



Study of TRAIL and SAHA Co-Treatment on Leukemia K562 Cell Line

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Abstract

TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) is an attractive agent being considered a potential cancer treatment. It attaches to its death receptors, leading many cancer cells to apoptosis. However, some malignancies indicate substantial resistance to TRAIL, challenging anticancer scientists. Herein, combination therapy with TRAIL plus SAHA (Suberoyl Anilide Hydroxamic Acid) was conducted to evaluate the capability of SAHA to overcome TRAIL resistance in the leukemia K562 cell line. First, the IC₅₀ for SAHA was calculated (2 μM) at 12, 24, 48, and 72 h of treatment using MTT assay. Second, the K562 cells were treated with concentrations of 50 and 100 nM of TRAIL and 2 μM of SAHA separately and together for 24, 48, and 72 h and the survival of these cells was evaluated by Flowcytometry following the annexin-V and PI staining. To demonstrate the non-toxicity of the combined treatment for normal cells, the HEK-293 cell line was treated with the TRAIL 100 nM and SAHA 2 μM combined and separated at the same periods. In the end, by performing real-time PCR, the amount of candidate genes' expression implicated in TRAIL resistance, and the levels of BCR-ABL expression was measured. The drug dosages were not toxic to normal cells. SAHA plus TRAIL strongly triggered apoptosis in K562 cells after 24, 48, and 72 h of exposure. Furthermore, it was shown that DR4, DR5, and CHOP expressions were enhanced, and PI3K, Akt, ERK, STAT3, c-FLIPL, NF-κB, and BCR-ABL expressions were decreased by SAHA in K562 cells. Our study indicated that SAHA combined with TRAIL can increase the sensitivity of K562 leukemic cells to TRAIL by suppressing intracellular anti-apoptotic molecules and augmenting the expressions of DR4/DR5 and CHOP.

Keywords Hematologic Malignancy · TRAIL · SAHA · K562 · Apoptosis · Resistance

Introduction

Leukemia is a frequent cancer in children and adults that develops when abnormalities in standard cell regulatory systems lead to the uncontrolled growth of hematopoietic stem cells in the bone marrow [1]. Although leukemia patients are already receiving therapies, their five-year survival rate is still not very high, particularly for those at high risk and living in developing countries [2]. Several cancer treatment options are available today, including cell

therapy, radiation therapy, chemotherapy, and surgery [3]. However, the side effects of these treatments, such as cardiotoxicity, can cause numerous fatalities [4].

Therefore, it is crucial to develop modalities with the lowest complications and maximum efficacy. In this light, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL or Apo2L), a TNF (Tumor Necrosis Factor) superfamily member, is a remarkable therapeutic drug that selectively causes tumor cells to undergo the extrinsic apoptotic pathway [5]. TRAIL interacts with the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), and Osteoprotegerin (OPG) [6]. Due to their intracellular death domain (DD), which conveys the apoptotic signal, death receptors (DR4/DR5) possess agonistic characteristics. On the other hand, DcR1, DcR2, and OPG don't have DD and function as regulatory receptors [7]. When TRAIL attaches to its death receptors, the receptors get trimerized, forming the death-inducing signaling complex (DISC). The adapter protein FADD (Fas-associated protein with death domain) helps procaspase-8/10 recruitment by translocating

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to the DISC and interacting with the DD [8]. TRAIL is produced as a type II TNF transmembrane protein, but after being cleaved by proteases, its extracellular domain can function as a soluble cytokine [9].

Despite expectations that TRAIL therapy will be among the most successful cancer therapies, resistance to TRAIL therapy continues to be a problem for developing anticancer techniques. To get around this issue, TRAIL compounds have been tested for years to generate synergism or sensitize cancer cells resistant to treatment [10]. The main reasons most primary hematologic tumors resist TRAIL-mediated apoptosis include overexpression of anti-apoptotic proteins (like c-FLIP), TRAIL decoy receptor expression, and DR4/DR5 underexpression [11]. Resistance of cancer cells to TRAIL may also be afforded through activating anti-apoptotic pathways such as NF- κ B, PI3K/Akt, ERK, and signal transducer and activator of transcription 3 (STAT3) [12, 13]. On the other hand, C/EBP homologous transcription factor (CHOP), an essential player in the ER stress-mediated apoptosis pathway, can regulate the transcriptional level of DR5 expression and increase TRAIL-induced apoptosis in resistant cells [14, 15]. Given this, CHOP plays a vital role in ER-mediated apoptosis by upregulating DR5 in human cancer cells [16].

According to the TRAIL-resistance mechanisms, agents that can kill tumors and have fewer side effects, such as Histone deacetylase inhibitors, can be used combined with TRAIL to sensitize TRAIL-resistant cells. Histone deacetylases (HDACs) are a class of enzymes that eliminate acetyl groups from histones and control gene expression through chromatin modifications [17]. HDACs exhibit oncogenic behavior in several cancer types, which sparked the use of HDAC inhibitors (HDACi) as anticancer medications [18]. SAHA (Suberoyl Anilide Hydroxamic Acid or Vorinostat) is an HDAC inhibitor that generally inhibits class I/II HDAC enzymes. However, Class III HDACs are not blocked by vorinostat [19]. SAHA is the first FDA-approved HDACi for treating Cutaneous T Cell Lymphoma (CTCL), and it can cause apoptosis in hematological and solid malignancies through both extrinsic and intrinsic apoptosis pathways [19, 20].

This research investigated whether or not SAHA could increase the sensitivity of K562 cells to TRAIL-induced apoptosis. Furthermore, we assessed DR4/5, NF- κ B, PI3K, Akt, ERK, STAT3, c-FLIPL (long isoform), and CHOP expression at mRNA levels, which are potential genes related to the demonstration of TRAIL resistance in tumor cells to understand how they influenced the K562 cells' resistance to TRAIL. In addition to the mentioned genes, we measured BCR-ABL expression at mRNA levels to see how this combination treatment could impact the K562 cell's growth capacity.

Materials and Methods

Cell Culture

The K562 cell line was acquired from the Immunology Research Center (Tabriz, Iran). These cells were cultured in RPMI-1640 (Gibco, Paisley, UK) media containing 10% V/V FBS (fetal bovine serum) and penicillin/streptomycin (100 mg/ml) in an incubator at 37 °C and 5% CO₂. The HEK-293 cell line was acquired from the Immunology Research Center (Tabriz, Iran). DMEM (Gibco, Paisley, UK) media, supplemented with 10% V/V FBS, was used to culture these cells in an incubator at 37 °C and 5% CO₂.

Compounds

A 0.5 mg/ml stock solution of rhTRAIL (recombinant human TRAIL, Merck, Darmstadt, Germany) was created in water. Besides, dimethyl sulfoxide (DMSO) was used to make a 50 mM stock solution of SAHA (Sigma, Aldrich), which was stored at -20 °C. Dimethyl sulfoxide and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) substances were obtained from Sigma-Aldrich, and RPMI-1640 media was procured from Gibco (Carlsbad, CA).

The Evaluation of Cell Survival

To determine the IC₅₀ concentration of SAHA in K562 cells, after being seeded 1×10^4 cells/100 μ L in each well of a 96-well plate, the cells were treated with 0–8 μ M SAHA concentration. Using an Epoch ELISA reader, the wells' optical density (OD) was assessed at 570 nm. Following the addition of 20 μ L of the MTT reagent after 12, 24, 48, and 72 h of the treatment. Ultimately, SAHA's IC₅₀ concentration was calculated using GraphPad Prism version 9.4.0.

Assessment of Apoptosis Using Flow Cytometry

The apoptotestTM-FITC Kit (Dako, Glostrup, Denmark) was used to evaluate the amount of apoptosis in the treated groups. After treating the K562 cells with rhTRAIL 50 and 100 nM, SAHA 2 μ M, and rhTRAIL 50 and 100 nM plus SAHA 2 μ M for 24, 48, and 72 h, the percentage of apoptosis in the K562 cells was calculated following the addition of 5 μ L PI (Propidium iodide) and 10 μ L FITC (Annexin V-fluorescein isothiocyanate). Also, to demonstrate the non-toxicity of the combined treatment for normal cells, the HEK-293 cell line was treated with the TRAIL 100 nM and SAHA 2 μ M combined and separated at the same periods, and the apoptosis percentage was evaluated. Finally, the outcomes were assessed using version 10.8.1 of FlowJo software.

Table 1 Primer pairs of candidate genes for real-time PCR

Gene	Primer
DR4	Forward Sequence GTGTGGGTTACACCAATGCTTCC Reverse Sequence CCTGGTTTGCACGACATGCTG
DR5	Forward Sequence AGCACTCACTGGAATGACCTCC Reverse Sequence GTGCCTTCTTCGCACTGACACA
c-FLIPL	Forward Sequence AGTGAGGCGATTGACCTGCTC Reverse Sequence CCTCACCAATCTCTGCCATCAG
NF- κ B	Forward Sequence GCAGCACTACTTCTTGACCACC Reverse Sequence TCTGCTCCTGAGCATTGACGTC
STAT3	Forward Sequence CTTTGAGACCGAGGTGTATCACC Reverse Sequence GGTCAGCATGTTGTACCACAGG
CHOP	Forward Sequence GGTATGAGGACCTGCAAGAGGT Reverse Sequence CTTGTGACCTCTGCTGGTTCTG
PI3K	Forward Sequence GAAGCACCTGAATAGGCAAGTCG Reverse Sequence GAGCATCCATGAAATCTGGTCGG
Akt	Forward Sequence TGGACTACCTGCACTCGGAGAA Reverse Sequence GTGCCGAAAAGGTCTTCATGG
ERK	Forward Sequence TGGCAAGCACTACCTGGATCAG Reverse Sequence GCAGAGACTGTAGGTAGTTTCGG
BCR-ABL	Forward sequence CCAGGTGTATGAGCTGCTAGAG Reverse sequence GTCAGAGGGATTCCACTGCCAA

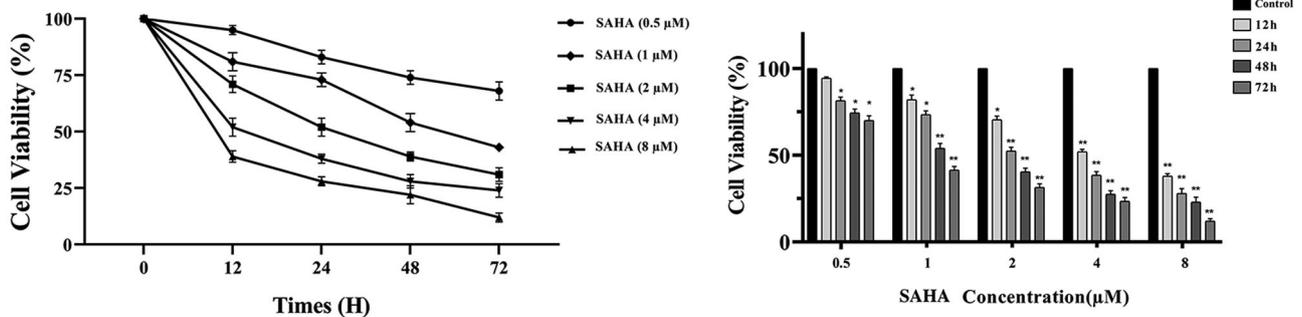


Fig. 1 The cytotoxic effect of SAHA on K562 cell proliferation. After exposure to different concentrations of SAHA (0–8 μ M) for 12, 24, 48, and 72 h, the IC_{50} values in K562 cells were calculated by applying

MTT assays. The mean \pm SEM of three separate tests is represented. IC_{50} : The half maximal inhibitory concentration; MTT Methyl Thiazolyl Tetrazolium assay, S.E.M Standard error of the mean

Quantitative Real-Time PCR

For BLASTing each primer sequence provided in Table 1, Primer-blast software on the NCBI website was employed. Initially, total RNA was extracted using Trizol reagent (Invitrogen, Milan, Italy). Then, the PrimeScriptTM reagent Kit (Takara, Japan) was used to generate cDNA (complementary DNA). Real-time PCR in three replications was performed for each sample using the RealQ plus 2x Master Mix Green (Ampliqon, Herlev, Denmark). For internal control, ACTB was used.

Statistical Analysis

For data comparison and analysis, two-way ANOVA with post hoc Tukey and Dunnett and multiple comparison tests

were applied using GraphPad Prism version 9.4.0. Next, the outcomes of three separate experiments were provided as the mean \pm SEM. P -values < 0.05 were counted as statistically meaningful.

Results

Effects of SAHA on K562 Cell Proliferation

Based on the results, SAHA inhibits the proliferation of K562 cells during all treatment periods. To determine the cytotoxic effect of SAHA on K562 cells, the IC_{50} value was calculated using the MTT assay. The IC_{50} concentration for K562 cells was calculated at 2 μ M after 24 h of treatment (Fig. 1).

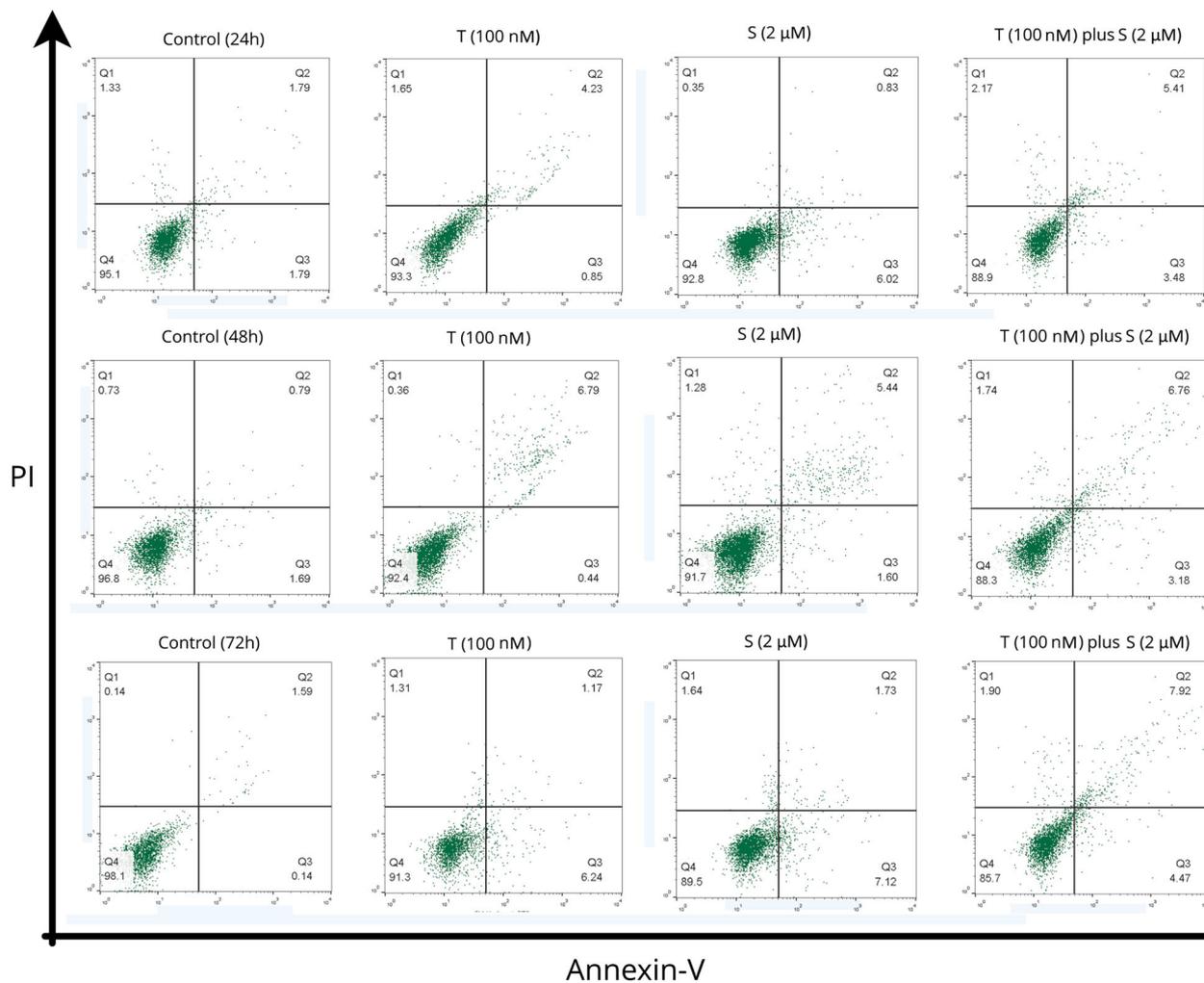


Fig. 2 Apoptosis of the HEK-293 cells treated with TRAIL (100 nM) and SAHA (2 μM) separately and together after 24, 48, and 72 h of exposure. Following Annexin-V and PI staining, cell death was calculated using FACS analysis, as detailed in the “materials and methods”

Effects of SAHA and TRAIL on HEK-293 Cells

The used dosages of drugs (TRAIL 100 nM and SAHA 2 μM combined and separated) have shown no toxic effects on the HEK-293 cell line as normal cells. As indicated in Fig. 2, these cells have shown no significant increase in the apoptosis percentage after treatment at any time.

Effects of SAHA and TRAIL on K562 Cells' Resistance to TRAIL

Comparing the target cells' apoptosis to the control group at any point during the research, the 50 nM and 100 nM doses of TRAIL exhibited no detectable impact under the circumstances utilized in this study. But the K562 cells revealed a considerable increase in their vulnerability to TRAIL-induced cytolysis, as appraised by Annexin-V and PI staining experiments at 24, 48, and 72 h, following

SAHA treatment at its IC_{50} concentration (2 μM) (Fig. 3A–D). The effects of SAHA and TRAIL at 24, 48, and 72 h after exposure to K562 cells were examined, both alone and in combination, to ascertain if SAHA plus TRAIL was more effective than SAHA alone. Based on the findings, SAHA could increase K562 cells' sensitivity to TRAIL (Fig. 3A–D, Table 2).

Impacts of SAHA and TRAIL on the Expression of DRs

The interaction between TRAIL and death receptors (DR4/DR5) is known to trigger apoptosis. The expression amount of Death Receptor 4 and 5 was measured by real-time PCR to discover whether or not the cytotoxic effect of SAHA is associated with an augmentation in the cell surface expression of these molecules in K562 cells. Results demonstrated no significant change in DR4 expression after

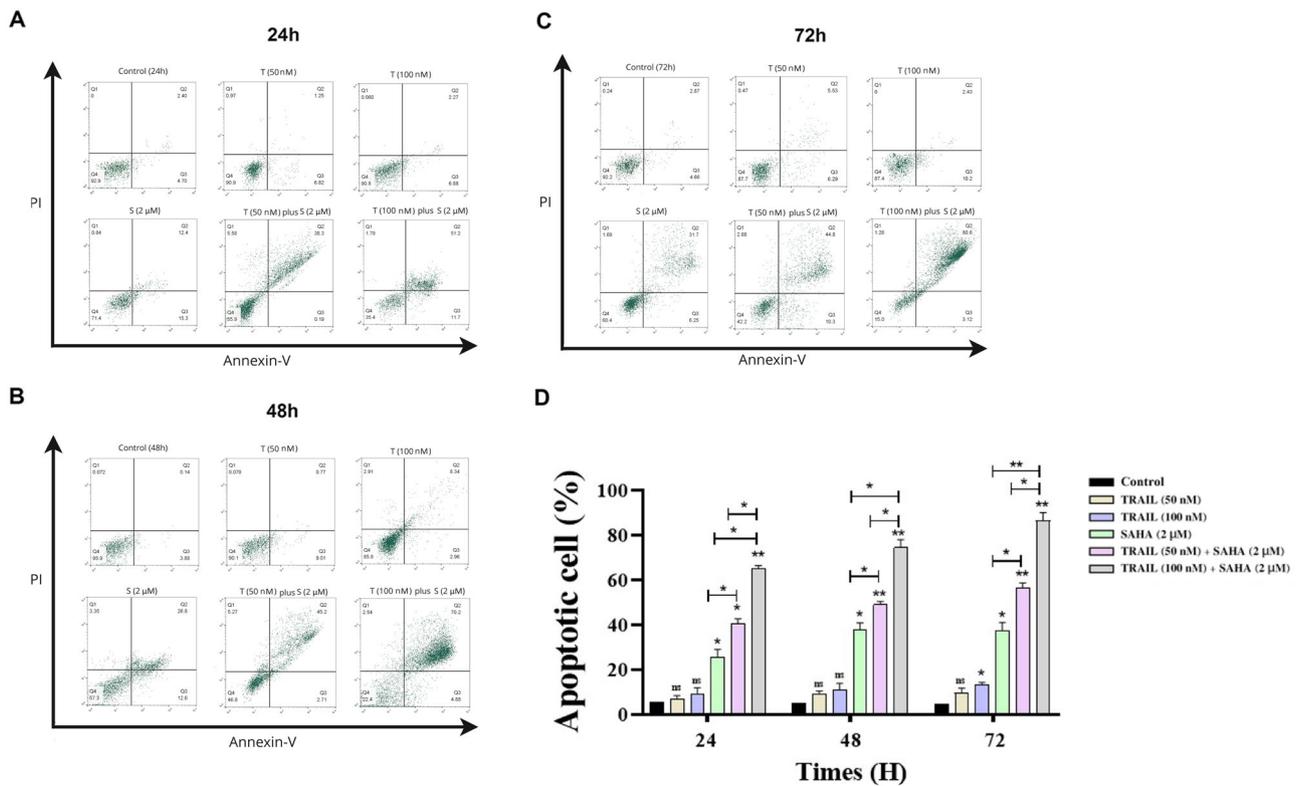


Fig. 3 Apoptosis of the K562 cells treated with TRAIL (50 nM and 100 nM) and SAHA (2 μM) both separately and together after 24(A), 48(B), and 72(C) hours of exposure. Following Annexin-V and PI staining, cell death was calculated using FACS analysis, as detailed in the “materials and methods.” Apoptosis percentage of the K562 cells

treated with TRAIL (50 nM and 100 nM) and SAHA (2 μM) both separately and in combination after 24, 48, and 72 h of exposure(D). The mean ± SEM of three separate tests is represented. *P*-values < 0.05 were regarded as statistically meaningful [*P* < 0.05 (*), *P* < 0.01 (**)]

Table 2 Apoptosis percentage of the K562 cells treated with TRAIL (50 nM and 100 nM) and SAHA (2 μM) both separately and in combination after 24, 48, and 72 h of exposure

Groups	24 h	48 h	72 h
Control	5.98 ± 1.78	5.01 ± 1.34	4.66 ± 0.97
TRAIL (50 nM)	7.18 ± 1.88	9.54 ± 1.76	9.66 ± 2.4
TRAIL (100 nM)	9.28 ± 2.78	11.54 ± 2.61	13.96 ± 1.12
SAHA (2 μM)	25.66 ± 2.91	37.18 ± 2.3	39.56 ± 3.01
SAHA (2 μM) + TRAIL (50 nM)	40.09 ± 2.76	49.43 ± 2.54	56.067 ± 2.81
SAHA (2 μM) + TRAIL (100 nM)	64.39 ± 2.41	76.24 ± 3.89	87.81 ± 3.92

24 h. However, after 48 and 72 h of treatment, the expression of DR4 increased slightly (Fig. 4A). However, a dose-dependent elevation in the expression of DR5 was discovered after exposure to SAHA (Fig. 4B).

Impacts of SAHA and TRAIL on c-FLIPL Expression

It is believed that c-FLIP acts as an anti-apoptotic factor associated with resistance to apoptosis-inducing

substances. Thus, after exposure to TRAIL and SAHA separately and together for various durations, the expression levels of c-FLIPL in K562 cells were assessed. There was no discernible alteration in the c-FLIPL expression after TRAIL-only treatment at any time. Conversely, after K562 cells were exposed to SAHA alone and SAHA combined with TRAIL, the c-FLIPL expression levels were dramatically reduced at all times (Fig. 4C). Therefore, the effect of the combinational therapy of SAHA-TRAIL on c-FLIPL expression was strongly substantiated by the analysis of the expression levels of c-FLIPL in cells that had been treated with both TRAIL and SAHA.

Impacts of SAHA and TRAIL on CHOP Expression

The expression of CHOP, as an apoptotic factor, was assessed after 24, 48, and 72 h following the exposure of K562 cells to TRAIL and SAHA alone and together. After 24 h of treatment, they had no noticeable impact on CHOP expression. However, the expression rate of CHOP increased dramatically after being treated for 48 and 72 h with SAHA and TRAIL (Fig. 4D).

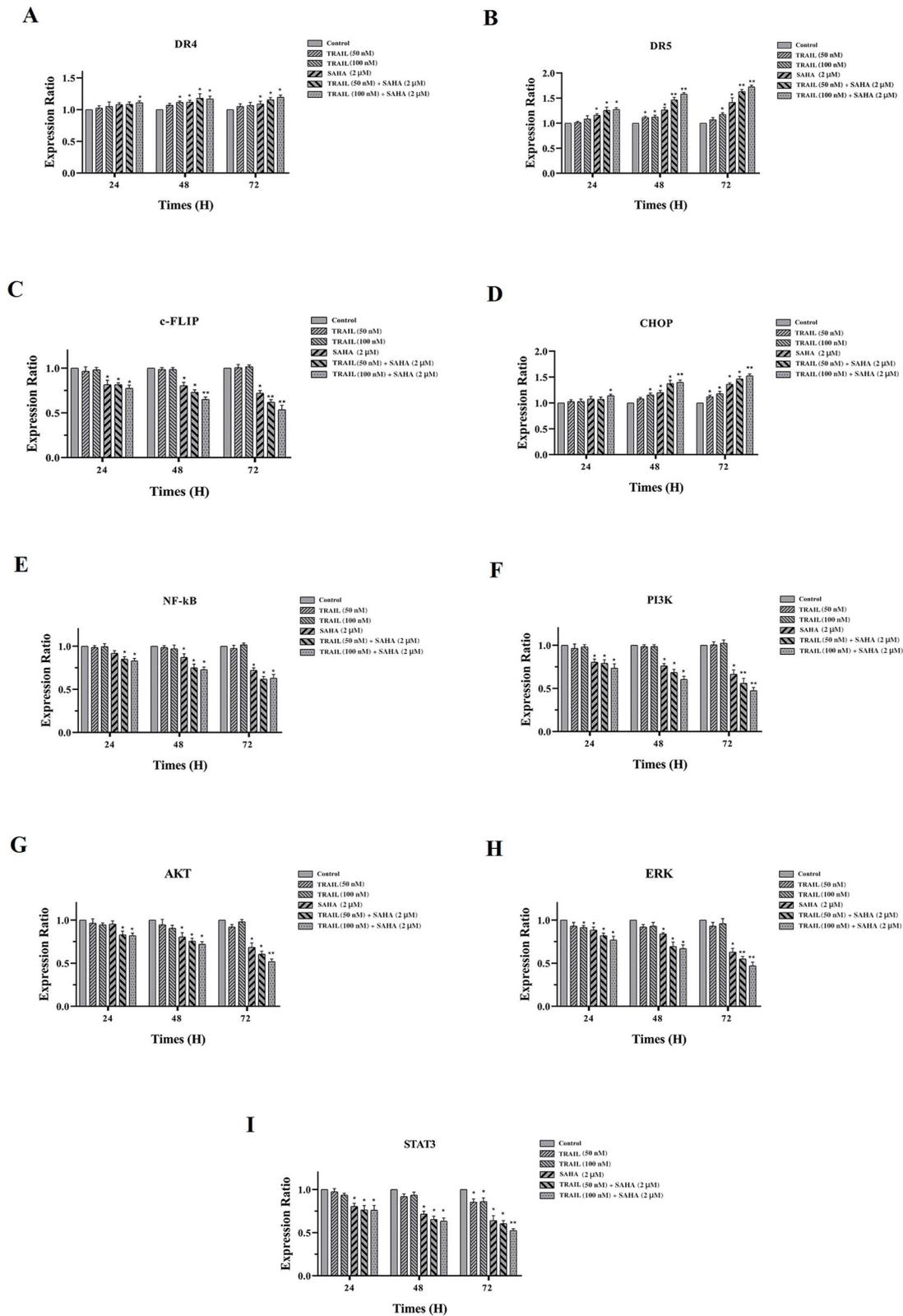
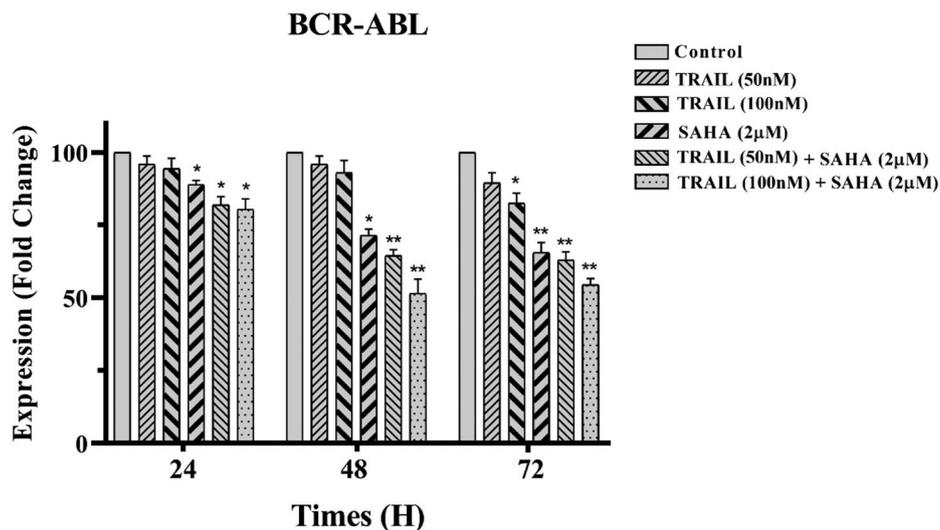


Fig. 4 Results of Real-Time PCR for the analysis of DR4 (**A**), DR5 (**B**), c-FLIP (**C**), CHOP (**D**), NF- κ B (**E**), PI3K (**F**), Akt (**G**), ERK (**H**), and STAT3 (**I**) expression in K562 cells after 24, 48, and 72 h of treatment with TRAIL (50 and 100 nM) and SAHA (2 μ M) alone and

in combination. β -actin was used as a normalizer. The mean \pm SEM of three separate tests is represented. P -values < 0.05 were regarded as statistically meaningful [$P < 0.05$ (*), $P < 0.01$ (**)]

Fig. 5 Results of Real-Time PCR for the analysis of BCR-ABL expression in K562 cells after 24, 48, and 72 h of treatment with TRAIL (50 and 100 nM) and SAHA (2 μ M) alone and in combination. β -actin was used as a normalizer. The mean \pm SEM of three separate tests is represented. *P*-values < 0.05 were regarded as statistically meaningful [*P* < 0.05 (*), *P* < 0.01 (**)]



Effects of SAHA and TRAIL on the Expression of Several Anti-Apoptotic Genes of Signaling Pathways

There have been reports of many proteins preventing the apoptotic process. Consequently, K562 was exposed to SAHA and TRAIL (alone and in combination) for varying lengths of time. Afterward, the mRNA rates of several anti-apoptotic proteins were assessed by real-time PCR. Those cells treated with TRAIL alone (50 and 100 nM) showed no apparent alteration in the expression of NF- κ B, PI3K, Akt, and ERK at any time. However, treating K562 cells with SAHA (2 μ M) and SAHA plus TRAIL significantly reduced the expression of these genes after 24, 48, and 72 h (Fig. 4E–H). STAT3 levels did not considerably alter after K562 cells were treated with TRAIL alone for 24 and 48 h. Although, after 72 h of TRAIL alone treatment, STAT3 expression in K562 cells slightly decreased. However, treatment of K562 cells with SAHA and the SAHA-TRAIL combination significantly reduced STAT3 expression at all times (Fig. 4I).

Impacts of SAHA and TRAIL on BCR-ABL Expression

BCR-ABL expression was measured after 24, 48, and 72 h of treatment. There was no significant reduction in the expression of this translocation after exposure to TRAIL-only at 24 and 48 h. Although, a slight decrease was observed after 72 h of TRAIL alone treatment. Treating K562 cells with SAHA alone and SAHA combined with TRAIL showed a noticeable drop in BCR-ABL expression at mRNA levels in these cells at all times (Fig. 5).

Discussion

The ability of TRAIL to initiate apoptosis in different tumor cells while having no similar impact on normal cells led to

its immediate acceptance as a promising cancer therapy following its discovery [21]. Since TRAIL's death receptors (DR4 and DR5) are weakly expressed on the surface of normal human cells, it is regarded as a harmless and safe agent for them [6]. Also, TRAIL performs a crucial function in the immune response to cancer, including NK cell- and IFN- γ -dependent tumor surveillance [22]. Studies have shown that TRAIL can activate apoptosis independently of the p53 gene, which makes it a promising therapeutic option for cancer patients whose p53 genes are frequently mutated [23]. However, many cancer cells have resisted TRAIL's apoptotic effects. Even cancers that were previously responsive to TRAIL-mediated apoptosis may gain resistance with repeated exposure [24]. It has been discovered that various types of tumor cells have functional deficiencies in DR4 and DR5. These cancerous cells lack DR4 and/or DR5 on their surface despite the expression of their protein. Therefore, the absence of surface DR4 and DR5 can cause tumors to resist targeted TRAIL treatments [25]. In addition, increasing data show that CHOP directly regulates the accumulation of DR5 mRNA, and its activation stimulates DR5 transcription, suggesting a relationship between CHOP and TRAIL receptors, which can play a prominent role in sensitizing tumor cells to TRAIL [26]. Also, studies on leukemia cells showed that inhibiting NF- κ B enhanced susceptibility to apoptosis triggered by TRAIL, providing evidence for the significance of NF- κ B activity in resistance to apoptosis [27]. Many studies suggest that other signaling molecules, such as PI3K, Akt, ERK, and STAT3, can dramatically develop resistance to TRAIL in many cancerous cells [28–32]. For instance, according to earlier research, the activation of STAT3 in hepatocyte carcinoma cells is probably a critical factor in developing TRAIL resistance, or another study showed that TRAIL resistance can result from ERK activation in breast cancer cells. ERK activation prevents caspase-8 and Bid

from being processed [13, 33]. Besides, the significant anti-apoptotic regulator c-FLIP, which attaches to FADD, caspase 8 or 10, and DR5, prevents apoptosis by forming an Apoptosis Inhibitory Complex (AIC) and so induces resistance to TRAIL through inhibiting caspase cascades [34, 35].

Different approaches have been used to defeat resistance to TRAIL. Combination therapy is one of the most popular tactics [36]. Thus, we used SAHA, a Histone deacetylase inhibitor, combined with TRAIL to investigate whether TRAIL resistance could be defeated. Many human malignancies have been demonstrated to have excessive HDAC expression [37]. Therefore, targeting HDAC inhibition to create innovative anticancer therapies is reasonable. Vorinostat (SAHA) substantially affects cellular acetylation patterns and induces growth halt and death in various malignant cells at dosages that are not lethal to normal cells [38]. Additionally, prior studies showed that SAHA could sensitize TRAIL-resistant cancerous breast cells by upregulating DR4/5 expression [39]. Hence, we selected SAHA as an appropriate TRAIL combo medication to highlight potential routes of action for this histone deacetylase inhibitor and offer suggestions for unexplored areas of clinical research.

Here, we demonstrated that SAHA could make K562 cells sensitive to TRAIL. Comparing cells exposed to SAHA or TRAIL separately to cells treated with both substances showed a considerable boost in the number of apoptotic cells, indicating that SAHA significantly augmented TRAIL's anti-tumor actions. Our current study showed that SAHA alone and SAHA with TRAIL upregulated DR4, DR5, and CHOP as the apoptotic proteins while reducing the expression of PI3K, AKT, STAT3, and ERK as the anti-apoptotic molecules. Also, suppressing c-FLIP expression suggested that SAHA may evoke apoptosis in K562 cells by activating the caspase cascade. Combining SAHA with TRAIL implies that SAHA sensitizes TRAIL-mediated apoptosis by impairing the apoptosis-blocking molecule c-FLIP and inhibiting NF- κ B activation. In this regard, because of the correlation between NF- κ B activation and overexpression of c-FLIP, it seems inhibiting NF- κ B expression suppresses c-FLIP expression, which results in activating the extrinsic pathway of apoptosis in K562 cells [40]. The findings reported here may provide insight into the potential use of histone deacetylase inhibitors like SAHA and TRAIL to cause apoptosis in leukemic cells.

In addition to the pathways and genes we discussed, increasing evidence demonstrates that SAHA can show antiproliferative impacts on BCR-ABL-positive cells. Nimmanapalli et al. indicated that SAHA could reduce BCR-ABL product at mRNA and protein levels, leading K562 cells to apoptosis [41], which was confirmed through

our investigation (Fig. 5). Our study suggests that reducing the BCR-ABL expression by SAHA decreases the proliferative capacity of K562 cells, which may potentially be associated with the increased sensitivity of these cells to TRAIL-mediated apoptosis. Accordingly, as well as the genes and pathways we assessed, it seems TRAIL-SAHA combined therapy can be an appealing course to lead BCR-ABL-positive cells to apoptosis.

Conclusion

Our research shows that SAHA raised the levels of DR4 and DR5 and lowered the levels of anti-apoptotic proteins like PI3K, AKT, STAT3, ERK, c-FLIP, and NF- κ B. This made leukemia K562 cells more likely to die through TRAIL-mediated apoptosis. Furthermore, SAHA could reinforce TRAIL-mediated apoptosis in K562 cells by increasing CHOP expressions implicated in upregulating DR5 expressions. Thus, it appears that in addition to having the ability to stimulate apoptosis in leukemic cells, histone deacetylase inhibitors, such as SAHA, can operate as sensitizing agents to reduce leukemic cells' resistance to therapeutic drugs. In conclusion, additional study is needed to determine the effects of HDAC inhibitors and their combination therapy with TRAIL to achieve specific treatment results in TRAIL-based leukemia therapy.

Data Availability

The data for this research is available by contacting the corresponding author.

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Author Contributions The study was designed and guided by [Saeed Solali]. Material preparation, data collection and analysis were performed by [Amirarsalan Alaei]. The first draft of the manuscript was written by [Amirarsalan Alaei] and [Masoud Mohammad Mirzapour]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. The student research committee approved this research. Tabriz university of medical sciences, Tabriz, Iran (IR.TBZMED.VCR.REC.1402.063)

Informed consent For this type of study, informed consent is not required.

Consent for publication For this type of study, consent for publication is not required.

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