Apoptosis
DOI 10.1007/s10495-010-0521-9

ORIGINAL PAPER

Histone deacetylase inhibitors synergistically potentiate death receptor 4-mediated apoptotic cell death of human T-cell acute lymphoblastic leukemia cells

Eun-Sil Sung · Aeyung Kim · Joon Seong Park · Junho Chung · Myung-Hee Kwon · Yong-Sung Kim

Abstract Cell-death signaling through the pro-apoptotic tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors, death receptor 4 (DR4) and DR5, has shown tumor-selective apoptotic activity. Here, we examine susceptibility of various leukemia cell lines (HL-60, U937, K562, CCRF-CEM, CEM-CM3, and THP-1) to an anti-DR4 agonistic monoclonal antibody (mAb), AY4, in comparison with TRAIL. While most of the leukemia cell lines were intrinsically resistant to AY4 or TRAIL alone, the two T-cell acute lymphoblastic leukemia (T-ALL) lines, CEM-CM3 and CCRF-CEM cells, underwent synergistic caspase-dependent apoptotic cell death by combination of AY4 or TRAIL with a histone deacetylase inhibitor (HDACI), either suberoylanilide hydroxamic acid (SAHA) or valproic acid (VPA). All of the combined treatments synergistically downregulated several anti-apoptotic proteins (c-FLIP, Bcl-2, Bcl-X_L, XIAP, and survivin) without significant changing the expression levels of pro-apoptotic proteins (Bax and Bak) or the receptors (DR4 and DR5). Downregulation of c-FLIP to activate caspase-8 was a critical step for the synergistic apoptosis through both extrinsic and intrinsic apoptotic pathways. Our results demonstrate that the HDACIs have synergistic effects on DR4-specific mAb AY4-mediated cell death in the T-ALL cells with comparable competence to those exerted by TRAIL, providing a new strategy for the targeted treatment of human T-ALL cells.

Keywords Death receptor 4 · Agonistic antibody · TRAIL · Histone deacetylase inhibitor · Apoptosis

Introduction

Ideal anti-cancer therapeutics would induce cell death selectively for tumor cells but not normal cells. In this regard, pro-apoptotic tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors, death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2), are attractive targets for the development of agonistic anti-cancer biologics. Stimulation of DR4 and DR5 by the cognate ligand TRAIL or agonistic monoclonal antibodies (mAbs) can selectively kill tumor cells by eliciting intracellular apoptotic cell death signaling both in vitro and in vivo with little cytotoxicity in normal cells [1, 2]. Activation of DR4 and DR5 by agonist-induced clustering recruits the adaptor protein Fas-associated death domain (FADD) and then pro-caspase-8 to form the death-inducing signaling complex (DISC) [1–3]. Activated caspase-8 by DISC formation can trigger the extrinsic apoptotic pathway by directly activating the downstream effector caspases,

Electronic supplementary material The online version of this article (doi:10.1007/s10495-010-0521-9) contains supplementary material, which is available to authorized users.

E.-S. Sung · A. Kim · Y.-S. Kim (✉)
Department of Molecular Science and Technology, Ajou University, San5, Woncheon-dong, Yeongtong-gu, Suwon 443-749, Korea
e-mail: kimys@ajou.ac.kr

J. S. Park
Department of Hematology-Oncology, Ajou University School of Medicine, Suwon 442-721, Korea

J. Chung
Department of Biochemistry and Molecular Biology and Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799, Korea

M.-H. Kwon
Department of Microbiology, Ajou University School of Medicine, Suwon 442-721, Korea

Published online: 26 June 2010

such as caspase-3, -6, and -7. Caspase-8 can also cleave Bid and the cleaved Bid (tBid) cause translocation of Bax and/or Bad to mitochondria to activate the intrinsic/mitochondrial apoptotic pathway [1]. In some cells (designated Type I cells), caspase-8 activation is sufficient to activate the effector caspases to execute apoptosis via the extrinsic pathway, whereas, in other cells (designated Type II cells), amplification of the extrinsic pathway through the intrinsic pathway is needed to commit the cells to apoptotic cell death [2, 3].

However, most malignant tumors, including hematological malignant leukemia and lymphoma, often remain resistant to TRAIL receptor agonists alone, the underlying mechanisms of which can be varied depending on the cell types [4–7]. Potential mechanisms that inhibit the extrinsic pathway include downregulation, deletion and/or mutation of DR4/DR5, decreased caspase-8 expression, and overexpression of cellular FLICE-inhibitory protein (c-FLIP) [2, 3]. c-FLIP competes with procaspase-8 for binding to FADD to inhibit procaspase-8 activation [8]. Conversely, the intrinsic apoptotic pathway can be inhibited by upregulation of pro-survival Bcl-2/Bcl-X_L; upregulation of inhibitors of apoptosis proteins (IAPs), such as X-linked IAP (XIAP) and survivin; sustained activation of phosphatidylinositol-3-kinase (PI3K)/NFκB; or downregulation of pro-apoptotic Bax/Bak [9]. These mechanisms thus can contribute to resistance to DR4/DR5-mediated cell death. Chemotherapeutic agents can counteract these resistance mechanisms by regulating downstream components in the extrinsic or intrinsic apoptotic pathways, which would enhance TRAIL- or anti-DR4/DR5 mAb-induced apoptosis of normally resistant tumor cells, including primary leukemia cells and established leukemia cell lines [2, 3]. For example, subtoxic dosages of chemotherapeutic agents with different mechanisms of action, such as DNA damaging agents (doxorubicin and etoposide) [4, 10, 11], proteasome inhibitors (MG132) [12], phosphatidylinositol-3-kinase (PI3K)/Akt inhibitor (perifosine) [13], and NFκB pathway inhibitor (SN50) [14, 15], potentiated TRAIL-mediated cell death in various types of leukemia cells by modulating the extrinsic or intrinsic pathways. In particular, histone deacetylase inhibitors (HDACIs) have shown extensive synergistic cell death-inducing activity in combination with TRAIL [3, 16]. Valproic acid (VPA) significantly increased TRAIL-sensitivity of patient-derived primary chronic lymphocytic leukemia (CLL) cells by downregulating c-FLIP [17] and in an established chronic myeloid leukemia (CML) cell line by increasing expression of DR4 and DR5 [18]. Suberoylanilide hydroxamic acid (SAHA) also augmented TRAIL-mediated cytotoxicity in various established acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and chronic myeloid leukemia (CML) cells by amplifying the extrinsic and intrinsic pathways [19–21]. HDACIs combined with agonistic mAbs specific to DR4 or DR5 have also shown synergistic apoptosis-inducing activity in various solid tumors [22] and in primary CLL cells [23, 24].

The receptor-specific agonistic mAb studies demonstrated that, while various solid tumors were more sensitive to DR5-mediated apoptosis than DR4-induced apoptosis [25, 26], primary CLL cells underwent apoptotic cell death almost exclusively through DR4, not DR5 [23, 24, 27]. However, there have been limited studies of how DR4- or DR5-mediated signaling alone or in combination with chemotherapeutic agents can contribute to apoptosis for other leukemia cells, such as ALL, AML, and CML cells. Thus evaluation and detailed mechanistic studies of DR4- and DR5-selective cell death in these other leukemia cell lines are necessary to differentiate between the specific roles of DR4 and DR5 and to identify optimal combinations of receptor agonists and chemotherapeutic agents for targeted cancer therapy.

We have recently reported the development of a murine mAb, AY4 (IgG1, κ), which binds specifically to the extracellular domain of DR4 and induces caspase-dependent apoptotic cell death in various types of cancer cells without significant toxicity to normal human hepatocytes [28]. Although AY4 recognizes regions of DR4 distinct from those bound by TRAIL, AY4 stimulation of DR4 can activate both the extrinsic and intrinsic apoptotic pathways in AY4-sensitive tumor cells [28, 29]. In this study, we further investigate the specific role of DR4 in inducing apoptosis by studying the susceptibility of various leukemia cell lines to AY4 alone or in combination with various chemotherapeutic agents, in comparison with TRAIL. We found that most leukemia cell lines are intrinsically resistant to AY4 or TRAIL alone, but two T-cell acute lymphoblastic leukemia (T-ALL) cell lines become substantially sensitized by combined treatment of AY4 or TRAIL with a HDACI, either SAHA or VPA, by synergistic amplification of both the extrinsic and intrinsic apoptotic pathways.

Materials and methods

Materials

Soluble TRAIL (residues 114–281 with a C-terminal 6× His-tag; described as TRAIL hereafter) and mAb AY4 were prepared as previously described [28]. SAHA, doxorubicin, and etoposide were from Sigma (St Louis, MO); VPA and MG132 were from Calbiochem (San Diego, CA); SN50 was from BIOMOL (Plymouth Meeting, PA). SAHA, doxorubicin, etoposide, and MG132 were dissolved in DMSO (Sigma), and VPA and SN50 were

dissolved in H_2O. The pan-caspase inhibitor Z-VAD-fmk, caspase-8 inhibitor Z-IETD-fmk, and caspase-9 inhibitor Z-LEHD-fmk were from Santa Cruz Biotechnology (Santa Cruz, CA).

Rabbit anti-PARP, anti-Bid, anti-Bcl-X_L, and anti-XIAP antibodies were from Cell Signaling Technology (Danvers, MA); mouse anti-Bcl-2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-caspase-8 and anti-Bak antibodies were from BD Pharmingen (San Diego, CA); rabbit anti-caspase-3 antibody was from Assay Designs (Ann Arbor, Michigan); rabbit anti-survivin was from Calbiochem (San Diego, CA); rabbit anti-β-actin was form BETHYL Laboratories, Inc. (Montgomery, TX); mouse anti-Bax antibody was from Abcam (Cambridge, MA); mouse anti-FLIP antibody was from ALEXIS Biochemicals (Plymouth Meeting, PA); goat anti-rabbit horseradish peroxidase-conjugated antibody was from ZYMED Laboratories (South San Francisco, CA); goat anti-mouse horseradish peroxidase-conjugated antibody was from Cell Signaling Technology.

Cell lines and cell cultures

Non-adherent human leukemia cell lines HL-60 (acute promyelocytic leukemia, AML), U937 (monoblastic leukemia), K562 (chronic myelocytic leukemia, CML), CCRF-CEM (acute T-lymphoblastic leukemia, T-ALL), CEM-CM3 (acute T-lymphoblastic leukemia, T-ALL), and THP-1 (acute monocytic leukemia, AML) were purchased from ATCC (American Type Culture Collection, Manassas, VA) or KCLB (Korean Cell Line Bank, Korea). HL-60, U937, K562, CCRF-CEM, and THP-1 cell lines were cultured in RPMI1640 (WELGENE, Korea) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (GIBCO/Invitrogen, Carlsbad, CA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (WELGENE, Korea). CEM-CM3 cells were cultured in DMEM (WELGENE) supplemented with 20% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator and routinely screened for Mycoplasma contamination.

Cell viability assays

Cells, seeded at a density of 2×10^4 cells/well in 96-well plates, were cultured for ~24 h and then treated with medium, mAb AY4 (10 μg/ml), or TRAIL (0.5 μg/ml) for the indicated periods in figure legends. The cytotoxicity of chemotherapeutic agents for various leukemia cells were determined by incubating the cells for 24 h with increased dosages of each chemotherapeutic agent as follow, 0–10 μM of doxorubicin, 0–200 μM of etoposide, 0–100 μM of MG132, 0–200 μM of SAHA, 0–10 μM of SN50, and 0–30 mM of VPA. The cytotoxicity of ABT-737 (Selleck Chemicals, Houston, TX), a BH3 mimetic [30] for CCRF-CEM cells was examined by treating the cells with various concentrations of ABT-737 (0.001–100 μM) for 24 h (Supplementary Fig. S2). For combined treatment of either mAb AY4 or TRAIL with a chemical agent, cells were co-treated for 24 h with either AY4 (10 μg/ml) or TRAIL (0.5 μg/ml) in combination with an individual chemical agent at a subtoxic dosage. Effects of caspase inhibitor were examined by first pretreating cells for 0.5 h with Z-VAD-fmk, Z-IETD-fmk or Z-LEAD-fmk before subsequent treatment with the other agents. Cell viability was analyzed using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)-based cell growth determination kit (Sigma) and data were presented as a percentage relative to untreated control cells using the following formula: cell viability (%) = (absorbance of sample treated cells − blank wells)/(absorbance of untreated wells − blank wells) × 100 [31, 32]. The results are presented as mean ± standard deviation (SD) values from at least three independent experiments. The cytotoxicity data of chemotherapeutic agents were analyzed by using a four parameter sigmoidal curve fit on Sigma plot software (SPSS Inc.) to calculate IC_{50}, the concentration of an anti-cancer agent giving a 50% reduction in cell viability [28, 31]. The IC_{50} values are presented as mean ± SD of triplicate determinations (Supplementary Table S1).

Western blotting analysis

Cells (5×10^5 cells/well) plated in 6-well plates were treated with medium, AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM), VPA (1 mM), doxorubicin (0.2 μM), etoposide (1 μM), MG132 (1 μM), or SN50 (0.1 μM) alone as well as in combination for 12 or 24 h, as specified in the figure legends. A standard procedure for Western blotting was performed as described previously [28, 32]. The horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used to visualize proteins via an enhanced chemiluminescence system (ECL, Thermo Scientific Inc, Rockford, IL).

Flow cytometry

For apoptosis detection and quantification, cells were treated as specified in the figure legends, stained with Annexin-V-FITC and propidium iodide (PI) (BD Pharmingen) and then analyzed by a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), as described previously [28, 29].

Cell-surface expression levels of DR4 and DR5 were determined by flow cytometry after indirect immunofluorescent labeling of cells using rabbit anti-TRAIL-R1/DR4

or TRAIL-R2/DR5 mAbs (Koma Biotechnology, Korea) followed by goat anti-rabbit FITC-conjugated IgG (PIERCE) [28]. To investigate the effects of SAHA and VPA on expression of DR4 and DR5, CEM-CM3 and CCRF-CEM cells were treated with SAHA (1 μM) or VPA (1 mM) for 24 h before flow cytometry analysis as described above.

RNA interference

Small interfering RNA (siRNA) oligonucleotides were synthesized at Bioneer Co. (Daejeon, Korea). The mRNA sequences targeted by c-FLIP$_L$ siRNA was 5′-AAGGAA CAGCTTGGCGCTCAA-3′, which was used in a previous study [33]. An unrelated siRNA with a sequence of 5′-AATTCTCCGAACGTGTCACGT-3′ was also employed as a control [33]. CCRF-CEM cells were transfected with 2 μM of siRNA by electroporation using MicroPorator MP-100 according to manufacturer's instruction (NanoEn Tek Inc., Korea) [31], and downregulation of c-FLIP$_L$ expression was examined by Western blotting after incubation for 48 h. After 24 h of siRNA transfection, the cells (2×10^4 cells/well in 96-well plate) were treated with AY4 (10 μg/ml) or TRAIL (0.5 μg/ml) alone or in combination with SAHA (1 μM) or VPA (1 mM) for 24 h prior to MTT assay.

Statistical analysis

Statistical significance was analyzed by Student's t test (two-sided) with P values < 0.05 considered significant. Synergistic cytotoxicity was determined by isobolographic analysis [34].

Results

Responses of various leukemia cell lines to AY4 or TRAIL alone

The anti-DR4 agonistic mAb AY4 efficiently induces apoptotic cell death of the TRAIL-sensitive leukemia cell lines Ramos (Burkitt's lymphoma) and Jurkat (acute T-cell leukemia), but not of TRAIL-sensitive HL-60 (AML) [28]. To further explore the anti-tumor activity of AY4 for other leukemia cells, a variety of leukemia cell lines, such as U937 (monoblastic leukemia), K562 (CML), CCRF-CEM (ALL), CEM-CM3 (ALL), and THP-1 (AML) were treated with AY4, in comparison with TRAIL. The cells were incubated with 10 μg/ml AY4 or 0.5 μg/ml TRAIL for 24 h, under the condition of which AY4 and TRAIL alone induced more than 60% of cell death for AY4- and TRAIL-sensitive cells, respectively [28, 29]. All tested leukemia cells, except for CEM-CM3, which underwent ~28% cell death by MTT assay, were highly resistant (less than 15% cell death) to AY4 treatment on its own (Fig. 1). In contrast, TRAIL significantly induced cytotoxicity in HL-60 (~50%) and U937 (~35%) cells and moderately induced cytotoxicity in CEM-CM3 cells (~22%), but showed only slight effects on cell viability of K562 (~15%), THP-1 (~15%) and CCRF-CEM (~10%) cells (Fig. 1), in good agreement with previous reports [4, 5, 20, 21, 23, 27, 28]. TRAIL can engage DR4 and/or DR5 to transduce intracellular apoptotic signaling [1]. The much higher sensitivity of HL-60 and U937 cells to TRAIL than DR4-specific mAb AY4 suggested that DR5 is a preferential receptor for the two cells in TRAIL-induced apoptosis, consistent with previous results [23, 35]. Particularly, HL-60 cells underwent monocytic maturation rather than apoptosis by DR4 stimulation through NFκB pathway [35].

Screening of chemotherapeutic agents for synergistic effects on AY4- and TRAIL-mediated cytotoxicity in various leukemia cell lines

Since most of the established leukemia cell lines examined here were highly resistant to AY4 treatment, we combined AY4 treatment with chemotherapeutic agents with different mechanisms of action [3], such as DNA damaging agents (doxorubicin, etoposide), a proteasome inhibitor (MG132), an NFκB inhibitor (SN50), and HDACIs (SAHA, VPA), to investigate whether any combinations can enhance AY4 response, in comparison with TRAIL. The six chemotherapeutic agents mentioned have been reported to augment TRAIL-induced apoptosis in various types of tumor cells, including primary leukemia cells from patients [3]. Subtoxic doses of each chemotherapeutic agent for each cell line were determined to be the concentrations at which cytotoxicity was between 5–15% in the cells after incubation for 24 h (Supplementary Fig. S1), and there was a slight variation in subtoxic dose among the cell lines, as described in the legend for Fig. 1. The subtoxic dosages of each agent were very similar to those previously used in combination with TRAIL to induce synergistic cell death of various tumor cells [11, 12, 14, 17, 20, 21, 23]. The cells were treated for 24 h with subtoxic dosage of each chemical agent alone or in combination with either AY4 (10 μg/ml) or TRAIL (0.5 μg/ml) prior to the determination of cell viability. While combined treatments of either AY4 or TRAIL with etoposide, doxorubicin, SN50, or MG132 exhibited moderate or merely additive cytotoxicity for all tested leukemia cells, SAHA and VPA significantly enhanced both AY4- and TRAIL-induced cell death in CEM-CM3 and CCRF-CEM cells beyond what may be attributed to additive effects (Fig. 1).

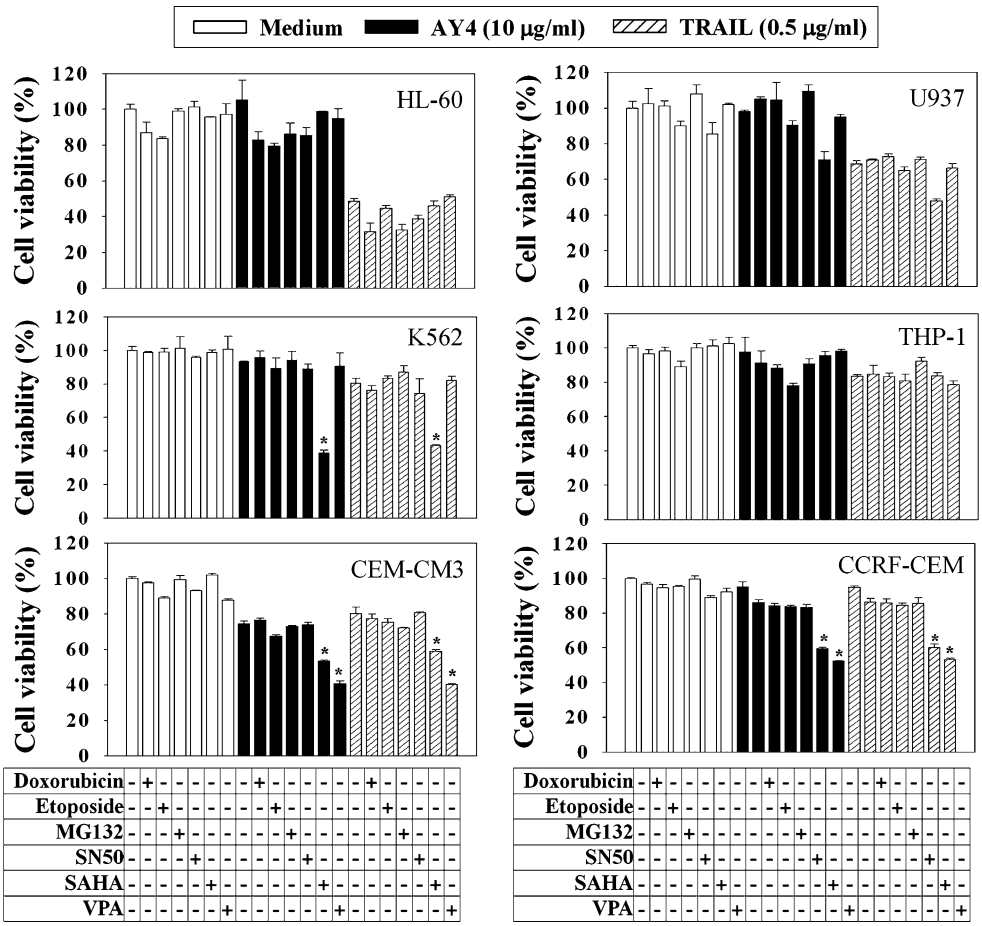

Fig. 1 Sensitivity of various leukemia cell lines to anti-DR4 agonistic mAb AY4, TRAIL, or chemotherapeutic agents, treated alone or in combination. The six cell lines were left untreated (medium) or treated for 24 h with AY4 (10 μg/ml), TRAIL (500 ng/ml) or subtoxic concentrations of chemotherapeutic agents as follows: doxorubicin (0.1 μM for U937, THP-1; 0.2 μM for HL-60, K562, CEM-CM3, CCRF-CEM), etoposide (1 μM for HL-60, U937, THP-1, CEM-CM3, CCRF-CEM; 10 μM for K562), MG132 (1 μM for all cell lines), SAHA (1 μM for HL-60, U937, CEM-CM3, CCRF-CEM; 2 μM for THP-1; 10 μM for K562), SN50 (0.1 μM for all cell lines), and VPA (1 mM for all cell lines). The cells were also treated for 24 h with combinations of chemotherapeutic agents with either AY4 or TRAIL using the above concentrations for each agent. Cell viability was assessed by MTT assay. *Asterisk* (*) indicates the combinations showing much higher cytotoxicity than would be expected from additive cytotoxicity of the two agents. *Error bars*, SD

Combining two-fold greater dosages of etoposide, doxorubicin, SN50, or MG132 with either AY4 or TRAIL resulted in only additive cytotoxicity without synergistic effects (data not shown). Interestingly, SAHA substantially potentiated both AY4- and TRAIL-mediated cell death in K562 cells, while VPA had no such synergy. This difference between SAHA and VPA may be due to the fact that SAHA can inhibit all of four classes of HDACs while VPA can inhibit only Class I and IIa HDACs [16]. Different classes of HDACIs have also previously been found to induce diverged synergistic effects on TRAIL-induced apoptosis in K562 and Jurkat cells [27]. SAHA and VPA failed to enhance TRAIL-induced cytotoxicity beyond mere additivity in TRAIL-sensitive HL-60 and U937 cells, probably because subtoxic concentrations of the HDACIs negligibly contributed to the apoptosis-inducing effects excessively exerted by TRAIL alone in the cells [20, 21]. Taken together, the results show that, among the six chemotherapeutic agents tested, SAHA and VPA substantially enhance AY4- or TRAIL-mediated cytotoxicity only in CEM-CM3 and CCRF-CEM cells.

SAHA and VPA synergize both AY4- and TRAIL-induced apoptosis in CEM-CM3 and CCRF-CEM cells

To determine whether the enhanced cytotoxicity observed with the combined treatments was truly synergistic, CEM-CM3 cells were treated with serial concentrations of AY4 (0.4–10 μg/ml), TRAIL (0.02–0.5 μg/ml), SAHA (0.25–2 μM), or VPA (0.25–2 mM) as a single agent or in their combined forms at various concentration ratios for the isobolographic analysis [34]. The concentrations of each

single agent required to cause ~50% cytotoxicity were determined and then used to get normalized fractional inhibitory concentrations of each agent used in the combined treatments with the similar cytotoxicity. In the isobolographic analysis, the straight line joining the two points obtained from treatment with each single agent alone represents the additive effect expected by combining the two agents [34]. As shown in Fig. 2a, the experimental measurements of the four combined treatments (AY4 with SAHA or VPA and TRAIL with SAHA or VPA) were well below the theoretical additive line. These data demonstrated that, in CEM-CM3 cells, the potentiated cytotoxic effects of AY4 or TRAIL treatment in conjunction with the HDACIs resulted from synergistic cytotoxic interactions. The synergistic cytotoxic effect of all the combined treatments was also observed in CCRF-CEM cells by isobolographic analysis (data not shown).

Synergistic effects of either SAHA or VPA on AY4- and TRAIL-induced cell death were further confirmed by an apoptotic cell death assay, which was monitored by Annexin V-FITC and PI analysis using flow cytometry. Compared with treatment by single agent alone, the combined treatments induced significant levels of Annexin V-FITC-positive staining in CEM-CM3 and CCRF-CEM cells (Fig. 2b), indicative of the synergistic apoptotic cell death. These results clearly show that SAHA and VPA synergistically potentiate both AY4- and TRAIL-induced apoptotic cell death for CEM-CM3 and CCRF-CEM cells. In K562 cells, SAHA, but not VPA, significantly synergized with the apoptosis-inducing activity of both AY4 and TRAIL (Fig. 2b), consistent with the data from MTT assay (Fig. 1).

Cell-surface expression levels of DR4 and DR5 are not closely correlated with AY4 or TRAIL-induced cytotoxicity

We next used flow cytometry to determine whether cell-surface expression levels of DR4 and DR5 in leukemia cells can explain their differential responses to AY4- and TRAIL-induced cytotoxicity. CEM-CM3 cells that were moderately sensitive to AY4 expressed much lower levels of DR4 than the cell lines that were highly resistant to AY4, such as K562 and THP-1 (Fig. 3a). DR5 expression

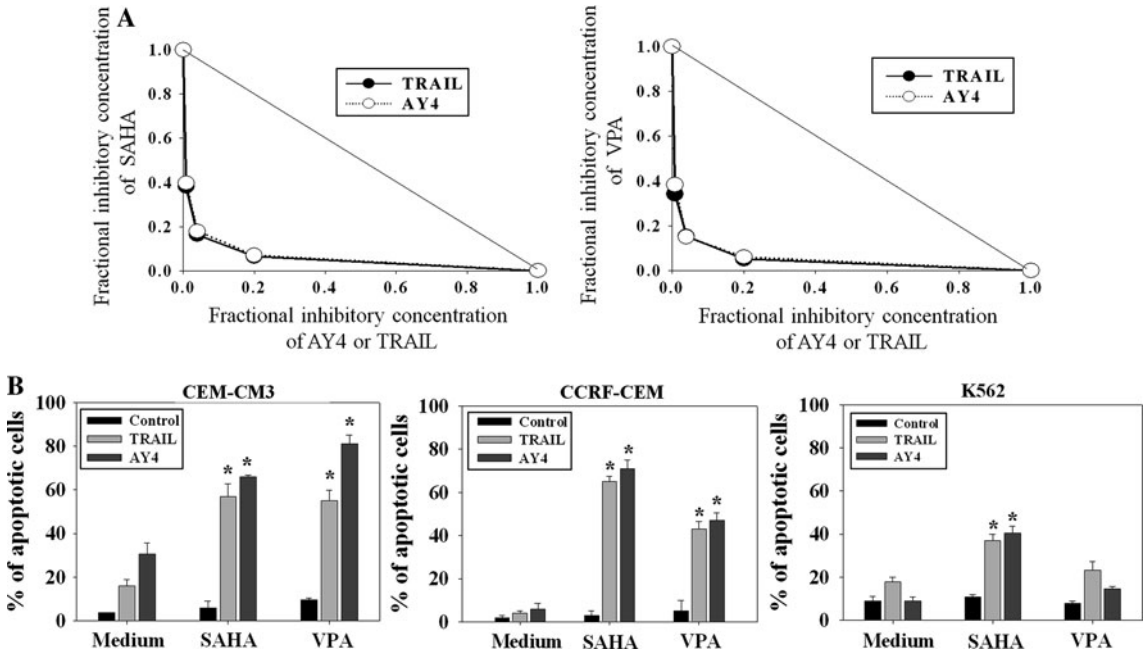

Fig. 2 Synergistic cytotoxicity by combining treatment of AY4 or TRAIL with either SAHA or VPA. **a** Isobolographic analysis of synergistic cytotoxicity by the combined treatments in CEM-CM3 cells. CEM-CM3 cells were treated for 24 h with AY4 (0.4–10 μg/ml), TRAIL (0.02–0.5 μg/ml), SAHA (0.25–2 μM), or VPA (0.25–2 mM) as a single agent, or in combinations of AY4 plus either SAHA or VPA and TRAIL plus either SAHA or VPA at various concentration ratios. Isobolograms were obtained showing ~50% cytotoxicity, as described in detail in the text. The diagonally drawn *solid line* represents the theoretical additive line of the combined treatments, and the symbols indicate the experimental values. **b** Synergistic apoptosis was assessed by Annexin-V staining for the combined treatments in CEM-CM3, CCRF-CEM, and K562 cells. The cells were treated with medium, AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM for CEM-CM3 and CCRF-CEM cells; 10 μM for K562), or VPA (1 mM) alone or in combination for 24 h (as indicated in each panel). After dual staining of the cells with Annexin-V-FITC and PI followed by flow cytometry analysis, the percentages of Annexin-V-FITC positive cells (% of apoptotic cells) were plotted. *Asterisk* (*), $P < 0.05$ (compared to the additive value of the corresponding two single agents)

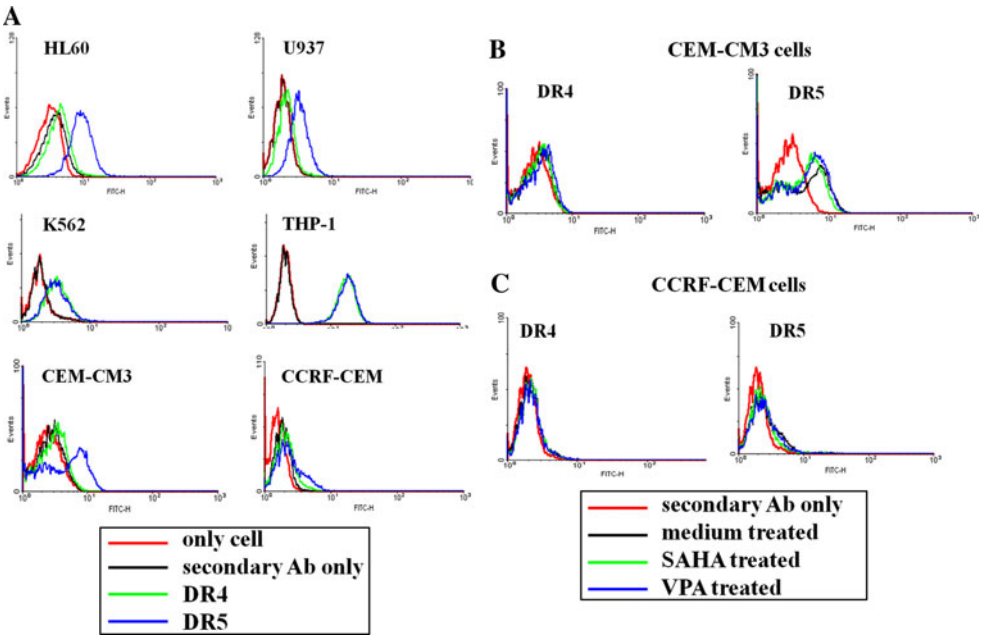

Fig. 3 Analysis of cell-surface expression levels of DR4 and DR5 in various leukemia cells. **a** Surface expression of DR4 (*green lines*) and DR5 (*blue lines*) was measured by flow cytometry analysis on various human leukemia cell lines. Commercial DR4- and DR5-specific goat mAbs were used for the analyses by comparing negative control cells that were unlabeled (*red lines*) or labeled by only secondary antibody (*black lines*). **b**, **c** CEM-CM3 (**b**) and CCRF-CEM (**c**) cells were incubated for 24 h with SAHA (1 μM) or VPA (1 mM) and then cell-surface expression levels of DR4 and DR5 were measured as above

levels in almost TRAIL-resistant K562, CEM-CM3, and CCRF-CEM cells were slightly lower than or comparable to those of TRAIL-sensitive HL-60 and U937 cells. Even though THP-1 cells exhibited the highest expression levels of both DR4 and DR5 among the cell lines examined, they were highly resistant to AY4, TRAIL, and any combined treatment with the tested chemotherapeutic agents. A previous report showed that TRAIL-resistant THP-1 cells become sensitized to TRAIL-induced apoptosis when treated in combination with a phosphatidylinositol-3-kinase (PI3K)/Akt signaling inhibitor, perifosine [13]. Thus the cell-surface expression levels of DR4 and DR5 in leukemia cells are not strictly correlated with susceptibility to AY4- and TRAIL-induced cell death, in agreement with previous reports [11, 28, 36].

Many studies have attributed synergistic cytotoxicity by combining TRAIL or anti-DR5 agonistic mAbs with HDACIs to upregulation of DR4 or DR5 expression on the cell surface [17, 18, 21, 37]. When treated with SAHA (1 μM) or VPA (1 mM) for 24 h, both CEM-CM3 and CCRF-CEM cells exhibited little or no change in DR4 and DR5 cell surface expression levels, compared with the untreated control cells (Fig. 3b, c). These data suggest that upregulation of the pro-apoptotic receptors DR4 and DR5 is not involved in the synergistic cell death of CEM-CM3 and CCRF-CEM cells by the combined treatment of AY4 or TRAIL with either SAHA or VPA. Previous studies have also shown that combining TRAIL with HDACIs, such as SAHA and depsipeptide, synergistically induces apoptosis in various leukemia cell lines (U937, Jurkat, HL-60, and K562 cells) without significantly changing DR4 and DR5 expression levels [20, 23]. Thus other factors at downstream of the receptors may be involved in the synergistic apoptosis by the combined treatments.

SAHA and VPA synergize with both AY4- and TRAIL-mediated cell death by amplifying both extrinsic and intrinsic apoptotic pathways in CEM-CM3 and CCRF-CEM cells

To understand how SAHA and VPA synergistically enhanced AY4- and TRAIL-mediated apoptotic cell death in CEM-CM3 and CCRF-CEM cells, Western blotting was used to analyze the activation of various intracellular proteins involved in apoptosis. Cells were exposed to AY4, TRAIL, SAHA, and VPA, singly or in combination, for 12 or 24 h prior to Western blotting. In CEM-CM3 cells, which were moderately sensitive to both AY4 and TRAIL, either agonist alone exhibited only moderate cleavage of procaspase-9, procaspase-3 and poly(ADP-ribose) polymerase (PARP) after 12 h incubation, despite of negligible activation of caspsae-8 and Bid at the same period (Fig. 4a). However, the longer incubation of 24 h significantly activated all of the pro-apoptotic molecules examined (Fig. 4a). In CCRF-CEM cells, however, no significant activation of the pro-apoptotic molecules was observed even after 24 h treatment by AY4 and TRAIL as a single agent (Fig. 4b), consistent with the almost complete resistance of CCRF-CEM cells to the single agent treatments (Fig. 1). Treatment of SAHA or VPA alone at subtoxic concentrations exhibited negligible activation of the pro-apoptotic molecules even after 24 h incubation in both cell lines. In contrast, co-treatment of the HDACIs with either AY4 or TRAIL induced more significant

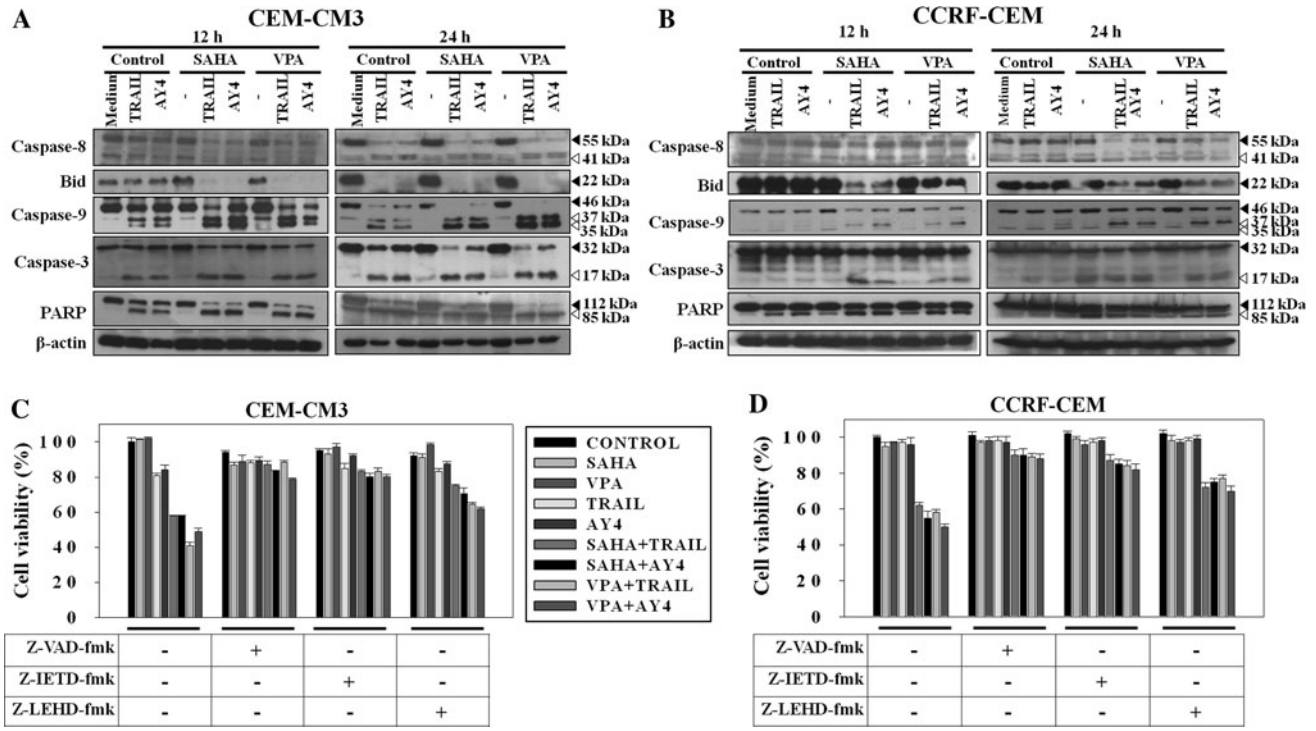

Fig. 4 Combined treatment of AY4 or TRAIL with either SAHA or VPA induces caspase-dependent apoptotic cell death. **a**, **b** Detection of proteolytic processing of apoptotic molecules by Western blotting of CEM-CM3 (**a**) and CCRF-CEM (**b**) cells, which were treated with medium, AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM), or VPA (1 mM) alone or in combination for 12 or 24 h (as indicated in each panel). Pro-forms (*black arrowhead*) and cleaved forms (*white arrowhead*) of caspase-8, Bid, caspase-9, caspase-3, and PARP are indicated. The *β*-actin protein levels are included as a control for protein loading. **c**, **d** Effect of caspase inhibitors on cell viability of CEM-CM3 (**c**) and CCRF-CEM (**d**) cells. The cells were pretreated for 0.5 h with the pan-caspase inhibitor Z-VAD-fmk (30 μM), the caspase-8 inhibitor Z-IETD-fmk (30 μM), or the caspase-9 inhibitor Z-LEHD-fmk (30 μM) and then further incubated for 24 h with medium, AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM), or VPA (1 mM) alone or in combination (as indicated in each panel) prior to the determination of cell viability by MTT assay. *Error bars*, SD

activation of caspase-8, caspase-3, Bid, caspase-9, and PARP, from 12 h incubation in both cell lines, compared with those of each agent alone (Fig. 4a, b). The pro-apoptotic molecules were more substantially activated by the combination treatments in CEM-CM3 that were more susceptible to both AY4 and TRAIL than CCRF-CEM cells (Figs. 1, 4).

Noticeably, the four combined treatments induced activation of Bid and caspase-9 in both cell lines, suggesting that the extrinsic pathway triggered by the receptor agonists are connected to the mitochondrial/intrinsic pathway. To further dissect the respective contributions of the extrinsic and intrinsic pathways in apoptotic cell death of CEM-CM3 and CCRF-CEM cells, the cells were pretreated for 0.5 h with the pan-caspase inhibitor Z-VAD-fmk (30 μM), the caspase-8 inhibitor Z-IETD-fmk (30 μM), or the caspase-9 inhibitor Z-LEHD-fmk (30 μM) [28] prior to co-treatment of AY4 or TRAIL with either SAHA or VPA for 24 h. The presence of Z-VAD-fmk or Z-IETD-fmk conferred almost complete resistance to all of the combined treatments for both cell lines, whereas Z-LEHD-fmk only partially protected the cells from the combined treatments (Fig. 4c, d), suggesting that the combined treatments induced caspase-dependent apoptotic cell death in the two cells. These results further suggested that caspase-8 activation is a critical first step in the activation of both the extrinsic and intrinsic apoptotic pathways and that the two pathways simultaneously contribute to caspase-dependent apoptotic cell death. Taken together, our results demonstrated that both SAHA and VPA synergistically sensitize CEM-CM3 and CCRF-CEM cells to AY4- and TRAIL-mediated apoptotic cell death by amplifying both the extrinsic and intrinsic pathways.

Combined treatment of AY4 or TRAIL with either SAHA or VPA downregulates expression level of c-FLIP in CEM-CM3 and CCRF-CEM cells

In AY4- and TRAIL-resistant CCRF-CEM cells, caspase-8 was activated only by the combined treatment with SAHA or VPA, but not by either agent alone, even up to 24 h (Fig. 4b). Even in CEM-CM3 cells that were moderately sensitive to both AY4 and TRAIL, the combined treatments induced faster and more significant activation of

caspase-8 than any single agent treatment (Fig. 4a). It had been previously observed that HDACIs enhance caspase-8 activation by downregulating c-FLIP, a competitive inhibitor of caspase-8 for DISC formation, in TRAIL-induced apoptosis in various leukemia cell lines [17, 18, 20, 21, 37]. Those findings prompted us to determine whether SAHA and VPA, in combination with AY4 or TRAIL, can also decrease c-FLIP expression in CEM-CM3 and CCRF-CEM cells. Single agent treatment with AY4 or TRAIL, but neither SAHA nor VPA, slightly reduced c-FLIP$_L$ expression levels after 24 h incubation in moderately sensitive CEM-CM3 cells. In contrast, both CEM-CM3 and CCRF-CEM cells treated in combination of AY4 or TRAIL with either SAHA or VPA exhibited faster and more dramatic decreases in c-FLIP$_L$ expression levels, as seen after 12 and 24 h incubation (Fig. 5). These results suggested that the combined treatments downregulate c-FLIP expression to facilitate caspase-8 activation at the DISC level.

To further assess the effect of c-FLIP$_L$ expression level on AY4- and TRAIL-mediated apoptosis, we reduced the expression of c-FLIP$_L$ by transfection of siRNA targeted specifically for c-FLIP$_L$ into CCRF-CEM cells before treatments of AY4 and TRAIL individually or in combination with either SAHA or VPA. Compared with an irrelevant siRNA, the c-FLIP$_L$-specific siRNA efficiently reduced the expression level of c-FLIP$_L$ in CCRF-CEM cells (Fig. 6a). Downregulation of c-FLIP$_L$ efficiently sensitized CCRF-CEM cells to the single agent treatment of AY4 or TRAIL, exhibiting comparable cell death-inducing activities to those obtained by the combined treatments of AY4 or TRAIL with either SAHA or VPA (Fig. 6b). In the setting of c-FLIP$_L$ downregulation (i.e., CCRF-CEM cells transfected with c-FLIP$_L$-specific siRNA), however, no synergistically enhanced cytotoxicity was observed by the combined treatments (Fig. 6b). These results suggested that downregulation of c-FLIP$_L$ to activate caspase-8 is a critical step for the synergistic apoptosis exerted by the combinations of AY4 and TRAIL with either SAHA or VPA.

Combined treatment of AY4 or TRAIL with either SAHA or VPA downregulates expression levels of anti-apoptotic proteins in CEM-CM3 and CCRF-CEM cells

Since our results with caspase-9 inhibitors suggested that the intrinsic/mitochondrial pathway is involved in combination-mediated synergistic cytotoxicity, we further examined how SAHA and VPA regulate the expression levels of several proteins closely associated with the intrinsic/mitochondrial pathway, such as the anti-apoptotic

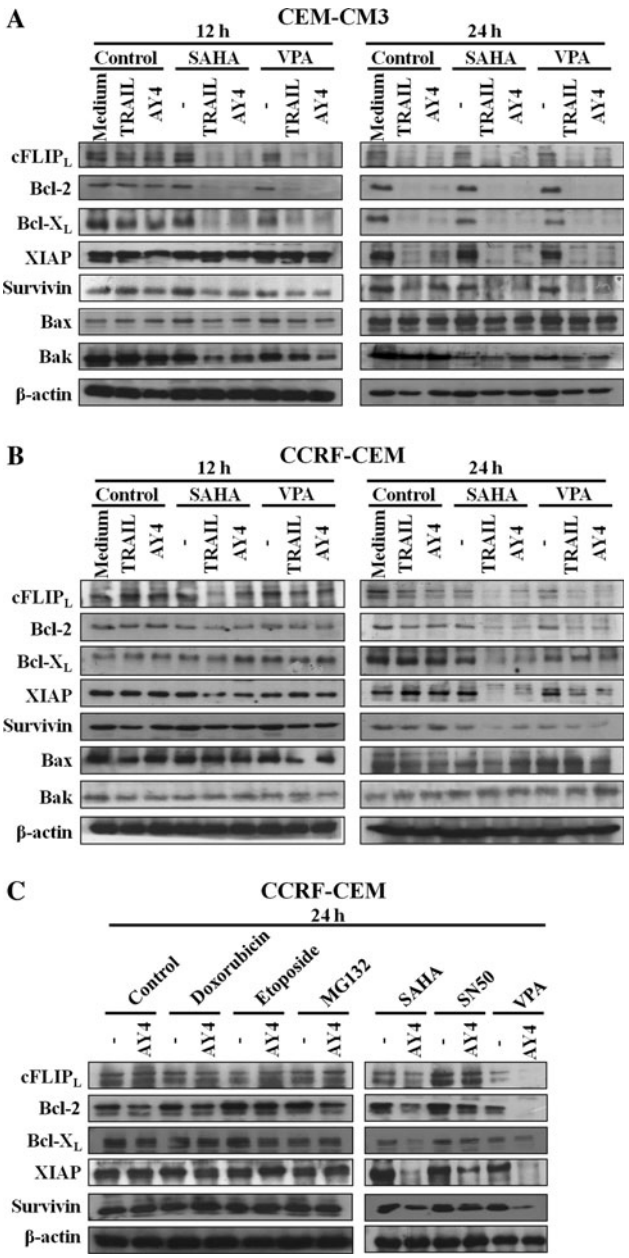

Fig. 5 Combinations of AY4 or TRAIL with either SAHA or VPA synergize apoptotic cell death of CEM-CM3 and CCRF-CEM cells by amplifying both the extrinsic and intrinsic apoptotic pathways. **a**, **b** The expression levels of anti-apoptotic (c-FLIP$_L$, Bcl-2, Bcl-X$_L$, XIAP, and survivin) and pro-apoptotic (Bax and Bak) molecules were detected by Western blotting in CEM-CM3 (**a**) and CCRF-CEM (**b**) cells. Cells were treated with medium, AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM), and VPA (1 mM) alone or in combination for 12 or 24 h (as indicated in each panel). **c** The expression levels of the anti-apoptotic molecules were monitored by Western blotting in CCRF-CEM cells, which were treated for 24 h with medium (control) or the chemotherapeutic agent alone or in combination with AY4 (10 μg/ml) (as indicated in the panel). The subtoxic concentration of each chemotherapeutic agent was used as follows: doxorubicin (0.2 μM), etoposide (1 μM), MG132 (1 μM), SAHA (1 μM), SN50 (0.1 μM) or VPA (1 mM). In **a**–**c**, the *β*-actin protein levels are included as a control for protein loading

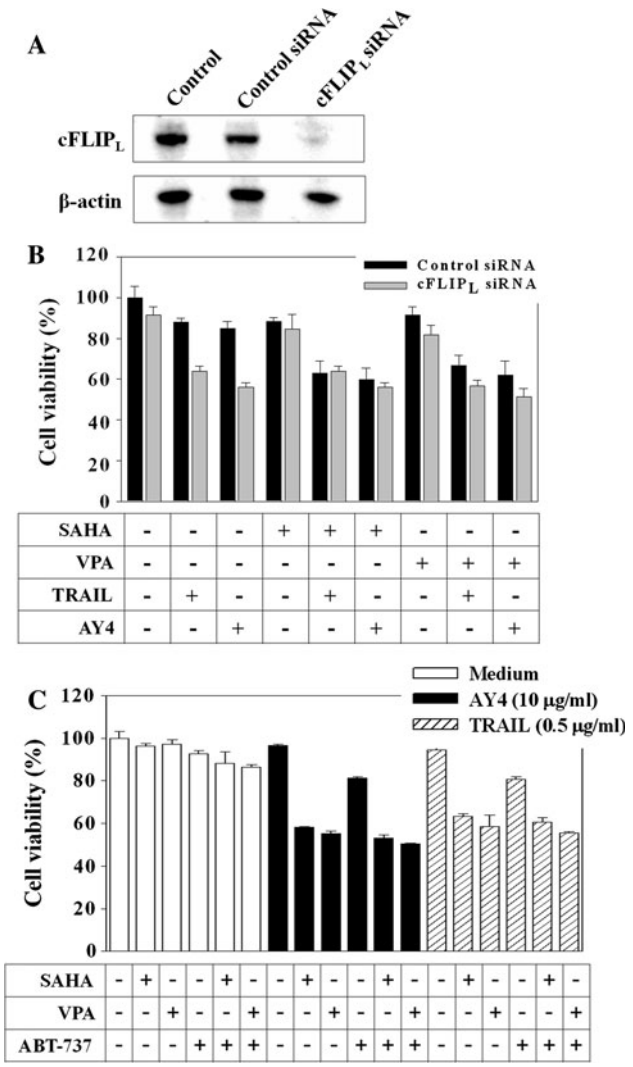

Fig. 6 Downregulation of c-FLIP is a critical step for the synergistic apoptosis exerted by the combinations of AY4 and TRAIL with either SAHA or VPA. **a** Downregulation of c-FLIP$_L$ in CCRF-CEM cells by siRNA technique. After transfection with c-FLIP$_L$-specific (c-FLIP$_L$ siRNA) or irrelevant (control siRNA) siRNA, the cells were cultured for 48 h prior to Western blotting analysis. Untransfected cells (control) were also included as a control. **b** Effect of c-FLIP$_L$ downregulation on the cell viability of CCRF-CEM cells by various treatments. After 24 h of siRNA transfection, the cells were treated with AY4 (10 μg/ml) or TRAIL (0.5 μg/ml) alone or in combination with SAHA (1 μM) or VPA (1 mM) for 24 h prior to MTT assay (as indicated in the panel). **c** Effect of ABT-737 on the cell viability of CCRF-CEM cells by treatments of AY4 (10 μg/ml), TRAIL (0.5 μg/ml), SAHA (1 μM), VPA (1 mM), or ABT-737 (10 μM) alone or in combination for 24 h prior to MTT assay (as indicated in the panel)

proteins of Bcl-2, Bcl-X_L, XIAP, and survivin, and the pro-apoptotic proteins of Bax and Bak (Fig. 5a, b). In CEM-CM3 cells, only the combined treatments of AY4 and TRAIL with SAHA or VPA substantially decreased the expression levels of Bcl-2 and Bcl-X_L, without significant changes in the levels of XIAP, survivin, Bax, and Bak, within the first 12 h (Fig. 5a). After 24 h, however, either AY4 or TRAIL, alone or in combination with one of the two HDACIs, substantially downregulated Bcl-2, and Bcl-X_L, XIAP, and survivin, but not Bax and Bak. Neither SAHA nor VPA alone could affect the expression levels of Bcl-2, Bcl-X_L, XIAP, or survivin, even after 24 h incubation (Fig. 5a). Hence, more rapid downregulations of Bcl-2 and Bcl-X_L observed upon the combined treatments can be attributed to synergistic effects between AY4/TRAIL and SAHA/VPA.

In CCRF-CEM cells that were more resistant to AY4 and TRAIL than CEM-CM3 cells, neither AY4, TRAIL, SAHA, nor VPA as a single agent had any effect on the expression levels of the measured pro- and anti-apoptotic molecules even after 24 h incubation (Fig. 5b). However, the combined treatment of AY4 or TRAIL with either SAHA or VPA significantly downregulated expression levels of Bcl-2, Bcl-X_L, XIAP, and survivin after 24 h incubation, but not at 12 h (Fig. 5b). Expression of pro-apoptotic Bax and Bak in CCRF-CEM cells was not affected by any of the treatments within the incubation periods tested. Our data in both CEM-CM3 and CCRF-CEM cells suggested that the combined treatments decrease the expression levels of the anti-apoptotic proteins, but have no effect on the pro-apoptotic molecules.

To indirectly assess how much downregulation of Bcl-2/Bcl-X_L contributes to the synergistic apoptosis induced by the combined treatments, we examined how ABT-737, a small-molecule BH3 mimetic that binds to and antagonizes Bcl-2/Bcl-X_L [30], affects the AY4- or TRAIL-induced apoptosis in CCRF-CEM cells. Co-treatment of AY4 or TRAIL with a subtoxic dosage of ABT-737 (10 μM) showed reasonably increased cell death-inducing efficiency in CCRF-CEM cells by the additive effects of the two agents, compared with those of the single agent alone (Fig. 6c). The combinations of SAHA (1 μM) or VPA (1 mM) with ABT-737 (10 μM) also showed only additively enhanced cytotoxicity by the two agents. However, the presence of ABT-737 did not further potentiate the synergistic cytotoxicity induced by the combined treatment of AY4 or TRAIL with either SAHA or VPA (Fig. 6c). These results suggested that downregulation of Bcl-2/Bcl-X_L only marginally contributes to the synergistic apoptosis exerted by the combination of AY4 or TRAIL with either SAHA or VPA.

Combined treatment of AY4 with the other agents does not significantly affect expression level of anti-apoptotic proteins in CCRF-CEM cells

Among the six chemotherapeutic agents tested, only SAHA and VPA potentiated AY4-induced apoptotic cell death in CEM-CM3 and CCRF-CEM cells (Fig. 1). To elucidate why the other agents (doxorubicin, etoposide, MG132, and

SN50) did not show the synergistic effects with AY4-mediated cell death, we examined the regulation of anti-apoptotic proteins (c-FLIP$_L$, Bcl-2, Bcl-X$_L$, survivin, and XIAP) in CCRF-CEM cells after treatments for 24 h with the chemical agents alone or in combination with AY4 (Fig. 5c). Unlikely SAHA and VPA, the other agents even in the combination with AY4 failed to decrease substantially the expression levels of c-FLIP$_L$, Bcl-2, Bcl-X$_L$, survivin, and XIAP in CCRF-CEM cells, which may explain why the four agents did not cooperate with the AY4-mediated cell death. Although highly toxic concentrations of etoposide (50 μM) and doxorubicin (50 μM) was reported to decrease the expression levels of XIAP and survivin in HL-60, U937, and Jurkat cells [4], their subtoxic concentrations (1 μM etoposide, 0.2 μM doxorubicin) showed little effects on the expression levels of anti-apoptotic proteins in CCRF-CEM cells (Fig. 5c). Collectively, these results suggested that downregulation of the anti-apoptotic proteins is a determining factor in the synergistic apoptotic cell death mediated by DR4 and/or DR5 stimulation in some leukemia cells, including CCRF-CEM cells.

Discussion

The differential susceptibilities of various leukemia cell lines to TRAIL and anti-DR4 agonistic mAb AY4 could not be directly explained by differences in their surface expression levels of DR4 and DR5 (Figs. 1, 3), suggesting that other factors regulate the sensitivity at the downstream signaling of the receptors. However, it seems that some leukemia cells undergo apoptotic cell death through signaling by either pro-apoptotic DR4 or DR5, but not both, as has been found for various solid tumors [25, 26]. The substantial sensitivity of HL-60 (AML) and U937 (monoblastic leukemia) cells to TRAIL-mediated cytotoxicity, but not to AY4, suggests that DR5 is the main receptor that transduces apoptotic signaling in the leukemia cells. Conversely, CEM-CM3 (T-ALL) cells from this study and Jurkat (T-ALL) cells from our previous study [28] were more sensitive to DR4-mediated cell death stimulated by AY4 rather than TRAIL, though CCRF-CEM T-ALL cells were highly resistant to both AY4 and TRAIL. Previous reports have also shown that some CLL cells were sensitive to anti-DR4 agonistic mAbs, but were resistant to anti-DR5 agonistic mAbs [23, 24] or DR5-selective TRAIL mutants [27]. Thus, it seems that DR4-mediated apoptosis may be more potent in lymphocytic leukemia than myeloid leukemia cells. Taken together, these results reinforce the necessity of determining which receptor plays the more dominant role in death-receptor mediated cell death of targeted tumor cells, including leukemia.

Our studies combining AY4 or TRAIL treatment with six anti-cancer agents revealed that only SAHA and VPA, both HDAC inhibitors, synergistically potentiated both AY4- and TRAIL-mediated cell death in CEM-CM3 and CCRF-CEM T-ALL cells, but not in the other cell lines examined (Figs. 1, 2). Exceptionally, K562 cells were significantly sensitized to co-treatment of AY4 or TRAIL in combination with SAHA, but not with VPA. The other compounds, doxorubicin, etoposide, MG132, and SN50, showed only modest or negligible additive cytotoxic effects when combined with AY4 or TRAIL treatment in all tested leukemia cell lines. In contrast to SAHA and VPA, the four chemical agents as a single agent or in combination with AY4 did not reduce the expression levels of the anti-apoptotic proteins in CCRF-CEM cells (Fig. 5c). Other works, however, have shown that these agents synergistically cooperate with TRAIL or anti-DR4/DR5 agonistic mAbs in other leukemia and lymphoma lines. Doxorubicin, a DNA damaging agent, enhanced TRAIL-induced cell death of the Burkitt lymphoma cell line BJAB by facilitating DR5 clustering on the cell surface [10]. Doxorubicin also showed synergistic cytotoxicity with anti-DR4 agonistic mAb HGS-ETR1 and anti-DR5 agonistic mAb HGS-ETR2 in some primary and established lymphoma cells [11]. Likewise, etoposide and doxorubicin showed synergistic effects with TRAIL by selectively increasing DR5 expression, but not DR4 expression, in HL-60 and Jurkat cells [4]. The proteasome inhibitor MG132 and NFκB pathway inhibitor SN50 effectively cooperated with TRAIL to induce apoptosis in CLL cells by increasing cell surface expression of both DR4 and DR5 [12] and multiple myeloma cells through downregulation of Bcl-X$_L$ [14], respectively. However, the present study revealed that these agents failed to show any cooperative effects with AY4 or TRAIL in all tested leukemia cells. These results suggest that comprehensive screening of chemotherapeutic agents for synergistic combined treatment with TRAIL or anti-DR4/DR5 agonistic mAbs are required for targeted treatment of particular leukemia and lymphoma cell types.

HDACIs, which are promising, tumor-killing anti-cancer agents with low toxicity against normal cells, can also potentiate TRAIL-induced apoptosis in leukemia cells through multiple cellular mechanisms that affect both the extrinsic and intrinsic apoptotic pathways in various tumor types [3, 16]. SAHA has been approved by the U.S. Food and Drug Administration for the treatment of cutaneous T cell lymphoma and other various HDACIs are now under Phase I/II clinical trials as a single agent or in combination with other agents [16, 38]. Our study demonstrated that the HDACIs, SAHA and VPA, significantly enhance both AY4- and TRAIL-induced caspase-dependent apoptotic cell death in CEM-CM3 and CCRF-CEM cells, mainly

through downregulation of anti-apoptotic proteins (c-FLIP, Bcl-2, Bcl-X_L, XIAP, and survivin) without significantly changing expression levels of the pro-apoptotic proteins Bax and Bak or the death receptors DR4 and DR5. Almost complete and substantial inhibitions of synergistic cell death by the caspase-8 and caspase-9 inhibitors, respectively (Fig. 4c, d), suggest that caspase-8 activation is a critical step for the synergistic apoptosis through both the extrinsic and intrinsic apoptotic pathways. Caspase-8, an initiator caspase in the death receptor pathway, can directly activate effector caspases, such as caspase-3, to activate the extrinsic pathway or cleave Bid to activate the mitochondrial pathway, resulting in activation of caspase-9 and then effector caspases [2, 3]. Caspase-8 activation induced by our combined treatments occurred via downregulation of c-FLIP rather than upregulation of DR4 and DR5 in both CCRF-CEM and CEM-CM3 cells (Figs. 3, 4, 5). DISC activation by c-FLIP downregulation without upregulation of DR5 was also previously observed in CLL cells co-treated with TRAIL and a HDACI, depsipeptide [39]. c-FLIP structurally resembles caspase-8 but lacks proteolytic activity, and it blocks death receptor-mediated signaling by preventing caspase 8 activation at the DISC formed by DR4 and/or DR5 stimulation [8]. Many previous reports have shown that HDACIs enhance TRAIL-induced apoptosis by downregulating c-FLIP in various tumor types, such as by sodium butyrate in human colon carcinoma cells [40], by VPA in primary CLL cells isolated from patients [17] and by LAQ824 in Jurkat and HL-60 ALL cells [37]. In both CCRF-CEM and CEM-CM3 cells, compared with the single agent treatments, the combination of AY4 or TRAIL with SAHA or VPA triggered much faster and more substantial decreases in c-$FLIP_L$ expression, which in turn increased caspase-8 activation and Bid cleavage to subsequently activate both the extrinsic and intrinsic apoptotic pathways. No synergistic effects of the combined treatments in the c-$FLIP_L$ downregulated CCRF-CEM cells also highlighted that c-FLIP downregulation plays a main role in the synergistic apoptosis (Fig. 6b). Thus, by inhibiting DR4/DR5-mediated signaling at the DISC level, upregulation of c-FLIP seems to a major resistance mechanism against AY4 and TRAIL-mediated cell death in CCRF-CEM and CEM-CM3 cells, and possibly other leukemia cell lines. However, synergistic apoptosis of other leukemia cell lines (U937, Jurkat, and HL-60) by treatment with TRAIL plus sodium butyrate or SAHA was not due to c-FLIP downregulation, but rather attributed to activation of the intrinsic pathway through Bid cleavage and downregulation of the pro-survival molecules Bcl-2/Bcl-X_L [20].

The ratio between pro- and anti-apoptotic Bcl-2 family proteins at the mitochondrial level can determine cellular fate in response to external stimuli in malignant tumors, including leukemia cells [1, 2, 4]. The anti-apoptotic proteins Bcl-2 and Bcl-X_L associate with the mitochondrial outer membrane and stabilize its integrity, inhibiting mitochondrial release of pro-apoptotic factors (e.g., cytochrome c and Smac). In contrast, the pro-apoptotic proteins Bax and Bak destabilize the outer mitochondrial membrane, increasing the potential of releasing pro-apoptotic factors into the cytosol [3, 9]. Overexpression of the pro-survival proteins Bcl-2/Bcl-X_L is closely correlated with resistance to TRAIL receptor-mediated apoptosis in various tumor types [41], including leukemia cells [4]. Our results showed that, in both CEM-CM3 and CCRF-CEM cells, compared with either AY4 or TRAIL alone, combined treatment of AY4 or TRAIL with either SAHA or VPA induced much faster and more dramatic downregulation of Bcl-2 and Bcl-X_L without significantly changing the expression levels of Bax and Bak (Fig. 5a, b). This decreased ratio of anti-apoptotic proteins to pro-apoptotic proteins in the cells seems to be one of the molecular determinants for the combined treatments to efficiently trigger apoptotic cell death. Similar results were observed in various leukemia cell lines by combined treatment of TRAIL with various HDACIs, including SAHA and VPA [20, 21].

The IAP family of proteins, including XIAP, cIAP-1, cIAP-2, and survivin, can protect cells against apoptosis at a downstream level of mitochondria by directly binding to and inhibiting caspase-9 and -3 [1–3]. High levels of XIAP and survivin in various patient-derived acute leukemia cells were closely associated with poor responses to anti-cancer drug-induced apoptosis in vitro and poor clinical prognostic outcomes [7]. As a result, reduced expression of XIAP and/or survivin in various leukemia cells exposed to combined treatment of TRAIL with various HDACIs is thought to contribute to the synergistic apoptosis-inducing activity observed [20, 21]. In our results, the downregulation of XIAP and survivin was clearly observed in CCRF-CEM cells after the longer incubation of 24 h, but not in 12 h, only when AY4 or TRAIL treatment was combined with either SAHA or VPA. In moderately AY4- and TRAIL-sensitive CEM-CM3 cells, XIAP and survivin downregulation was also detected after 24 h incubation with AY4 or TRAIL either alone or in combinations with an HDACI. These results suggest that downregulation of XIAP and survivin by the combined treatments increases caspase-9 and caspase-3 activity in both CEM-CM3 and CCRF-CEM cells, contributing to the observed increase in apoptotic cell death.

Down-regulations of Bcl-2/Bcl-X_L and XIAP/survivin at the mitochondrial and its downstream levels, respectively, may eventually cooperate each other to increase the activations of caspase-9 and caspase-3 in the combined treatments of both cells (Figs. 4, 5). Substantial inhibition of

synergistic cell death by the caspase-9 inhibitor (Fig. 4c, d) indicated that not only are the effector caspases directly activated by caspase-8 through the extrinsic pathway, but effector caspase activation is also enhanced by the intrinsic pathway. Caspase-3, activated by the intrinsic pathway, cleaves procaspase-8 and amplifies its activation by means of a positive feedback loop between casapase-8 and caspase-3 [1]. Augmented activation of caspase-9 and/or effector caspases by downregulations of Bcl-2/Bcl-X_L and XIAP/survivin may also collaborate with increased caspase-8 activation provided by c-FLIP down-regulation, resulting in the synergistic caspase-dependent apoptotic cell death by the combined treatments. In this aspect, both CEM-CM3 and CCRF-CEM cells behave as type II cells in response to both AY4- and TRAIL-mediated apoptosis, requiring HDACIs, such as SAHA and VPA, to amplify the extrinsic pathway through the mitochondrial pathway to cause efficient cell death.

In summary, our data demonstrate that AY4-mediated apoptosis signaling through DR4 can cooperate with SAHA and VPA to induce synergistic cell death of CEM-CM3 and CCRF-CEM T-ALL cells by downregulating anti-apoptotic proteins (c-FLIP, Bcl-2, Bcl-X_L, XIAP, and survivin) without significant influences on the expression levels of pro-apoptotic proteins (Bax, Bak, DR4 and DR5). The mechanisms by which this occurs are almost identical with those exerted by TRAIL combined with the HDACIs in the same cell lines. These results suggest that, when combined with SAHA or VPA, AY4-mediated signaling exclusively through DR4 is functionally comparable to TRAIL-mediated signaling, which can engage DR4 and/or DR5 in T-ALL cells. Finally, our results suggest that combined treatment of anti-DR4 agonistic mAb AY4 with SAHA or VPA can be used as a new therapeutic strategy for targeted treatment of human T-ALL cells.

Acknowledgments This work was supported by grants from the basic research (KRF-2007-313-D00248 and 2009-0064411), and Priority Research Centers Program (2009-0093826) from the National Research Foundation of the Ministry of Education, Science and Technology, and the "GRRC" Project of the Gyeonggi Provincial Government, Republic of Korea.

Conflict of interest None of the authors have any conflicts of interest in connection with this manuscript.

References

1. Ashkenazi A (2008) Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov 7:1001–1012
2. Fox NL, Humphreys R, Luster TA, Klein J, Gallant G (2010) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-1 and receptor-2 agonists for cancer therapy. Expert Opin Biol Ther 10:1–18
3. Johnstone RW, Frew AJ, Smyth MJ (2008) The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 8:782–798
4. Wen J, Ramadevi N, Nguyen D, Perkins C, Worthington E, Bhalla K (2000) Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. Blood 96:3900–3906
5. Hao XS, Hao JH, Liu FT, Newland AC, Jia L (2003) Potential mechanisms of leukemia cell resistance to TRAIL-induced apoptosis. Apoptosis 8:601–607
6. Hasegawa H, Yamada Y, Harasawa H et al (2005) Sensitivity of adult T-cell leukaemia lymphoma cells to tumour necrosis factor-related apoptosis-inducing ligand. Br J Haematol 128:253–265
7. Adida C, Recher C, Raffoux E et al (2000) Expression and prognostic significance of survivin in de novo acute myeloid leukaemia. Br J Haematol 111:196–203
8. Thome M, Schneider P, Hofmann K et al (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386:517–521
9. Thorburn A, Behbakht K, Ford H (2008) TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them. Drug Resist Updates 11:17–24
10. Dumitru CA, Carpinteiro A, Trarbach T, Hengge UR, Gulbins E (2007) Doxorubicin enhances TRAIL-induced cell death via ceramide-enriched membrane platforms. Apoptosis 12:1533–1541
11. Georgakis GV, Li Y, Humphreys R et al (2005) Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br J Haematol 130:501–510
12. Kabore AF, Sun J, Hu X, McCrea K, Johnston JB, Gibson SB (2006) The TRAIL apoptotic pathway mediates proteasome inhibitor induced apoptosis in primary chronic lymphocytic leukemia cells. Apoptosis 11:1175–1193
13. Tazzari PL, Tabellini G, Ricci F et al (2008) Synergistic proapoptotic activity of recombinant TRAIL plus the Akt inhibitor perifosine in acute myelogenous leukemia cells. Cancer Res 68:9394–9403
14. Mitsiades CS, Treon SP, Mitsiades N et al (2001) TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 98:795–804
15. Park SJ, Kim MJ, Kim HB et al (2009) Cotreatment with apicidin overcomes TRAIL resistance via inhibition of Bcr-Abl signaling pathway in K562 leukemia cells. Exp Cell Res 315:1809–1818
16. Batty N, Malouf GG, Issa JP (2009) Histone deacetylase inhibitors as anti-neoplastic agents. Cancer Lett 280:192–200
17. Lagneaux L, Gillet N, Stamatopoulos B et al (2007) Valproic acid induces apoptosis in chronic lymphocytic leukemia cells through activation of the death receptor pathway and potentiates TRAIL response. Exp Hematol 35:1527–1537
18. Iacomino G, Medici MC, Russo GL (2008) Valproic acid sensitizes K562 erythroleukemia cells to TRAIL/Apo2L-induced apoptosis. Anticancer Res 28:855–864
19. Tsapis M, Lieb M, Manzo F et al (2007) HDAC inhibitors induce apoptosis in glucocorticoid-resistant acute lymphatic leukemia cells despite a switch from the extrinsic to the intrinsic death pathway. Int J Biochem Cell Biol 39:1500–1509
20. Rosato RR, Almenara JA, Dai Y, Grant S (2003) Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces

mitochondrial damage and apoptosis in human leukemia cells. Mol Cancer Ther 2:1273–1284
21. Shankar S, Singh TR, Fandy TE, Luetrakul T, Ross DD, Srivastava RK (2005) Interactive effects of histone deacetylase inhibitors and TRAIL on apoptosis in human leukemia cells: involvement of both death receptor and mitochondrial pathways. Int J Mol Med 16:1125–1138
22. Nawrocki ST, Carew JS, Douglas L, Cleveland JL, Humphreys R, Houghton JA (2007) Histone deacetylase inhibitors enhance lexatumumab-induced apoptosis via a p21Cip1-dependent decrease in survivin levels. Cancer Res 67:6987–6994
23. MacFarlane M, Inoue S, Kohlhaas SL et al (2005) Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. Cell Death Differ 12:773–782
24. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM (2005) TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. Cancer Res 65:11265–11270
25. Kelley RF, Totpal K, Lindstrom SH et al (2005) Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. J Biol Chem 280:2205–2212
26. van der Sloot AM, Tur V, Szegezdi E et al (2006) Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. Proc Natl Acad Sci USA 103:8634–8639
27. Inoue S, Mai A, Dyer MJ, Cohen GM (2006) Inhibition of histone deacetylase class I but not class II is critical for the sensitization of leukemic cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Cancer Res 66:6785–6792
28. Sung ES, Park KJ, Lee SH et al (2009) A novel agonistic antibody to human death receptor 4 induces apoptotic cell death in various tumor cells without cytotoxicity in hepatocytes. Mol Cancer Ther 8:2276–2285
29. Lee SH, Park DW, Sung ES, Park HR, Kim JK, Kim YS (2010) Humanization of an agonistic anti-death receptor 4 single chain variable fragment antibody and avidity-mediated enhancement of its cell death-inducing activity. Mol Immunol 47:816–824
30. Huang S, Sinicrope FA (2008) BH3 mimetic ABT-737 potentiates TRAIL-mediated apoptotic signaling by unsequestering Bim and Bak in human pancreatic cancer cells. Cancer Res 68:2944–2951
31. Park KJ, Lee SH, Kim TI et al (2007) A human scFv antibody against TRAIL receptor 2 induces autophagic cell death in both TRAIL-sensitive and TRAIL-resistant cancer cells. Cancer Res 67:7327–7334
32. Park KJ, Lee SH, Lee CH et al (2009) Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem Biophys Res Commun 382:726–729
33. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG (2005) Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther 4:2026–2036
34. Chou TC (2008) Preclinical versus clinical drug combination studies. Leuk Lymphoma 49:2059–2080
35. Secchiero P, Milani D, Gonelli A et al (2003) Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF-alpha promote the NF-kappaB-dependent maturation of normal and leukemic myeloid cells. J Leukoc Biol 74:223–232
36. Pukac L, Kanakaraj P, Humphreys R et al (2005) HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. Br J Cancer 92:1430–1441
37. Guo F, Sigua C, Tao J et al (2004) 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
38. Zhang C, Richon V, Ni X, Talpur R, Duvic M (2005) Selective induction of apoptosis by histone deacetylase inhibitor SAHA in cutaneous T-cell lymphoma cells: relevance to mechanism of therapeutic action. J Investig Dermatol 125:1045–1052
39. Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ, Cohen GM (2004) Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. Cell Death Differ 11(Suppl 2):S193–S206
40. Hernandez A, Thomas R, Smith F et al (2001) Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. Surgery 130:265–272
41. Fulda S, Meyer E, Debatin KM (2002) Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene 21:2283–2294