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HDAC inhibitors with different gene regulation activities depend on the mitochondrial pathway for the sensitization of leukemic T cells to TRAIL-induced apoptosis

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ABSTRACT

Epigenetic modifications commonly associated with tumor development, such as histone deacetylation, may influence the resistance of some tumor cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) by regulating gene transcription of components of the TRAIL signalling pathway. In the present study we have analyzed the effect of six different histone deacetylase inhibitors (HDACi), belonging to the four classic structural families, on TRAIL-induced apoptosis in leukemic T cell lines. Non-toxic and functional doses of all HDACi but apicidin, similarly sensitized different leukemic T cell lines to TRAIL-induced apoptosis, while they showed no effect on the resistance of normal T lymphocytes. Sensitizing doses of vorinostat, valproic acid, sodium butyrate and MS-275 regulated the expression of TRAIL-R2, c-FLIP and Apaf-1 in leukemic cells while TSA modulated only the expression of Apaf-1. The synergistic effect of all HDACi and TRAIL was inhibited in Bcl-2-overexpressing leukemic T cells. Thus, different HDACi may affect the expression of different TRAIL-related genes, but regulation of the mitochondrial pathway seems to be essential for the TRAIL sensitizing effect of HDACi in leukemic T cells. Overall, HDACi represent a promising and safe strategy in combination with TRAIL for treatment of T-cell leukaemia.

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) is a type II transmembrane protein, belonging to the tumor necrosis factor (TNF) superfamily, that induce apoptosis upon binding to its specific death domain-containing receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 [1–3]. Apoptotic TRAIL signalling is initiated by oligomerization of TRAIL death receptors and subsequent recruitment of the intracellular adapter molecule Fas-associated death domain protein (FADD) and procaspases-8 and -10, thereby forming the death-inducing signalling complex (DISC). Activation of caspase-8 in the DISC allows the initiation of a cascade of events that leads to apoptotic cell death [4,5] either by directly activating effector caspases or by Bid cleavage and engagement of the mitochondrial death pathway.

Despite the selective antitumor activity of TRAIL, a large number of cancer cells are resistant to this death ligand. Resistance can occur by different ways since regulation of TRAIL-induced apoptosis is exerted at many stages along its signalling pathway. Several intracellular molecules have been described to block the apoptotic effect of TRAIL, such as the cellular Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein (c-FLIP) that competes with caspase-8 for binding to FADD [6,7]; Bcl-2 and

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Bcl-x_L that impede the activation of the mitochondrial pathway [8]; or the inhibitors of apoptotic proteins XIAP, c-IAP1 and c-IPA2 that inhibit active caspases [9,10].

During the last few years, several studies have focused on the development of therapeutic strategies aimed at overcoming resistance to TRAIL. One of these strategies involves the pharmacologic inhibition of histone deacetylases (HDAC), enzymes implicated in the epigenetic modifications that regulate the expression of genes during cancer development and progression [11]. Several HDAC inhibitors (HDACi), from natural and synthetic origin, have been described to induce cell cycle arrest, differentiation and apoptosis in human tumor cells and non-human models of cancer [12,13]. These HDACi have different chemical structures so that they can be classified in four groups: (i) hydroxamic acids, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA, Vorinostat); (ii) benzamides, such as MS-275; (iii) short-chain fatty acids, such as valproic acid (VPA) and sodium butyrate (NaB); (iv) cyclic peptides, such as apicidin and depsipeptide [12,14]. The hydroxamic acids are the most potent HDACi showing activity on class I (HDAC1, 2, 3 and 8) and class II (HDAC4, 5, 6, 7, 9 and 10) HDAC. In contrast, MS-275, apicidin and the short-chain fatty acids VPA and NaB are more potent inhibitors of class I HDAC and exhibit little or no activity against class II isoforms [15,16].

Histone acetylation by HDACi affects the expression of many genes [17], some of which are involved in the TRAIL signalling pathway. Accordingly, it has been shown that HDACi down-regulate anti-apoptotic factors, such as c-FLIP, Bcl-2, Bcl-x_L and XIAP, while increase the expression of pro-apoptotic proteins, such as caspase-8, caspase-3, Bid, Bim, Bax or Bak, in different solid and hematopoietic tumor types [18–21]. In addition, TRAIL itself and its pro-apoptotic receptor TRAIL-R2 have been reported to be regulated by HDACi in myeloid leukaemia cells, thus mediating the apoptotic effect of these inhibitors [22,23].

HDACi induce histone acetylation in both tumor and normal cells, but, similarly to TRAIL, they have shown selective antitumor activity [12,24]. However, the mechanism of this selective activity is not known and few studies have determined the effects of the combined treatment with HDACi and TRAIL in non-transformed cells [21,23,25,26]. The present study was designed to compare and better understand the effects of HDACi from different chemical classes on the induction of apoptosis by TRAIL in leukemic T cells and normal T lymphocytes. We analyzed the susceptibility to TRAIL-induced apoptosis upon pre-treatment with HDACi in both cell types. Furthermore, we determined the expression of several proteins involved in the TRAIL signalling pathway, such as components of the DISC and factors of the mitochondrial death pathway, in normal and leukemic T cells in response to HDACi. Our results show that different HDACi may differ in their mechanism of action and their efficacy for regulating TRAIL-mediated apoptosis in leukemic T cells, but their sensitizing effect involves the mitochondrial apoptotic pathway. In addition, none of them is able to sensitize normal T lymphocytes to TRAIL.

2. Materials and methods

2.1. Reagents and antibodies

Human recombinant TRAIL was prepared as described previously [1]. Valproic acid (VPA), trichostatin A (TSA), MS-275, sodium butyrate (NaB), phytohemagglutinin and mouse anti-β-actin were from Sigma–Aldrich (St. Louis, MO). Apicidin was obtained from Calbiochem (Darmstadt, Germany). Suberoylanilide hydroxamic acid (SAHA, Vorinostat, Zolinza®) was generously provided by Merck Research Laboratories (Boston, MA). Z-VAD-FMK, a wide spectrum caspase inhibitor, was from Bachem (Bubendorf, Suiza). Anti-cFLIP monoclonal antibody NF6 and mouse anti-human TRAIL receptor-2 antibody were purchased from Alexis Biochemicals (San Diego, CA). Mouse anti-human CD28 was from eBioscience (San Diego, CA). Anti-human caspase-8 monoclonal antibody was purchased from Cell Diagnostica (Munster, Germany). Caspase inhibitors Z-IETD-FMK and Z-LEHD-FMK, anti-human caspase-9 monoclonal antibody and monoclonal anti-human Apaf-1 were from R&D Systems (Minneapolis, MN). Anti-human caspase-3 polyclonal antibody was obtained from Stressgen (Ann Arbor, MI). Polyclonal antibody anti-histone H4 acetylated was obtained from Upstate Biotechnology (Lake Placid, NY).

2.2. Cells and cell culture

Blood samples, obtained from healthy donors by informed consent, were collected into citrate tubes. Peripheral blood T lymphocytes were then isolated and activated as previously described [27].

The human leukemic T cell lines Jurkat, CEM-6 and MOLT-4 were kindly provided by Dr. Abelardo López-Rivas (CABIMER, Sevilla, Spain). They were all maintained in culture in RPMI 1640 medium with 10% fetal bovine serum, 1 mM L-glutamine, penicillin and streptomycin at 37 °C in a humidified 5% CO_2, 95% air incubator. Jurkat cells stably overexpressing Bcl-2 were generously provided by Dr. Jacint Boix (Departamento de Ciencias Médicas Básicas, Universidad de Lleida, Spain) and maintained in culture medium with 1 mg/ml G418 sulfate (Sigma Chemical Co.).

2.3. Determination of apoptotic cells

Hypodiploid apoptotic cells were detected by flow cytometry according to published procedures [28]. Briefly, cells were washed with PBS, fixed in cold 70% ethanol, and then stained with propidium iodide while treating with RNase. Quantitative analysis of sub-G1 cells was carried out in a FACScan cytometer using the Cell Quest software (BD Biosciences).

2.4. Flow cytometric analysis of histone acetylation

Histone acetylation was analyzed as previously reported [29]. In brief, after 4 h treatment with HDACi cells were washed and fixed for 20 min in 1% formaldehyde in PBS on ice. Cells were then permeabilized with 0.1% Triton

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X-100 in PBS for 10 min at room temperature, washed with PBS containing 1% BSA, and incubated with 10% normal goat serum in PBS for 30 min. Subsequently, samples were incubated with 0.1 μg/ml anti-acetylated histone H4 monoclonal antibody for 1 h at room temperature and, after washing, stained with goat anti-rabbit fluorescein isothiocyanate-conjugated antibody (1:1000; Caltag Laboratories, Burlingame, CA) for 1 h at room temperature in the dark. Fluorescence of acetylated histone H4 was determined in a FACScan cytometer using the Cell Quest software (BD Biosciences).

2.5. Immunoblot detection of proteins

For detection of cytosolic proteins, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 1% NP-40) for 30 min. Proteins were resolved on SDS–PAGE minigels and detected as reported previously [30].

2.6. Cytofluorometric analysis of TRAIL receptors

To detect TRAIL-R2 receptor at the cell surface, control or treated cells were incubated with primary antibody (5 μg/ml) for 30 min at 4 °C. After washing with PBS to remove unbound primary antibody, cells were incubated with goat anti-mouse fluorescein isothiocyanate-conjugated antibody (1:1000; Caltag Laboratories, Burlingame, CA) for 30 min at 4 °C. Cells were then washed, resuspended in PBS and analyzed in a FACScan flow cytometer.

2.7. Real-time PCR

Total RNA was extracted from cells with Trizol Reagent (Invitrogen) as recommended by the supplier. cDNAs were synthesized from 3 μg of total RNA by using M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primer in a total volume of 20 μl. Reverse transcription was performed at 37 °C for 50 min followed by 15 min at 70 °C for inactivation.

Real-time PCR analysis was carried out with 1 μl cDNA using iQ SYBR Green Supermix (BioRad) and the iCycler iQ detection system (BioRad) according to the manufacturer's instructions. Samples were analyzed in triplicate and mRNA expression was normalized to 18S rRNA and quantified by the comparative cycle threshold (Ct) method.

PCRs were done using the following specific primers: TRAIL-R2, forward 5′-TTGCATCAGTTAGGGATACTGGG-3′ and reverse 5′-CAAAACCAACATTGATTCTTCAATAC-3′; ~~c-FLIP$_L$, forward 5′-AATTCAAGGCTCAGAAGCGA-3′ and reverse 5′-GGCAGAAACTCTGCTGTTCC-3′; c-FLIP$_S$, forward 5′-AATGTTCTCCAAGCAGCAATCC-3′ and reverse 5′-CCAAGAATTTTCAGATCAGGACAAT-3′;~~ rRNA 18s, forward 5′-GATATGCTC

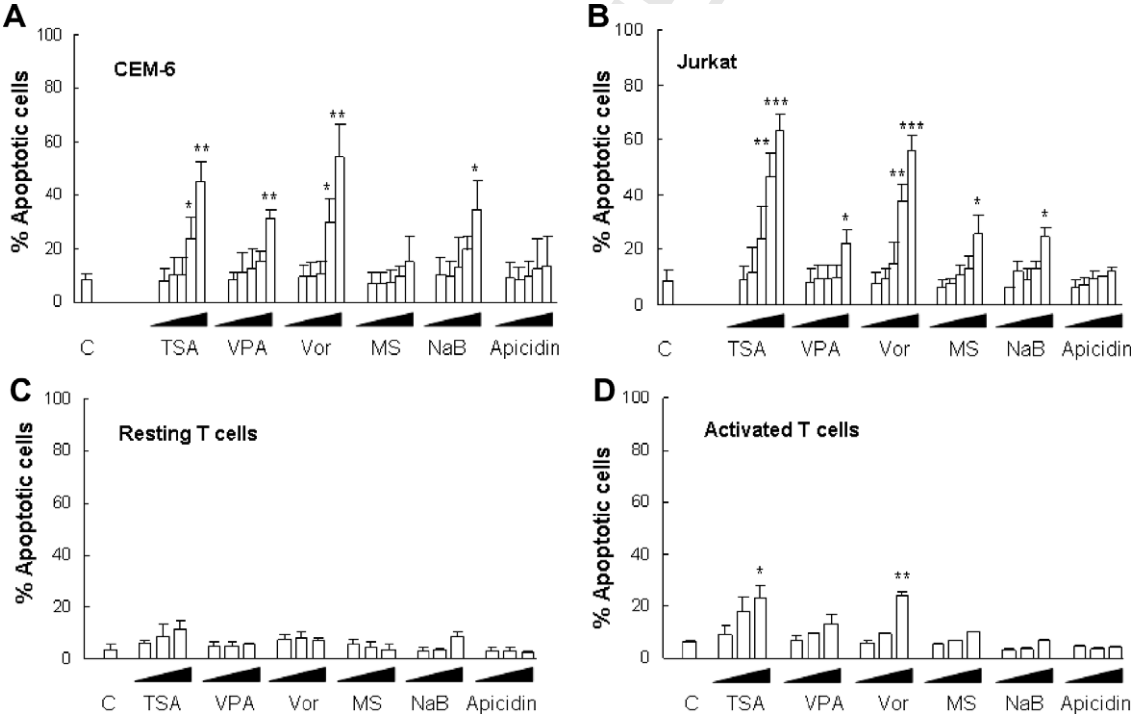

Fig. 1. Induction of apoptosis by HDACi in leukemic and normal T cells. (A) CEM-6 and (B) Jurkat cells were treated without (control, C) or with different doses of HDACi: TSA 10, 30, 50, 75 and 100 ng/ml; VPA 0.5, 0.75, 1, 2.5 and 5 mM; vorinostat (Vor) 0.1, 0.3, 0.5, 1 and 2 μM; MS-275 (MS) 0.5, 0.75, 1, 2.5 and 5 μM; NaB 0.1, 0.3, 0.5, 1 and 5 mM; and apicidin 10, 25, 50, 100 and 250 nM for 24 h. (C) Resting and (D) activated T cells were incubated for 24 h with the three lower doses of TSA and the three higher doses of the rest of HDACi mentioned above. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show SD from three independent experiments in (A and B) or three different donors in (C and D). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared to control cells.

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ATGTGGTGTTG -3′ and reverse 5′-AATCTTCTTCAGTCGCTC-CA-3′. The PCR cycling conditions were as follows: 95 °C for 5 min; then 40 cycles at 95 °C for 30 seg, 57 °C for 30 seg and 72 °C for 45 seg; and a final extension at 72 °C for 10 min.

2.8. Statistical analysis

The data were analyzed with unpaired Student's *t*-tests (two-tailed) by using GraphPad Prism 4 for Windows. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Non-toxic doses of different HDACi induce histone acetylation in both leukemic and normal T lymphocytes

We analyzed the induction of apoptosis in CEM-6 and Jurkat cells in response to treatment with the hydroxamic acids TSA and vorinostat, the benzamide MS-275, the aliphatic acids VPA and NaB, and the cyclic peptide apicidin. The range of doses used for each HDACi was established according to previous reports [19,27,31,32]. Similar results were found in both cell lines (Fig. 1A and B). All HDACi showed a dose-dependent apoptotic effect except for apicidin. In addition, no significant apoptosis was induced by MS-275 in CEM-6 cells. The higher percentages of apoptotic cells were observed after treatment with high doses of the pan-HDACi vorinostat and TSA. Next, we determined the induction of apoptosis in primary resting and activated T lymphocytes upon incubation with the three lower doses of TSA and the three higher doses of the rest of HDACi. Resting T cells were highly resistant to HDACi-mediated apoptosis, whereas high doses of both hydroxamic acids, TSA and vorinostat, showed a slight and significant toxicity against activated T cells (Fig. 1C and D).

Doses of HDACi (10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat, 1 μM MS-275, 0.5 mM NaB and 50 nM apicidin) were selected on the basis of their low and no toxicity for leukemic and normal T lymphocytes, respectively, and analyzed for their ability to enhance the levels of acetylated histone H4. Flow cytometric analyses demonstrated that all HDACi treatments increased H4 acetylation in leukemic T cells, reaching maximum acetylation level after 4 h of treatment (Fig. 2A and data not shown). Furthermore, the selected non-toxic doses of HDACi significantly increased the level of histone acetylation in resting T lymphocytes, with the exception of vorinostat (Fig. 2B). In the case of activated T cells, we also observed a low, but not significant, increase in histone H4 acetylation in response to all HDACi but apicidin (Fig. 2B).

3.2. Different HDACi potentiate TRAIL-induced apoptosis in leukemic T cell lines but not in normal T cells

We had previously shown that the HDACi valproic acid increased the susceptibility of the T-lymphoblastic leukemic CEM-6 cell line to TRAIL, while it did not alter the resistance of resting and activated normal T cells to TRAIL [27]. Now, we determined the ability of the different HDACi to increase TRAIL-mediated apoptosis in three different leukemic T cell lines, namely CEM-6, Jurkat and MOLT-4, when used at the above selected non-toxic concentrations. Pre-treatment for 4 h with all HDACi, except for apicidin, potentiated the apoptotic effect of TRAIL in the three cell lines, with CEM-6 showing a greater variability in the response to the different HDACi (Fig. 3A–C). Apicidin also enhanced TRAIL-induced apoptosis in Jurkat cells, but to a lesser degree when compared to the rest of HDACi (Fig. 3B). However, it had a significant although minimal effect and no significant effect in CEM-6 and MOLT-4 cell lines, respectively (Fig. 3A and C). We simultaneously analyzed the sensitivity to TRAIL-induced apopto-

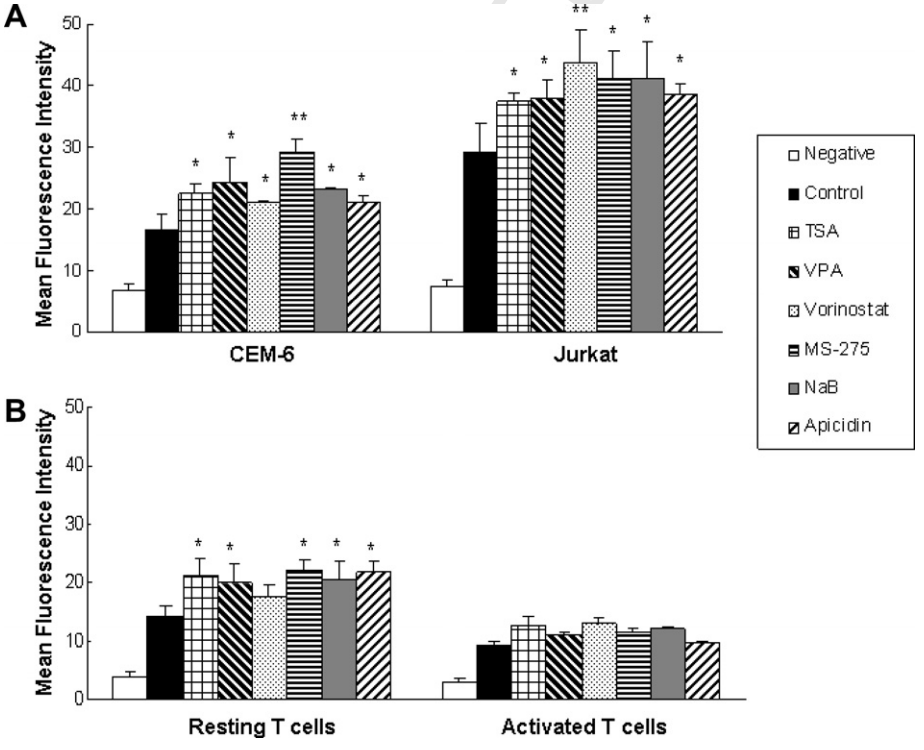

Fig. 2. HDACi increase histone acetylation levels in leukemic and normal T lymphocytes. (A) CEM-6, Jurkat cells, (B) resting and activated T cells were incubated without (control) or with 10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat, 1 μM MS-275, 0.5 mM NaB and 50 nM apicidin for 4 h. Histone H4 acetylation was analyzed by flow cytometry as described in Section 2. Negative represent background fluorescence with secondary antibody. Error bars show SD from three independent experiments in (A), or three different donors in (B). $^{*}p < 0.05$; $^{**}p < 0.01$, compared to control cells.

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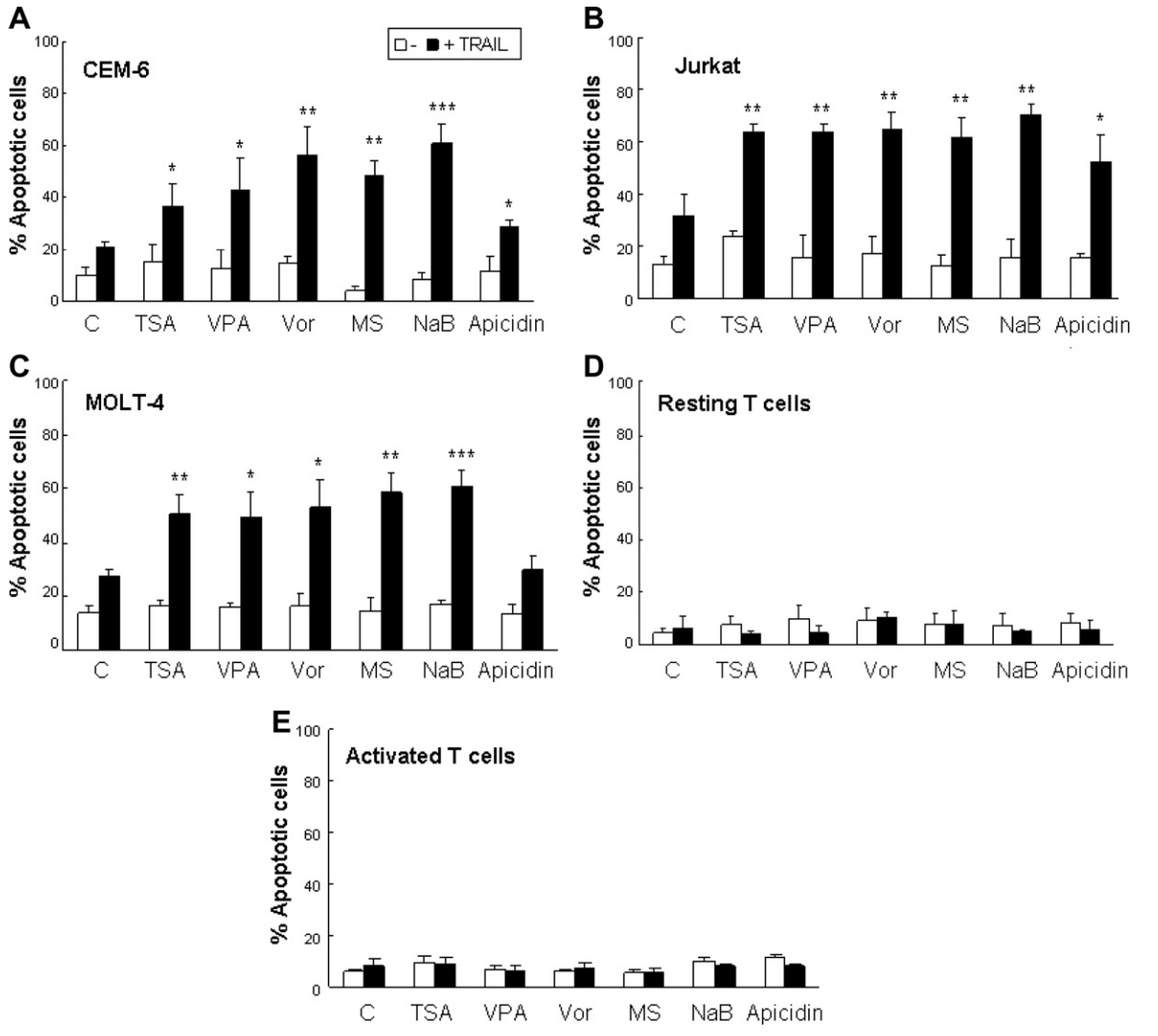

Fig. 3. Effect of HDACi on TRAIL-induced apoptosis in leukemic and normal T lymphocytes. (A) CEM-6, (B) Jurkat, (C) MOLT-4, (D) resting and (E) activated T cells were preincubated in the absence (control, C) or in the presence of non-toxic doses of HDACi (10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat (Vor), 1 μM MS-275 (MS), 0.5 mM NaB and 50 nM apicidin) for 4 h. After preincubation, cells were treated with or without recombinant TRAIL (100 and 250 ng/ml for leukemic and normal T cells, respectively) for 24 h. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show SD from three independent experiments in (A–C) or three different donors in (D and E). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared to TRAIL alone.

sis in primary T lymphocytes upon pre-treatment with HDACi. Strikingly, none of the inhibitors altered the resistance to TRAIL in either resting or activated T cells (Fig. 3D and E).

To further characterize the apoptotic cell death induced by the combined treatment with HDACi and TRAIL in leukemic T cells, we analyzed the activation of caspase-8, -9 and -3. All HDACi, except for apicidin, enhanced TRAIL-induced activation of caspases in CEM-6 cells (Fig. 4A). The same results were obtained in MOLT-4 cells (data not shown). In contrast, apicidin induced a similar increase in caspases activation to that observed with the rest of HDACi in Jurkat cells, as shown in Fig. 4A where the effect of apicidin is compared with that of NaB. These data correlate with those obtained in Fig. 3 and ~~indicates~~ that, in contrast to other HDACi, apicidin may selectively regulate TRAIL-induced apoptosis in some, but not all, leukemic T cells.

Previous studies have suggested the involvement of both the extrinsic and the intrinsic pathways in the synergistic effect of TRAIL and HDACi in human myeloid ~~leukemia~~ cell lines [19,33]. To confirm these observations in leukemic T cells, we compared the effect of specific caspase-8 and caspase-9 inhibitors (Z-IETD-FMK and Z-LEHD-FMK respectively) with that of the pan-caspase inhibitor Z-VAD-FMK, in the induction of apoptosis by HDACi and TRAIL in Jurkat cells. Not only the wide spectrum inhibitor Z-VAD, but also the caspase-8 and caspase-9 inhibitors were able to completely block apoptosis in response to the combined treatment with HDACi and TRAIL (Fig. 4B).

3.3. Components of the death receptor signalling pathway are differentially regulated by different HDACi in leukemic and normal T lymphocytes

To study the mechanism by which HDACi selectively modulate the sensitivity of leukemic T cells to TRAIL-induced apoptosis, we compared their effect on the expression of several pro- and anti-apoptotic proteins in both leukemic and normal T cells. At the selected sublethal doses, all HDACi slightly up-regulated the expression of TRAIL-R2 receptor, except for TSA and apicidin in CEM-6 cells and TSA in Jurkat cells (Fig. 5A). In

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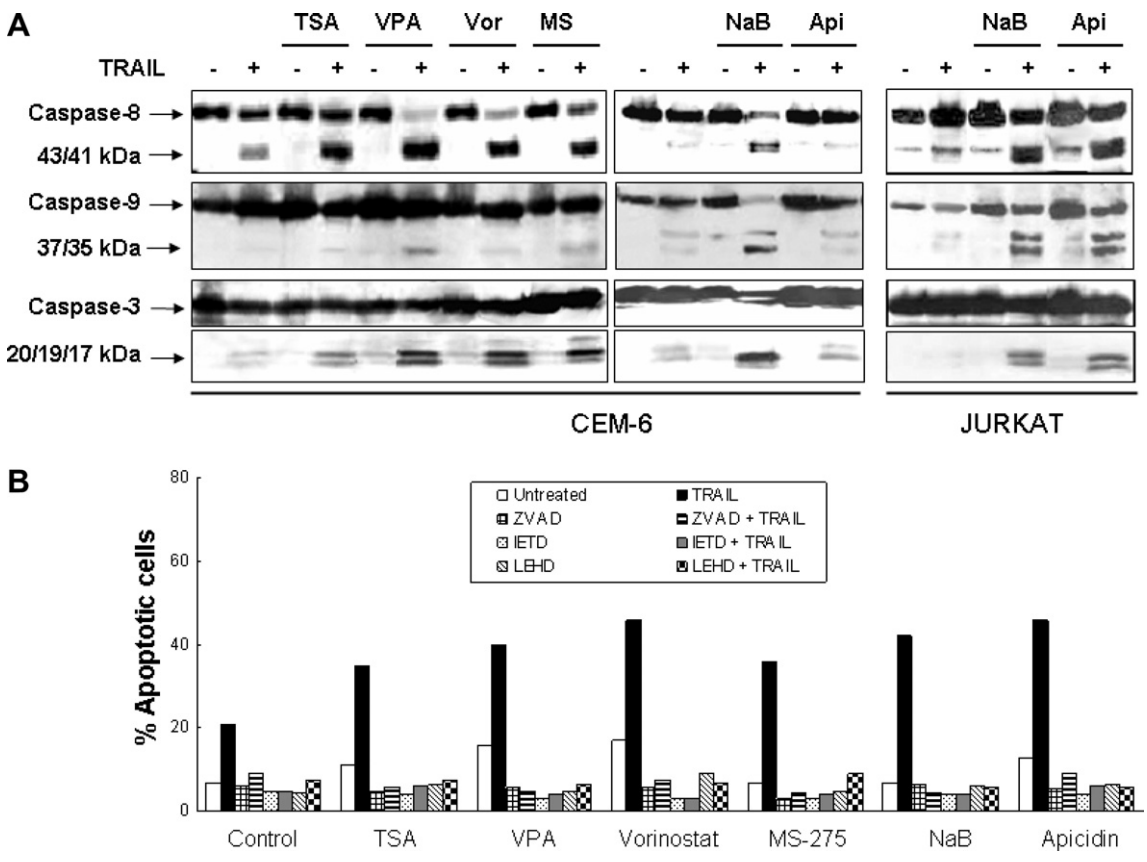

Fig. 4. HDACi enhance TRAIL-induced caspase activation in leukemic T cells. (A) CEM-6 and Jurkat cells were preincubated for 4 h with 10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat (Vor), 1 μM MS-275 (MS), 0.5 mM NaB or 50 nM apicidin (Api) before treatment without or with 100 ng/ml recombinant TRAIL for 20 h. Activation of caspase-8, -9 and -3 was determined by Western-blot. (B) Jurkat cells were preincubated for 1 h in the absence or in the presence of the caspase inhibitors Z-VAD-FMK, Z-IETD-FMK or Z-LEHD-FMK (20 μM) and then treated with HDACi for 4 h before adding TRAIL, as in (A). The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Results shown are representative of at least three independent experiments.

contrast, after treatment with the same non-toxic concentrations of HDACi there was no up-regulation of TRAIL-R1 expression (data not shown). We also analyzed the regulation of TRAIL-R2 expression in primary T cells. Both resting and activated T lymphocytes expressed barely detectable levels of TRAIL-R2 [27] and no change in the expression was observed upon treatment with HDACi (data not shown). To further confirm the regulation of TRAIL-R2 in leukemic T cell lines we determined mRNA receptor expression by real-time PCR. We observed that all HDACi increased the expression of TRAIL-R2 mRNA in Jurkat cells but the effect of TSA was minimal, compared to the rest of HDACi (Fig. 5B). In CEM-6 and MOLT-4 cells, not only TSA but also apicidin exerted a negligible effect in the expression of TRAIL-R2 mRNA (Fig. 5B and data not shown). These results are in agreement with that of surface TRAIL-R2 analysis and suggest that the ability to regulate gene expression may vary between cell lines and HDACi.

Next, we determined the expression of different factors involved in the extrinsic apoptotic pathway, such as caspase-8, FADD and c-FLIP. We found no changes in the levels of caspase-8 and FADD (Fig. 4A and data not shown). Regarding the anti-apoptotic protein c-FLIP, all HDACi, except for TSA and apicidin, seemed to reduce the expression of the long isoform, c-FLIP$_L$, in leukemic T cell lines, but the expression of the short isoform, c-FLIP$_S$, could not be detected by Western-blot (data not shown). We further analyzed the expression of several proteins involved in the mitochondrial apoptotic pathway and no changes were observed in the levels of Bcl-2, Bcl-x$_L$, Mcl-1, XIAP, c-IAP1, c-IAP2, Bim or Smac/DIABLO upon treatment with any of the HDACi (data not shown). In contrast, we found a clear increase in the expression of Apaf-1 in response to treatment with all HDACi but apicidin in all cell lines (Fig. 6A). As expected, HDACi did not regulate the levels of Apaf-1 in primary normal T lymphocytes (Fig. 6B).

3.4. HDACi do not increase TRAIL-induced apoptosis in leukemic T cells overexpressing Bcl-2

To establish the requirement of mitochondrial signals in the induction of apoptosis by HDACi and TRAIL in leukemic T cells, we examined the effect of this combined treatment in Jurkat cells overexpressing the anti-apoptotic protein Bcl-2. Prevention of TRAIL-mediated apoptosis by Bcl-2 is complex as it depends on the dose of TRAIL as well as the levels of Bcl-2 expression [8,34]. We found that overexpression of Bcl-2 failed to protect Jurkat cells from TRAIL-induced apoptosis at the dose used in this study (Fig. 7A and B). However, no significant sensitizing effect of HDACi was observed in Bcl-2-overexpressing cells (Fig. 7B) thus suggesting that regulation of mitochondrial factors and signals may play an essential role in the synergistic effect of HDACi and TRAIL. Interestingly, apicidin did not facilitate TRAIL-induced apoptosis in mock-transfected Jurkat cells, used as control of Bcl-2-overexpressing cells (Fig. 7A), which may be related to the existence of different Jurkat subclones that derived from different sources. In addition, we compared the effect on caspase activation in Jurkat Bcl-2 cells of two HDACi (i.e., vorinostat and TSA) showing different ability to regulate the expression of TRAIL-R2 and c-FLIP, that is, factors of the TRAIL signalling pathway acting upstream of mitochondrial events. As it was expected, pre-treatment with vorinostat, but not with TSA, increased TRAIL-induced activation of caspase-8 in Bcl-2 overexpressing cells (Fig. 7C). Even though, neither of them was able to

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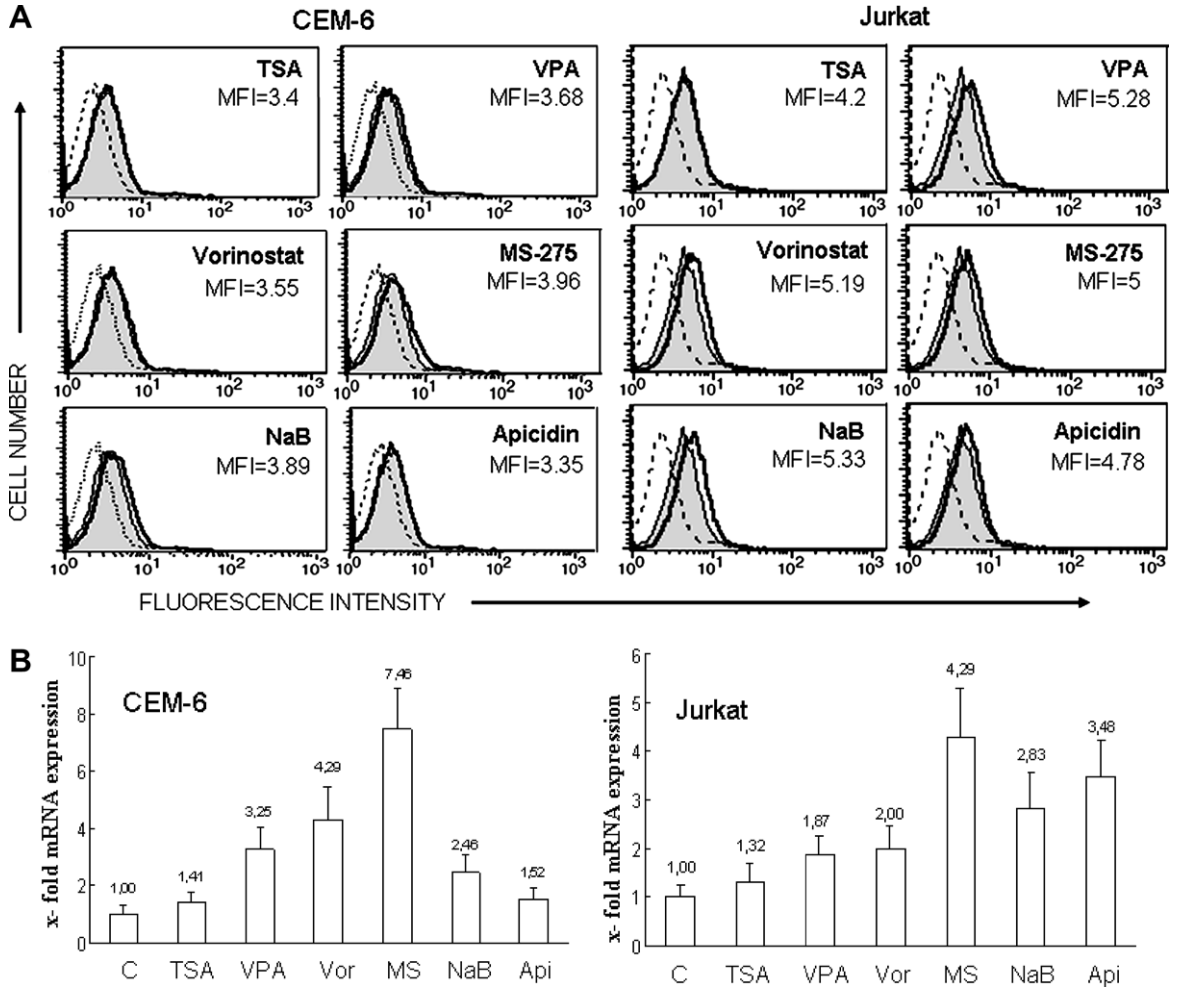

Fig. 5. HDACi up-regulate the expression of TRAIL-R2 in leukemic cells. (A) Cell surface TRAIL-R2 receptor expression was analyzed by flow cytometry in CEM-6 and Jurkat cells after treatment without (shaded peaks) or with (unshaded peaks) 10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat, 1 μM MS-275, 0.5 mM NaB or 50 nM apicidin for 20 h. Dashed lines represent background fluorescence with secondary antibody alone. MFI, median fluorescence intensity for TRAIL-2 expression in cells treated as indicated. The MFI values of untreated CEM and Jurkat cells were 3.4 and 4.25, respectively. (B) TRAIL-R2 mRNA levels were determined by real-time PCR in CEM-6 and Jurkat cells after treatment with HDACi as in (A) for 15 h. Experiments were done in triplicate and results were normalized to the expression of 18S rRNA for all samples. Error bars show SD from triplicate samples. Data shown in all panels are representative of at least two independent experiments.

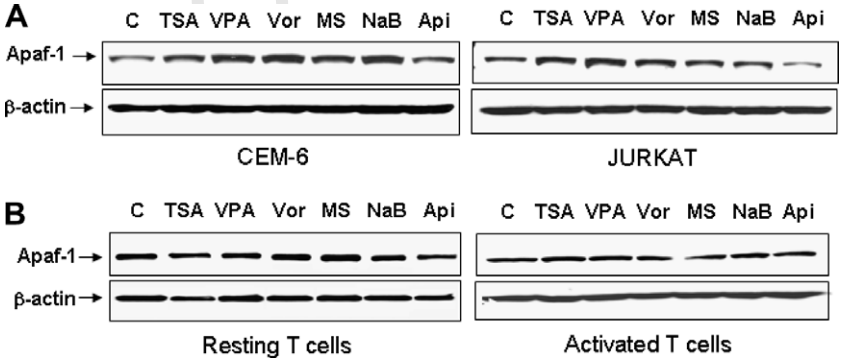

Fig. 6. Apaf-1 is up-regulated in response to treatment with HDACi in leukemic T cells. (A) CEM-6, Jurkat, (B) resting and activated T cells were treated with 10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat (Vor), 1 μM MS-275 (MS), 0.5 mM NaB or 50 nM apicidin (Api) for 20 h. Apaf-1 protein expression was determined by Western-blot and β-actin was used as control of loaded protein. Data shown are representative of at least three independent experiments.

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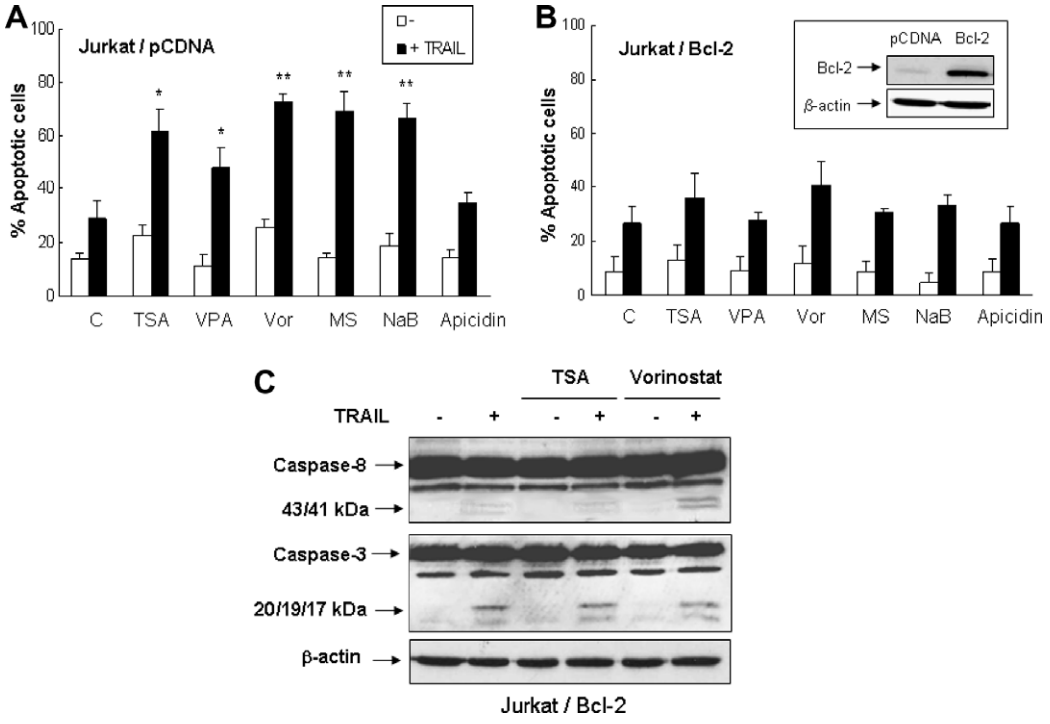

Fig. 7. Bcl-2 overexpression inhibits HDACi mediated sensitization to TRAIL in Jurkat cells. (A) Mock-transfected Jurkat cells (Jurkat/pCDNA) and (B) Bcl-2-overexpressing Jurkat cells (Jurkat/Bcl-2) were preincubated without (control, C) or with 10 ng/ml TSA, 1 mM VPA, 0.5 μM vorinostat (Vor), 1 μM MS-275 (MS), 0.5 mM NaB or 50 nM apicidin for 4 h. After preincubation, cells were treated with or without 100 ng/ml recombinant TRAIL for 20 h. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show SD from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. Inset in figure (B) shows levels of Bcl-2 and β-actin in mock-transfected and Bcl-2-overexpressing Jurkat cells, as determined by Western-blot. (C) Activation of caspase-8 and -3 was determined by Western-blot in Jurkat/Bcl-2 cells incubated with or without 100 ng/ml TRAIL for 20 h after pre-treatment with 10 ng/ml TSA or 0.5 μM vorinostat for 4 h. β-actin was used as control of loaded protein. Data shown are representative of three independent experiments.

enhance caspase-3 activation in response to TRAIL, in agreement with data of sub-G1 apoptotic cells (Fig. 7B and C).

4. Discussion

HDAC inhibitors have been recently proposed as a valuable therapeutic strategy to improve the sensitivity of tumor cells to TRAIL-induced apoptosis by regulating the expression of pro- and anti-apoptotic factors. To date, most of the studies have focused on the effects of two or three HDACi in some tumor cell types [18–21,32]. The variability in the results from different groups suggests that the pattern of gene regulation by HDACi may depend not only on the cell type but also on the chemical structure of the inhibitor. Few studies have investigated the effect of HDACi on the sensitivity of human leukemic T cells to TRAIL-mediated apoptosis, and all of them used the Jurkat cell line as the model of T-cell leukaemia [19,32,35]. Here, for the first time, we have simultaneously compared the ability of six different HDACi, belonging to the four classic structural families, to potentiate TRAIL-induced apoptosis in three leukemic T cell lines. With the exception of apicidin, pre-treatment with all HDACi increased the percentage of apoptotic cells and the activation of caspases-8, -9 and -3 induced by TRAIL in CEM-6, Jurkat and MOLT-4 cells. Interestingly, apicidin only regulated TRAIL sensitivity in a certain subclone of Jurkat cells.

The analysis of several genes of the extrinsic and the intrinsic apoptotic pathways revealed that the pro-apoptotic factors TRAIL-R2 and Apaf-1 were up-regulated, and the anti-apoptotic protein c-FLIP down-modulated, in different leukemic T cell lines in response to treatment with vorinostat, VPA, NaB and MS-275. In contrast, TSA only regulated the expression of Apaf-1. In agreement with our results, the expression of TRAIL-R2 has been previously reported to be regulated by vorinostat, NaB and MS-275 in Jurkat cells [19,32]. Additionally, these authors describe the regulation of TRAIL-R2 in response to TSA but discrepancies between these reports and our data may be due to the different doses of TSA used. Our most interesting finding about TSA is that non-toxic doses of this HDACi may potentiate TRAIL-induced apoptosis in leukemic T cells without affecting the expression of TRAIL-R2. These results also suggest that regulation of Apaf-1, and therefore the mitochondrial pathway, may be involved in the sensitization to TRAIL-induced apoptosis. In relation with this hypothesis, we demonstrated that overexpression of the anti-apoptotic protein Bcl-2 inhibited the synergistic effect of all HDACi on TRAIL-mediated apoptosis, without affecting the induction of apoptosis by TRAIL alone. In this respect, our results are similar to that obtained by Shankar et al. with Jurkat cells overexpressing either Bcl-2 or Bcl-x_L, although they observed inhibition of apoptosis induced by TRAIL alone and in combination with HDACi [19]. In

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contrast, other authors have described the enhancement of TRAIL-induced apoptosis in Bcl-x_L-overexpressing Jurkat cells by high doses of TSA [25]. It is possible that, in addition to the dose of TRAIL and the expression levels of Bcl-2 proteins [8,34], the dose of HDACi used may affect the response of Bcl-2-overexpressing cells. Our results further showed that HDACi did not potentiate TRAIL-induced apoptosis in Jurkat Bcl-2 cells even when a clear increase in apical caspase-8 activation occurred, as it was observed after pre-treatment with vorinostat. These are interesting results since they confirm that, regardless of the regulation of TRAIL-R2 and c-FLIP expression, modulation of mitochondrial signals also play a key role in the sensitizing effect of HDACi in leukemic T cells. In addition, they explain why TSA synergizes with TRAIL in the absence of regulation of proteins involved in the extrinsic apoptotic pathway. The increase in TRAIL-induced caspase-8 activation observed after pre-treatment with TSA in leukemic T cells is probably due to a mitochondria-mediated amplification feedback loop.

Concerning apicidin our results are striking as it induced histone acetylation in a similar way to other HDACi in both Jurkat and CEM cells but its sensitizing effect was only evident in Jurkat cells by regulating the expression of TRAIL-R2. To our knowledge, there is no other published data about the effect of apicidin in combination with TRAIL, apart from a recent report by Park et al. describing the sensitization of K562 erythroleukemia cells to TRAIL-induced apoptosis through a caspase-dependent mitochondrial pathway [36]. Interestingly depsipeptide, an HDACi from the same structural family as apicidin, has been shown to up-regulate the expression of TRAIL-R2 and to increase DISC formation in Jurkat cells [25], which could support our results. However, we cannot rule out the possibility that apicidin may be regulating other apoptosis-related factors that contribute to its sensitizing effect. Further studies on the effects of apicidin in different cell lines will help clarify the selective effect of this HDACi but, on the whole, results with apicidin suggest that this HDACi is not the one of choice for facilitating TRAIL-induced apoptosis in leukemic T cells.

Differences found in the mechanism of action of HDACi and their ability to enhance TRAIL-induced apoptosis in leukemic T cells may come from their different selectivity against diverse classes of HDAC [15,16]. In addition, different HDACi, even belonging to the same structural group, show different selectivity and potency toward different isoforms of the same HDAC class. For instance, MS-275 does not inhibit the class I isoform HDAC8; and TSA seems to be more potent against some class II isoforms than vorinostat. Considering all these data it is tempting to speculate that, except for HDAC8, class I HDAC play a major role in the regulation of the sensitivity to TRAIL-induced apoptosis in leukemic T cells, which is in agreement with a previous study in chronic lymphocytic leukaemia cells [37]. Interestingly, enhanced expression of class I rather ~~than~~ class II HDAC isoforms seems to be associated with cell survival and worse tumor prognosis [24,38]. We have determined the expression of some HDAC isoforms in leukemic T cell lines. Concerning class I HDAC, the results of this study indicate that Jurkat cells express similar levels of HDAC1 but lower levels of HDAC2 and HDAC3 than CEM-6 and MOLT-4 cell lines (data not shown). These are interesting results as the response to HDACi is similar in all cell lines. However, they might explain the peculiar behaviour of apicidin, which is a selective inhibitor of HDAC2 and HDAC3 [16]. We can hypothesize that apicidin is less potent than other HDACi and, therefore, it is only able to show activity in cells with low levels of HDAC, that is, Jurkat cells.

Several HDACi are currently in clinical trials as anti-cancer drugs. In particular, vorinostat has been approved for the treatment of cutaneous manifestations in patients with cutaneous T cell lymphoma who have progressive, persistent or recurrent disease on or following two systemic therapies [38,39]. Although the molecular mechanism responsible for the selective action of HDACi in cancer cells is not completely understood, global chromatin alterations associated with oncogenic transformation might at least in part account for their different activity against tumor and normal cells. In addition, alterations in the expression and function of HDAC enzymes have been found in many human cancers [24]. Regarding the therapeutic potential of combined treatment with HDACi and TRAIL, few studies have paid attention to the outcome of this combination in normal cells [21,25,26,32]. We had previously reported that valproic acid did not regulate TRAIL resistance in primary T cells [27]. Now, we have simultaneously analyzed the effect of the six already mentioned HDACi in primary resting and activated T lymphocytes. The effect in the last ones is interesting as they are similar to tumor cells in terms of proliferative potential. Higher doses of TSA and vorinostat showed low toxicity toward activated T lymphocytes, which might be associated with their activity as broad-spectrum inhibitors. It is not surprising that resting and activated T lymphocytes showed different sensitivity to these HDACi as their mechanisms of apoptosis regulation are different [27,40]. In contrast, at the selected doses that are effective in potentiating TRAIL-induced apoptosis in leukemic T cells, HDACi neither showed toxicity nor regulated TRAIL resistance in primary T lymphocytes, despite their ability to increase histone acetylation in these cells. Overall, our data emphasize the necessity of thoroughly selecting specific HDACi that show safety and efficacy in combined therapeutic strategies with TRAIL for the treatment of T-cell ~~leukemic~~.

Conflict of interest

The authors declare that they have no conflict of interest.

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