The role of oxidative stress in apoptosis induced by the histone deacetylase inhibitor suberoylanilide hydroxamic acid in human colon adenocarcinoma HT-29 cells

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Abstract. Histone deacetylase inhibitors (HDACIs) activate genes that promote cell cycle arrest and apoptosis in a number of tumor cells. This study showed that suberoylanilide hydroxamic acid (SAHA), a potent and commonly used HDACI, induced apoptosis in human colon adenocarcinoma HT-29 cells in a time- and dose-dependent manner. This effect was accompanied by the induction of oxidative stress, dissipation of mitochondrial transmembrane potential and activation of executioner caspases. Moreover, SAHA increased the levels of phosphorylated active forms of p38 and JNK. The addition of either the antioxidant N-acetyl-cysteine or the specific inhibitor of NADPH oxidase diphenylene iodonium chloride reduced the cytotoxic effects of SAHA in HT-29 cells, suggesting that the induction of oxidative stress represents a crucial event in the apoptotic mechanism. In addition, SAHA up-regulated the death receptor DR5, inducing the activation of caspase-8 with the consequent cleavage of Bid. Furthermore, SAHA down-regulated $FLIP_L$ and Akt, two proteins which exert an inhibitory role in apoptosis.

Introduction

Reversible histone acetylation/deacetylation is one of the key mechanisms involved in the epigenetic control of gene expression (1). Acetylation depends on histone acetyl-transferases, which acetylate lysine residues at the N-terminal tails of core histones, neutralizing their positive charges. Deacetylation is catalyzed by histone deacetylases (HDACs), a family of different enzymes, which remove acetyl groups causing DNA to wrap more tightly around the histone octamer. This event is associated with a relatively compact and inaccessible chromatin state, which generally correlates with lower gene transcription.

The histone deacetylase inhibitors (HDACIs) act by promoting the acetylated forms of core histones, weakening the histone-DNA interactions and inducing the relaxation of the chromatin structure, followed by transcriptional activation of genes involved in the control of cell proliferation, differentiation and apoptosis (2). Since the anti-proliferative and pro-apoptotic effects are more pronounced in tumor cells than in normal cells, HDACIs have recently emerged as promising chemotherapeutic agents (3,4).

HDACIs belong to a heterogeneous class of compounds that includes derivatives of short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides (5).

Hydroxamic acids have attracted the attention of researchers because of their high antiproliferative properties in many tumor types *in vitro* and *in vivo*. Specifically, suberoylanilide hydroxamic acid (SAHA) has been shown to inhibit the growth of transformed human cells, including erythroleukemia and lymphoma cells (6), as well as cultured cell lines obtained from different solid cancers (7,8). In addition, a clinical investigation demonstrated that SAHA exhibits a high therapeutic potential in the treatment of various malignancies at doses that were well-tolerated by patients (9).

Another notable property of SAHA is its ability to exert antitumor effects in a synergistic manner with various compounds such as TRAIL (10,11) and anticancer drugs, such as topoisomerase (12,13) and proteasome inhibitors (14). Recently, we demonstrated that in human hepatoma cells SAHA strongly induces apoptosis showing also a clear synergistic effect with the proteasome inhibitor bortezomib (15). In contrast, SAHA, alone or in combination with bortezomib, was found to be ineffective in inducing apoptosis in primary human hepatocytes (15).

The biochemical mechanism through which SAHA induces apoptosis is under active investigation. It has been shown that SAHA alters the expression of a large number of genes which can be involved in apoptosis or cell survival mechanisms (16).

Colorectal carcinoma represents one of the main causes of cancer-related death in the world. Currently available protocols of treatment include the antimetabolite 5-fluorouracil, topoisomerase inhibitors and platin derivatives (17,18).

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However, this tumor appears to develop resistance to chemotherapy owing to a variety of survival mechanisms, including the enhanced efflux of drugs and alterations in drug metabolism (19).

Additionally, HDACIs have been considered as potential chemotherapeutic agents against colorectal carcinoma based on the observation that high dietary fiber intake has been associated with a reduced risk for this type of neoplasia. This effect is ascribed to n-butyrate, a histone deacetylase inhibitor, which is produced in the gastrointestinal tract (20,21). Furthermore, histone acetylation has been associated with enhanced radiocytotoxicity in certain colorectal carcinoma cell lines (22).

In our study, we analyzed the cytotoxic activity of SAHA in the colon cancer HT-29 cell line. We provide evidence that SAHA is particularly efficacious in inducing apoptosis in HT-29 cells through a mechanism involving the production of reactive oxygen species (ROS), dissipation of mitochondrial transmembrane potential and the activation of executioner caspases.

Materials and methods

Chemicals and reagents. SAHA was obtained from Italfarmaco S.p.a. (Milan, Italy). Benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD) was from Promega (Italy). N-acetylcysteine (NAC) and diphenylene iodonium chloride (DPI) were supplied by Sigma (St. Louis, MO). Each antibody used was purchased from Cell Signalling (Beverly, MA, USA), except Bid antibody (Santa Cruz Bio, Santa Cruz, CA, USA) and pro-caspase-6 antibody (Sigma). Stock solutions of SAHA were prepared in DMSO and opportunely diluted in the culture medium. The final concentration of DMSO never exceeded 0.04%.

Cell culture conditions. Human colon adenocarcinoma HT-29 cells were grown as monolayers in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 2.0 mM L-glutamine. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were seeded at 60-70% confluence. After plating, cells were allowed to adhere overnight and then treated with the compounds.

Cell cycle analysis. The cell cycle phase distribution was evaluated through flow cytometric analysis. After exposure to the drugs, cells were harvested by trypsinization and incubated for 2-3 h at 4°C in a hypotonic solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.01% Nonidet P-40 and 10 μg/ml RNase A. The cell cycle profiles were analysed by Epics XL flow cytometer (Beckman Coulter) using Expo32 software. Apoptosis was determined by evaluating the percentage of events accumulated in the sub-G0/G1 position, according to Darzynkiewicz *et al* (23).

Measurement of ROS production. ROS production was measured by using 2',7'-dichlorofluorescindiacetate (H₂DCFDA, Molecular Probes, Eugene, OR), an indicator for ROS which is converted into a fluorescent compound after the removal of the acetate groups by intracellular esterases and oxidation by ROS.

After treatment, the cells were gently washed once with warm buffer HBSS/Ca/Mg and incubated with 5 μM H₂DCFDA dye at 37°C for 30 min. The cells were trypsinized, re-suspended in a buffer and analysed by flow cytometry at the excitation and emission setting of 488 and 525 nm, respectively, using Expo32 software. The percentage of cells showing a higher fluorescence, which reflects ROS production, was determined by comparison with untreated controls.

Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$). Mitochondrial transmembrane potential was measured by using 3,3-dihexyloxacarbocyanine (DiOC₆, Molecular Probes, Eugene, OR), a fluorochrome which exclusively emits within the spectrum of green light and accumulates in the mitochondrial matrix under the influence of $\Delta\psi_m$.

HT-29 cells were harvested by trypsinization, treated with 40 nM DiOC₆ for 20 min at 37°C, washed with phosphate-buffered saline and analysed by flow cytometry using the excitation and emission setting of 488 and 525 nm, respectively. The percentage of cells showing lower fluorescence, reflecting the loss of $\Delta\psi_m$, was determined by comparison with untreated controls.

Western blot analysis. Whole cell extracts were prepared by dissolving the cells in lysis buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, pH 7.4) for 30 min at 4°C in the presence of a mix of protease inhibitors (25 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium ortovanadate, 10 mM NaF, 25 μg/ml leupeptin, 0.2 mM sodium pyrophosphate). Cells were then sonicated three times for 10 sec and the lysates centrifuged at 15,000 x g for 20 min at 4°C. Equal amounts of protein samples (60 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for the detection with specific antibodies. Immunoblots were detected as previously reported (24). The correct protein loading was verified by means of Ponceau red staining and actin immunodetection. Bands were quantified by densitometric analysis using SMX Image software.

Results

The effects of the histone deacetylase inhibitor SAHA on HT-29 cell viability. Fig. 1 shows the effects of SAHA on the cell cycle distribution of HT-29 cells. Experiments were performed by means of flow cytometric analysis after staining of DNA with propidium iodide. The percentage of cells with subdiploid DNA confined to the sub-G0/G1 phase of the cell cycle was assumed as apoptosis index. Treatment with SAHA increased this percentage in a dose-dependent manner, so that at 36 h the percentage of cells in the sub-G0/G1 phase reached 92.5% with 5 μM SAHA (Fig. 1A).

In time-dependent experiments, we demonstrated that in the first phase of treatment (4-8 h) 5 μM SAHA induced an accumulation of HT-29 cells in the G2/M phase of the cell cycle. For longer periods, it was observed as a progressive increase in the percentage of cells confined to the sub-G0/G1 phase, which reached 44% at 24 h and 92% at 36 h of
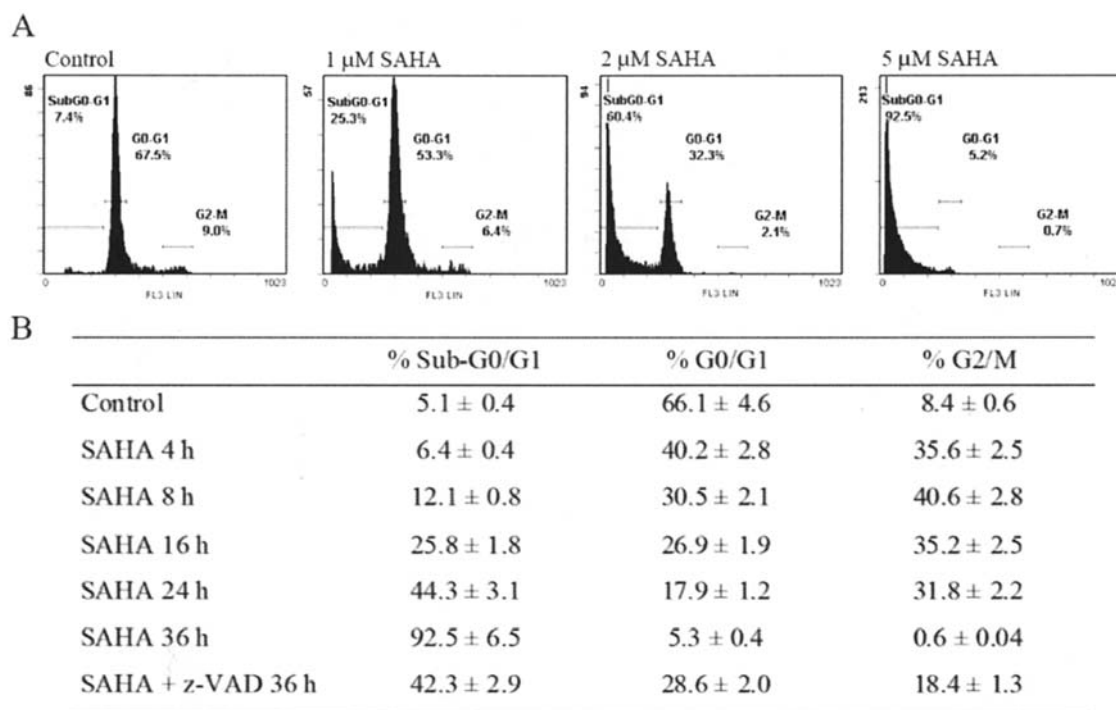
A

B

	% Sub-G0/G1	% G0/G1	% G2/M
Control	5.1 ± 0.4	66.1 ± 4.6	8.4 ± 0.6
SAHA 4 h	6.4 ± 0.4	40.2 ± 2.8	35.6 ± 2.5
SAHA 8 h	12.1 ± 0.8	30.5 ± 2.1	40.6 ± 2.8
SAHA 16 h	25.8 ± 1.8	26.9 ± 1.9	35.2 ± 2.5
SAHA 24 h	44.3 ± 3.1	17.9 ± 1.2	31.8 ± 2.2
SAHA 36 h	92.5 ± 6.5	5.3 ± 0.4	0.6 ± 0.04
SAHA + z-VAD 36 h	42.3 ± 2.9	28.6 ± 2.0	18.4 ± 1.3

Figure 1. The effects of SAHA on cell cycle distribution of HT-29 cells. Cells were incubated for 36 h (A) with the indicated doses of SAHA or for various times (B) with 5 μM of SAHA. After staining with propidium iodide, the percentage of cells confined in the different phases of the cell cycle was quantified by a flow cytometric analysis. The results are representative of four independent experiments ± SE.
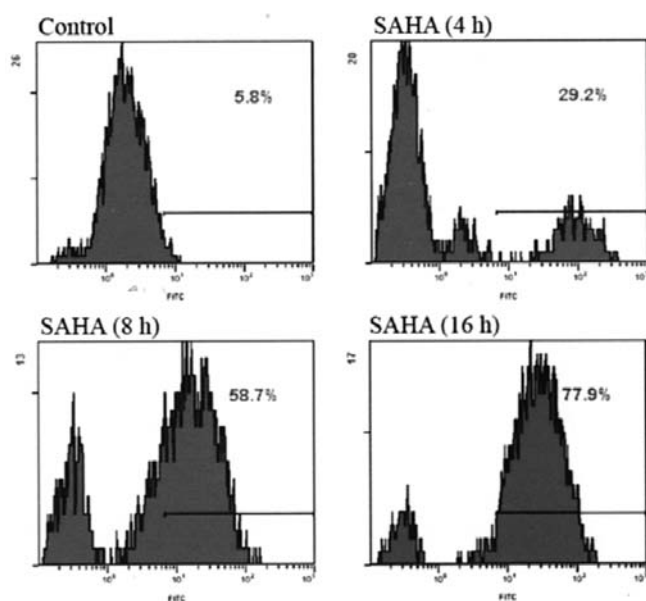

Figure 2. SAHA induced ROS production in HT-29 cells. Cells were treated for different times (4-16 h) with 5 μM of SAHA. The quantification of ROS was performed by flow cytometry with H_2DCFDA, as reported in Materials and methods. Untreated cells were assumed as the control. Data are representative of four separate experiments with similar results.

treatment. The addition of 100 μM z-VAD, a general caspase inhibitor, partially counteracted the effect induced by SAHA at 36 h of treatment, suggesting that caspase activities are involved in the cytotoxic effects induced by the drug (Fig. 1B).

The induction of oxidative stress and mitochondrial damage in SAHA-treated HT-29 cells. Previous studies have shown that HDACIs induce oxidative stress in transformed cells (25). In order to evaluate the involvement of oxidative stress in apoptosis induced by SAHA in HT-29 cells, we studied the effect of the drug on the production of ROS by using H_2DCFDA, a nonfluorescent lipophilic probe commonly employed to detect the cellular production of ROS. The removal of acetate groups by intracellular esterases and the ROS-dependent oxidation converts H_2DCFDA in a fluorescent compound detectable by flow cytometric analysis as a shift of the fluorescence histogram to the right. In HT-29 cells, treatment with 5 μM of SAHA caused a time-dependent increase in ROS-related fluorescence (Fig. 2). The effect reached the maximum after 16 h and thereafter, ROS production decreased (not shown).

It is well known that a possible consequence of ROS production is the loss of mitochondrial inner membrane potential ($\Delta\psi_m$) (26). Therefore, we investigated whether SAHA is capable of inducing dissipation of $\Delta\psi_m$ in HT-29 cells using a cytofluorimetric analysis which employs $DiOC_6$, a mitochondria-specific and voltage-dependent dye. The results indicated that 5 μM of SAHA induced a dissipation of $\Delta\psi_m$, as indicated by the increase in the percentage of cells with lower fluorescence. The effect started at 8 h and after 36 h ~80% of the HT-29 cells were depolarized (Fig. 3). The addition of 100 μM z-VAD markedly reduced the loss of $\Delta\psi_m$ induced by SAHA after 36 h of treatment.

In addition, we observed activation of caspase-9 as demonstrated by the reduction of the band corresponding to its
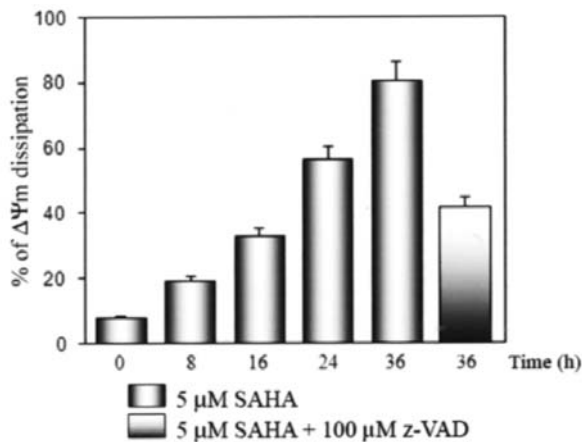
Figure 3. The effect of SAHA on mitochondrial transmembrane potential in HT-29 cells. Cells were treated for different times (8-36 h) with 5 μM of SAHA. Quantification of $\Delta\psi_m$ was performed by flow cytometry with the lipophilic dye $DiOC_6$, as reported in Materials and methods. Untreated cells were assumed as the control. The data are representative of three separate experiments with similar results.

inactive form and the appearance of the cleavage products of 37 and 35 kDa corresponding to its intermediate and active forms, respectively. This cleavage was evident after 16 h of treatment and at 24 h the intensity of the band at 35 kDa was ~three-fold the control value (Fig. 4). Such an effect seems to be correlated with the loss of $\Delta\psi_m$ induced by SAHA.

Activation of the apoptotic pathway in SAHA-treated HT-29 cells. To further investigate the putative molecular mechanism of the apoptotic activity of SAHA, we assessed the

expression of a number of pro- and anti-apoptotic factors in HT-29 cells exposed to the drug.

Some experiments were performed in order to evaluate the effect of SAHA on death receptors and their ligands. Western blot analysis showed that SAHA did not modify the expression of the FAS receptor and its ligand (not shown) while it induced up-regulation of DR5, another membrane death receptor, which is associated with the TNF-related apoptosis-inducing ligand (TRAIL). Fig. 4 shows that this effect was already evident after 8 h of treatment, then increased with time reaching at 24 h a level about two-fold higher than the control value. In addition, the effect was dependent on SAHA concentration (not shown).

Then, we analysed the effect of SAHA on death receptor-related caspase-8 and its specific inhibitor FLIP. Two major FLIP splicing variants are present in cells: a short, 26 kDa protein (FLIP$_S$) and a longer, 55 kDa form (FLIP$_L$), which shows a high homology to the prodomain of caspase-8 (27). Treatment with 5 μM of SAHA induced in HT-29 cells the processing of pro-caspase-8 with the appearance of the cleavage product of 43/41 kDa corresponding to its active form. The effect was already evident after 16 h of treatment and increased at 24 h. Moreover, Western blot analysis showed a progressive decrease in the level of FLIP$_L$ with almost the disappearance of the corresponding band after 24 h of treatment (Fig. 4). Appreciable variations in the levels of FLIP$_S$ were not observed.

In addition, a marked decrease in the native Bid protein (22 kDa) was observed in SAHA-treated HT-29 cells with the appearance of a 15 kDa band corresponding to its truncated active form (tBid). This effect can be considered as a consequence of caspase-8 activation.
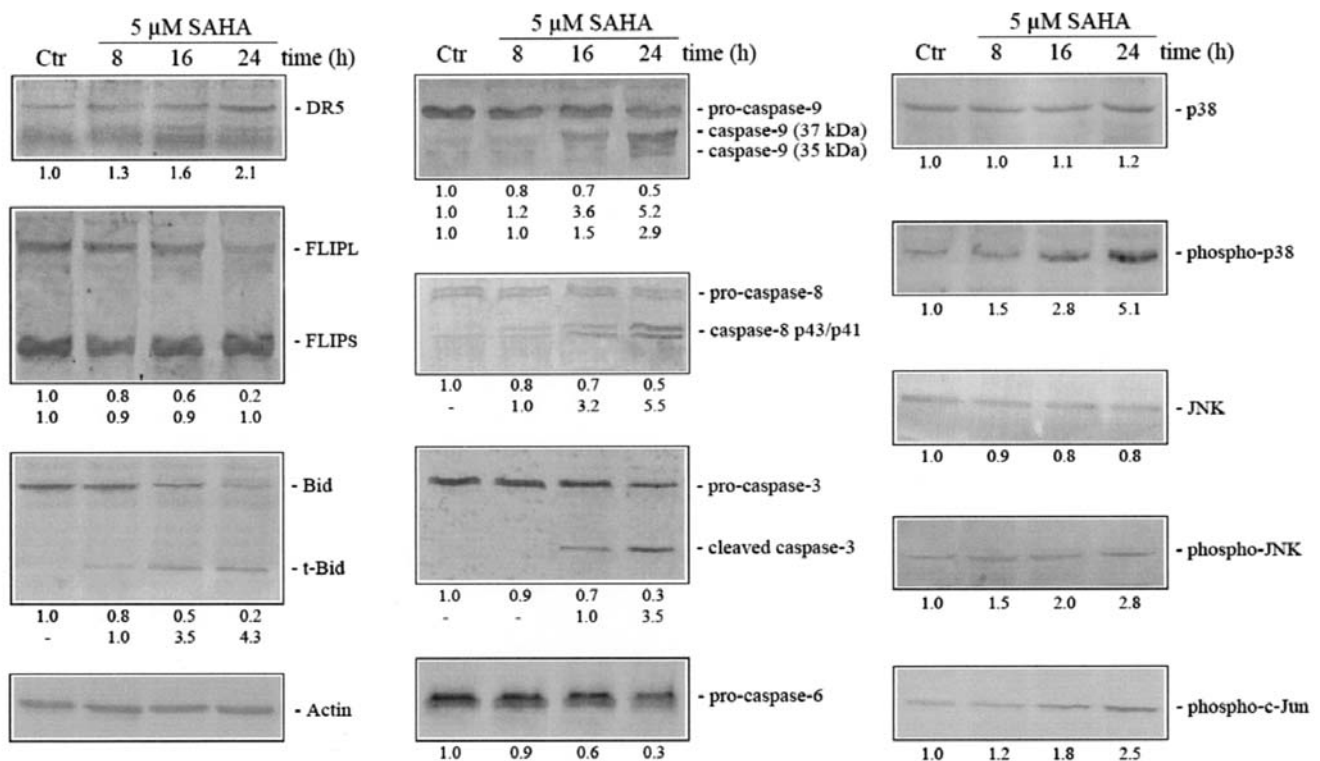

Figure 4. The effect of SAHA on apoptosis regulatory factors in HT-29 cells. Cells were treated for different times (8-24 h) with 5 μM of SAHA. Then, cell lysates were analysed by SDS-PAGE and Western blotting as indicated. Untreated cells were assumed as the control. The data are representative of four separate experiments with similar results.
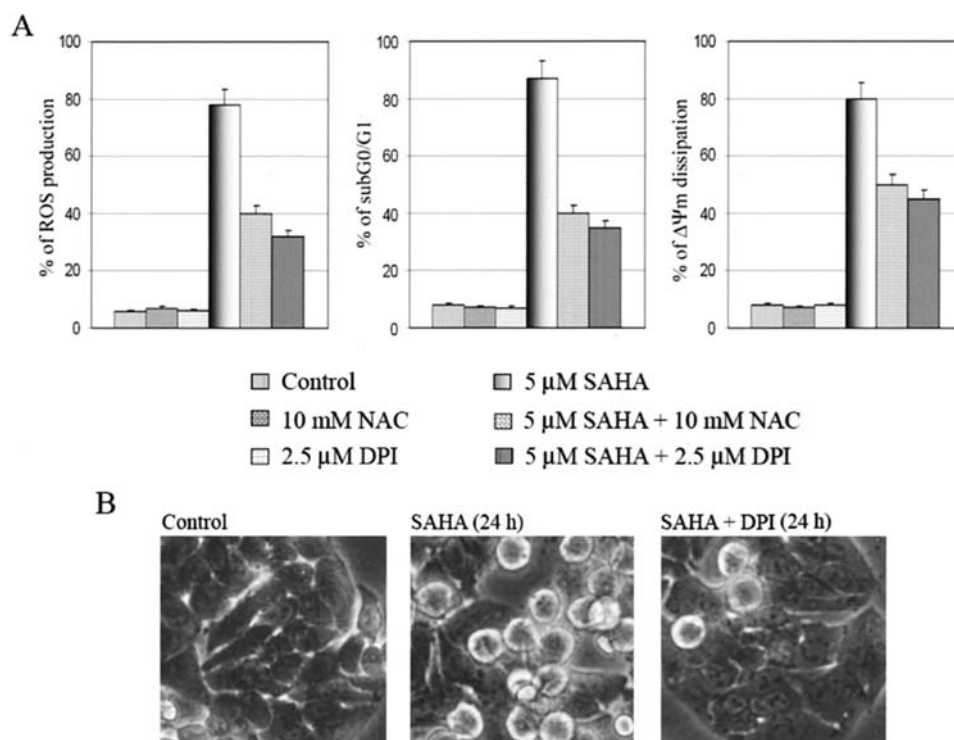
Figure 5. NAC and DPI prevented the effect of SAHA on ROS production, apoptosis and $\Delta\psi_m$ dissipation. (A) Cells were treated with 5 μM of SAHA in the presence of 10 mM NAC or 2.5 μM DPI. The effect on ROS production was evaluated at 16 h of treatment, while those on sub-G0/G1 accumulation and transmembrane potential dissipation at 36 h of treatment. (B) Morphological analysis of HT-29 cells treated for 24 h with 5 μM of SAHA in the presence or absence of 2.5 μM DPI. Data are representative of three separate experiments with similar results.
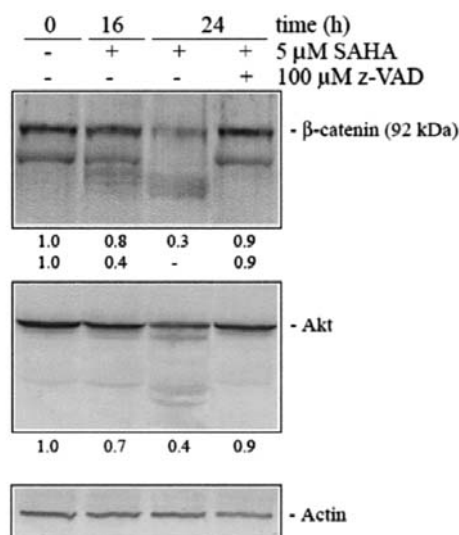

Figure 6. The effect of SAHA on the levels of ß-catenin and Akt. Cells were treated for 16 and 24 h with 5 μM of SAHA in the presence or absence of 100 μM z-VAD. Then, cell lysates were analysed by SDS-PAGE and Western blotting. Untreated cells were assumed as the control. The data are representative of four separate experiments with similar results.

The activation of extrinsic and mitochondrial pathways led to the cleavage of executioner proteases. Clear reductions in the levels of pro-caspase-3 and pro-caspase-6 were observed after SAHA treatment and after 24 h the intensities of the corresponding bands were ~30% of the respective control values. Moreover, the specific antibody for caspase-3 also evidenced the appearance of its cleaved active form of 17 kDa (Fig. 4).

The effect of N-acetylcysteine and diphenyleneiodonium on ROS production, DNA fragmentation and mitochondrial membrane potential dissipation in SAHA-treated HT-29 cells. To study the role of oxidative stress in SAHA-induced cell death, we analysed whether the antioxidant NAC or the inhibitor of NADPH oxidase DPI are capable of modifying the effects induced by SAHA on HT-29 cells. Fig. 5A shows that the addition of 10 mM NAC to 5 μM of SAHA reduced the percentage of cells with ROS-related fluorescence by ~50%. In the same conditions, it was observed that the percentage of treated cells confined to the sub-G0/G1 phase of the cell cycle decreased by ~55%. In concomitance, SAHA-induced dissipation of the mitochondrial inner membrane potential was reduced by the addition of NAC (Fig. 5A).

The addition of 2.5 μM DPI significantly diminished ROS generation in SAHA-treated cells, as well as the percentage of cells confined to the sub-G0/G1 phase of the cell cycle and the dissipation of mitochondrial transmembrane potential (Fig. 5A). Moreover, as demonstrated by microscopic analysis (Fig. 5B), DPI almost completely reverted the morphological changes induced by 24 h of treatment with SAHA in HT-29 cells, consisting in the isolation and detaching from culture substrate of cells with rounded profiles. These results strongly suggest the involvement of NADPH oxidase in SAHA-induced ROS production and apoptosis.

The activation of the p38/JNK pathway in SAHA-treated cells. It was reported that oxidative stress often causes the activation of the p38/JNK pathway (28). Western blot analysis demonstrated that treatment of HT-29 cells with 5 μM of SAHA caused a time-dependent increase in the levels of phosphorylated active forms of p38 and JNK, while we did not observe significant variations in the corresponding non-phosphorylated forms (Fig. 4). The level of phospho-c-Jun increased with SAHA treatment. These effects were already observed in the first phase of treatment (8 h) reaching the maximum at 24 h (~2.5-5-fold of the respective control values) (Fig. 4).

SAHA induced in HT-29 cells the down-regulation of ß-catenin and Akt. It is well known that HT-29 cells are characterized by high levels of ß-catenin and it has been suggested that this condition contributes to their de-regulated proliferation and resistance to apoptosis (29). Western blot analysis evidenced in these cells the presence of two bands, one which corresponds to the canonical 92 kDa form of the protein and the other at a lower molecular weight (~70 kDa). After treatment with 5 μM of SAHA for 16 h, a decrease in the levels of the two bands with the appearance of degradation forms of the protein was observed. The effect was more evident after 24 h of treatment when the two bands were markedly reduced and the degradation forms became prevalent. The addition of 100 μM of z-VAD completely counteracted the effects of SAHA on ß-catenin (Fig. 6, lane 4), indicating a role of caspases on ß-catenin degradation.

We analysed the effect of SAHA on the serine/treonine kinase Akt, a protein which is involved in cell survival and inhibition of apoptosis (30). Western blot analysis showed that treatment with SAHA induced a reduction in Akt level. The decrease was modest after 16 h of treatment and became remarkable at 24 h (Fig. 4). In this case, the effect was reverted by the addition of z-VAD (Fig. 6).

Discussion

SAHA, a chemotherapeutic drug capable of inhibiting HDAC activity, can regulate gene expression and induce cell cycle arrest or cell death in many tumor systems (16). This study showed that colorectal carcinoma HT-29 cells express a high sensitivity to SAHA-induced apoptosis. In particular, SAHA promoted apoptosis in HT-29 cells with a dose- and time-dependent mechanism, which was partially prevented by z-VAD, a pan-specific inhibitor of caspase activity. Therefore, we conclude that caspases seem to be involved in the apoptotic pathway induced by SAHA.

Our results also provide evidence that SAHA induces the production of ROS in HT-29 cells. Since the antioxidant NAC prevented the cytotoxic effect of the drug, we suggest that ROS plays a crucial role in SAHA-induced apoptosis. The production of ROS was correlated with the dissipation of membrane mitochondrial potential, as suggested by the observation that NAC partially prevented the mitochondrial injury induced by SAHA. Moreover, the finding that DPI, an inhibitor frequently used to block NADPH oxidase, reduced the apoptotic effect induced by SAHA, led to the conclusion that ROS production can be dependent on NADPH oxidase

activation, as recommended by many authors who correlated this activity with apoptosis (31).

Furthermore, our results revealed that the exposure of cells to SAHA stimulated the phosphorylation of p38 and JNK, two enzymes which are generally associated with ROS-dependent apoptosis. We hypothesize that the activation of p38 and JNK could be correlated with the mitochondrial membrane potential dissipation via the phosphorylation and inactivation of Bcl-2, as demonstrated by Seo *et al* (32). Thus, ROS, through p38 and JNK activation could induce mitochondrial injury with the consequent activation of caspase-9 and -3.

However, our results also suggest a possible involvement of the extrinsic pathway in SAHA-induced apoptosis. In this connection, we observed that SAHA increased the level of the TRAIL receptor DR5 and contemporaneously diminished the level of FLIP$_\text{L}$, an endogenous inhibitor of caspase-8, although the drug did not modify the level of the FAS receptor and that of its ligand. Therefore, the activation of the TRAIL pathway could be responsible for the activation of caspase-8, which was observed after treatment with SAHA, though it is not possible to exclude that this event was a consequence of a feedback loop related to caspase-9 or -3 activation (33). In addition, it seems likely that the activation of caspase-8 was responsible for the cleavage of Bid, a pro-apoptotic factor of the Bcl-2 family, with the production of its truncated active form, which is well known in preventing the antiapoptotic action of Bcl-2 at the level of a mitochondrial membrane (34). The observation that z-VAD partially reduced the effect of the SAHA on $\Delta\psi_\text{m}$ dissipation supports the close relationship among the activation of caspase-8, the cleavage of Bid and the dissipation of mitochondrial potential. Therefore, mitochondrial damage induced by SAHA in HT-29 cells could be dependent on two different mechanisms: the production of ROS with the activation of p38 and JNK and the activation of caspase-8 with cleavage of Bid.

The expression of the survival factor Akt is increased in many cancers including colorectal carcinoma (30) and may confer resistance to certain anticancer drugs. Our experiments demonstrated that the level of this protein was affected by SAHA treatment. Since Akt is responsible for antiapoptotic effects, it seems probable that its decrease can favour the induction of apoptosis in HT-29 cells. Moreover, the level of ß-catenin, which contributes to the de-regulated proliferation of colorectal carcinoma, was strongly reduced after treatment with SAHA. This event, which was observed in the second phase of the treatment and was prevented by z-VAD, could be a consequence of caspase-3 activation, as demonstrated in other cells (35).

In conclusion, our study indicates that in HT-29 cells, the main experimental model of colon carcinoma, the apoptotic mechanism induced by the inhibitor of histone deacetylase SAHA involves mitochondrial events mediated by oxidative stress and the activation of the extrinsic apoptotic pathway. Altogether, our results confirm that the drug can be considered as a promising new therapeutic strategy for human cancers.

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