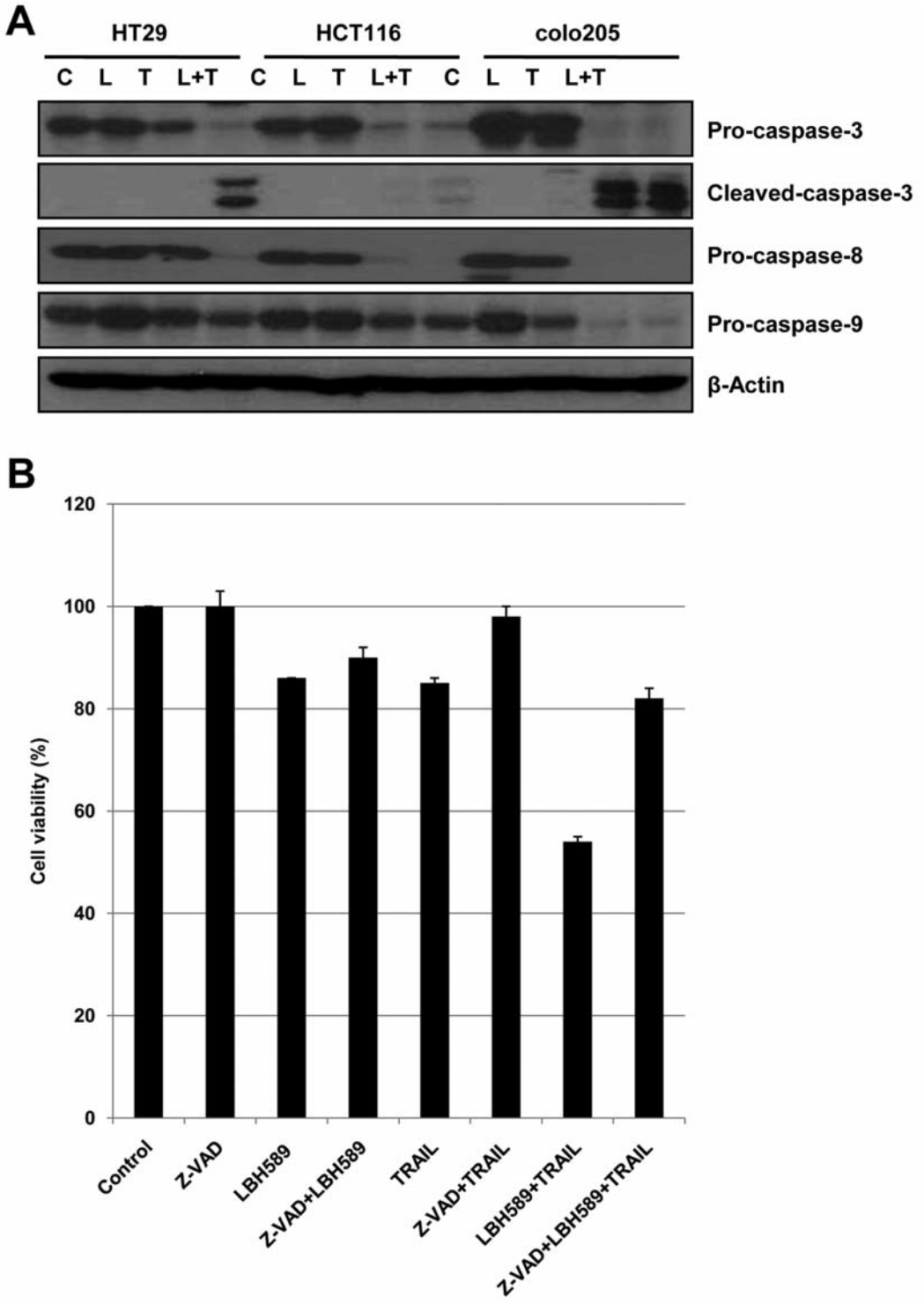

Figure 3. *Synergistic effects of TRAIL, LBH589, or TRAIL plus LBH589 on the activation of caspases. A: Protein expression of caspases. Cells were treated with 10 ng/ml of TRAIL, 50 nM of LBH589, or TRAIL plus LBH589 at these concentrations for 24 hours. Whole-cell lysates were analyzed by Western blotting. β-Actin was used as an internal control. C, Controls (untreated cells); L, 50 nM of LBH589; T, 10 ng/ml of TRAIL; L+T, 10 ng/ml of TRAIL+50 nM of LBH589. B: The synergism shown by TRAIL and LBH589 depended on caspase activation. HT29 cells were treated for 24 hours with 100 ng/ml of TRAIL alone, 50 nM of LBH589 alone, or TRAIL plus LBH589 at these concentrations with/without pretreatment of 1 hour with the pancaspase inhibitor Z-VAD at 100 μM. Cell viabilities were determined using MTT assays. Untreated cells were used as controls. Results are the means of three independent experiments.*

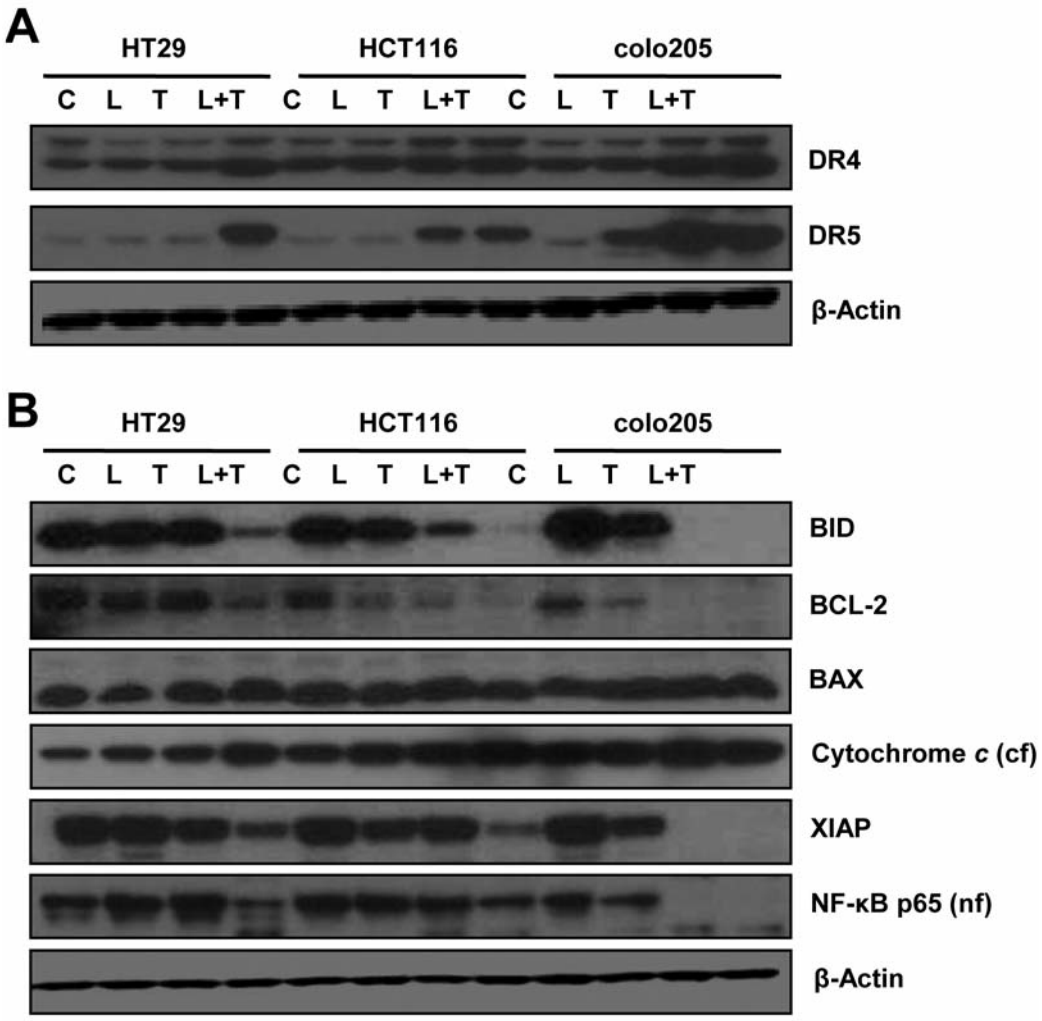

Figure 4. *Effects of TRAIL, LBH589, or TRAIL plus LBH589 on the expressions of TRAIL death receptors and of apoptosis-related proteins. A: Expressions of DR4 and DR5. B: Changes in the levels of pro- and anti-apoptotic proteins. Cells were treated with 10 ng/ml of TRAIL, 50 nM of LBH589, or TRAIL plus LBH589 at these concentrations for 24 hours. Whole cell lysates were analyzed by Western blotting. β-Actin was used as an internal control. C, Control (untreated cells); L, 50 nM of LBH589; T, 10 ng/ml of TRAIL; L+T, 10 ng/ml of TRAIL + 50 nM of LBH589; cf, cytosolic fraction; nf, nuclear fraction.*

colon cancer cells (a TRAIL-resistant cell line) TRAIL and LBH589 (at nanomolar concentrations) exhibited synergism in terms of apoptosis induction. Furthermore, the proportions of apoptosis observed were similar when TRAIL was dosed at 10 or 100 ng/ml with 50 nM LBH589. HCT116 and colo205 colon cancer cells were sensitive to TRAIL and the degree of apoptosis was dose-dependent. Interestingly, low-dose TRAIL and high-dose TRAIL plus LBH589 had similar apoptotic effects in these cells. These results reveal that the 10 ng/ml TRAIL plus 50 nM LBH589 is sufficient to overcome TRAIL resistance and can function at lower TRAIL doses for TRAIL-sensitive cells. The recent treatment trend for inoperable or relapsed colon cancer is toward continued chemotherapy with or without targeting drugs until disease progression. Thus intractable drug toxicity and resistance are major treatment obstacles. However, although TRAIL and LBH589 are non-toxic to normal cells, we have concerns about liver toxicity, and therefore, low-dose TRAIL is justly acceptable.

DR5 induction is importantly involved in the mechanisms of drug-mediated augmentation and TRAIL-sensitization induced apoptosis (32, 33). In the present study, we found that neither TRAIL nor LBH589 enhanced DR5 expression in the TRAIL-resistant colon cancer cell line, but that DR5 was expressionally up-regulated when cells were treated with TRAIL plus LBH589, which suggests that the mechanism underlying the LBH589-mediated augmentation of TRAIL-induced apoptosis is similar to that of previously investigated drugs in colon cancer cell lines (33).

The intrinsic pathway is essential for TRAIL-induced apoptosis (34). BCL-2 family members, such as, BCL-2 and BAX, have been suggested to regulate TRAIL-induced apoptosis, and HDACis are known to enhance TRAIL-induced apoptosis in some cancer cells by modulating BCL-2 family members (32, 34, 35). In the present study, the truncation of BID and the down-regulation of BCL-2 were profound when the TRAIL-resistant colon cancer cell line was treated with TRAIL plus LBH589, but BAX expression was not changed.

IAP family members, like XIAP, have also been suggested to regulate TRAIL induced apoptosis. XIAP is involved in the inactivations of caspase-3, -7, and -9 (36). Furthermore, in recent reports, HDACis, such as LBH589 and LAQ824, have been found to augment TRAIL-induced apoptosis by downregulating XIAP in various cancer cells (31, 37). In the present study, we observed marked down-regulation of XIAP in TRAIL-sensitive and -resistant colon cancer cell lines treated with TRAIL plus LBH589. It would appear that XIAP down-regulation is associated with the synergism shown by LBH589 and TRAIL in colon cancer cell lines.

The constitutive activation of NF-κB has been detected in several types of cancer, including colorectal cancer, and this has been implicated in the development of resistance to TRAIL and chemotherapeutics (38). Furthermore, NF-κB is also known to be activated by TRAIL (39). In the present study, NF-κB expression was found to be slightly elevated in the TRAIL-resistant colon cancer cell line, and this was markedly reduced after TRAIL plus LBH589 treatment, which suggests that LBH589 suppresses NF-κB activation by TRAIL or by chemotherapeutic agents.

Summarizing, here we report that LBH589 (an HDACi) enhances TRAIL-induced apoptosis in human colon cancer cell lines, especially those resistant to TRAIL-induced apoptosis. Furthermore, the overall effects of low- and high-dose TRAIL plus LBH589 were similar. This finding provides initial evidence regarding the potential effectiveness of low-dose TRAIL plus LBH589 for the treatment of patients with non-resectable or metastatic colon cancer. Accordingly, we recommend that this combination be further evaluated in animal models of colon cancer and that it be considered for clinical trials. In addition, our findings show that DR5 induction is an initial event during LBH589-mediated augmentation or LBH589-mediated sensitization to TRAIL-induced apoptosis, and suggest that this is associated with changes in the expression of pro- and anti-apoptotic proteins.

References

1 Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin *55*: 74-108, 2005.

2 Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger and Starling N: Colorectal cancer. Lancet *375*: 1030-1047, 2010.

3 Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J and Dixit VM: The receptor for the cytotoxic ligand TRAIL. Science (Washington DC) 276: 111-113, 1997.

4 Ashkenazi A and Dixit VM: Death receptor: signaling and modulation. Science (Washington DC) *281*: 1305-1308, 1998.

5 Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C and Smith CA: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity *3*: 673-682, 1995.

6 Marsters SA, Pitti RM, Donahue CJ, Ruppert S, Bauer KD and Ashkenazi A: Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. Curr Biol *6*: 750-752, 1996.

7 Griffith TS, Chin WA, Jackson GC, Lynch DH and Kubin MZ: Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol *161*: 2833-2840, 1998.

8 Lee NS, Cheong HJ, Kim SJ, Kim SE, Kim CK, Lee KT, Park SK, Baick SH, Hong DS, Park HS and Won JH: *Ex vivo* purging of leukemia cells using tumor necrosis factor-related apoptosis-inducing ligand in hematopoietic stem cell transplantation. Leukemia *17*: 1375-1383, 2003.

9 Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med *5*: 157-163, 1999.

10 El-Deiry WS: The TRAIL to an anticancer agents. Drug Resist Updat 2: 79-80, 1999.

11 Keane MM, Ettenberg SA, Nau MM, Russell EK and Lipkowitz S: Chemotherapy augments TRAI- induced apoptosis in breast cancer cells. Cancer Res *59*: 734-741, 1999.

12 Gliniak B and Le T: Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity *in vivo* in enhanced by the chemotherapeutic agent CPT-11. Cancer Res *59*: 6153-6158, 1999.

13 Kim K, Fisher MJ, Xu SQ and El-Deiry WS: Molecular determination of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res *6*: 335-346, 2000.

14 Byun JH, Hong YS, Cheong HJ, Kim SJ, Lee NS, Won JH, Hong DS and Park HS: Effect of arsenic trioxide in TRAIL (tumor necrosis factor-related apoptosis inducing ligand)-mediated apoptosis in multiple myeloma cell lines. Cancer Res Treat *35*: 472-477, 2003.

15 Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR and Strom SC: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med *6*: 564-567, 2000.

16 Prince HM, Bishton MJ and Johnstone RW: Panobinostat (LBH589): a potent pan-deacetylase inhibitor with promising activity against hematologic and solid tumors. Future Oncol *5*: 601-612, 2009.

17 Bolden JE, Peart MJ and Johnstone RW: Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov *5*: 769-784, 2006.

18 Xu WS, Parmigiani RB and Marks PA: Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*: 5541-5552, 2007.

19 Peart MJ, Smyth GK, van Laar RK Bowtell DD, Richon VM, Marks PA, Holloway AJ and Johnstone RW: Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci USA *102*: 3697-3702, 2005.

20 Bhalla KN: Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. J Clin Oncol *23*: 3971-3993, 2005.

21 Shao W, Growney JD, Feng Y, Wang P, Yan-Neale Y, O'Connor G, Kwon P, Yao Y, Fawell S and Atadja P: Potent anticancer activity of the pan-deacetylase inhibitor panobinostat (LBH589) as a single agent in *in vitro* and *in vivo* tumor models. AACR Annual Meeting, April 12-16, 2008, San Diego, CA, USA (poster presentation).

22 Atadja P: Development of the pan-DAC inhibitor panobinostat (LBH589): Successes and challenges. Cancer Letters *280*: 233-241, 2009.

23 Bucur O, Ray S, Bucur MC and Almasan A: APO2 ligand/tumor necrosis factor-related apoptosis-inducing ligand in prostate cancer therapy. Front Biosci *11*: 1549-1568, 2006.

24 Guo F, Sigua C, Tao J, Bali P, George P, Li Y, Wittmann S, Moscinski L, Atadja P and Bhalla K: Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. Cancer Res *64*: 2580-2589, 2004.

25 Kelley SK and Ashkenazi A: Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol *4*: 333-339, 2004.

26 Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest *104*: 155-162, 1999.

27 Rowinsky EK: Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activiating agents. J Clin Oncol *23*: 9394-9407, 2005.

28 Ren YG, Wagner KW, Knee DA, Aza-Blanc P, Nasoff M and Deveraux QL: Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. Mol Biol Cell *15*: 5064-5074, 2004.

29 Van Geelen CM, Pennarun B, Ek WB, Le PT, Spierings DC, De Vries EG and De Jong S: Downregulation of active caspase 8 as a mechanism of acquired TRAIL resistance in mismatch repair-proficient colon carcinoma cell lines. Int J Oncol *37*: 1031-1041, 2010.

30 Fandy TE, Shankar S, Ross DD, Sausville E and Srivastava RK: Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma. Neoplasia *7*: 646-657, 2005.

31 Symanowski J, Vogelzang N, Zawel L, Atadja P, Pass H and Sharma S: A histone deacetylase inhibitor LBH589 downregulates XIAP in mesothelioma cell lines which is likely responsible for increased apoptosis with TRAIL. J Thorac Oncol *4*: 149-160, 2009.

32 Kauh J, Fan S, Xia M, Yue P, Yang L, Khuri FR and Sun SY: c-FLIP degradation mediates sensitization of pancreatic cancer cells to TRAIL-induced apoptosis by the histone deacetylase inhibitor LBH589. PLoS One *5*: e10376, 2010.

33 Elrod HA and Sun SY: Modulation of death receptors by cancer therapeutic agents. Cancer Biol Ther *7*: 163-173, 2007.

34 Wang S: The promise of cancer therapeutics targeting the TNF-related apoptosis-inducing ligand and TRAIL receptor pathway. Oncogene *237*: 6207-6215, 2008.

35 Huang S, Okumura K and Sinicrope FA: BH3 mimetic obatoclax enhances TRAIL-mediated apoptosis in human prostate cancer cells. Clin Cancer Res 15: 150-159, 2009.

36 Deveraux QL, Takahashi R, Salvesen GS and Reed JC: X-Linked IAP is a direct inhibitor of cell-death proteases. Nature *388*: 300-304, 1997.

37 Vogler M, Durr K, Jovanovic M, Debatin KM and Fulda S: Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. Oncogene *26*: 248-257, 2007.

38 Jani TS, DeVecchio J, Mazumbar T, Agyeman A and Houghton JA: Inhibition of NF-kB signaling by quinacrine is cytotoxic to human colon carcinoma cell lines and is synergistic in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or oxaliplatin. J Biol Chem *285*: 19162-19172, 2010.

39 Ammann JU, Haag C, Kasperczyk H, Debatin KM and Fulda S: Sensitization of neuroblastoma cells for TRAIL-induced apoptosis by NF-kappaB inhibition. Int J Cancer *124*: 1301-1311, 2009.

Received July 11, 2011
Revised June 5, 2011
Accepted June 6, 2011