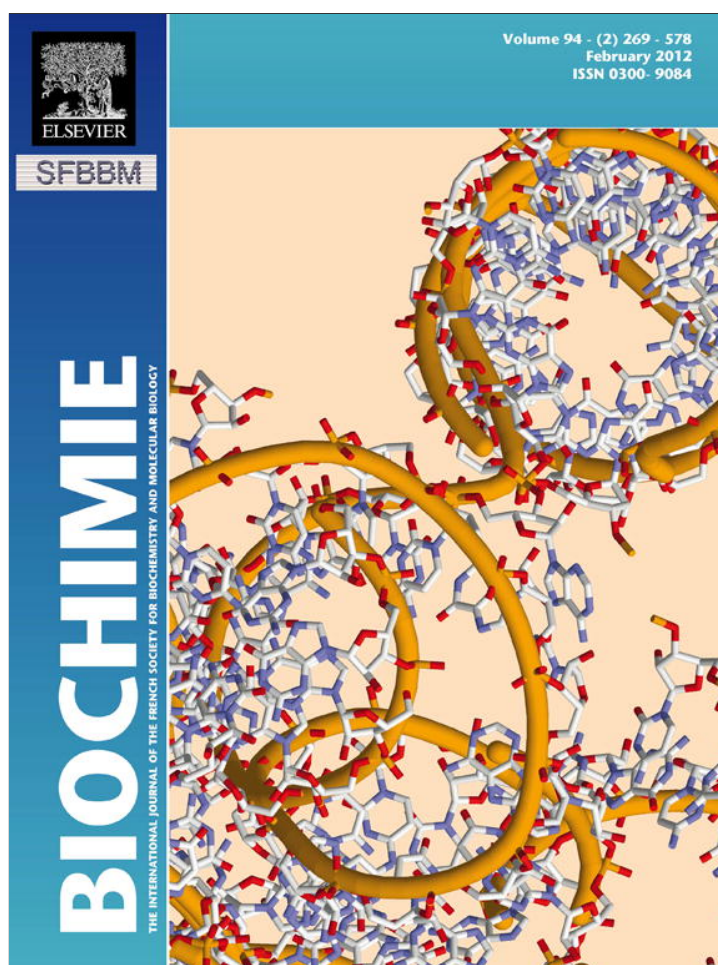


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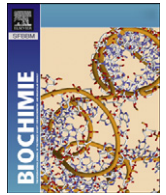
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Research paper

SAHA/TRAIL combination induces detachment and anoikis of MDA-MB231 and MCF-7 breast cancer cells

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ABSTRACT

SAHA, an inhibitor of histone deacetylase activity, has been shown to sensitize tumor cells to apoptosis induced by TRAIL, a member of TNF-family. In this paper we investigated the effect of SAHA/TRAIL combination in two breast cancer cell lines, the ER α -positive MCF-7 and the ER α -negative MDA-MB231. Treatment of MDA-MB231 and MCF-7 cells with SAHA in combination with TRAIL caused detachment of cells followed by anoikis, a form of apoptosis which occurs after cell detachment, while treatment with SAHA or TRAIL alone did not produce these effects. The effects were more evident in MDA-MB231 cells, which were chosen for ascertaining the mechanism of SAHA/TRAIL action. Our results show that SAHA decreased the level of c-FLIP, thus favouring the interaction of TRAIL with the specific death receptors DR4 and DR5 and the consequent activation of caspase-8. These effects increased when the cells were treated with SAHA/TRAIL combination. Because z-IEDT-fmk, an inhibitor of caspase-8, prevented both the cleavage of the focal adhesion-kinase FAK and cell detachment, we suggest that activation of caspase-8 can be responsible for both the decrement of FAK and the consequent cell detachment. In addition, treatment with SAHA/TRAIL combination caused dissipation of $\Delta\Psi_m$, activation of caspase-3 and decrement of both phospho-EGFR and phospho-ERK1/2, a kinase which is involved in the phosphorylation of BimEL. Therefore, co-treatment also induced decrement of phospho-BimEL and a concomitant increase in the dephosphorylated form of BimEL, which plays an important role in the induction of anoikis.

Our findings suggest the potential application of SAHA in combination with TRAIL in clinical trials for breast cancer.

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1. Introduction

Breast cancer is the most common form of cancer diagnosed in women. Despite the beneficial effects of estrogens in women's health, there is a plethora of evidence that suggests an important role of these hormones, particularly 17 β -estradiol (E2), in the development and progression of breast cancer [1,2].

Estrogens are known to mediate their diverse functions through two specific intracellular receptors, estrogen receptor (ER)-alpha and -beta [3]. Estrogen receptors occur in about two-thirds of breast tumors, and the presence of ER seems to be an important

prognostic factor in breast cancer [4]. The more favourable prognosis of ER-positive tumors appears to be due to their better response to anti-hormone therapy as compared with ER-negative tumors [5]. Endocrine therapy for hormone-dependent breast cancers is based on the employment of estrogen antagonists, such as tamoxifen [6], which inhibits the binding of estrogens to their receptors, or aromatase inhibitors, which prevent estrogens synthesis [7]. Tumors that are negative for estrogen receptors are poorly differentiated, associated with a higher recurrence rate and unresponsive to endocrine therapy [8].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF)-family, is a promising candidate for cancer therapeutics due to its ability to induce apoptosis selectively in cancer cells [9]. TRAIL activates apoptosis upon binding to specific cell-surface death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) in a wide variety of human tumor cell lines [10]. Moreover, TRAIL and some agonist antibodies are currently studied in patients in clinical phases I and II trials [11,12].

Several tumor cells, including breast cancer cells, are resistant to TRAIL [13,14]. However, resistance to TRAIL-mediated apoptosis can

Abbreviations: DR4, Death receptor 4; DR5, Death receptor 5; ER, estrogen receptor; EGFR, epidermal growth factor receptor; ERK1/2, Extracellular Signal-Regulated Kinase1/2; FAK, Focal Adhesion Kinase; c-FLIP, FLICE inhibitory protein; HDACI, histone deacetylase inhibitor; z-IEDT-fmk, carbobenzoxy-Ile-Glu-Thr-Asp-fluoromethylketone; poly-HEMA, poly(2-hydroxyethylmethacrylate); SAHA, suberoylanilide hydroxamic acid; TRAIL, TNF-related apoptosis-inducing ligand.

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be overcome by combining TRAIL with many compounds, such as anticancer cytotoxic drugs, proteasome inhibitors, or histone deacetylase (HDAC) inhibitors [15–17].

HDAC inhibitors are a new class of anticancer drugs, which have shown significant antiproliferative activity against a spectrum of hematological and solid tumors [18,19]. Our previous studies demonstrated the ability of suberoylanilide hydroxamic acid (SAHA), an inhibitor of HDAC, to sensitize human hepatocarcinoma cells to TRAIL-induced apoptosis by enhancing the expression of DR5 and decreasing the level of the anti-apoptotic factor c-FLIP [20].

It has been reported that MDA-MB231 cells, which are ER α -negative breast cancer cells, exhibit a reduced sensitivity to TRAIL-induced apoptosis [21]. However HDAC inhibitors are capable of sensitizing MDA-MB231 cells to TRAIL [22,23]. The present study shows that SAHA/TRAIL co-treatment induced detachment of MDA-MB231 cells, followed by anoikis, a specific form of apoptosis observed in cells deprived of the adhesion-derived signals. Co-treatment induced detachment and anoikis also in MCF-7 cells, which are ER α -positive cells, although the effects were less evident than in MDA-MB231 cells. Our results show that these effects were correlated with a number of events, such as the decrements in the levels of c-FLIP, FAK, EGFR and phospho-BimEL, and the concomitant increment in dephospho-BimEL, the form which exerts a fundamental role in the induction of anoikis [24].

2. Materials and methods

2.1. Chemicals and reagents

TRAIL signalling has been activated using a soluble human recombinant TRAIL/APO2L, containing the residues of aminoacids from 114 to 281 of natural TRAIL. This compound was purchased from Pepro Tech. (EC Ltd, London, UK) and is reported in this paper as "TRAIL". AG1478, a selective inhibitor of the EGFR tyrosine kinase, was supplied from Calbiochem UK. z-IEDT-fmk from Clontech (Palo Alto, CA). All the other compounds were from Sigma (St. Louis, MO). SAHA was kindly provided by Italfarmaco S.p.a. (Milan, Italy). Stock solutions of SAHA were dissolved in DMSO and diluted in culture medium. In each experimental condition, DMSO never exceeded 0.04% and this percentage was not toxic and did not interfere with cell growth.

2.2. Cell cultures, cell viability and cell death assay

MDA-MB231 cells and MCF-7, obtained from "Istituto Scientifico Tumori" (Genoa, Italy), were grown as monolayers in DMEM medium supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) and 1 mM sodium pyruvate, at 37 °C in a humidified atmosphere containing 5% CO₂. After plating on 6-well plates, cells were allowed to adhere overnight and then treated with chemicals or vehicle only. When the cells were treated with SAHA/TRAIL combination, analyses were performed after separation of detached from adherent cells. The number of viable cells was determined by trypan blue exclusion and counting on a haemocytometer.

Distribution of cells throughout the cell cycle phases was ascertained by flow cytometric analysis using a Beckman Coulter Epics XL cytometer as previously reported [19]. In addition, apoptotic cells were detected by flow cytometry using the annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen; San Diego, CA), according to the manufacturer's instructions.

The presence of condensed chromatin was assessed using Hoechst 33342 staining. In brief, cells were exposed to 2.5 μ g/mL of Hoechst 33342 at 37 °C for 30 min. Then, Hoechst solution was removed and the cells were rinsed with PBS and incubated in fresh

medium in the presence of the drugs. Nuclear morphology was observed under a fluorescence microscope. Apoptotic cells appear as a strong bright blue color due to the chromatin condensation characteristic of apoptotic cells, while normal healthy cells appear as a uniform blue.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured by using 3,3-dihexyloxycarbocyanine (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 $\Delta\Psi_m$. Cells were incubated with 40 nM DiOC6 for 20 min at 37 °C, washed twice with PBS and analysed by flow cytometry on Beckman Coulter Epics XL flow cytometer with excitation and emission settings of 488 and 525 nm respectively. The percentage of cells showing a lower fluorescence, reflecting loss of mitochondrial transmembrane potential, was determined by comparison with untreated control using Expo32 software. Carbonyl cyanide m-chlorophenylhydrazone (CCCP, 50 μ M), a protonophore that completely de-energizes mitochondria by dissipating the transmembrane potential, was used as a positive control for maximum $\Delta\Psi_m$ disruption.

2.3. Cell detachment assays

Tissue culture plates were coated with poly-HEMA [poly(2-hydroxyethylmethacrylate); Sigma–Aldrich, Castle Hill, NSW, Australia] as follows: 1 ml of 10 mg/ml of poly-HEMA in 95% ethanol was applied to each well and allowed to dry. After drying, the wells were washed in PBS and stored dry until used. Cells were plated onto poly-HEMA-coated wells at 1×10^5 cells per well in a six-well plate. At the indicated times, cells were harvested from poly-HEMA-coated wells by centrifugation.

2.4. Western blotting analysis

Cell lysates were prepared as reported [25]. Protein samples (30 μ g/lane) were subjected to SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for detection with specific antibodies. Pro-caspase-8, pro-caspase-3 and Bim antibodies were obtained from Cell Signalling Technology (Beverly, MA); DR4 and DR5 antibodies from ProSci Incorporated (San Diego, CA); all the other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein bands were developed by Enhanced ChemiLuminescence (Pierce, Rockford, IL) and their intensity was quantified by using Quantity One quantification analysis software (Bio-Rad, Hercules, CA). The correct protein loading was ascertained by means of both red Ponceau staining and immunoblotting for actin.

2.5. Statistical analysis

Data are presented as means \pm S.D. from at least three independent experiments. Statistical analysis of the data was performed by Student's *t*-test. *P*-values <0.05 were considered statistically significant.

2.6. Down-regulation of endogenous c-FLIP and EGFR expression

Specific siRNAs against c-FLIP and EGFR, delivered by St Cruz Biotechnology (Santa Cruz, CA) as pools of double-stranded RNA oligonucleotides, were transfected into the cells (2×10^5 cells) at a final concentration of 80 nM siRNA in the presence of 6 μ l Met-afectene Pro (Biontix, Martinsried/Planegg, GmbH) in a final volume of 1 ml serum-free DMEM. The reaction was stopped after 5 h of treatment, replacing the medium with DMEM + 20% FBS and the expressions of c-FLIP and EGFR were evaluated by western

blotting analysis after other 24 h of incubation. Fluorescein Conjugated siRNA, consisting in a scramble sequence, was used as a negative control.

3. Results

3.1. SAHA/TRAIL combination induced cell detachment and apoptosis of MDA-MB231 and MCF-7 cells

Preliminary experiments were performed to evaluate the effects exerted by SAHA and TRAIL in MDA-MB231 cells, a very aggressive and poorly differentiated breast cancer cell line, which is negative for ER α [26]. The effect of the compounds was also evaluated in MCF-7 cells, a more differentiated breast cancer cell line which is ER α -positive [26]. MDA-MB231 and MCF-7 cells were treated for various times with different concentrations of SAHA or TRAIL alone or in combination.

Treatment of MDA-MB231 or MCF-7 cells with SAHA (0.2–2 μ M) or TRAIL (10–50 ng/ml) alone did not induce cell detachment or any morphological change even after prolonged incubation (36–48 h) (not shown). Instead, when MDA-MB231 cells were exposed to combination of SAHA (2 μ M) and TRAIL (50 ng/ml), about 25% of cells were deprived of anchorage already at 8 h of treatment. The effect then increased with the time and at 24 h detached cells reached the percentage of 82% (Fig. 1A). Co-treatment also induced

detachment of MCF-7 cells, although the effect was observed after a longer period of treatment (12 h) (Fig. 1B). In both the cell lines detachment was markedly reduced by the addition of 100 μ M z-IEDT-fmk, the specific inhibitor of caspase-8, suggesting that this caspase activity is involved in detachment of MDA-MB231 and MCF-7 cells (Fig. 1A and B). It is interesting to note that during the first phase of co-treatment (0–12 h) detached cells were grouped in small viable clusters, but when the time of treatment was prolonged (14–24 h) the number of clustered cells diminished and signs of apoptosis appeared.

These considerations prompted us to analyze separately adherent from detached cells. The study of cell cycle distribution by flow cytometric analysis revealed that after 14h of treatment with SAHA/TRAIL combination a consistent percentage of detached MDA-MB231 cells was confined in subG0/G1 phase, a condition which is representative of cells with fragmented DNA, while treatment with SAHA or TRAIL alone did not induce any sign of apoptosis (Fig. 2A). Moreover, staining MDA-MB231 cells with Hoechst 33342 revealed that co-treatment induced in a high percentage of detached cells the presence of morphological changes typical of apoptosis, including chromatin condensation and nuclear fragmentation (Fig. 2 A, insets). These results were confirmed by flow cytometric analysis of cells stained with annexin V-FITC. Time course experiments showed that apoptotic effects were limited in the first phase of co-treatment (0–12 h), but

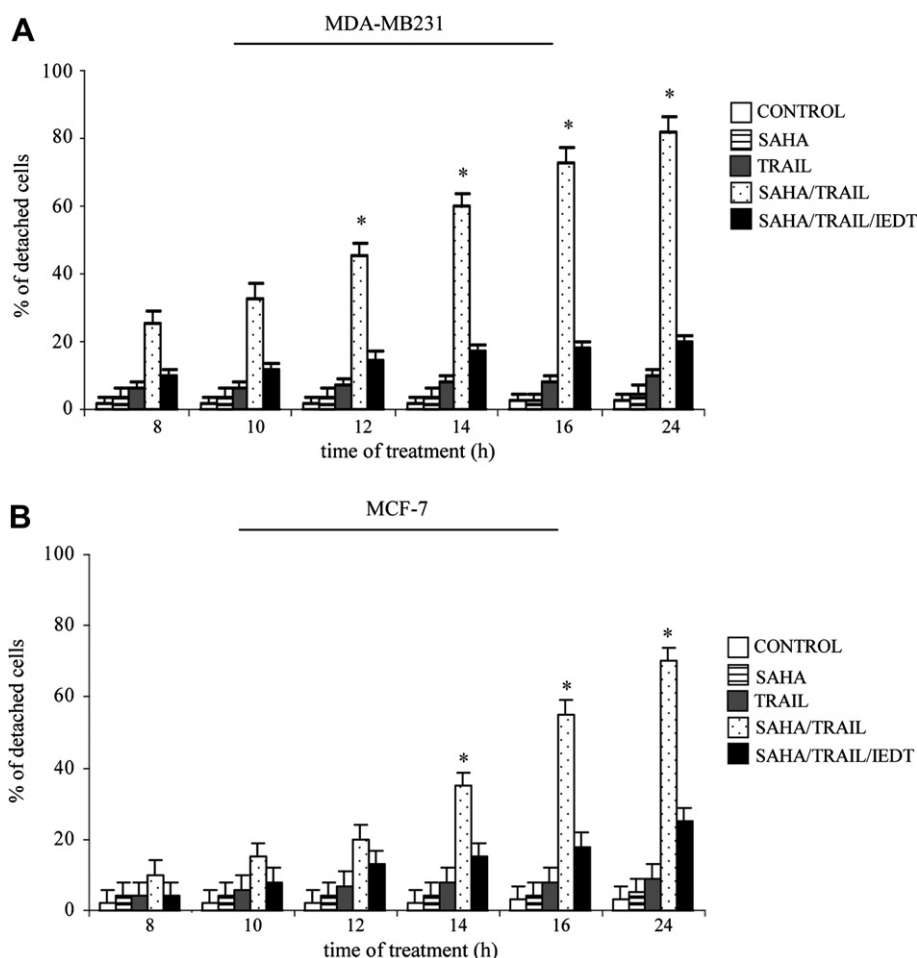


Fig. 1. SAHA/TRAIL combination induced detachment of MDA-MB231 (A) and MCF-7 (B). Cells were treated for different times with 2 μ M SAHA or 50 ng/ml TRAIL or SAHA/TRAIL combination without or with 100 μ M IEDT-fmk. Adherent and detached cells were counted in Neubauer chamber. Detached cells were expressed as percentage of the total cells. Results are the means \pm S.E. of three independent experiments. * P < 0.01 versus control.

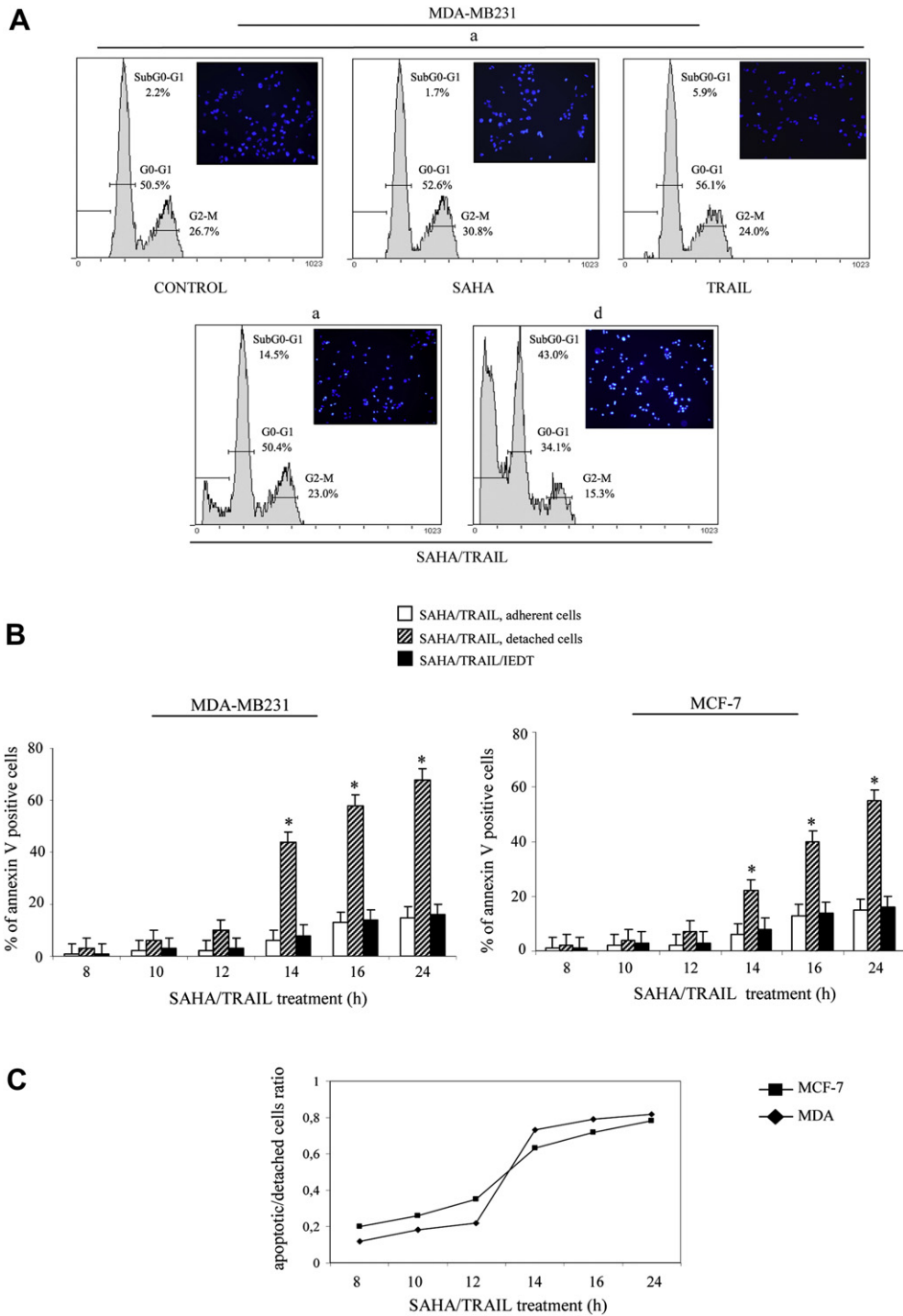


Fig. 2. SAHA/TRAIL combination induced anoikis in MDA-MB231 and MCF-7 cells. Cells were treated with 2 μ M SAHA or 50 ng/ml TRAIL or SAHA/TRAIL combination. Since treatment with SAHA/TRAIL combination induced detachment of cells, in this case the effects of the drugs were analysed in both adherent (a) and detached (d) cells. (A) Flow cytometric analysis of MDA-MB231 cells, treated with the drugs for 14 h, after staining with PI. The effects of the treatment on cell morphology observed in the insets were ascertained after staining with Hoechst 33342. Results are representative of three independent experiments. (B) Time course of the apoptotic effect induced by SAHA/TRAIL combination. Apoptotic cells were detected by flow cytometry using the annexin V-FITC kit. * $P < 0.01$ versus adherent cells. (C) Percentages of MDA-MB231 or MCF-7 apoptotic cells with respect to total detached cells at various times of co-treatment. (B and C), results are the means \pm S. E. of three independent experiments.

markedly increased between 14 and 24 h, and at 24 h apoptotic signs appeared in about 68% of treated cells. Differently in adherent cells only modest effects were observed also after prolonged treatments with drug combinations. Finally, the addition of 100 μ M z-IEDT-fmk prevented both detachment of cells and the apoptotic effects (Figs. 1 and 2B).

It is interesting to note that the percentage of detached cells showing apoptotic signs was very low at 8 h of treatment, then increased with the time of incubation so that at 24 h apoptotic signs were observed in almost all the detached cells (Fig. 2C). This result clearly suggested that apoptotic effect was secondary to cell detachment.

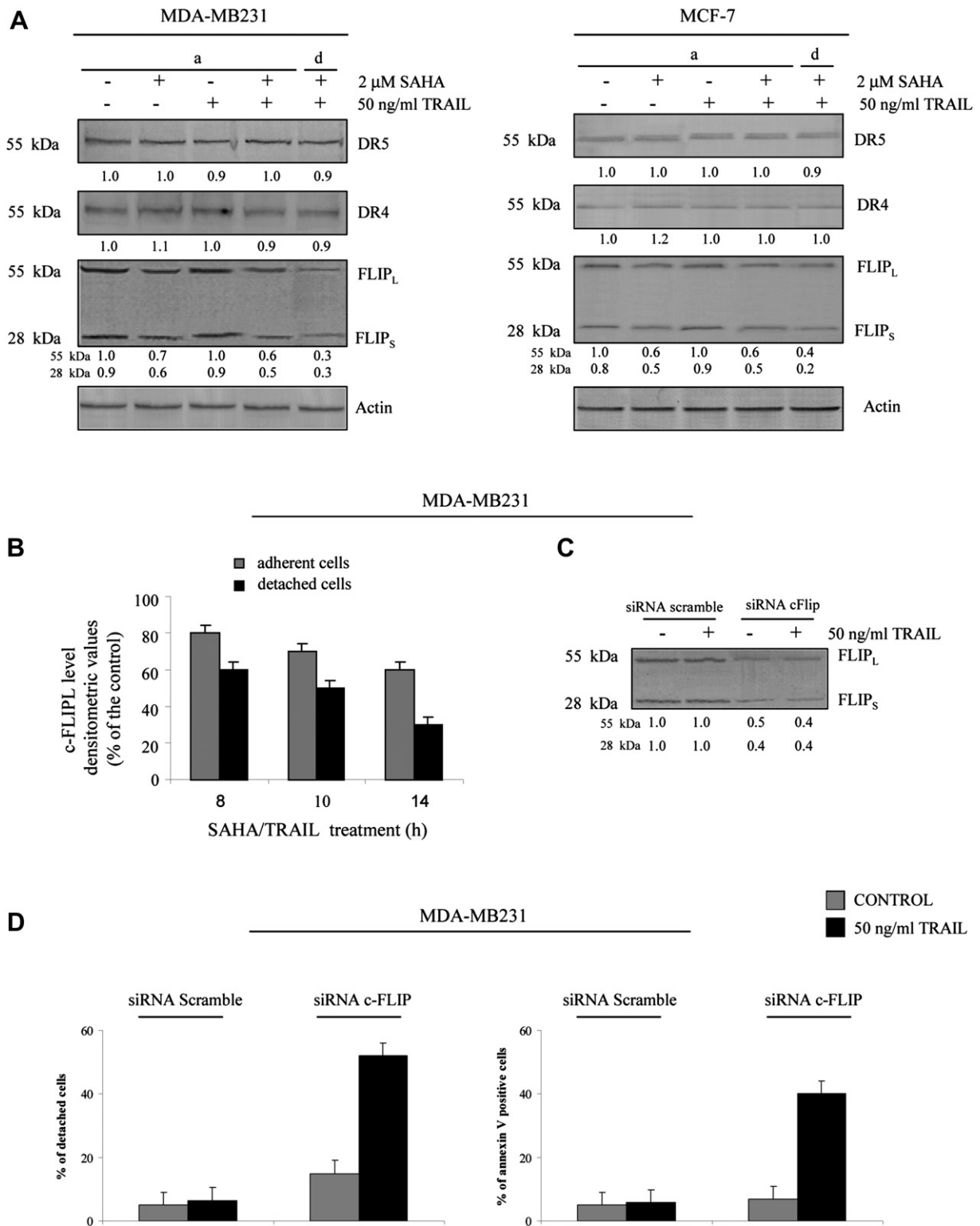


Fig. 3. The effect of SAHA and TRAIL on TRAIL receptors and c-FLIP in MDA-MB231 and MCF-7 cells. Cells were treated with SAHA or TRAIL or SAHA/TRAIL combination. At the end, cell extracts were prepared and analysed for Western blotting. In cells treated with SAHA/TRAIL combination the analyses were performed in both adherent (a) and detached (d) cells. (A) The effect exerted by treatment for 14 h on the levels of DR4, DR5 and c-FLIP. Detection of actin is enclosed to demonstrate equal loading of protein for each sample. Densitometric values are representative of three independent experiments. (B) Time course of the effect exerted by co-treatment on c-FLIP_L level. Densitometric values are expressed as percentage of control sample, which is represented by cells incubated in the absence of the drugs. (C and D) MDA-MB231 cells were transfected for 5 h with siRNA sequence directed against c-FLIP or with siRNA scramble and then incubated for other 24 h. (C) Western blotting analysis showing down-regulation of c-FLIP in c-FLIP silenced cells. (D) The effects induced in c-FLIP silenced cells by 14 h of treatment with TRAIL on cell detachment (left) and on apoptosis (right).

In MCF-7 cells the ratio “apoptotic/detached” cells increased with time similarly to MDA-MB231 cells (Fig. 2C), suggesting that also in MCF-7 cells apoptosis induced by SAHA/TRAIL combination was secondary to cell detachment. However, since the apoptotic effects were more evident in MDA-MB231 cells, these cells were chosen for ascertaining the mechanism of SAHA/TRAIL action.

3.2. The effects of SAHA and TRAIL on the expression of death receptors and c-FLIP

Sensitization of cancer cells to TRAIL-induced apoptosis can result either by the increase in the level of TRAIL receptors or by the decrease in the level of anti-apoptotic factors such as c-FLIP or IAPs [20,27]. Therefore, we performed western blotting experiments to evaluate the effects of the drugs on both TRAIL receptors and c-FLIP. Our results demonstrated (Fig. 3A) that MDA-MB231 cells exhibit both DR4 and DR5 receptors and high levels of c-FLIP_L and c-FLIP_S, the two different isoforms of c-FLIP. Also in MCF-7 cells we ascertained the presence of death receptors and c-FLIP proteins, but at lower levels (Fig. 3A). However, the expression of death receptors was not modified by the drugs in both the cell lines. Instead treatment with SAHA alone decreased the level of both c-FLIP_L and c-FLIP_S either in MDA-MB231 or MCF-7 cells (Fig. 3A). A more consistent effect was observed when the cells were treated with SAHA/TRAIL combination. Down-regulation of c-FLIP was found already at 8 h of incubation and the effect reached the maximum at 14 h (Fig. 3B).

In order to ascertain whether down-regulation of c-FLIP sensitizes MDA-MB231 cells to TRAIL-induced apoptosis, cells were transiently transfected with siRNA sequence directed against c-FLIP, inducing a decrease in c-FLIP content of approximately 50% (Fig. 3C). This down-regulation of c-FLIP sensitized the cells to TRAIL. In fact the addition of TRAIL to silenced cells induced cell detachment and apoptosis (Fig. 3D). These effects were not found when the cells were transfected with siRNA scramble.

3.3. The effects of SAHA and TRAIL on the level of integrins and FAK

It is well known that cell anchorage to extracellular matrix (ECM) is mediated mainly by integrins [28]. Upon integrin ligation, focal adhesion-kinase (FAK) is phosphorylated and interacts with several proteins, many of which stimulate proliferation and cellular survival [29]. The loss of integrin engagement eliminates these signals, thus committing detached cells to anoikis [30].

In our experiments we demonstrated that SAHA and TRAIL neither alone nor in combination modified the levels of $\alpha 5$ and $\beta 1$ integrin subunits (Fig. 4A), which are present at high levels in MDA-MB231 cells and that have been reported to exert a critical role in proliferation and migration of these cells [31–33]. Differently, SAHA/TRAIL co-treatment induced down-regulation of FAK (Fig. 4A). This effect, which was observed already at 8 h, increased with time reaching the maximum at 14 h (Fig. 4B). Down-regulation of FAK was much more pronounced in detached than in adherent cells and was not observed when the cells were treated with the single compounds. Moreover, the addition of z-IEDT-fmk, a specific caspase-8 inhibitor, prevented both the detachment of cells and the decrease in FAK level induced by co-treatment (Fig. 4A). This finding, in line with a previous observation [34], suggests an involvement of caspase-8 in FAK cleavage. Accordingly, in MDA-MB231 cells transfected with c-FLIP siRNA our results show that treatment with TRAIL caused both activation of caspase-8 (not shown) and down-regulation of FAK (Fig. 4C).

c-Src is a tyrosine kinase which is involved in the phosphorylation and activation of FAK [35]. By means of western blotting analysis we demonstrated that in MDA-MB231 cells SAHA and

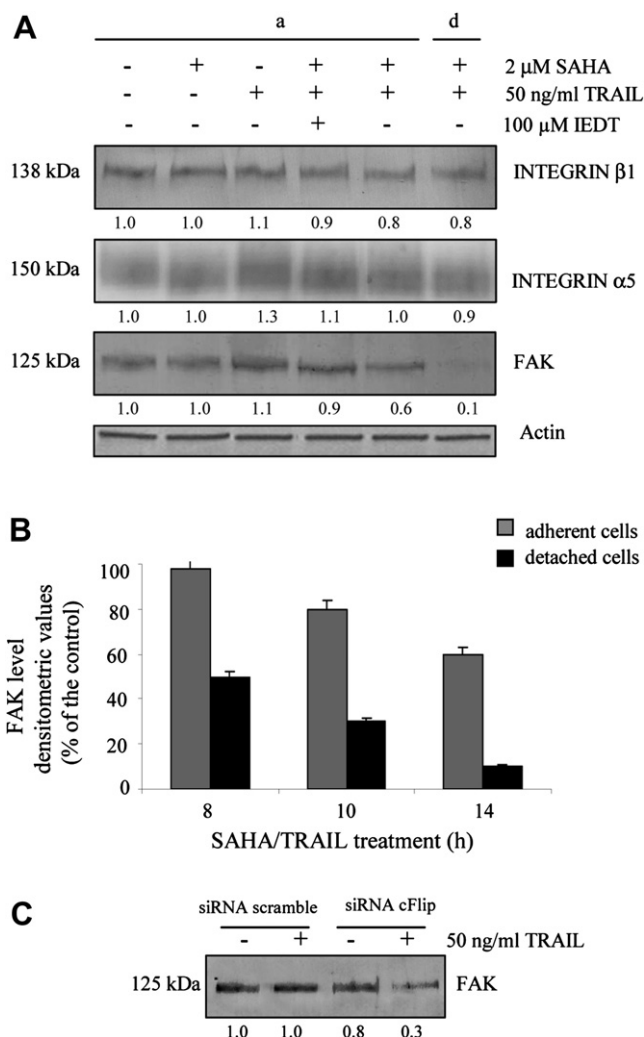


Fig. 4. The effects of SAHA and TRAIL on the levels of $\alpha 5$, $\beta 1$ integrins and FAK in MDA-MB231 cells. Cells were treated with SAHA or TRAIL or SAHA/TRAIL combination. After treatment, cells extracts were prepared and analysed for Western blotting. When the cells were treated with SAHA/TRAIL combination, analysis was performed both in adherent (a) and detached (d) cells. (A) The effect exerted by treatment for 14 h on the levels of $\alpha 5$, $\beta 1$ integrins and FAK. Detection of actin was enclosed to demonstrate equal loading of protein for each sample. Densitometric values is representative of three independent experiments. (B) Time course of the effect exerted by SAHA/TRAIL combination on the level of FAK in detached cells. Densitometric values are expressed as percentage of control sample, which is represented by cells incubated in the absence of the drugs. Values are the means \pm S. E. of three independent experiments. (C) MDA-MB231 cells were transfected for 5 h with siRNA sequence directed against c-FLIP or with scramble siRNA. After other 24 h of incubation the cells were treated for 8 h with TRAIL to evaluate the effect on the level of FAK.

TRAIL neither alone nor in combination modified the level of c-Src (not shown).

3.4. The effect of SAHA and TRAIL on BimEL

Bim, a proapoptotic member of Bcl-2 family, exists in three splice variants, short, long and extra long (BimS, BimL and BimEL, respectively). BimEL is the major species expressed, while the other forms are present at very low levels [36]. An important role in modulating BimEL activity is exerted by phosphorylation of the protein on Ser69 by ERK1/2 [37]. Phosphorylation of BimEL leads to ubiquitination and the consequent proteasomal degradation [38]. Instead, dephosphorylated form of BimEL, which is peculiar of cells

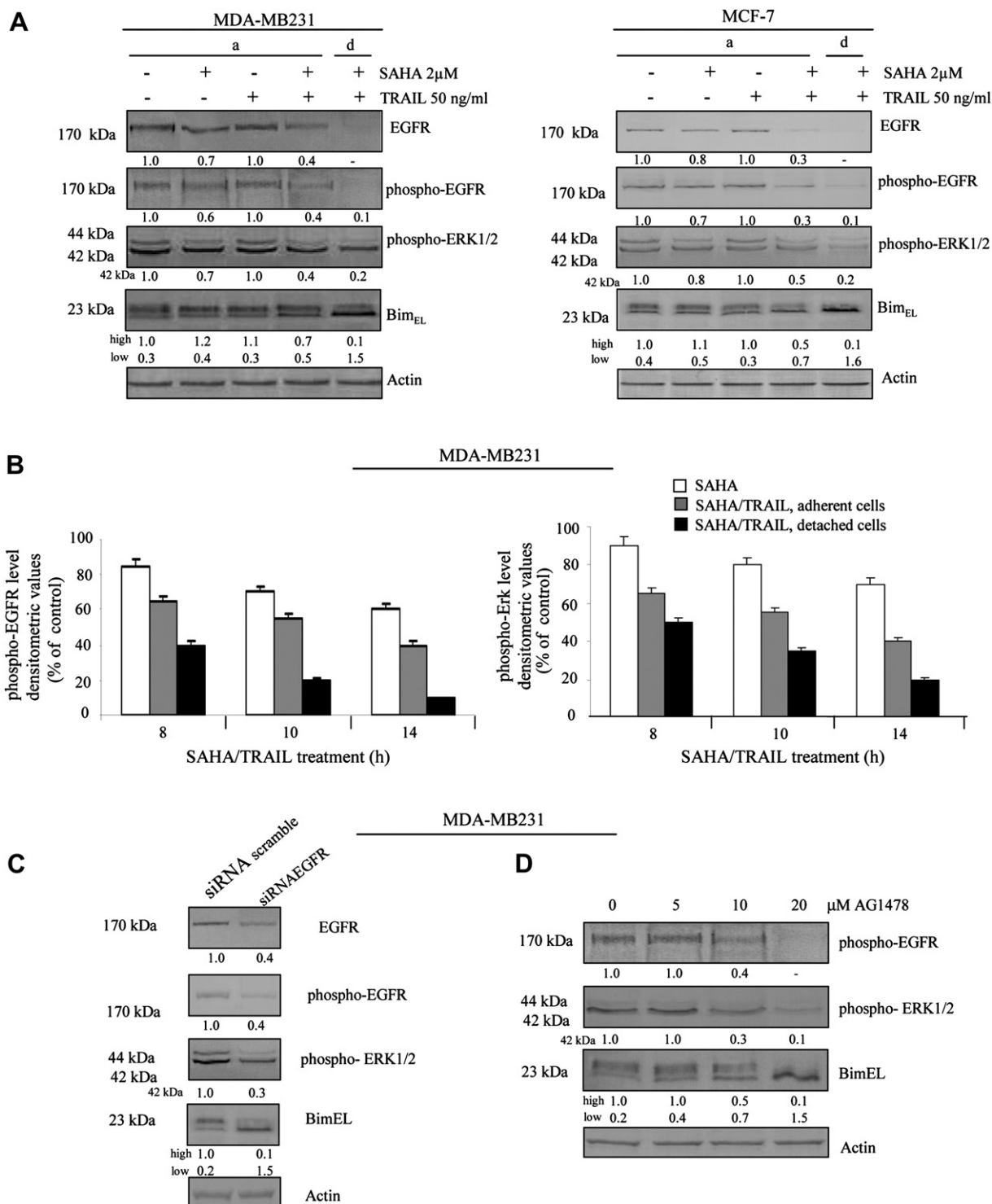
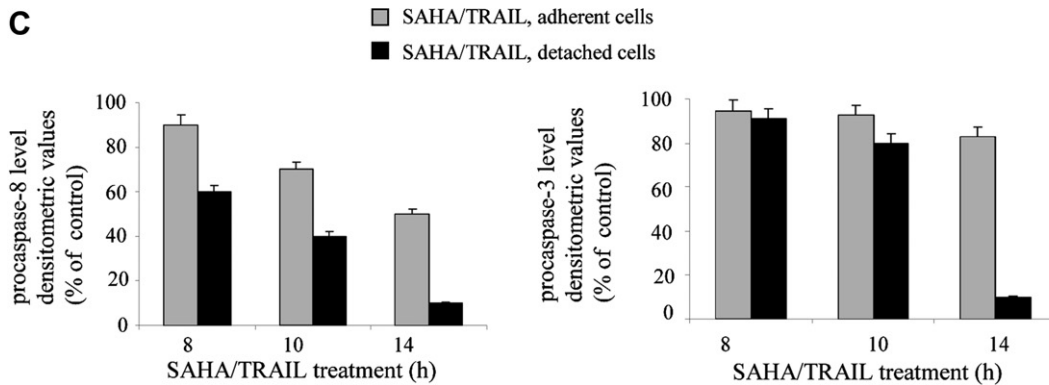
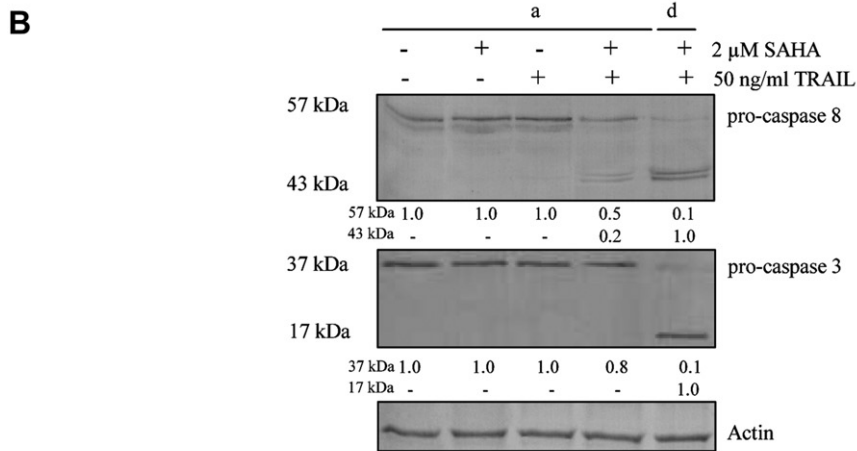
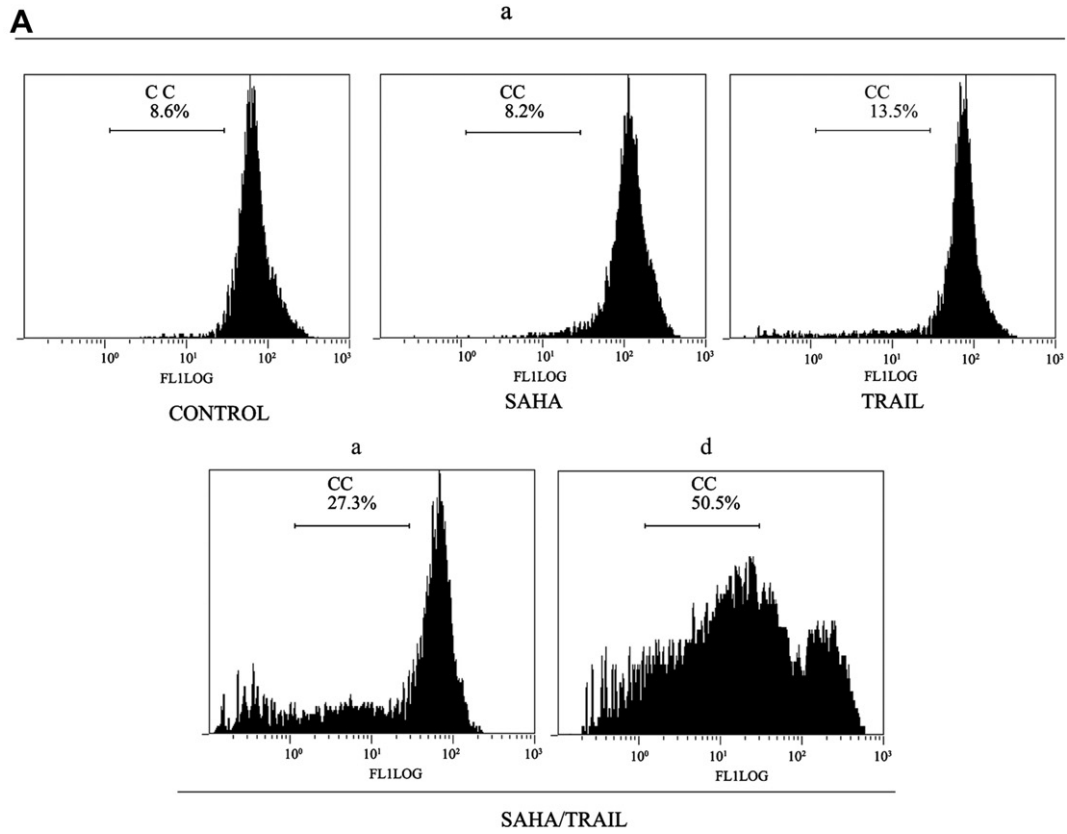


Fig. 5. The effects of SAHA and TRAIL on the levels of EGFR, phospho-EGFR, phospho-ERK1/2, and BimEL in MDA-MB231 and MCF-7 cells. Cells were treated with 2 μ M SAHA or 50 ng/ml TRAIL or SAHA/TRAIL combination. After treatment, cell extracts were prepared and analysed for Western blotting. When the cells were treated with SAHA/TRAIL combination, analysis was performed both in adherent (a) and detached (d) cells. (A) The effects of treatment for 14 h on the levels of EGFR, phospho-EGFR, phospho-ERK1/2 and BimEL. (B) Time course of the effects exerted by SAHA/TRAIL combination on the levels of phospho-EGFR and phospho-ERK1/2. Densitometric values, expressed as percentage of control, are the means \pm S. E. of three independent experiments. (C) MDA-MB231 cells were transiently transfected for 5 h with siRNA sequence directed against EGFR and employed in comparison with cells transfected with siRNA scramble to evaluate after other 24 h of incubation the effect of EGFR silencing on the level of EGFR, phospho-EGFR, phospho-ERK1/2 and BimEL. (D) The effect of treatment of MDA-MB231 cells for 8 h with various concentrations of AG1478 on the levels of phospho-EGFR, phospho-ERK1/2 and BimEL. (A, C and D). Detection of actin was enclosed to demonstrate equal loading of protein for each sample. Densitometric values are representative of three independent experiments.



addressed to anoikis [40], is capable of stimulating BAX oligomerization thus antagonizing Bcl-2 function [40].

Two bands of BimEL with different electrophoretic mobility were detected by western blotting analysis (Fig. 5A) in untreated MDA-MB231 and MCF-7 cells: a slow-migrating band at high intensity, which corresponds to phosphorylated form [41], and a fast-migrating one at low intensity, which is related to dephosphorylated protein [41]. In agreement with the observation of Bali et al. [42], our results demonstrated that treatment with SAHA alone caused a modest increase in the level of both the forms of BimEL either in MDA-MB231 or in MCF-7 cells. However, when the cells of both the lines were treated with SAHA/TRAIL combination (14 h) a marked decrease in the intensity of the band corresponding to phospho-BimEL together with a concomitant increase in the dephosphorylated form was observed. These changes were more pronounced in detached than in adherent cells (Fig. 5A).

3.5. Down-regulation of EGFR plays a role in anoikis induced by SAHA/TRAIL combination

Among the molecules which can mediate survival signals at the membrane level, epidermal growth factor receptor (EGFR) exerts an important role in stimulating the proliferation of MDA-MB231 cells [43]. It has been shown that dimerization and autophosphorylation of EGFR represent the initial and essential events in the activation of the receptor in response to EGF binding [44]. EGFR activation results in phosphorylation of ERK1/2 [44], which in turn phosphorylates BimEL [45].

As shown in Fig. 5A, high levels of both EGFR and phospho-ERK1/2 were detected in untreated MDA-MB231 cells, while in MCF-7, in agreement with the observation of Frogne et al. [46], low levels of these proteins were found. The treatment reduced the levels of EGFR, phospho-EGFR and phospho-ERK1/2. The decrements were already observed when the cells were treated with SAHA alone and became more consistent after SAHA/TRAIL treatment (Fig. 5A). The effects appeared in both the cell lines at 8 h of treatment and increased up to 14 h (Fig. 5B for MDA-MB231 cells).

These results led us to hypothesize that the increase in the dephosphorylated form of BimEL induced by SAHA/TRAIL combination can be a consequence of the inhibition of EGFR signalling. To confirm this hypothesis, MDA-MB231 cells were transfected with siRNA sequence directed against EGFR. In response to this treatment EGFR level diminished to about 40% of the control (Fig. 5C). In close correlation also the level of both phospho-EGFR and phospho-ERK1/2 decreased with respect to the control together with the level of phospho-BimEL, while dephosphorylated form of BimEL concomitantly increased (Fig. 5C). Similar results were obtained when MDA-MB231 cells were treated for 8 h with different concentrations of AG1478 (Fig. 5D), a specific tyrosine kinase inhibitor of EGFR [47]. Therefore, these results strongly suggest that down-regulation of EGFR, induced by SAHA-TRAIL combination, can be responsible for activation of BimEL and consequently for induction of anoikis.

3.6. The role of mitochondria in anoikis induced by SAHA/TRAIL combination

It has been reported that dephosphorylated form of BimEL binds Bax favouring its oligomerization [39]. In turn, Bax may interact

with mitochondrial proteins like VDAC and/or ANT to regulate the permeability transition pore (PTP) [48]. To evaluate whether treatment with SAHA/TRAIL combination induced dissipation of mitochondrial membrane potential, we utilized the fluorescent lipophilic dye DiOC6. As shown in Fig. 6A, co-treatment resulted in an increase in the percentage of depolarised cells. The effect was observed only in detached cells and was not induced in cells treated with the single compounds.

Finally, we ascertained by western blotting analysis that treatment of MDA-MB231 cells with SAHA/TRAIL combination induced activation of both caspases -8 and -3. As shown in Fig. 6B, co-treatment decreased the level of procaspase-8 with the concomitant appearance of the cleaved form of caspase-8. Moreover, co-treatment also induced cleavage of procaspase-3. However, cleavage of procaspase-8 was already observed at 8 h of treatment, while that of caspase-3 appeared only after a more prolonged period of incubation (14 h) (Fig. 6C). Activation of caspases-8 and -3 was also observed in MCF-7 treated with SAHA/TRAIL combination (not shown).

3.7. The effects of SAHA and TRAIL on MDA-MB231 cells cultured in poly-HEMA

Several studies report that MDA-MB231 cells are resistant to anoikis [39,41], because are capable of growing in an anchorage-independent manner. To confirm these data we carried out experiments in which adhesion of MDA-MB231 cells was prevented by culturing them in poly-HEMA, an inert polymer impeding cell attachment [49]. Our results show that MDA-MB231 cells, cultured in poly-HEMA, grew in cluster and did not present morphological alterations (control cells in Fig. 7A), confirming that MDA-MB231 cells survive when are deprived of anchorage. However, when the cells cultured in poly-HEMA were treated with SAHA/TRAIL combination, but not with SAHA or TRAIL alone, clear apoptotic signs were observed. In fact, cytofluorimetric analysis showed significant percentages of cells in subG0/G1 phase at 14 h of treatment. This effect was accompanied by morphological evidence of apoptosis (Fig. 7A).

Western blotting analysis showed that MDA-MB231 cells cultured in poly-HEMA contain high levels of EGFR, phospho-EGFR, phospho-ERK1/2, FAK and c-FLIP. The levels of these factors markedly lowered after treatment with SAHA/TRAIL combination, while the level of the dephosphorylated form of BimEL increased (Fig. 7B).

Finally, in order to clarify whether activation of EGFR signalling plays a role in anoikis resistance of MDA-MB231 cells, we cultured in poly-HEMA MDA-MB231 cells, which had been previously transfected with siRNA sequence directed against EGFR. Cytofluorimetric analysis demonstrated that down-regulation of EGFR sensitized MDA-MB231 cells to TRAIL induced apoptosis (Fig. 7C). In fact, when transfected cells were treated for 14 h with TRAIL alone a consistent percentage of cells (33%) resulted confined in subG0/G1 phase, whereas this event was not observed in cells transfected with siRNA scramble.

4. Discussion

Several breast cancer cell lines, such as ER α -negative MDA-MB231 cells and ER α -positive MCF-7 cells, exhibit resistance to

Fig. 6. The effects of SAHA and TRAIL on mitochondrial membrane potential and caspase activities in MDA-MB231 cells. Cells were treated with 2 μ M SAHA or 50 ng/ml TRAIL or SAHA/TRAIL combination. After co-treatment, analysis was performed both in adherent (a) and detached (d) cells. (A) Co-treatment for 14 h induced dissipation of mitochondrial membrane potential ($\Delta\Psi_m$), evaluated by flow cytometry using lipophilic dye DiOC6. Results are representative of three independent experiments. (B) Co-treatment for 14 h induced activation of caspases-8 and -3. The levels of the proteins were analysed by Western blotting using specific antibodies. Detection of actin was enclosed to demonstrate equal loading of protein for each sample. Densitometric values are representative of three independent experiments (C) Time course of the effects exerted by co-treatment on the levels of pro-caspases-8 and 3, analysed by western blotting. Densitometric values, expressed as percentage of control, are the means \pm S. E. of three independent experiments.

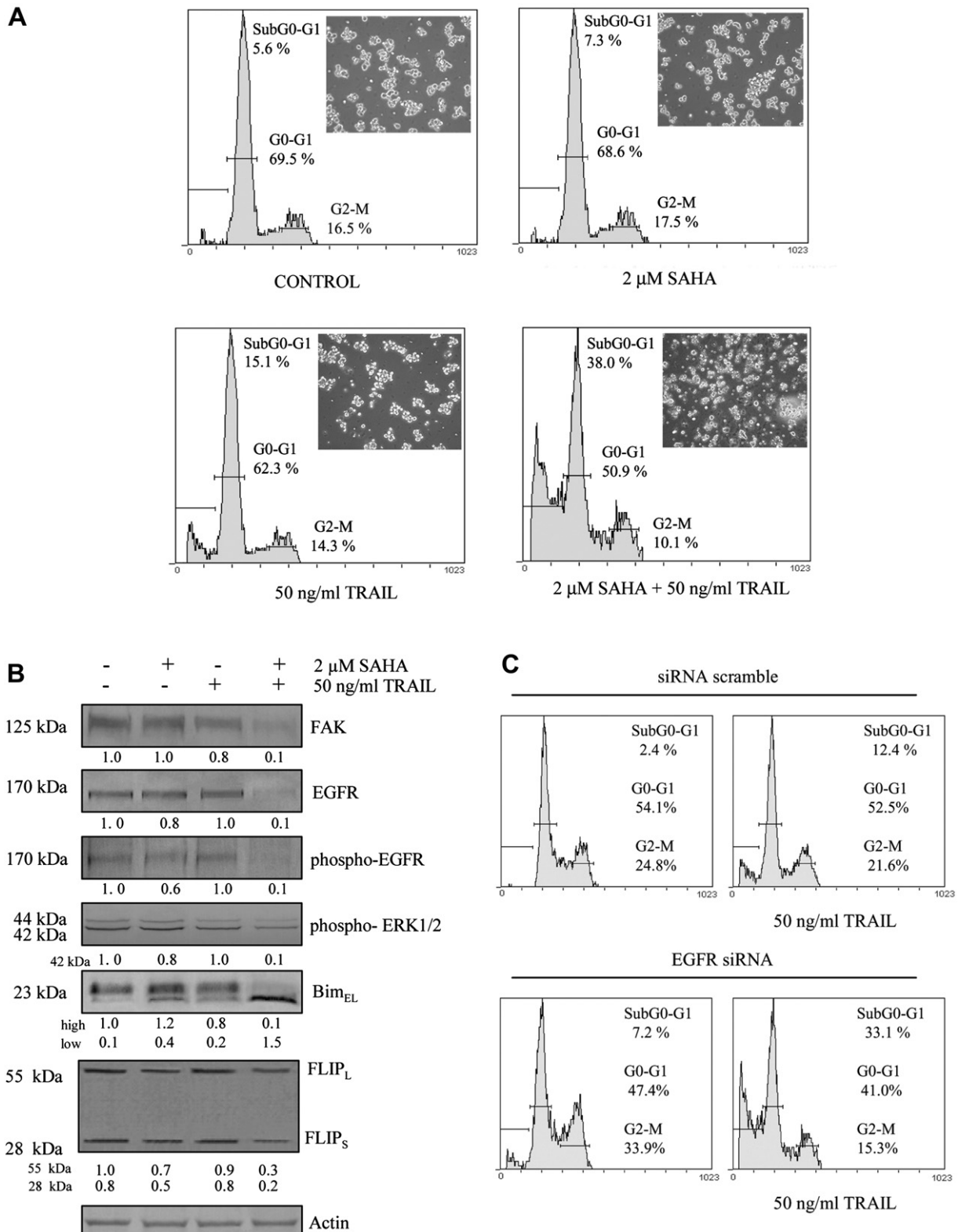


Fig. 7. The effects of SAHA and TRAIL on MDA-MB231 cells cultured on poly-HEMA. Cells were seeded on 6-well poly-HEMA-coated tissue culture dishes to produce non-anchored cells. After 24 h of growth, cells were treated with SAHA or TRAIL alone or in combination for another 14 h. (A) Flow cytometric analysis of MDA-MB231 cells, treated with the drugs for 14 h after staining with PI. The effects induced by treatment on cell morphology, as shown by optical microscope, were reported in the insets. Results are representative of three independent experiments. (B). The effects of co-treatment on the levels of FAK, EGFR, phospho-EGFR, phospho-ERK1/2, Bim_{EL} and c-FLIP, ascertained by western blotting analysis using specific antibodies. Detection of actin was enclosed to demonstrate equal loading of protein for each sample. Densitometric values are representative of three independent experiments. (C) MDA-MB231 cells were transfected for 5 h with siRNA sequence directed against EGFR or with siRNA scramble and then cultured on poly-HEMA. After other 24 h of incubation the cells were treated with TRAIL for 14 h to evaluate the effect on apoptosis.

apoptosis induced by TRAIL signalling [22,50]. This paper shows that SAHA, an effective inhibitor of histone deacetylases, sensitized both MDA-MB231 and MCF-7 cells to TRAIL-induced apoptosis, since treatment with SAHA/TRAIL combination induced detachment of cells and apoptosis of detached cells in both the cell lines.

Time course experiments demonstrated that already at 8 h of treatment with SAHA/TRAIL combination about 25% of MDA-MB231 cells were deprived of anchorage and grouped in viable clusters, while signs of apoptosis (chromatin condensation and fragmentation) appeared at 12–14 h of treatment and were observed only in detached cells. This event was peculiar of anoikis, a form of apoptosis that occurs in various normal cell types after detachment from their proper substratum [24]. Similar observations were also made for MCF-7 cells treated with SAHA/TRAIL combination.

In order to clarify how the treatment sensitizes the cells of both the lines to TRAIL-signalling pathway we ascertained that neither SAHA nor TRAIL, either alone or in combination, modified the level of both DR4 and DR5. However, treatment with SAHA alone decreased the level of the anti-apoptotic factor c-FLIP. Because c-FLIP prevents the activation of procaspase-8 at the DISC [51], its down-regulation was responsible for activation of caspase-8. Such an effect became more consistent when the cells were treated with SAHA/TRAIL combination.

Our results also demonstrated that SAHA and TRAIL either alone or in combination were unable to exert any effect on the levels of $\alpha 5$ and $\beta 1$ integrin subunits, which have been reported to be up-regulated in MDA-MB231 cells and seem to be involved in the survival and migration of these cells [31,32]. However, we cannot exclude that SAHA/TRAIL co-treatment modified the levels of other integrin subunits, since aberrant expression of several other integrins has been shown in breast cancer cell lines [52,53]. However, our results demonstrated that SAHA/TRAIL combination markedly reduced the level of FAK, the tyrosine kinase which is present at sites of integrin/ECM attachment and plays a key role in mediating integrin signalling [54]. The decrease in FAK level was already observed at 8 h of treatment and was prevented by IEDT-fmk, the specific inhibitor of caspase-8. This observation was in line with the finding that FAK is a target of caspases [34]. It has been reported that the inhibition of FAK expression with antisense oligonucleotides or dominant-negative leads to cell rounding, detachment and anoikis [54,55]. Thus, the cleavage of FAK by activation of caspase-8 could induce the disassembly of focal adhesion complex favouring cell detachment. Therefore, down-regulation of c-FLIP, which induced activation of caspase-8 and down-regulation of FAK, could be considered as an initial event to provoke detachment of the cells. In line with this consideration, silencing of c-FLIP in MDA-MB231 cells favoured detachment of cells induced by treatment with TRAIL alone.

In breast cancer cells EGF receptor is capable of favouring proliferation, migration and cell survival as well as inhibiting apoptosis [43]. It is known that MDA-MB231 cells contain a high level of EGFR [43]. This can be a consequence of the absence in these cells of estrogen receptor α , because it has been demonstrated an inverse relationship between the presence of estrogen receptor α and the level of EGFR [43]. This paper demonstrates that SAHA decreased the level of both EGFR and its phosphorylated form. This result is in line with the observation of Zhou et al. [56], showing that in breast cancer cells SAHA down-regulated EGFR by destabilizing its mRNA. Our results also showed that the decrease induced by SAHA was potentiated by addition of TRAIL, although the mechanism by which co-treatment can induce this effect is unknown at the moment.

It has been shown that down-regulation of EGFR reduces ERK1/2 phosphorylation with the consequent increase in the dephosphorylated and active form of BimEL, an effect which plays

an important role in the induction of anoikis [57]. Our results demonstrated that the same effects were determined in MDA-MB231 cells by the treatment with SAHA/TRAIL combination. Thus down-regulation of EGFR could be responsible for accumulation of the dephosphorylated form of BimEL. This conclusion was supported by the finding that EGFR down-regulation induced in MDA-MB231 cells by specific siRNA markedly reduced phosphorylated forms of EGFR, ERK1/2 and BimEL, while increased dephosphorylated form of BimEL. Similar results were observed using AG1478, a specific inhibitor of the tyrosine kinase activity of EGFR. In addition, because it is known that phosphorylation of ERK1/2 can be also dependent on FAK activation [58], it is possible that FAK cleavage, induced by co-treatment, could play a role in ERK1/2 inhibition and BimEL activation. Finally, in agreement with the observations of Frogne et al. [46], we demonstrated that MCF7 cells express low levels of EGFR, phospho-EGFR and phospho-ERK1/2. SAHA/TRAIL co-treatment markedly reduced the levels of these factors and concomitantly increased the dephosphorylated form of BimEL, thus suggesting that also in MCF-7 cells down-regulation of EGFR can be correlated with accumulation of the active form of BimEL.

It is known that BimEL in the dephosphorylated form exerts apoptotic function either by inhibiting the anti-apoptotic factor Bcl-2 or favouring BAX oligomerization [36]. Bcl-2 inhibition and BAX activation are responsible for the loss of mitochondrial membrane potential [59]. Thus, the increase in the dephosphorylated form of BimEL, induced by SAHA/TRAIL combination, might assume a particular relevance in the loss of $\Delta\psi_m$ and the consequent activation of caspase-3, observed in detached MDA-MB231 cells treated with the drugs in combination.

Many normal cell types, including epithelial cells, undergo anoikis upon detachment from their proper substratum [24]. In contrast, many malignant cells survive after cell detachment [60]. Anoikis-resistance contributes to metastasis allowing cancer cells to survive in the blood stream and invade distant sites [60]. It has been reported that MDA-MB231 cells are resistant to anoikis [41,61], an event which can be correlated with the activation of survival pathways [39,61]. Our results agree with this conclusion, since MDA-MB231 cells survive and proliferate when cultured in poly-HEMA. These cells contain high levels of both EGFR and FAK and low basal levels of the dephosphorylated form of BimEL. Moreover, down-regulation of EGFR sensitizes MDA-MB231 cells, cultured in poly-HEMA, to TRAIL-induced apoptosis. Therefore, our results suggest that high levels of EGFR can prevent MDA-MB231 cells from anoikis but do not exclude that also high levels of FAK can be responsible for anoikis resistance of MDA-MB231 cells. This conclusion is in line with recent observations showing that dual inhibition of FAK and EGFR cooperatively enhanced apoptosis in human breast cancer cell lines via inhibition of signalling that involve both AKT and ERK1/2 pathways [34]. Finally, our results show that treatment with SAHA/TRAIL combination induced apoptosis of MDA-MB231 cells cultured in poly-HEMA, suggesting that this drug combination was capable of restoring anoikis sensitivity of these cells. This effect was correlated with a marked decrease in the levels of FAK, EGFR and phospho-ERK1/2, as well as with an increase in the dephosphorylated form of BimEL.

It is known that ER α -negative forms of breast cancer are more aggressive than ER α -positive ones. Moreover, ER α -negative forms are unresponsive to endocrine therapy and resistant to anoikis. Our results demonstrate that SAHA/TRAIL combination is effective in inducing anoikis in both ER α -positive MCF-7 and ER α -negative MDA-MB231 breast cancer cells. The observation that treatment with SAHA and TRAIL restores sensitivity to anoikis in MDA-MB231 cells suggests a new and attractive pharmacological tool for the treatment of breast cancer.

Recently, it has been shown that SAHA/TRAIL combination inhibited the growth of xenograft tumors derived from MDA-MB468 cells, another ER α -negative breast cancer cell line [62]. We have in program a new study in order to ascertain in nude mice bearing tumors derived from MDA-MB231 cells the anticancer potential of SAHA/TRAIL combination, the molecular mechanism of this effect and the possible advantageous association with other antitumor factors.

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