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The histone deacetylase inhibitor suberoylanilide hydroxamic acid sensitises human hepatocellular carcinoma cells to TRAIL-induced apoptosis by TRAIL-DISC activation

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ABSTRACT

This paper shows that the histone deacetylase inhibitor SAHA sensitised at sub-toxic doses human hepatocellular carcinoma cells (HepG2, Hep3B and SK-Hep1) to TRAIL-induced apoptosis, while it was ineffective in primary human hepatocytes (PHHs).

In particular in HCC cells SAHA increased the expression of death receptor 5 (DR5) and caused a decrement of c-Flip. These two modifications provoked in the presence of TRAIL the rapid production of TRAIL-DISC and the activation of caspase-8. Consequently SAHA/TRAIL combination induced many apoptotic events, such as a cleavage of Bid into tBid, dissipation of mitochondrial membrane potential, activation of caspase-3 with the consequent cleavage of both NF-kB and Akt. The decrease in NF-kB level seemed to be responsible for the reduction in the content of IAP family antiapoptotic proteins while the decrease in Akt level caused a reduction in phospho-Bad. These events led to the activation of caspase-9, which contributed to the strong apoptotic activity of TRAIL.

Sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by SAHA may suggest new strategies for the treatment of liver tumours.

1. Introduction

Recently the interest of the researchers has been focused on TRAIL (Apo2L), a member of the TNF superfamily,[1] because this cytokine induces apoptosis in a number of tumour cell lines but is ineffective in the majority of normal cells.[2]

TRAIL can bind to five different receptors: four membrane-bound and one soluble receptor.[2,3] Two of these membrane receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), act as agonistic receptors, containing a cytoplasmic death domain (DD) through which TRAIL can transmit an apoptotic signal. Two other membrane receptors, decoy receptor 1 (DcR1/TRAIL-R3) and decoy receptor 2 (DcR2/TRAIL-R4), can also bind TRAIL, but act as antagonistic receptors, lacking an intact death domain. Finally the soluble receptor osteoprotegerin (OPG) binds TRAIL with low affinity.[3]

Binding of TRAIL to DR4 or DR5 results in trimerization of the receptors with the production of the death-inducing signalling complex (DISC). Through their intracellular death domains these receptors bind the adaptor protein FADD, which in turn recruits the initiator procaspase-8 to the DISC.[4] Within this complex, procaspase-8 is activated by autoproteolytic cleavage[5] with the consequent activation of effector caspases, such as caspase-3,[6] or cleavage of Bid and induction of the intrinsic apoptotic pathway.[6] Besides caspase-8, also caspase-10 can be recruited to the DISC,[7] although caspase-10

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seems to be activated in TRAIL-induced apoptosis, only when low doses of TRAIL are available.[8] Activation at the DISC of both caspases-8 and -10 can be inhibited by the cellular c-Flip, which is recruited to the DISC through FADD, thereby preventing the recruitment of caspases into the complex.[9]

NF-kB is a nuclear transcription factor which exerts a role in the control of apoptosis. This factor is assembled through the dimerization of two components selected of five different subunits: cRel, RelA (p65), RelB, p50 and p52.[10] NF-kB is predominantly found in the cytoplasm complexed with IkB-α protein. Degradation of IkB-α in response to stimuli is an obligatory step in NF-kB activation.[11] This degradation is mediated by the proteasome and requires phosphorylation of IkB-α at specific serine residues by IKK complex.[11] The released NF-kB translocates to the nucleus where it regulates the transcription of several genes involved in immune and inflammatory responses and in the control of cell proliferation, apoptosis, metastasis and angiogenesis.[12]

TRAIL has been shown, such as TNF-α, to be involved in the activation of NF-kB.[13,14] In particular TRAIL-DISC can recruit RIP-1, which causes activation of NIK and phosphorylation of IKK complex.[11] The consequent phosphorylation and cleavage of IkB-α permits the translocation of NF-kB to the nucleus, where in particular stimulates the expression of genes coding for IAP family of proteins.[15] It is noteworthy that c-Flip exerts an important role in defining whether TRAIL-DISC induces the activation of caspase-8 and apoptosis or activation of NF-kB. In fact c-Flip is not only an inhibitor of caspase-8[9] but it can recruit the proteins involved in the activation of NF-kB, favouring this process and consequently the survival of cells.[16]

It is interesting to note that the cells exhibit a different sensitivity to TRAIL. The scarce susceptibility of normal cells to TRAIL can be considered as a consequence of multiple conditions, including overexpression of the decoy receptors, low expression of the death receptors and also high levels of c-Flip and IAPs.[17]

Many tumour cells, including hepatoma cells,[18,19] exhibit resistance to the proapoptotic effect of TRAIL,[20] but their sensitivity to TRAIL can be increased by combining TRAIL with many compounds, such as anticancer cytotoxic drugs,[21] proteasome inhibitors[22,23] and HDAC inhibitors.[18,19,24,25] These findings have permitted to elaborate several new therapeutical strategies.[26]

We have previously demonstrated that various HDACIs are effective in inducing apoptosis in HepG2 cells.[27] Because little is known[18,19] about the sensitization to TRAIL induced by HDACIs in HCC cells, we have performed a research on this subject. Between the different HDACIs, we have employed for our experiments the suberoylanilide hydroxamic acid (SAHA), because this compound strongly sensitises HCC cells to TRAIL but exhibits a very low toxicity on primary human hepatocytes (PHH). In addition SAHA has shown promising clinical activity against various solid tumours at doses that have been well tolerated by patients.[28]

In this paper we demonstrate that SAHA sensitised HCC cells to TRAIL by inducing up-regulation of DR5 and down-regulation of c-Flip. Both these events led to the activation of TRAIL-DISC and caspase-8 with induction of apoptosis. The consequent activation of caspase-3 provoked cleavage of both NF-kB and Akt, which contributed to the strong apoptotic activity of TRAIL.

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 amino acids 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". z-VAD-fmk and DEVD-cho were purchased from Promega (Milan, Italy), IEDT-fmk from Clontech (Palo Alto, CA), z-LEHD-fmk from Imgenex (San Diego, CA). All the other compounds were purchased 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%, a percentage which was not toxic and did not interfere with cell growth.

2.2. Cell cultures, cell viability and cell death assay

HepG2 cells, obtained from "Istituto Scientifico Tumori" (Genoa, Italy), Hep3B and SK-Hep1 cells from the European Collection of Animal Cell Cultures (ECACC, Health Protection Agency, Porton Down, Wiltshire, UK) and PHH, from Cambrex Bio Science (Walkersville, MD), were cultured as described.[29,30] After plating either on 96-well plates or 100-mm culture dishes, cells were allowed to adhere overnight and then treated with chemicals or vehicle only. Cell viability was determined by the MTT quantitative colorimetric assay as described.[31]

Apoptotic cells were detected by flow cytometry analysis using the annexinV-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen; San Diego, CA), according to the manufacturer's instructions. In order to ascertain the presence of condensed chromatin and apoptotic bodies, cells were fixed in 3:1 methanol/acetic acid and incubated for 30 min with Hoechst 33258. After washing in PBS nuclear morphology was observed under a fluorescence microscope.

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analysed as described.[31]

2.3. Western blotting analysis

Cell lysates were prepared as reported.[32] Protein samples (30 μg/lane) were subjected to SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for detection with specific antibodies obtained 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 β-actin.

2.4. Down-regulation of endogenous c-Flip, Bid and p65 expression

Specific siRNAs against c-Flip, Bid and the component of NF-kB p65 were delivered by St Cruz Biotechnology (Santa Cruz, CA) as a pool of siRNAs and transfected into the cells (2×10^5 cells) at a final concentration of 80 nM siRNA in the presence of 6 μl Metafectene Pro (Biontex, Martinsried/Planegg, GmBH) in a six-well plate format with a final volume of 1 ml serum-free RPMI. The reaction was stopped after 5 h of treatment, replacing the medium with RPMI + 20% FBS and the expression of c-Flip, Bid and p65 were evaluated by Western blotting analysis after 48 h. Fluorescein conjugated siRNA, consisting in a scrambled sequence, was used as a negative control.

2.5. Transient transfections of Akt

Cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, for 24 h to reach approximately 60–80% confluence before transfection. Then cells (2×10^5/well) were transfected, according to the manufacturer's protocol (Invitrogen, Carlsbed, CA), with pUse empty vector, pUseAktCA (myristoylated-constitutively active Akt) or pUseAktDN (dominant negative Akt) construct, obtained from Upstate Biotechnology (Lake Placid, NY), in 1 ml serum-free RPMI in the presence of Metafectene Pro. After 5 h from transfection, the reaction was stopped replacing the culture medium with RPMI + 20% FBS and transfection efficacy was evaluated after 48 h by Western blotting.

2.6. Isolation and analysis of the TRAIL-DISC

One mg/ml purified recombinant TRAIL was incubated with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 1 h on ice. Then reaction was stopped by adding 1/10 volume of 1 M Tris–HCl at pH 7.5 and the sample was passed on Zeba desalt spin columns (Pierce, Rockford, IL) to remove unincorporated biotin from biotin-conjugated-TRAIL (bio-TRAIL).

HepG2 cells (1×10^6/ml) were treated for 16 h with 2 μM SAHA. Then the cells, resuspended in 200 μl medium, were incubated for 2 h with a high dose of biotinylated TRAIL (1 μg/sample). DISC formation was then stopped and unbound bio-TRAIL was removed by washing the cells with ice-cold PBS. Cells were then resuspended in 200 μl of lysis buffer containing protease inhibitors. After 60 min on ice, samples were centrifuged at 15,000*g* for 10 min at 4 °C and bio-TRAIL (1 μg/sample) was added to lysates from control cells. Then TRAIL-DISC was precipitated using 30 μl of streptavidin-agarose beads (Pierce) at 4 °C overnight. Precipitates were washed five times with lysis buffer and receptor complexes were eluted from the beads with 60 μl of the sample buffer. About 20 μl of the eluted complexes was employed for Western blotting.

2.7. NF-kB assay

NF-kB activity was quantified by using an ELISA-based assay (Trans-AM NF-kB; Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cell nuclear extracts were placed in 96-well plates, coated with an oligonucleotide containing the NF-kB consensus sequence, and the presence of active NF-kB was detected using specific antibodies for p65 and p50 subunits. A horseradish peroxidase (HRP)-conjugated secondary antibody was used to quantify NF-kB by a colorimetric procedure.

2.8. Real-time PCR analysis

Total cellular RNA prepared using RNeasy mini kit (Qiagen, Valencia, CA) was treated for 30 min at 37 °C with RQ1 RNase free DNase (Promega, Madison, WI) to remove residual genomic DNA before quantitative RT-PCR. The single-stranded cDNA (20 μl) was synthesised using the GeneAmp Kit for reverse transcriptase-polymerase chain reaction (Perkin–Elmer, Foster City, CA) as previously described.[33]

For real-time PCR analyses, each cDNA sample (5 μl) was amplified using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The following primers were used: DR5: sense: 5′-GCACTCACTGGAATGACCTC-3′; antisense: 5′-GCCTTCTTCGCACTGACAC-3′ (Ta = 55 °C); DR4: sense: 5′-CAGAACGTCCTGGAGCCTGTAAC-3′; antisense: 5′-ATGTCCATTGCCTGATTCTTTGTG-3′ (Ta = 63 °C); GAPDH: sense: 5′-TGACATCAAGAAGGTGGTGA-3′; antisense: 5′-TCCACCACCCTGTTGCTGTA-3′ (Ta = 55 °C).

All reactions were performed in triplicate. For each PCR, we checked linear range of a standard curve of serial dilutions. The relative quantification of DR5 or DR4 gene expression was evaluated after normalisation with the endogenous control, GAPDH. Data processing and statistical analysis were performed using IQ5 cycler software.

2.9. Statistical analysis

Data are presented as means ± SD 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.

3. Results

3.1. SAHA sensitises HCC cells to TRAIL-mediated apoptosis

In a previous paper we ascertained that the histone deacetylase inhibitor SAHA exerted a cytotoxic effect on HepG2 cells, in a dose-dependent manner, decreasing the number of cells by 50% after 36 h of treatment with 2.5 μM.[27] The present paper shows that sub-toxic doses of SAHA are capable of sensitising HCC cells to TRAIL. As shown in Fig. 1A, treatment with TRAIL alone (20–50 ng/ml) caused only modest effects in HepG2 cells, which were observed after 24 h of incubation, while TRAIL was ineffective in Hep3B and SK-Hep1 cells. However, a sub-toxic dose of SAHA (1 μM) did not induce any modification in the viability of all tested cell lines, but when 1 μM SAHA was added to 20 ng/ml TRAIL remarkable cytotoxic effects were observed in the three cell lines (Fig. 1A). The most striking effect appeared in HepG2 cells. After 16 h of incubation co-treatment reduced cell viability to 55% (Fig. 1A and B), then the effect increased with time

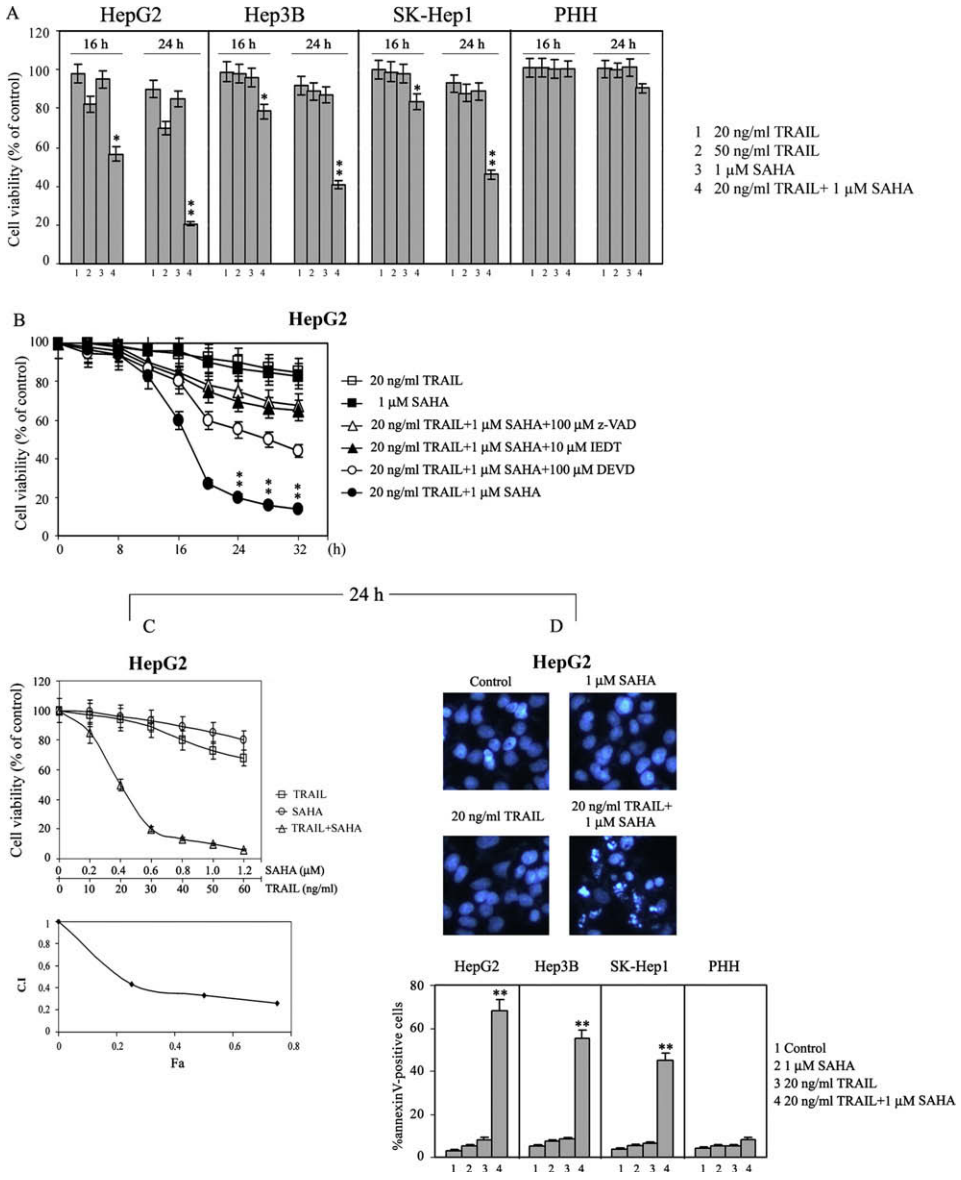

Fig. 1 – SAHA sensitises HCC cells to TRAIL-induced apoptosis. (A) Cytotoxic effects exerted on HepG2, Hep3B, SK-Hep1 cells and PHH by SAHA alone or in combinations with TRAIL. After treatment of cells for 16 or 24 h cell viability was evaluated by MTT assay. (B) Time-dependence of the cytotoxic effect exerted on HepG2 cells by 1 μM SAHA alone or in combination with 20 ng/ml TRAIL in the presence or absence of 100 μM z-VAD, 10 μM IETD or 100 μM DEVD. (A and B) Data are the means ± SD of three independent experiments performed in triplicate. *P < 0.05 and **P < 0.01 versus TRAIL alone. (C) Synergistic effects exerted on HepG2 cells by SAHA and TRAIL. HepG2 cells were treated for 24 h with SAHA/TRAIL combinations at concentrations which changed in a fixed ratio. At the end of treatment cell viability was measured by MTT assay. Data were employed to calculate combination index with respect to fraction affected (Fa). (D) Apoptotic effects induced by the treatment of HepG2, Hep3B, SK-Hep1 cells and PHH for 24 h with 1 μM SAHA, 20 ng/ml TRAIL or TRAIL/SAHA combination. Apoptotic morphology was evaluated after staining of the cells with Hoechst 33342. AnnexinV positive cells were quantified by flow cytometric analysis after double staining of cells with annexinV and PI. The results are representative of three independent experiments ± SD. **P < 0.01 versus TRAIL alone.

and after 24 h cell number decreased to about 20% of control. Minor effects were observed in both Hep3B and SK-Hep1 cells, since cell viability was reduced after 24 h of co-treatment to about 40% and 45% of the respective controls (Fig. 1A). Moreover, the cytotoxic effect exerted by SAHA/TRAIL combination was prevented by the broad spectrum caspase inhibitor z-VAD-fmk and the preferential caspase-8 inhibitor IEDT-fmk (Fig. 1B). A modest protective effect was instead observed in the presence of DEVD-cho (Fig. 1B), a preferential inhibitor of caspase-3 activity.

In order to ascertain synergistic interactions between SAHA and TRAIL, HepG2, Hep3B and SK-Hep1 cells were treated for 24 h with combinations of various doses of SAHA and TRAIL at a fixed ratio. Analysis of the combination index (CI)

with respect to the proportion of death cells (fraction affected, Fa) showed values of CI considerably less than 1.0 in HepG2 (Fig. 1C) as well as in Hep3B and SK-Hep1 cells (not shown), indicating a strong synergistic interaction between the two compounds.[34,35]

As shown in Fig. 1A, treatment with SAHA or TRAIL alone did not induce any sign of toxicity in PHH, while treatment with SAHA/TRAIL combination caused only a modest reduction in cell viability (–12%).

Many evidences suggested that the decrease in cell viability was caused by apoptosis. At first, using Hoechst 33258 staining we demonstrated that a high percentage of HepG2 cells, treated with 1 μM SAHA/20 ng/ml TRAIL combination, exhibited condensed/fragmented nuclei, while this aspect was not observed when the cells were treated with SAHA or TRAIL alone (Fig. 1D). Clear morphological signs of apoptosis were also observed in Hep3B and SK-Hep1 cells treated with SAHA/TRAIL combination (not shown).

Apoptosis was further evaluated by the analysis of annexinV-FITC and propidium iodide stained cells (Fig. 1D). Significant percentages of annexinV-FITC positive cells were observed in all the three HCC cell lines at 24 h of treatment with 1 μM SAHA/20 ng/ml TRAIL, while the percentages of PI positive (necrotic) cells were not significantly altered by the treatment. In contrast SAHA/TRAIL combination resulted ineffective in inducing any apoptotic effect in PHH.

In consideration of these results 1 μM SAHA and 20 ng/ml TRAIL were chosen to evaluate the effects exerted by combined treatment on HCC cells.

3.2. *The effect of SAHA on the expression of death receptors and c-Flip*

In order to understand how SAHA sensitises HepG2 cells to TRAIL-induced apoptosis we investigated the effect of SAHA on TRAIL receptors. Real-time PCR provided evidence that treatment with SAHA alone markedly increased the expression of DR5 mRNA, while it did not modify that of DR4 mRNA. The effect was already observed at 8 h of treatment (not shown). At 16 h (Fig. 2A) a two-fold increase in the expression of DR5 mRNA was observed with 1 μM SAHA and a fourfold increase with 15 μM. Lower effects were found after treatment with SAHA/TRAIL combination, while TRAIL alone did not produce any modification (Fig. 2A). Consistent with these results, Western blotting analysis (Fig. 2B) demonstrated that also DR5 protein increased after SAHA treatment. In contrast the expression of DR4 (Fig. 2B) and that of the decoy receptors DcR1 and DcR2 (not shown) were not modified by treatment with SAHA or TRAIL alone or in combination.

Afterwards other experiments were performed to establish the effect of treatment on the two spliced forms of c-Flip.[9] Fig. 2C shows that the treatment of HepG2 cells for 16 h with 1 μM SAHA alone decreased the level of both c-Flip$_S$ and $_L$ to 70% of control. This effect was in agreement with the observation of Pathil et al.[18] that ITF2357, another inhibitor of histone deacetylase, caused down-regulation of c-Flip in HepG2 cells. Moreover, a stronger effect was observed when the cells were treated with SAHA/TRAIL combination. In fact after 16 h of combined treatment the level of both the isoforms of c-Flip markedly decreased reaching a value corresponding to only 20% of control. The addition of z-VAD or IEDT prevented this further decrease. Therefore, such an effect seemed to be a consequence of activation of caspases, and in particular caspase-8. In contrast, the decrease induced by the treatment with SAHA alone was not prevented by z-VAD (Fig. 2C, comparison of lane 3 with lane 2) and therefore it can be considered independent on the caspase activity.

In addition, Fig. 2D shows that SAHA increased the expression of DR5 protein and caused down-regulation of c-Flip also in Hep3B and SK-Hep1 cells.

3.3. *The effect of SAHA on the production of TRAIL-DISC*

In order to ascertain whether sensitization with SAHA stimulated the production of TRAIL-DISC and the consequent activation of caspase-8, HepG2 cells were at first treated for 16 h with a low dose of SAHA (2 μM and then stimulated for 2 h with a high dose of biotinylated TRAIL (1 μg/sample) to induce rapidly the production of DISC and the consequent effects. After incubation, lysates were prepared and TRAIL-DISC was precipitated by means of streptavidin beads. In cells which were not stimulated during incubation, biotinylated-TRAIL was added after cell lysis in order to induce the production of DISC. Western blotting of the proteins eluted from the beads (Fig. 2E) showed that sensitisation by SAHA of cells treated with biotinylated-TRAIL (comparison of lane 4 with lane 3) resulted in a strong increase in the amount of DR5 recruited by TRAIL to DISC. It is noteworthy that in this condition also the recruitment of procaspase-8 to TRAIL-DISC markedly enhanced with the consequent activation of caspase-8. Moreover, treatment with biotinylated-TRAIL of SAHA-sensitised cells decreased the level of both c-Flip forms and RIP-1 in the TRAIL-DISC. These effects were prevented by both z-VAD and IEDT, suggesting that caspase-8 was involved in the cleavage of both c-Flip and RIP-1. However, it was not possible to demonstrate in this condition the activation of caspase-10 (not shown).

Finally the observation that a much lower content of DR5 was found when biotinylated-TRAIL was added to lysates of HepG2 cells rather than to intact cells (Fig. 2E, comparison of lane 2 with lane 4) strongly suggested that cell integrity and viability were necessary for the production of DISC.

3.4. *Down-regulation of c-Flip sensitised HCC cells to TRAIL-induced apoptosis*

In order to ascertain the possible effects determined by the lowering of c-Flip on the activation of TRAIL-DISC, HepG2 cells were transiently transfected with siRNA sequence directed against c-Flip. This treatment caused a reduction in c-Flip content of approximately 50% (Fig. 3A, comparison of lane 4 with lane 1). Down-regulation of c-Flip determined by siRNA transfection sensitised the cells to TRAIL-induced apoptosis. In fact when TRAIL was added to silenced cells a remarkable loss in cell viability was observed (Fig. 3B), which was accompanied by the appearance of morphological signs of apoptosis (Fig. 3C). In addition the treatment of silenced cells with TRAIL caused activation of caspase-8 and down-regulation

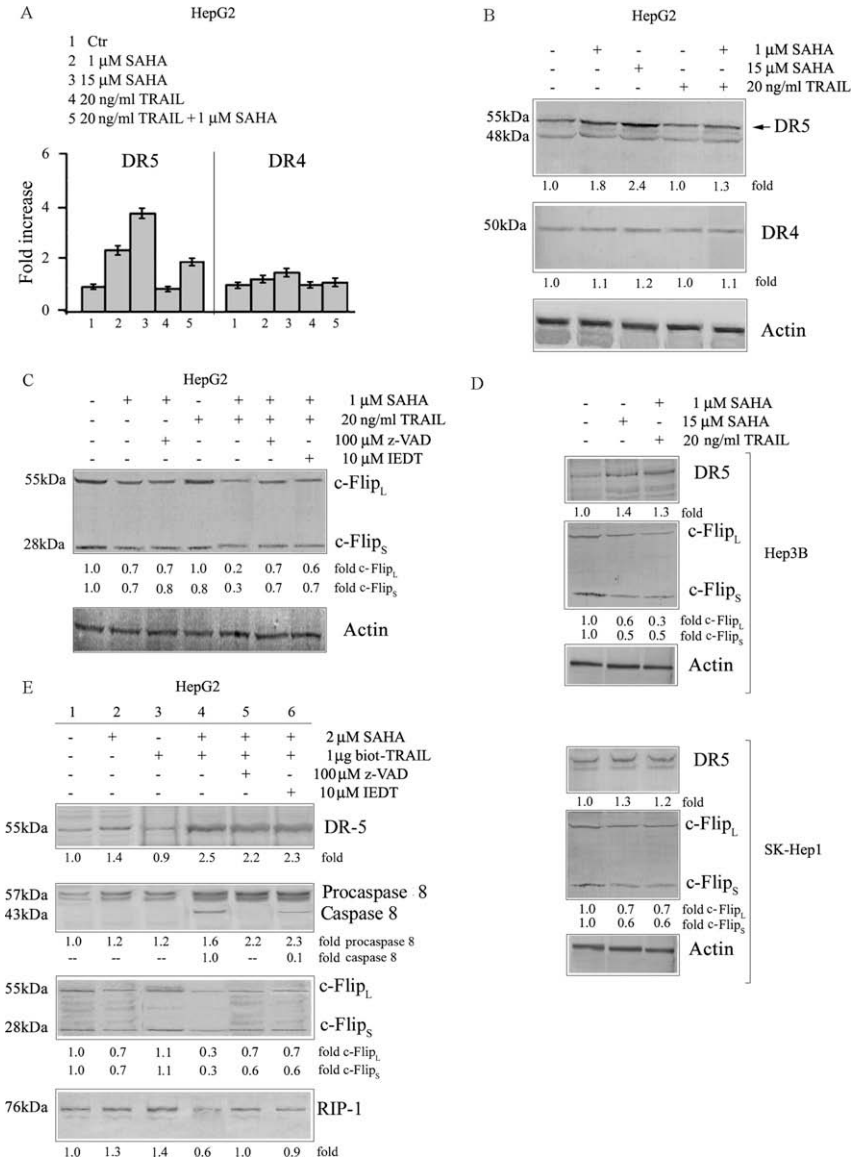

Fig. 2 – SAHA stimulates TRAIL signal in human HCC cells. HepG2 (A, B, C and E), Hep3B and SK-Hep1 (D) cells were treated for 16 h with TRAIL, SAHA or SAHA/TRAIL combination in the presence or absence of caspase inhibitors. (A) Real-time PCR analysis of the expression of DR5 or DR4 mRNA. Results are representative of three independent experiments ± SD. (B–D) Western blotting analysis to evaluate the levels of DR5 (B and D), DR4 (B) and c-Flip (C and D) proteins. (E) Immunoprecipitation analysis to evaluate the composition of TRAIL-induced DISC. HepG2 cells were preincubated for 16 h with 2 μM SAHA. Then the cells were stimulated with 1 μg/sample biotinylated-TRAIL for other 2 h. At the end, cells were lysed and biotinylated-TRAIL was added to the lysates of the unstimulated samples. TRAIL-DISC was precipitated with streptavidin-agarose beads and receptors complexes were eluted with sample buffer and analysed in order to ascertain the level of DR5, procaspase-8, c-Flip and RIP-1. Control is represented by cells treated with vehicle only. All Western blot experiments in B–E were repeated twice with similar results.

of RIP-1 (Fig. 3A, comparison lane 5 with lane 4). Obviously these effects were not found when TRAIL was added to cells treated with scramble siRNA (Fig. 3A, comparison lane 2 with lane 1). It seems likely that the effects on cell viability and the appearance of apoptotic signs, observed in Fig. 3B and C, were consequences of activation of caspase-8, since they were partially prevented by IEDT. As shown in Fig. 3D, TRAIL increased in non-transfected cells the level of both p65 and p50 NF-kB subunits. This result was in accordance with the observation that TRAIL stimulates the activation of NF-kB.[13] Such an effect was not observed when TRAIL was added to HepG2 cells treated with c-Flip siRNA (Fig. 3D).

These results suggest the conclusion that in HepG2 cells down-regulation of c-Flip favoured the activation of caspase-8 and induction of apoptosis, while prevented the activation of NF-kB induced by TRAIL.

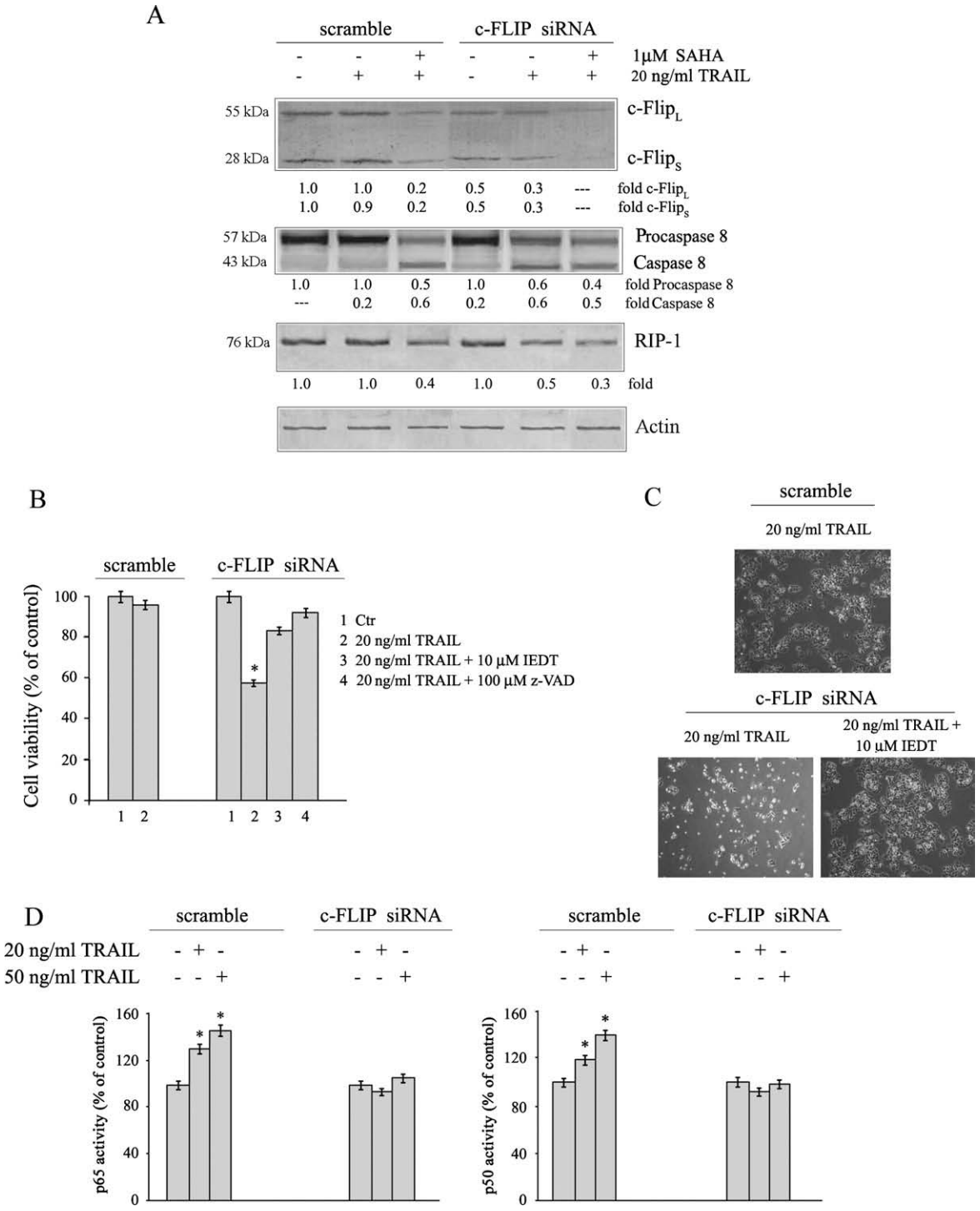

Fig. 3 – Down-regulation of c-Flip by specific siRNA sensitises HepG2 cells to TRAIL signal. HepG2 cells were transiently transfected with siRNA sequence directed against c-Flip and employed for the experiments in comparison with cells transfected with scrambled siRNA. (A) Western blotting analysis showing the activation of caspase-8 and RIP-1 cleavage induced by TRAIL in c-Flip silenced cells. (B) MTT assay showing HepG2 cells transfected with c-Flip siRNA and treated for 16 h with 20 ng/ml TRAIL in the presence or absence of 100 μM zVAD or 10 μM IEDT. Data are the means ± SD of three independent experiments performed in triplicate. *$P < 0.05$ versus control untreated cells. (C) Morphological effects induced by TRAIL in c-Flip silenced HepG2 cells after 16 h of treatment. (D) ELISA assay of NF-kB binding activity, showing that pre-treatment with c-Flip siRNA prevents in HepG2 cells activation of NF-kB induced by TRAIL. Nuclear extracts, prepared from untreated and treated cells, were employed to assay the binding of both p65 and p50, two members of NF-kB, to consensus binding sequence of NF-kB. Control is represented by cells treated with vehicle only. Results are representative of three independent experiments performed in triplicate ± SD. *$P < 0.05$ versus control untreated cells.

3.5. The effect of combined treatment on the activation of Bid and caspases

As shown in Fig. 4A treatment of HepG2 cells with SAHA/TRAIL combination induced the cleavage of Bid and that of both procaspase-8 and procaspase-3, while treatments with the single compounds were ineffective (not shown). The effects appeared at 8 h of treatment and increased at 24 h. It is interesting to note that IEDT prevented all these effects. Therefore, we suggest that caspase-8 was responsible for the cleavage of procaspase-3 and Bid. Also the activation of caspase-9, which occurred in the second phase of treatment (24 h), exerted a role in the apoptotic mechanism. This conclusion was suggested by the observation that z-LEHD, a specific inhibitor of caspase-9, also inhibited the cleavage of procaspase-3 (Fig. 4A).

Fig. 4B shows that SAHA/TRAIL combination caused a pronounced decrease in mitochondrial membrane potential ($\Delta\Psi_m$), while single treatment did not modify it (not shown). Such an effect was prevented by the addition of either z-VAD or IEDT. In order to ascertain the role of Bid in the induction of apoptosis we have transiently transfected HepG2 cells with siRNA sequence directed against Bid. siRNA transfection decreased the level of Bid by 50–60% and markedly reduced the effect of SAHA/TRAIL combination on procaspase-3, procaspase-9 (Fig. 4C), $\Delta\Psi_m$ (Fig. 4D) and apoptosis (Fig. 7), but not the effect on procaspase-8. These results suggest that the activation of caspase-8 induced cleavage of Bid with the production of tBid, which provoked dissipation of $\Delta\Psi_m$ and the consequent involvement of mitochondria in apoptotic events.

3.6. The effect of combined treatment on NF-kB and Akt

In order to ascertain a relationship between the inhibition of NF-kB activity and the induction of apoptosis, we measured at first the effect of the treatment on NF-kB DNA binding activity by using an ELISA-based assay which detects the binding of p65 and p50 subunits to NF-kB consensus sequence. As shown in Fig. 5A, stimulation of HepG2 with TRAIL alone enhanced both p65 and p50 NF-kB DNA binding activities, while 1 μM SAHA was ineffective. In contrast treatment with SAHA/TRAIL combination induced remarkable decreases in both p65 and p50. Experiments of time-dependence showed that the lowering of both p65 (Fig. 5B) and p50 (not shown) activities was modest in the first phase of treatment (4–12 h), but markedly increased in the second phase (16–32 h). Because either z-VAD or DEVD completely prevented these effects, while z-LEHD exerted only a modest action, we suggest that caspase-3 can be responsible for the cleavage of both the components of NF-kB. Western blotting analysis shows that treatment with SAHA/TRAIL combination decreased the level of both p65 and phospho-p65 in a time-dependent manner (Fig. 5C).

Another important survival factor that contributes to apoptotic signal resistance is the protein kinase B (PKB)/Akt. Akt is a Ser/Thr protein kinase, normally activated by growth factors in a PI3K-dependent manner, which is implicated in the inhibition of apoptosis and the stimulation of cellular growth[36] and is constitutively active in a variety of human tumours.[37,38] PKB/Akt activity protects against apoptosis through its phosphorylation and inhibition of pro-apoptotic mediators, such as Bad,[39] FOXO family members[40] and procaspase-9.[41] As shown in Fig. 5C, treatment with SAHA/TRAIL combination induced a time-dependent decrease in the level of both Akt and phospho-Akt. Also in this case caspase inhibitors markedly reduced these events.

Finally, also in Hep3B and SK-Hep1 cells SAHA/TRAIL combination decreased both p65 and Akt levels and their phosphorylated forms (Fig. 5D) in a time-dependent manner (not shown).

3.7. The effect of combined treatment on the members of IAP and Bcl-2 families of proteins

We investigated whether the SAHA/TRAIL combination can also determine down-regulation of the proteic factors involved in cell survival. In this connection, members of IAP and Bcl-2 families of proteins were analysed by Western blotting (Fig. 6). Treatment of HepG2 cells for 24 h with SAHA/TRAIL combination markedly decreased the level of a number of proteic factors, such as IAP1, IAP2, XIAP, survivin, Bcl-2, Bcl-X_L (Fig. 6A_2, lane 2 versus lane 1) and p-Bad (Fig. 6B_2, lane 2 versus lane 1), while treatment with SAHA or TRAIL alone did not produce any significant effect on their level (not shown). Because NF-kB has been shown to stimulate the expression of anti-apoptotic genes of IAP and Bcl-2 families,[10,15] we advanced the hypothesis that down-regulation of NF-kB, caused by combined treatment, can be responsible for the decrease in the level of these factors. To test this hypothesis, HepG2 cells were transfected for 5 h with siRNA sequence directed against p65 and then incubated for another 12 h. In these cells the level of p65 diminished to 40–45% of the value found in the control cells transfected with scramble siRNA (not shown). In correlation with this effect the level of many antiapoptotic factors (IAP1, IAP2, XIAP, survivin, Bcl-2 and Bcl-X_L) decreased with respect to the control by about 40–50% when the cells were incubated in the absence of drugs (Fig. 6A_1, lane 5 versus lane 3) and by 70–80% in the presence of SAHA/TRAIL combination (Fig. 6A_1, lane 6 versus lane 4). In addition, treatment for 12 h with drugs also increased in p65 depleted cells the effectiveness of the apoptosis (Fig. 7).

At 24 h of treatment combination SAHA/TRAIL induced remarkable effects either in cells transfected with p65 siRNA (Fig. 6A_2, lane 6) or in their control siRNA cells (Fig. 6A_2, lane 4). Consequently modest differences were found between the two types of cells regarding either the level of antiapoptotic factors or the activity of apoptosis (not shown).

In order to clarify whether the decrease in the level of phosphorylated Bad was a consequence of the down-regulation of Akt induced by SAHA/TRAIL combination, HepG2 cells were transfected for 5 h with the dominant negative form of Akt (DN-Akt) and then incubated for 12 h or 24 h without or with SAHA/TRAIL combination. At 12 h incubation without the addition of drugs the level of phospho-Bad decreased by 50% (Fig. 6B_1, lane 5) with respect to the cells transfected with empty vector control (Fig. 6B_1, lane 3). Treatment of these cells with SAHA/TRAIL combination caused at 12 h of incubation a further remarkable decrease in the level of phospho-Bad

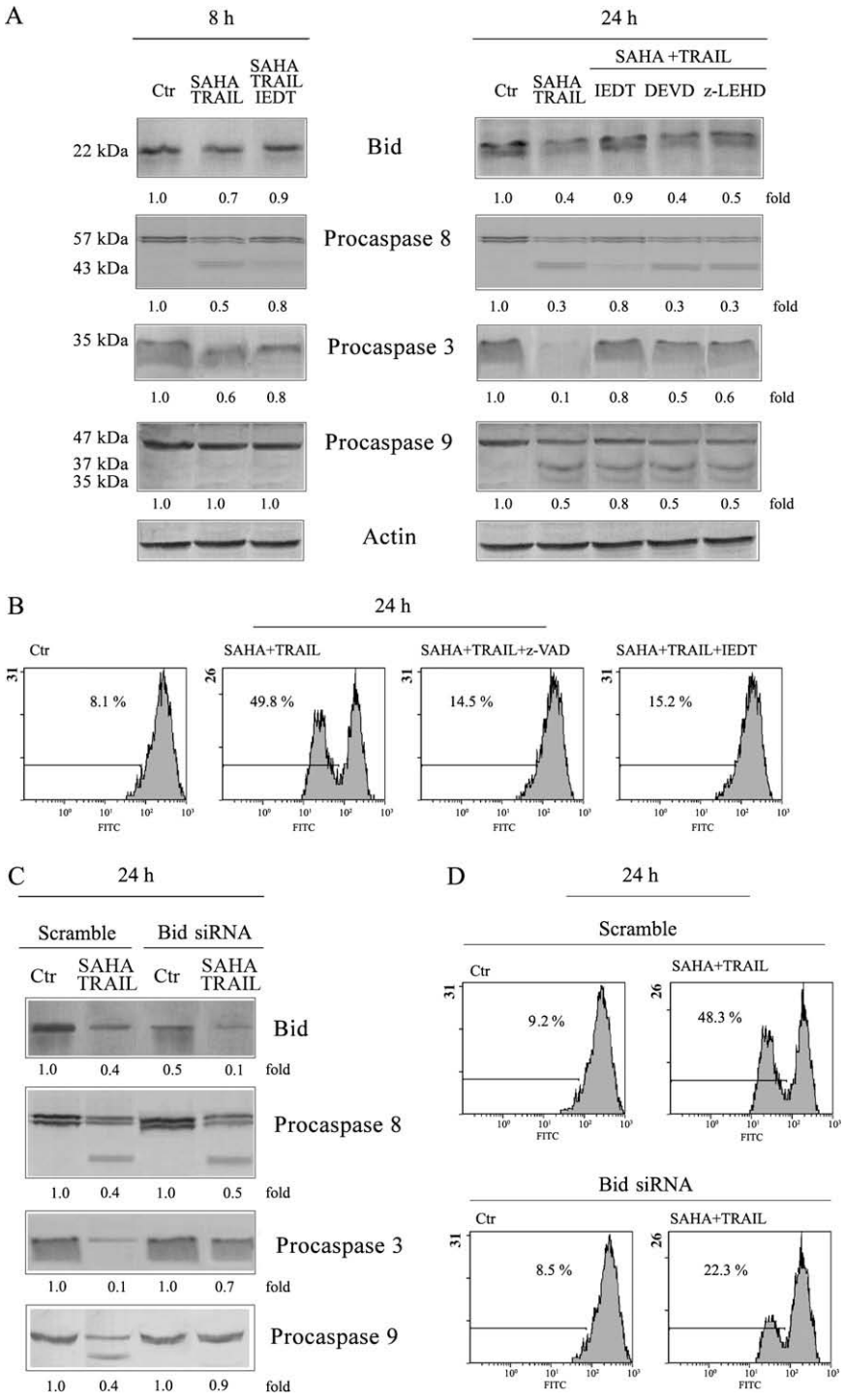

Fig. 4 – The effects of combined treatment on Bid, caspases and mitochondrial membrane potential. HepG2 cells were incubated for 8 h (A) or 24 h (A and B) with 1 μM SAHA in combination with 20 ng/ml TRAIL in the presence or absence of 10 μM IEDT, 100 μM DEVD and 10 μM z-LEHD. At the end, cell extracts were prepared and analysed for Western blotting (A) in order to ascertain the level of Bid, caspase-8, -3 and -9. Dissipation of mitochondrial membrane potential (B and D) was evaluated by flow cytometry using lipophilic dye DioC6 after 24 h of treatment. (C and D) HepG2 cells were transiently transfected with siRNA sequence directed against Bid and employed in comparison with cells transfected with scrambled siRNA to evaluate the effect of combined treatment on the level of Bid, procaspase-8, -3 and -9 (C) and on the mitochondrial membrane potential (D) after 24 h of treatment. Control is represented by cells treated with vehicle only. Results are representative of three independent experiments.

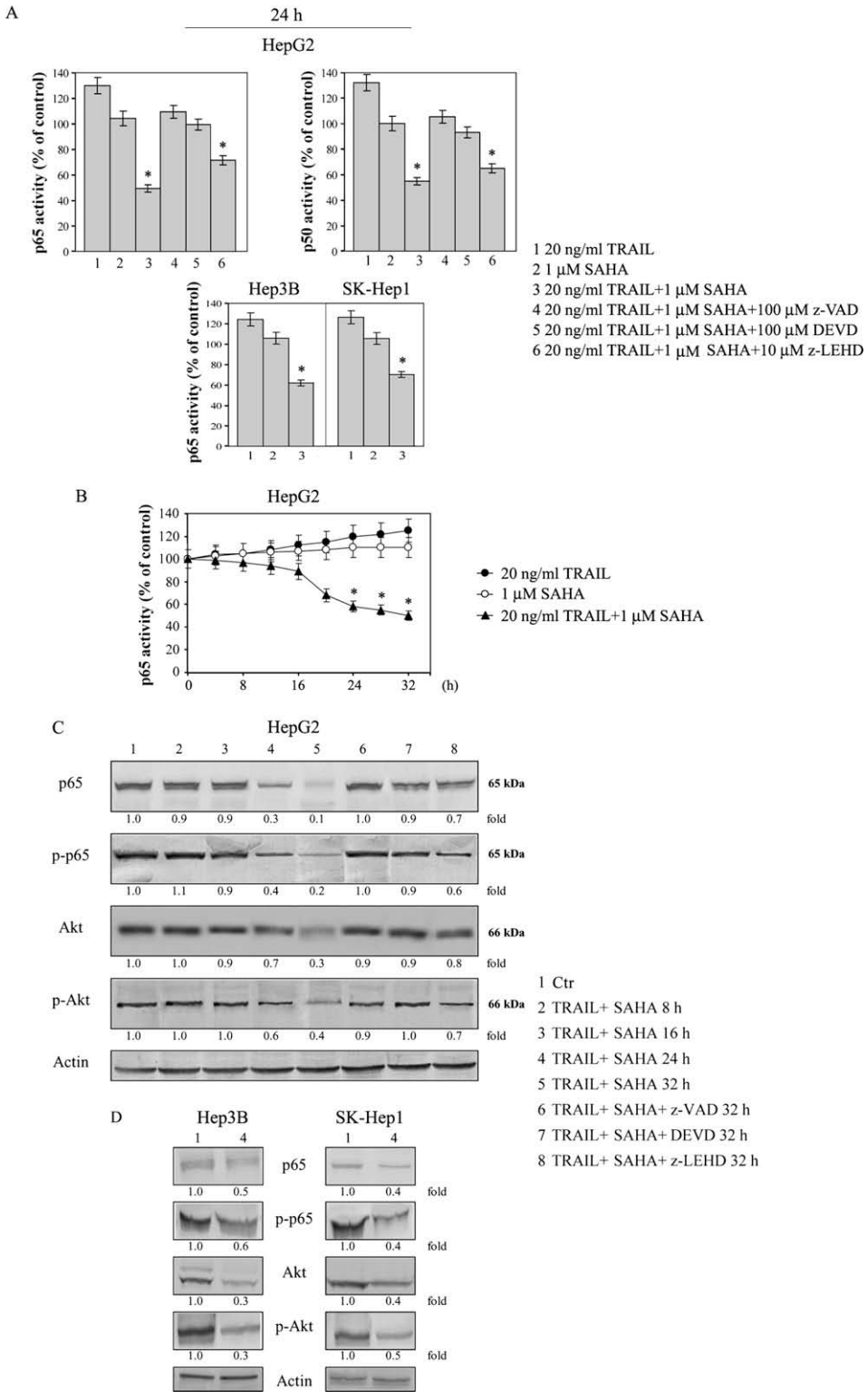

Fig. 5 – The effect of SAHA/TRAIL combination on NF-kB and Akt. HepG2, Hep3B and SK-Hep1 cells were treated with 1 μM SAHA alone or in combination with 20 ng/ml TRAIL in the presence or absence of 100 μM z-VAD, 100 μM DEVD or 10 μM z-LEHD for 24 h (A and D) or various times (B and C). At the end of treatment nuclear extracts were prepared and employed for different analyses. (A) The effect of various treatments on NF-kB activity determined by measuring with an ELISA kit the binding of NF-kB subunit p65 or p50 to consensus binding sequence of NF-kB. (B) The time course of the effect of various treatments on DNA-binding of NF-kB subunit p65. (A and B) Data are the means ± SD of three independent experiments performed in triplicate. *P < 0.05 versus control untreated cells. (C and D) Western blotting analysis showing the reduction of p-65, phosho-p65, Akt and phospho-Akt levels induced by SAHA/TRAIL combination. Control is represented by cells treated with vehicle only. All Western blot experiments were repeated twice with similar results.

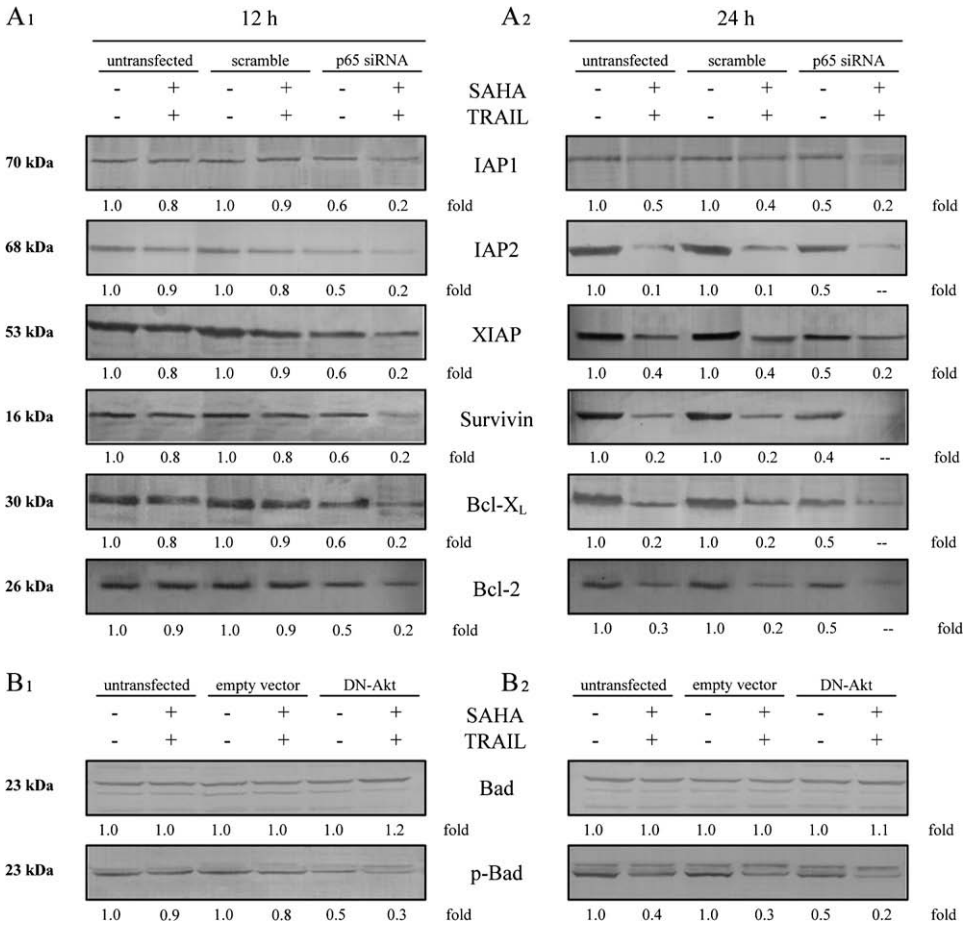

Fig. 6 – The effects of combined treatment on IAP and Bcl-2 family members. (A) HepG2 cells were incubated for 12 or 24 h with 1 μM SAHA alone or in combination with 20 ng/ml TRAIL. At the end, cell extracts were prepared and analysed for Western blotting in order to ascertain the level of IAP1, IAP2, XIAP, survivin, Bcl-X_L and Bcl-2. The results were compared with those obtained using HepG2 cells transiently transfected with p65 siRNA or scramble siRNA. (B) The effect of combined treatment on the level of Bad and phospho-Bad studied in HepG2 cells non-transfected or transiently transfected with DN-Akt or empty vector. All Western blot experiments were repeated twice with similar results.

(Fig. 6B_1, lane 6) while a concomitant modest increase was observed in the level of Bad.

Moreover, our results demonstrated that treatment for 12 h with SAHA/TRAIL combination induced a higher apoptotic effect in HepG2 cells transfected with DN-Akt and a lower effect in cells transfected with CA-Akt, which is a constitutively active form of Akt, thus suggesting that also the decrement in Akt level can play a role in apoptosis induced by SAHA/TRAIL combination (Fig. 7).

4. Discussion

Histone deacetylase inhibitors (HDACIs) induce apoptosis in many tumour cells with little associated toxicity.[28,42] In addition HDACIs have been shown to enhance the apoptosis-inducing potential of TRAIL in melanoma,[43] myeloma,[44] prostate[45] and breast tumour cells.[46]

This paper shows that SAHA, an effective inhibitor of histone deacetylases, sensitised human HCC cells (HepG2, Hep3B and SK-Hep-1) to TRAIL-induced apoptosis, but was ineffective in PHH. Previously, other authors[18,19,47] have demonstrated that various HDACIs sensitise HCC cells to TRAIL. In particular valproate and ITF have been shown to induce the activation of caspase-3 and a decrement in the level of the caspase-8-inhibitory protein c-Flip. However, the molecular mechanism by means of which HDACIs sensitise HCC to TRAIL is not clearly defined at the moment. Therefore, in order to contribute to the understanding of this mechanism, we at first investigated the effects of SAHA on the expression of death receptors in the three lines of HCC cells. We observed that low doses of SAHA strongly increased the expression of DR5 mRNA and protein but not that of DR4 or decoy receptors. Moreover, low doses of SAHA lowered the level of c-Flip. Therefore our results suggest that SAHA sensitised human hepatocarcinoma cells to TRAIL by means of a combination of two effects: up-regulation of DR5 and down-regulation of c-Flip.

In order to explain how SAHA causes these effects it is interesting to note that hyperacetylation of histones induced by HDACIs can de-repress gene expression increasing the susceptibility of tumour cells to apoptosis.[28] In particular HDACIs have been shown to increase the expression of many

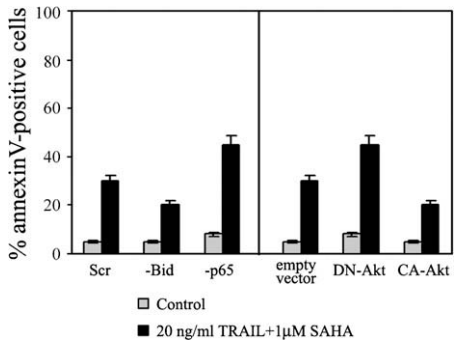

Fig. 7 – Effects induced by changes in Bid, p65 and Akt expression on apoptosis. Apoptotic effect induced by treatment with combination 1 μM SAHA/20 ng/ml TRAIL of HepG2 cells transiently transfected with siRNA for Bid, p65, DN-Akt or CA-Akt. Data are compared with those obtained in cells transfected with scramble siRNA or empty vector. After 12 h of treatment annexin-V positive cells were quantified by flow cytometric analysis after double staining of cells with annexin-V and PI. Control is represented by cells treated with vehicle only. Results are representative of three independent experiments ± SD.

proapoptotic and antiproliferative genes, including DR5 and TNF-α.[48] In addition hyperacetylation of histones can down-regulate the expression of antiapoptotic molecules, including the intracellular inhibitors of apoptosis c-Flip and survivin.[47]

The effects caused by low doses of SAHA on c-Flip and DR5 expression favoured the production and activation of TRAIL-DISC. Analysis of TRAIL-DISC revealed a marked enhancement of the recruitment of both DR5 and procaspase-8 when HepG2 cells were sensitised with SAHA and stimulated with biotinylated TRAIL. Moreover, the activation of caspase-8 seemed to be responsible for the cleavage of c-Flip and RIP-1, since these events were prevented by IEDT, a specific inhibitor of caspase-8. This conclusion is in accordance with the observation of other authors that active caspase-8 induces cleavage of c-Flip with the production of inactive products.[16]

In order to individuate the role of c-Flip in the activation of TRAIL-DISC we have transfected HepG2 cells with c-Flip siRNA and have observed in c-Flip depleted cells that TRAIL alone was sufficient to activate caspase-8. In addition down-regulation of c-Flip prevented the activation of NF-kB by TRAIL. These observations are in accordance with the statements that the increase of caspase-8/c-Flip ratio within the DISC favours caspase-8 activity and counteracts TRAIL-induced activation of NF-kB.[16] Similar considerations on the role exerted by c-Flip have been reported by Okano and colleagues.[49]

In addition our results show that caspase-8 exerted a crucial role in apoptosis induced in HepG2 cells by SAHA/TRAIL combination, since this activity was involved either in the activation of caspase-3 and in the cleavage of Bid to tBid. Both these effects appeared at 8 h, in the first phase of treatment, and were prevented by IEDT.

Bid takes part in the apoptotic mechanism described in this paper. Production of tBid, caused by caspase-8, was responsible for the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) with the consequent activation of caspase-3. This conclusion was suggested by the observation that down-regulation of the expression of Bid by siRNA prevented the loss of $\Delta\Psi_m$ as well as counteracted the reduction of procaspase-3 and reduced the degree of apoptosis induced by combined treatment. Therefore, in the first phase of treatment activation of caspase-3 can be considered only as a close consequence of caspase-8 activity. Instead, in the second phase of treatment, the activation of caspase-3 can also result from the induction of the intrinsic pathway of apoptosis, consequent to dissipation of mitochondrial membrane potential, provoked by the production of tBid.

Our results clearly demonstrate that the activation of caspase-3 provoked cleavage of both NF-kB and Akt. NF-kB has been shown by many authors[10,15] to up-regulate the activity of genes encoding for IAPs, Bcl-2 and Bcl-X_L, while Akt is responsible for phosphorylation of Bad.[39] We suggest that the decrease in the levels of IAPs, Bcl-2 and Bcl-X_L proteins which was observed in the second phase of treatment can be correlated with the decrement of NF-kB while the down-regulation of phospho-Bad can be considered as a consequence of the decrement of Akt. These conclusions are in agreement with our results showing that the down-regulation of p65 induced by siRNA diminished in HepG2 cells the level of IAPs (IAP1, IAP2, XIAP, survivin) and that of Bcl-2 and Bcl-X_L. In addition transfection of HepG2 cells with DN-Akt induced a marked decrease in the level of phospho-Bad.

Finally, another interesting aspect is that the cleavage of NF-kB, leading to the down-regulation of IAPs, can induce caspase-9 activation. Moreover, also cleavage of Akt, a kinase which is involved in the phosphorylation and inactivation of caspase-9,[41] can be responsible for caspase-9 activation. Such an event induced new stimulation of caspase-3 activity and further cleavage of NF-kB and Akt. The involvement of caspase-9 was strongly suggested by the observation that the inhibitor of caspase-9 z-LEHD diminished the activation of caspase-3 and counteracted the reduction of both NF-kB and Akt induced by the treatment.

Our results taken together strongly suggest that NF-kB and Akt exert a fundamental role in the protection of HepG2 cells against apoptosis. Therefore, we conclude that the cleavage of these factors dependent on caspase-3 represented a crucial event in the induction of apoptosis by treatment with SAHA/TRAIL combination.

Hepatocellular carcinoma cell lines are resistant to anticancer agents. In particular they display resistance to TRAIL-mediated cell death.[18,50] However, this paper shows that tumour liver cells can be sensitised to TRAIL by treatment with SAHA, while this compound is unable to sensitise normal hepatocytes. Moreover, our results show that down-regulation of NF-kB and Akt exerted an important role in the induction of TRAIL-mediated apoptosis. Therefore, we suggest that both NF-kB and Akt inhibitors can contribute to sensitise tumour liver cells to TRAIL, improving the efficacy of the therapeutical approach.

Conflict of interest statement

None declared.

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