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ORIGINAL ARTICLE

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Trichostatin A sensitizes TRAIL-resistant myeloma cells by downregulation of the antiapoptotic Bcl-2 proteins

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Abstract *Purpose*: In this study, we have investigated the effect of trichostatin A (TSA) pretreatment on the cytotoxicity of TRAIL in TRAIL-resistant myeloma cells. *Methods and Results*: MM1S myeloma cells exhibited resistance to TRAIL-induced apoptosis even at high doses of TRAIL and was sensitive to low doses of TSA. Sequential treatment of myeloma cells with TSA followed by TRAIL enhanced TRAIL cytotoxicity. TSA induced the transcription of TRAIL death receptor DR5 without affecting the transcription of DR4 and the decoy receptors; DcR1 and DcR2. However, the surface expression of both DR4 and DR5 was not modulated by TSA treatment. TSA treatment repressed the transcription and downregulated the expression of the antiapoptotic Bcl-2 proteins; Bcl-2 and Bcl-X_L. Surprisingly, the effect of TSA on the proapoptotic Bcl-2 proteins was mixed, the two isoforms of PUMA (α, β), Noxa, Bax were downregulated, while Bim was upregulated. Although MM1S cells showed higher expression level of $FLIP_S$ than other TRAIL-sensitive myeloma cells, the enhancing effect of TSA was not accompanied by $FLIP_S$ downregulation. *Conclusion*: In conclusion, TSA sensitized TRAIL-resistant myeloma cells by downregulating the antiapoptotic Bcl-2 protein without altering $FLIP_S$ expression.

Keywords TSA · FLIP · TRAIL · Bcl-2 family · Apoptosis

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Introduction

TNF-related apoptosis-inducing ligand (TRAIL) has shown efficacy and selectivity as antitumor agent. However, different cell lines exhibited TRAIL resistance. The resistance against TRAIL was attributed to different causes like FLICE inhibitory protein (FLIP) overexpression [1], loss of caspase-8 expression [2], death receptors mutation [3] or overexpression of Bcl-2 or Bcl-X_L [4].

Bcl-2 family proteins play a crucial role in apoptosis induction. The proapoptotic members like Bax, Bad, Bak and the BH3-only members (Bim, Bid, Noxa, PUMA) induce apoptosis through their mitochondrial translocation with the consequent release of apoptogenic factors from the mitochondria like cytochrome c and apoptosis-inducing factor (AIF) [5]. The antiapoptotic members like Bcl-2 and Bcl-X_L were shown to hinder apoptosis by inhibiting the release of the apoptogenic factors from the mitochondria. Previous reports have shown that agents that upregulate the expression of the proapoptotic members and/or downregulate the expression of the antiapoptotic Bcl-2 proteins can sensitize chemotherapy-resistant or TRAIL-resistant cells [6].

FLICE inhibitory protein has two different isoforms; $FLIP_L$ and $FLIP_S$, both forms have a structural homology to caspase-8 but lack an active catalytic domain. $FLIP_L$ and $FLIP_S$ overexpression were shown to confer TRAIL-resistance [7, 8]. Treatment of osteosarcoma cells with histone deacetylase (HDAC) inhibitors downregulated FLIP expression and consequently sensitized the cells to TRAIL [9].

Histone deacetylase inhibitors modify the architecture of chromatin and consequently modify gene expression. Accordingly, the hypothesis that HDAC inhibitors could simultaneously induce proapoptotic genes and repress antiapoptotic genes was developed. These changes in gene expression could be utilized for sensitizing or enhancing the apoptotic potential of chemotherapeutic agents or other anticancer agents like TRAIL.

In this study, we have used the HDAC inhibitor derived from hydroxamic acid; TSA, to sensitize TRAIL-resistant cells. TSA did not alter $FLIP_S$ expression or TRAIL death receptors surface expression. TSA repressed the transcription and downregulated the expression of the antiapoptotic Bcl-2 proteins. The effect of TSA on the proapoptotic Bcl-2 members was mixed, where PUMA, Noxa, Bax were downregulated and Bim was upregulated. This report shows that TSA can sensitize TRAIL-resistant cells through downregulation of the antiapoptotic Bcl-2 members without altering $FLIP_S$ expression.

Materials and methods

Reagents

The antibodies used were as follows: goat polyclonal antibodies for Noxa, PUMA β/δ and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal antibodies for Bax (Santa Cruz Biotechnology), β-Tubulin (Upstate, Charlottesville, VA, USA) and mouse polyclonal antibody for $FLIP_{S/L}$ (Santa Cruz Biotechnology). Rabbit polyclonal antibodies for Bcl-X_L, Bcl-2, Bim (Santa Cruz Biotechnology) and PUMA-α (Imgenex, San Diego, CA, USA). TRAIL was purchased from BIOMOL (Plymouth Meeting, PA, USA), TSA from Alexis biochemicals (Carlsbad, CA, USA) and all other chemicals used were of analytical grade from Fisher Scientific (Suwanee, GA, USA) or Sigma (St. Louis, MO, USA).

Cell culture

MM1S and ARP-1 cells were kindly provided by Dr. R. Fenton (School of Medicine, University of Maryland, Baltimore, MD, USA). Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS (GIBCO) in a humidified incubator supplied with 5% carbon dioxide. IL-6 was added to stimulate the growth of ARP-1 cells.

XTT colorimetric assay

XTT cell proliferation assay is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability, where XTT (tetrazolium salt) is metabolized by viable cells only to a water soluble formazan dye that can be measured spectrophotometrically. Briefly, 100 µl of cell suspension (1×10^6 cells/ml) was added to a 96-well plate, 100 µl of different concentrations of the drugs (TSA or TRAIL) were added to the wells and cells were incubated for various time points in a humidified incubator at 37°C and 5% CO_2. A mixture of 50 µl of XTT (0.2 mg/ml) and 2.5 µM phenazine methosulfate (PMS) dissolved in RPMI was added to each well and the cells were further incubated for 4 h at 37°C. Finally, the absorbance of the dye was measured spectrophotometrically at 450 and 690 nm as a reference wave length. To detect the role of different caspases in the cytotoxic effect of HDAC inhibitors, MM1S cells were pretreated with 30 µM of the pan-caspase inhibitor Z-VAD-FMK (Calbiochem, La Jolla, CA, USA) for 2 h prior to TSA treatment.

Propidium iodide (PI) staining for apoptosis assay

Propidium iodide is a fluorescent nucleic acid binding dye that binds preferentially to double-stranded nucleic acids, allowing fluorescent intensity to be used as indicator of the cellular DNA content. The assay was performed as per the manufacturer's instruction (Roche Applied Science, Indianapolis, IN, USA). PI stained cells were analyzed using a Beckton Dickinson FACScan flow cytometer and cells in the sub G1 peak were considered apoptotic.

DR4/DR5 staining for flow cytometry

MM1S cells (1×10^6) were fixed with 2% paraformaldehyde for 10 min at room temperature, washed twice with phosphate buffered saline (PBS), incubated with DR4 or DR5 mouse primary antibody (R&D Systems) for 1 h, washed twice with PBS and finally incubated with FITC-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 30 min in the dark. Fluorescence was analyzed using Becton Dickinson FACScan flow cytometer.

RT-PCR analysis

RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using the Access RT-PCR system (Promega, Madison, WI, USA) as per the manufacturer's instructions. Bcl-2 and Bcl-X_L primers used were as follows, sense: 5′TTCTTTGAGTTCGGTGGGGTC3′; antisense: 5′TGC ATATTTGTTTGG-GGCAGG3′ and sense: 5′TTGGA CAATGGACTGG TTGA3′; antisense: 5′GTAGAG TGGA TGGTCAGTG-3′, respectively. PUMA primers were, sense: 5′CAGACTGTGAATCCTGTGCT3′; antisense: 5′ ACAGTATCTTACAGGCTGGG3′. Noxa primers were, sense: 5′ GTGCCCTTGGAAACGGA GA3′ and antisense: 5′CCAGCCGCCCAGTCTAAT CA3′. Bim primers were, sense: 5′ATGAG-AAGATC CTCCCTGCT3′ and antisense: 5′AATGCATTCTCCA CACCAGG3′. Bax primers were, sense: 5′AAGAAG CTGAGCGAGTGT3′ and antisense: 5′GGAG-GAAG TCCAATGTC3′. DR4 primers were, sense: 5′ACAGC AATGGGAACATAG-CC3′ and antisense: 5′GTCAC TCCAGGGCGTACAAT3′. DR5 primers were, sense: 5′TGCAGCCGTAGTCTTGATTG3′ and antisense:

5′GCACCAAGTCTG-CAAAGTCA3′. DcR1 primers were, sense: 5′GTTTGTTTGAAAGACTTCACTGTG3′ and antisense: 5′GCAGGCGTTTCTGTCTGTGG-GAAC3′. DcR2 primers were, sense: 5′CTTCAGGA AACCAGAGCTTCCCTC3′ and antisense: 5′TTCTCC CGTTTGCTTA-TCACACGC3′. Proper loading was confirmed by using the following specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense: 5′TCTGCCCCCT CTGCTGATGC3′; antisense: 5′CCACCACCCTGTTGCTGTAG3′.

Results

TSA pretreatment sensitizes TRAIL-resistant myeloma cells

TNF-related apoptosis-inducing ligand has shown efficacy as an anticancer agent both in vitro and in vivo [10, 11]. However, some cell lines exhibit endogenous resistance to TRAIL because of the expression of different proteins that confer resistance to TRAIL [12]. MM1S myeloma cell line exhibited a high resistance profile against a high dose of TRAIL (250 ng/ml) and was sensitive to a low dose of TSA (Fig. 1a).

Trichostatin A is known for its transcriptional regulation of different genes; therefore, pretreatment of TRAIL-resistant cell lines with TSA could downregulate the expression of these genes and sensitize TRAIL-resistant cells. Pretreatment of MM1S cells with different doses of TSA followed by TRAIL treatment augmented TRAIL cytotoxicity with maximal enhancing effect at 100 nM TSA (Fig. 1b).

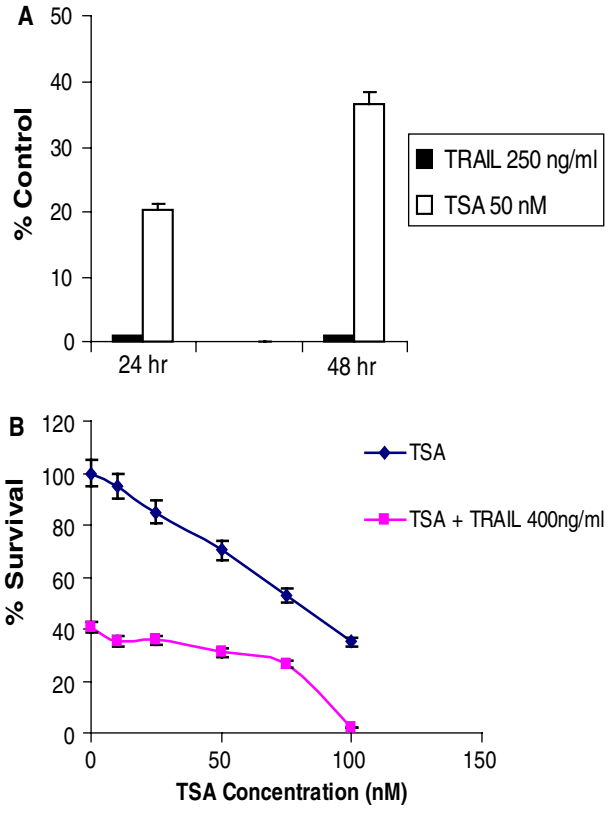

Fig. 1 Trichostatin A pretreatment sensitizes MM1S TRAIl-resistant cells. **a** MM1S cells were treated with TRAIL (250 ng/ml) or TSA (50 nM) for 24 and 48 h and propidium iodide (*PI*) staining was used to estimate apoptosis. Percentage of control indicates apoptosis relative to the untreated control samples. Data represent mean ± SD for three replicates. **b** MM1S cells were either treated with different concentrations of TSA alone for 48 h or pretreated for 24 h with different concentrations of TSA followed by treatment with TRAIL (400 ng/ml) for another 24 h. The percentage of survival was determined by XTT assay as described under methods. Data represent the mean for 4 replicates ±SD

TSA sensitizes TRAIL-resistant MM1S cells by downregulating the expression of Bcl-2 and Bcl-X_L

The Bcl-2 proteins family plays an essential role in apoptosis induction. Bcl-2 proteins can be divided into two categories; the proapoptotic and the antiapoptotic members. Overexpression of the antiapoptotic members like Bcl-2 and Bcl-X_L has been shown to block apoptosis induction [13]. TSA treatment of TRAIL-resistant MM1S myeloma cells repressed the transcription of both Bcl-2 and Bcl-X_L after 6 h (Fig. 2a). The expression of both proteins was also downregulated after 24 h of treatment (Fig. 2b). These observations are in concordance with the observed TSA-sensitizing effect.

TRAIL receptors surface expression is not modulated by TSA

TNF-related apoptosis-inducing ligand is recognized by five different receptors. Although there is an evidence for a lack of correlation between TRAIL receptors expression (DR4/DR5) and sensitivity to TRAIL [14], mutations in DR4/DR5 have been shown to induce TRAIL-resistance [3]. TSA treatment of MM1S cells induced the transcription of DR5 receptor without affecting the transcription of DR4 and the decoy receptors DcR1 and DcR2 (Fig. 3a). However, the surface expression of DR5 was not upregulated after TSA treatment for 24 (Fig. 3b) or 48 h (data not shown). DR4 surface expression also did not show any significant change after 24 or 48 h of treatment with TSA (data not shown).

TSA-induced cytotoxicity is caspase-independent

Caspases play an essential role in apoptosis induction; however, apoptosis induction and cell death can proceed through different mechanisms that are caspase-independent. Pretreatment of MM1S myeloma cells with the pan-caspase inhibitor Z-VAD-FMK did not attenuate

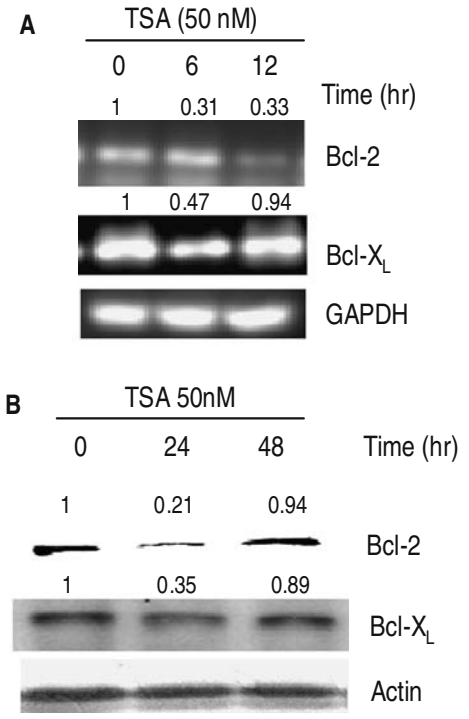

Fig. 2 Trichostatin A represses the transcription and downregulates Bcl-2 and Bcl-X_L expression. **a** MM1S cells were treated with TSA (50 nM) for 6 and 12 h. RT-PCR was done as described under methods to quantitate the mRNA levels of Bcl-2 and Bcl-X_L. GAPDH was used as a loading control. **b** MM1S cells were treated with TSA (50 nM) for 24 and 48 h. The protein expression of Bcl-2 and Bcl-X_L was quantified by Western blotting. Actin was used as a loading control. The *numerals* above the bands indicate their relative intensities after densitometric analysis

the cytotoxicity of TSA (Fig. 3c), indicating that TSA cytotoxicity is caspase-independent.

TSA modulates the expression of the Bcl-2 proapoptotic members

The proapoptotic members of the Bcl-2 family regulate apoptosis induction. TSA treatment of MM1S myeloma cells produced a mixed effect on the proapoptotic Bcl-2 members. While the expression of Bim and Bid was upregulated, the expression of other proteins like PUMA (α and β), Bax and Noxa were downregulated (Fig. 4a). The transcriptional induction of Bim and the transcriptional repression of PUMA, Bax and Noxa (Fig. 4b) were consistent with the observed proteins expression.

FLIP expression is not modulated by TSA

FLICE Inhibitory protein is a known mediator for TRAIL-resistance [7]. TRAIL-resistant MM1S cells showed a relatively higher expression of $FLIP_S$ than the ARP-1 TRAIL-sensitive [15] myeloma cells (Fig. 5a). TSA pretreatment could possibly alter the expression of $FLIP_S$ and consequently sensitize MM1S cells. However, the expression of $FLIP_S$ was not downregulated after TSA treatment for 24 or 48 h (Fig. 5b).

Discussion

In this study, we have investigated the effect of TSA pretreatment on the cytotoxic effect of TRAIL in TRAIL-resistant MM1S myeloma cells. TSA enhanced the cytotoxicity of TRAIL without affecting the surface expression of the death receptors DR4/DR5. TSA repressed the transcription and downregulated the expression of the antiapoptotic members of the Bcl-2 family proteins. The effect of TSA on the Proapoptotic members of the Bcl-2 family was mixed and only Bim and Bid were upregulated. TSA treatment did not alter the expression of $FLIP_S$ in MM1S myeloma cells, indicating that the observed enhancing effect of TSA on TRAIL cytotoxicity is attributed to the downregulation of the antiapoptotic members of the Bcl-2 family and is $FLIP_S$ independent.

TNF-related apoptosis-inducing ligand is a promising anticancer candidate; however, some cells showed resistance against TRAIL. Resistance to TRAIL can be explained by different reasons like FLIP overexpression [16, 17], mutated death receptors [3], constitutively active Akt/protein kinase B [18], loss of caspase-8 expression [2, 19] and overexpression of Bcl-2 or Bcl-X_L [4, 20]. We have shown here that MM1S myeloma cells exhibited TRAIL-resistance and a high dose of TRAIL induced only 2–4% apoptosis. However, pretreatment with TSA significantly enhanced TRAIL cytotoxicity. The same effect was reported in breast cancer [21] and other groups reported it in leukemia cells [22] and melanoma cells [23].

The Bcl-2 family proteins are crucial mediators of TRAIL-induced apoptosis. Several reports have shown that Bcl-2 or Bcl-X_L can hinder TRAIL-induced apoptosis [4, 20]. Here, we report the transcriptional repression and protein downregulation of both Bcl-2 and Bcl-X_L after TSA treatment. Other groups have reported the selective modulation of the proapoptotic and antiapoptotic members of the Bcl-2 family by HDAC inhibitors [23, 24] in melanoma cells and we have also reported the same effect in TRAIL-sensitive myeloma cells [15]. The mixed effect of TSA on the proapoptotic Bcl-2 family proteins is elusive, the upregulation of Bim and Bid is in support of the observed TRAIL-enhanced effect and agrees with the findings of others [23, 25]. The transcriptional repression and downregulation of the different isoforms of PUMA, Bax and Noxa after TSA treatment do not contradict with the observed TRAIL-enhancing effect, because these effects were accompanied by the upregulation of Bim and the downregulation of Bcl-2 and Bcl-X_L.

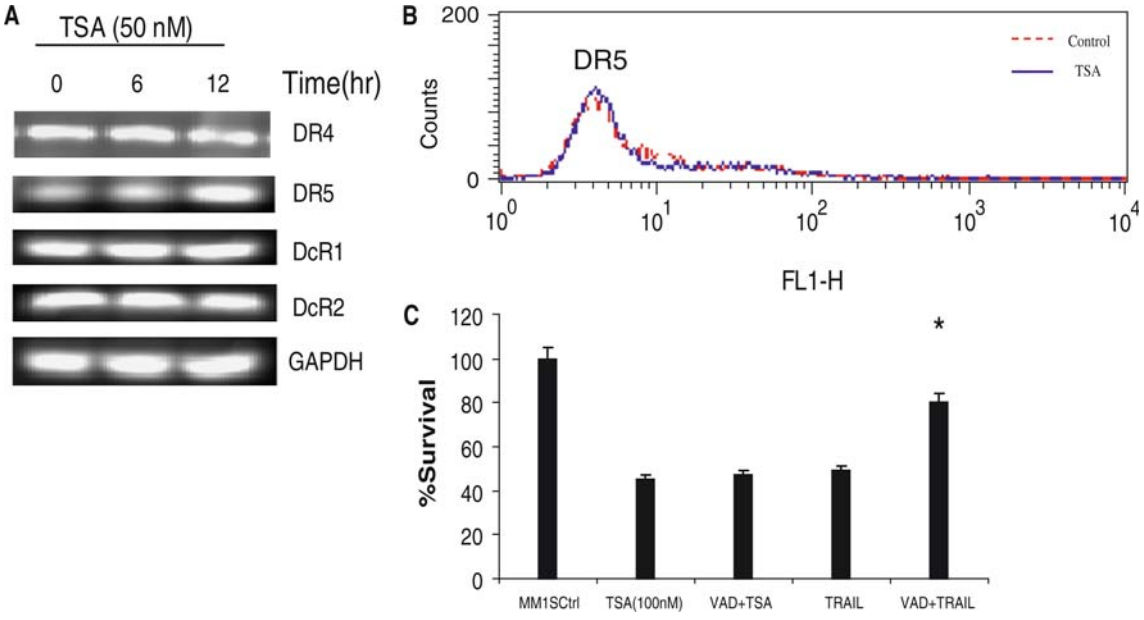

Fig. 3 Trichostatin A induces DR5 transcription without upregulating its surface expression. **a** MM1S cells were treated with TSA (50 nM) for 6 and 12 h and RT-PCR was done as described under methods to quantitate the mRNA of TRAIL receptors. GAPDH was used as a loading control. **b** MM1S cells were treated with TSA (50 nM) for 24 h, immunostained with DR5 antibody as described under methods and analyzed by flow cytometry. **c** MM1S cells were either pretreated with 30 μM of Z-VAD-FMK (*VAD*) for 2 h or left untreated, then treated with TSA (100 nM) or TRAIL (400 ng/ml) (positive control) for 24 h. XTT cell proliferation assay was used to estimate cell survival and percentage of survival was estimated as a percentage of the value of the untreated control. Data represent the mean for 4 replicates ±SD. * indicates significant difference from TRAIL treated cells at $P < 0.05$

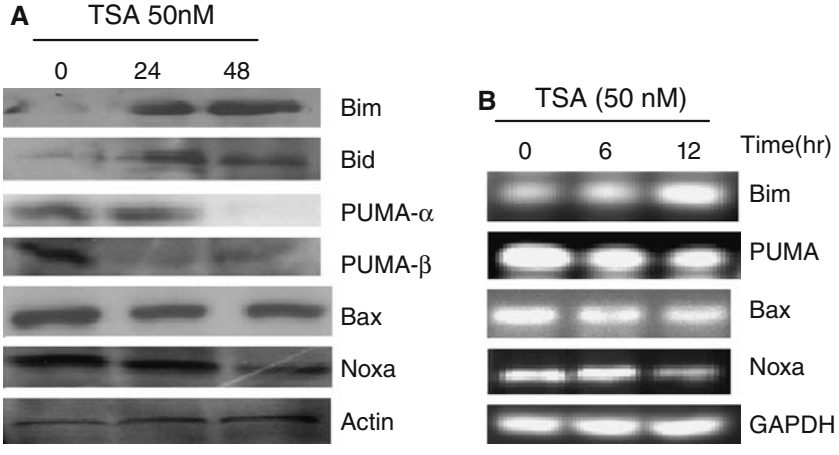

Fig. 4 The mixed effect of TSA on the proapoptotic Bcl-2 family members. **a** MM1S cells were treated with TSA (50 nM) for 24 and 48 h and Western blotting was used to quantify protein expression. Actin was used as a loading control. **b** MM1S cells were treated with TSA (50 nM) for 6 and 12 h and RT-PCR was performed to quantify the mRNA levels. GAPDH was used as a loading control

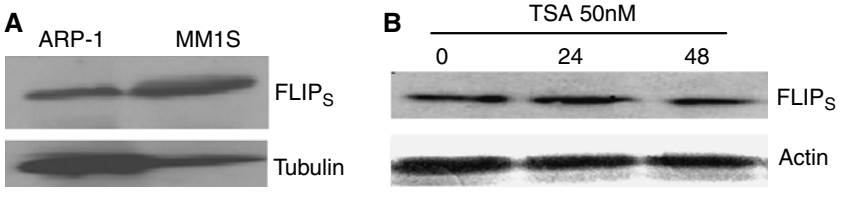

Fig. 5 Trichostatin A did not modulate $FLIP_S$ expression. **a** Protein extracts from ARP-1 and MM1S cells were analyzed for $FLIP_S$ expression using Western blotting. Tubulin was used as a loading control. **b** MM1S cells were treated with TSA (50 nM) for 24 or 48 h and the expression of $FLIP_S$ was quantified by Western blotting. Actin was used as a loading control

TNF-related apoptosis-inducing ligand death receptors (DR4/DR5) play an essential role in TRAIL-induced apoptosis. In this report we could not detect any change in the surface expression of DR4 or DR5 after TSA treatment, despite the transcriptional induction of DR5. This observation indicates that TSA enhances TRAIL cytotoxicity in a death receptor-independent manner. A previous report has shown also a similar observation in leukemia cells [22]. It is worth mentioning here that we have reported DR4/DR5 surface upregulation in TRAIL-sensitive myeloma cells after TSA or SAHA treatment [15], indicating that the effect of HDAC inhibitors on death receptors is cell type specific.

Caspases play a central role in mediating cell death. The cytotoxic effect of TSA was caspase-independent, which is in agreement with the results obtained by using SAHA, a hydroxamic acid derived HDAC inhibitor, in myeloma cells [26].

FLICE inhibitory protein is known for its role in mediating TRAIL-resistance. Although TRAIL-resistant MM1S cells showed relatively higher expression of $FLIP_S$ than TRAIL-sensitive ARP-1 myeloma cells, TSA did not downregulate the expression of $FLIP_S$, indicating that the enhancing effect of TSA on TRAIL cytotoxicity is $FLIP_S$ independent. This observation is in agreement with the effect of butyrates on both isoforms of FLIP in leukemia cells [27]. However, Other groups have shown that other HDAC inhibitors can downregulate FLIP expression [9, 28].

In summary, we have evaluated the effect of pretreatment of TRAIL-resistant myeloma cells with TSA on TRAIL cytotoxicity. The enhancing effect of TSA pretreatment on TRAIL cytotoxicity was not accompanied by surface upregulation of TRAIL death receptors (DR4/DR5) or FLIP dowregulation. However, the enhancing effect was associated with downregulation of the antiapoptotic members of the Bcl-2 family proteins and upregulation of the BH3 only domain protein, Bim.

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