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The multikinase inhibitor Sorafenib induces apoptosis and sensitises endometrial cancer cells to TRAIL by different mechanisms

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ABSTRACT

Sorafenib induces apoptosis and enhances Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-induced cell killing of tumoural cells. We have investigated the effects of the multikinase inhibitor Sorafenib alone or in combination with TRAIL and agonistic Fas antibodies on endometrial carcinoma cells. We have also focused on the search of the differential molecular mechanisms by which Sorafenib induces cell death and the ones involved in sensitisation to TRAIL. In the present study, we show that Sorafenib induces apoptosis of both endometrial cancer cell lines and human primary cultures and sensitises these cells to TRAIL and agonistic Fas antibodies (aFas)-induced apoptosis. However, Raf/MEK/ERK inhibition by Sorafenib was not responsible for Sorafenib cell death or TRAIL sensitisation of endometrial cancer cells. Sorafenib treatment correlated with a downregulation of both FLICE-Inhibitory Protein (FLIP) and myeloid cell leukaemia-1 (Mcl-1), caused by a proteasomal degradation of both proteins. We evaluated the contribution of FLIP and Mcl-1 downregulation in apoptosis triggered by Sorafenib alone or Sorafenib plus TRAIL. Interestingly, cell death caused by Sorafenib was mediated by downregulation of Mcl-1, but not by FLIP. In contrast, we found that Sorafenib sensitisation of endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis was dependent on FLIP but not on Mcl-1 downregulation. Altogether, we discern the dual mechanisms by which Sorafenib causes cell death from those involved in death receptor sensitisation.

1. Introduction

The Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and Fas Ligand (FasL) belong to the pro-apoptotic cytokines of the Tumour Necrosis Factor (TNF) superfamily. TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells[1,2] indicating that it may become a promising anticancer agent.[3,4] TRAIL-based therapies are under current clinical trials in Phases I and II.[5] However, an increasing number of tumoural cell types display resistance

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to TRAIL-mediated cell killing. To circumvent such resistance, combinatorial therapies to inhibit molecular determinants involved in inhibition of apoptosis triggered by TRAIL would be very important. TRAIL triggers apoptosis by binding one of its functional receptors DR4 or DR5.[6] Engagement of DR4 or DR5 receptors leads to the formation of a Death-Inducing Signalling Complex (DISC). The Death Domain (DD) of these receptors recruits Fas Associated DD-containing protein (FADD) which in turn binds pro-caspase-8. After recruitment to the DISC, pro-caspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signalling.[7–9] One of the key regulators of apoptosis triggered by either FasL or TRAIL is the FLICE-Inhibitory Protein (FLIP).[10,11] High levels of FLIP are found in many tumoural tissues including endometrial carcinoma. We have previously shown that FLIP plays a critical role in the regulation of sensitivity of endometrial carcinoma cells (ECC) to TRAIL-induced apoptosis. In this previous work, we demonstrated that siRNA mediated inhibition of FLIP sensitised endometrial cancer cells to TRAIL-induced apoptosis.[12] FLIP shares a high degree of homology with caspase-8, and contains two Death Effector Domains (DEDs) and a defective caspase-like domain that lacks proteolytic activity. Thus, high levels of FLIP compete with caspase-8 and displace its binding to FADD, which results in inhibition of apoptosis.

Sorafenib (also known as Bay 43-9006, Nexavar) was initially identified as a Raf-1 inhibitor, but subsequent studies revealed that Sorafenib is a multikinase inhibitor with activity over several kinases, including B-Raf on its wild type and V600 mutated forms; tyrosine kinase receptors such as platelet–derived growth factor, vascular-endothelial growth factors 1 and 2, c-Kit, FLT3 or Ret.[13,14] Sorafenib is currently administered as a chemotherapeutic agent to patients with advanced renal cell carcinoma and there are ongoing clinical trials for melanoma, hepatocellular carcinoma and non-small cell lung cancer.[13,14]

Recent findings show that Sorafenib may enhance TRAIL-induced cell killing on cancer cells.[15–17] The proposed molecular mechanisms by which Sorafenib sensitises cancer cells to TRAIL include downregulation of the myeloid cell leukaemia-1 (Mcl-1),[15] downregulation of Mcl-1 together with FLIP protein levels[17] or a transcriptional reduction of c-IAP2 and Mcl-1.[16] Moreover, the role of Raf kinase activity and its downstream kinases, MAPK/ERK kinase (MEK) and Mitogen-Activated Protein Kinase/Extracellular-Regulated Kinase (MAPK/ERK), as a mechanistic effector of Sorafenib anti-tumour effects is uncertain.

Here, we demonstrated that Sorafenib-induced apoptosis in endometrial carcinoma cell (ECC) lines and sensitised ECC and primary cultures from endometrial carcinoma patients to TRAIL-induced apoptosis. Long-term exposure to Sorafenib alone triggered apoptosis of ECC. However, short-exposure periods to Sorafenib had no killing effects, but dramatically enhanced TRAIL- and agonistic Fas (aFas) antibody-induced apoptosis. Then, we focused on the search of differential molecular mechanisms by which Sorafenib induces cell death and also the ones involved in sensitisation to TRAIL. Sorafenib sensitisation to TRAIL was independent of B-Raf kinase activity or MEK/ERK inhibition. Sorafenib sensitisation correlated with downregulation of FLIP protein levels. Sorafenib mediated FLIP reduction was not caused by transcriptional repression of FLIP but by proteasome degradation, since co-treatment with proteasome inhibitors completely prevented reduction of FLIP levels. Accordingly, FLIP overexpression was sufficient to inhibit Sorafenib sensitisation to TRAIL. In contrast, overexpression of Mcl-1, which effectively prevents apoptosis induced by Sorafenib, did not prevent cells from TRAIL plus Sorafenib-induced apoptosis. Because of the given importance of Sorafenib and TRAIL in cancer therapy, we exposed primary cultures obtained from biopsies of patients with endometrial carcinoma to TRAIL plus Sorafenib. Accordingly with the results obtained in cell lines, Sorafenib sensitised such cancer cells to apoptosis and reduced both Mcl-1 and FLIP levels.

2. Materials and methods

2.1. Reagents, plasmids and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to Tubulin and anti-Flag M2 were from Sigma (St Louis, MO). Kinase inhibitors PD98059, DRB and apigenin, proteasome inhibitor MG-132, monoclonal antibody to caspase-8 and human recombinant TRAIL were from Calbiochem (La Jolla, CA). Antibody to caspase-9 and cleaved caspase-3 were obtained from Cell Signalling (Beverly, MA). Monoclonal antibody to FLIP (NF6) and aFas antibody were purchased from Alexis Corp (Lausen, Switzerland). Antibody to Mcl-1 was purchased from BDbiosciences (San Jose, CA). Antibody to PARP was from Neomarkers. Anti-B-Raf antibody was from SantaCruz Biotechnology, Inc. (SantaCruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden). BAY 43-9006 (Sorafenib) was provided by Bayer Pharmaceuticals (New Haven, CT). Bid inhibitor (BI-6C9) was from Sigma.

Lentiviral vector containing Flag-tagged mouse FLIP cDNA was a gift from Dr. Joan Comella (Dept de Bioquímica i Biologia Molecular, Universitat Autonoma de Barcelona, Barcelona). The pCDNA3 vector encoding Mcl-1 cDNA was a generous gift from Dr. Isabel Marzo.

2.2. Cell lines, culture conditions and transfection

The Ishikawa 3-H-12 cell line (IK) was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr. Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95/2 and HEC-1A cells were a gift from Dr. Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbeco's modified Eagles Medium (DMEM) (Sigma) supplemented with 10% Foetal Bovine Serum (Invitrogen, Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicilin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% CO_2.

When indicated, transfection plasmid constructs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.

2.3. Sample collection and explant culture of endometrial adenocarcinoma

Endometrial carcinoma samples were collected in the operating room of the Department of Gynaecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (JP). A specific informed consent was obtained from each patient, and the study was approved by the local Ethics Committee. Tissue was collected in DMEM, chopped into 1 mm pieces and incubated with collagenase in DMEM for 1.5 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 10 ml pipette and a 1 ml blue tip and resuspended in 2 ml of fresh DMEM medium. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM medium and tissue was allowed to sediment, via gravity, for 5 min. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% Foetal Bovine Serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% of penicilin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37 °C with saturating humidity and 5% CO_2.

2.4. Lentiviral production and infection

Oligonucleotides to produce Mcl-1 plasmid based short hairpin (sh)RNA were cloned into the FSV vector using AgeI-BamHI restriction sites. shRNA target sequences were GCGTGCAGCGCAACCACGAGA for Mcl-1 1.1 shRNA and CTGGGGCAGGATTGTGACTCT for Mcl-1 2.3 shRNA. To produce infective FLIP overexpressing lentiviral particles, 293T cells were co-transfected by the calcium phosphate method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA producing vector (FSV) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles for 3–4 days in the same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 min at 1000 rpm and filtered through a 0.45 μM filter (Millipore). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24–48 h in the presence of medium containing lentiviral particles. After this period, the medium was replaced with fresh medium and cells were incubated for two additional days to allow endogenous protein knock-down or protein overexpression.

2.5. Cell viability assays and assessment of apoptosis

Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated on M96 well plates at 15×10^3 cells per well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent and lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmon, CA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under an epifluorescence microscope (Leica Microsystems).

The cytotoxicity assay kit was purchased from Roche Diagnostics (Indianapolis, IN, USA). Cells were first plated as described previously in the Materials and Methods cell culture section. After treatment, 100 μl of supernatant were transferred in a M96 multiwell plate. To determine LDH activity, 100 μl of freshly prepared reaction mixture were added to each well and incubated for up to 30 min at 15–25 grades as described by the manufacturer's instructions. Absorbance was measured at 490 nm with a reference wavelength of 600 nm. The percentage of cytotoxicity for each well was referenced to positive control cells permeabilised with triton 1%.

2.6. Western blot analysis

Endometrial adenocarcinoma cell lines were washed with cold PBS and lysed with lysis buffer (2% SDS, 125 mM Tris–HCL pH 6.8). Protein concentrations were determined with the Protein Assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST (20 mM Tris–Hcl pH7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4 °C. The signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

2.7. RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from Ishikawa (IK) cells treated as indicated using Trizol reagent (Invitrogen). One microgram of total RNA was converted into cDNA using Taqman Reverse Transcription Reagents (Applied biosystems P/N N808-0234) according to the manufacturer's recommendations. Two microlitres of the reverse transcription reaction were used as a template for the real-time detection of human FLIP expression using TaqMan Technology on an Applied Biosystems 7000 sequence detection system. Gene expression quantitation was performed in separate tubes (singleplex) for both target gene and endogenous control gene using the primer and probe sequences for human FLIP and GUSB obtained commercially from Applied Biosystems Assay-on-Demand™ Gene (ABI P/N 4331182:Hs00236002_m1, and ABI P/N 4326320E). The reaction was performed with 10 μl Taqman® Universal PCR Master Mix No AmpErase® UNG 2X (P/N 4324018), 1 μl 20X Assay-on-Demand™ Gene and 2 μl of complementary DNA (cDNA) diluted in RNase-free water adjusted to 20 μl volume reaction. The thermal cycler conditions were UNG activation 2 min at 50 °C, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (repeat 40 times) on ABI7000. Triplicate CT values were analysed with Quantitative Relative software using the comparative CT (ΔΔCT) method as described by the manufacturer. The amount of target($2^{-\Delta\Delta CT}$) was obtained by normalising to an endogenous reference gene (GUSB). Results are presented as a relative mRNA amount compared to the untreated samples.

3. Results

3.1. *Sorafenib induces apoptotic cell death of ECCs*

To begin with, we explored the sensitivity of endometrial carcinoma cell lines to Sorafenib-induced cell killing. For this purpose, we exposed IK, HEC-1A, RL-95/2 and KLE endometrial carcinoma cell lines to increasing doses of Sorafenib and we evaluated cytotoxicity by LDH release after 24 or 48 h. Sorafenib induced a dose-dependent release of LDH of all four cell lines. It is worth mentioning that IK, RL-95/2 and HEC-1A displayed maximum cytotoxicity at 24 h of Sorafenib exposure whereas KLE did not show a significant increase in cytotoxicity until 48 h of treatment (Fig. 1A). Because we observed similar effects on cytotoxicity over all cell lines, we chose IK cells to further analyse caspase activation and PARP processing. A time-course treatment of IK cells induced detectable caspase-3, caspase-9 and PARP processing after 12 and 24 h of exposure to 20 μM Sorafenib (Fig. 1B). The above results indicate that Sorafenib induces apoptotic cell death of endometrial cell lines.

3.2. *Sorafenib sensitises endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis*

Next, we investigated whether Sorafenib may sensitise resistant cells to TRAIL- and Fas-induced apoptosis. As demonstrated above, Sorafenib alone triggered apoptosis at 24 or 48 h of treatment. However, 8 h of treatment with Sorafenib alone caused a slight increase of cytotoxicity (Fig. 1C). To analyse whether Sorafenib may sensitise ECCs to death receptor-induced apoptosis, we exposed IK cells to 20 μM Sorafenib in the presence or absence of aFas or TRAIL. After 8 h of treatment, we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining and we assessed caspase processing and activation by Western blot to initiator caspases-8, -9, and the effector caspase-3. After 8 h, Sorafenib alone caused only a slight increase in apoptotic IK cells, but co-treatment with either aFas or TRAIL plus Sorafenib induced a marked increase in the number of nuclei displaying apoptotic morphology as assessed by Hoechst (Fig. 2A) and the processing of caspases-8, -9 and -3 (Fig. 2B). Similar results were obtained with KLE cells (Fig. 2A and B).

These results demonstrate that Sorafenib not only induces apoptosis but also sensitises endometrial cancer cells to TRAIL and aFas apoptosis.

3.3. *Sorafenib sensitisation to TRAIL is independent of B-Raf and MEK/ERK kinase activity*

One of the substrates of Sorafenib inhibitory activity is B-Raf, which regulates the activation of the MAPK/ERK pathway. Therefore, we examined whether Sorafenib sensitisation to TRAIL was caused by inhibition of the ERK/MAPKs. Treatment of both IK and KLE cells resulted in a significant decrease of ERK phosphorylation, suggesting that Sorafenib inhibited ERK/MAPK kinase signalling (Fig. 3A). Next, we analysed whether apoptosis sensitisation by Sorafenib was the result of inhibition of B-Raf kinase activity or the downstream MEK/ERK kinases. To assess this point, we first infected IK cells with a plasmid encoding a wild type B-Raf or a kinase-dead B-Raf K483M mutant (B-Raf KD). After 48 h, cells were exposed to TRAIL or aFas and we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining, and we assessed caspase processing and activation by Western blot to initiator caspases-8, -9, and the effector caspase-3. B-Raf K483 M neither caused an increase in the number of apoptotic nuclei nor the activation of any of the caspases analysed (Fig. 3B). As a control for sensitisation to TRAIL, we infected parallel cultures with lentiviruses carrying shRNA against FLIP. Similarly, treatment of cultures with the specific MEK inhibitor UO126 failed to sensitise IK cells to TRAIL- or aFas-induced apoptosis as assessed by Hoechst staining or caspase activation (Fig. 3C). As a control, we treated parallel cultures with DRB which we have previously demonstrated to sensitise ECCs to TRAIL and aFas.[18]

The above results suggest that the mechanism of sensitisation to TRAIL or aFas is independent of inhibition of B-Raf kinase activity or inactivation of the MEK/ERK signalling cascade.

3.4. *Sorafenib triggers proteasome-mediated degradation of FLIP and Mcl-1*

Having demonstrated that the effects of Sorafenib on ECC seem to be independent of MEK/ERK signalling, we focused our investigations on the search of mechanisms by which Sorafenib kills ECC and sensitises to death receptor apoptosis. Recent evidences point to Mcl-1 as an important molecule involved in regulation of both apoptosis induced by Sorafenib and apoptosis triggered by the combination of Sorafenib plus TRAIL. Moreover, we have previously demonstrated that FLIP is critical in the regulation of TRAIL-induced apoptosis of ECC.[12,18] These evidences enabled us to check whether Soranefib may regulate FLIP and Mcl-1. For this purpose, we performed a time-course analysis of expression of both FLIP and Mcl-1 of IK cells treated with Sorafenib. Both Mcl-1 and FLIP expression was markedly reduced within the first 24 h of treatment with Sorafenib (Fig. 4A and B). In contrast, the levels of Bcl-XL did not change at any time-point analysed. Of note, the decrease of FLIP expression was a rapid event and it became evident after 3 h of Sorafenib treatment. Such downregulation coincided with the rapid sensitisation of IK cells to TRAIL and aFas. Similar results were obtained when KLE cells were treated for 24 or 48 h with Sorafenib (data not shown).

Next, we investigated the mechanisms by which Sorafenib regulates FLIP and Mcl-1 levels. The levels of endogenous FLIP protein can be controlled transcriptionally but, recent evidences also suggest that endogenous FLIP protein levels may be regulated by the ubiquitin-proteasome system. To ascertain whether FLIP levels are transcriptionally regulated, we performed real-time PCR on mRNA extracted from IK cells treated with Sorafenib for 2 or 9 h. As a control, parallel cultures were treated for 9 h with DRB or apigenin which, as we have recently demonstrated, reduce FLIP mRNA levels.[18] Sorafenib treatment did not reduce the levels of FLIP mRNA, suggesting that Sorafenib regulates FLIP protein at the post-transcriptional level (Fig. 4C).

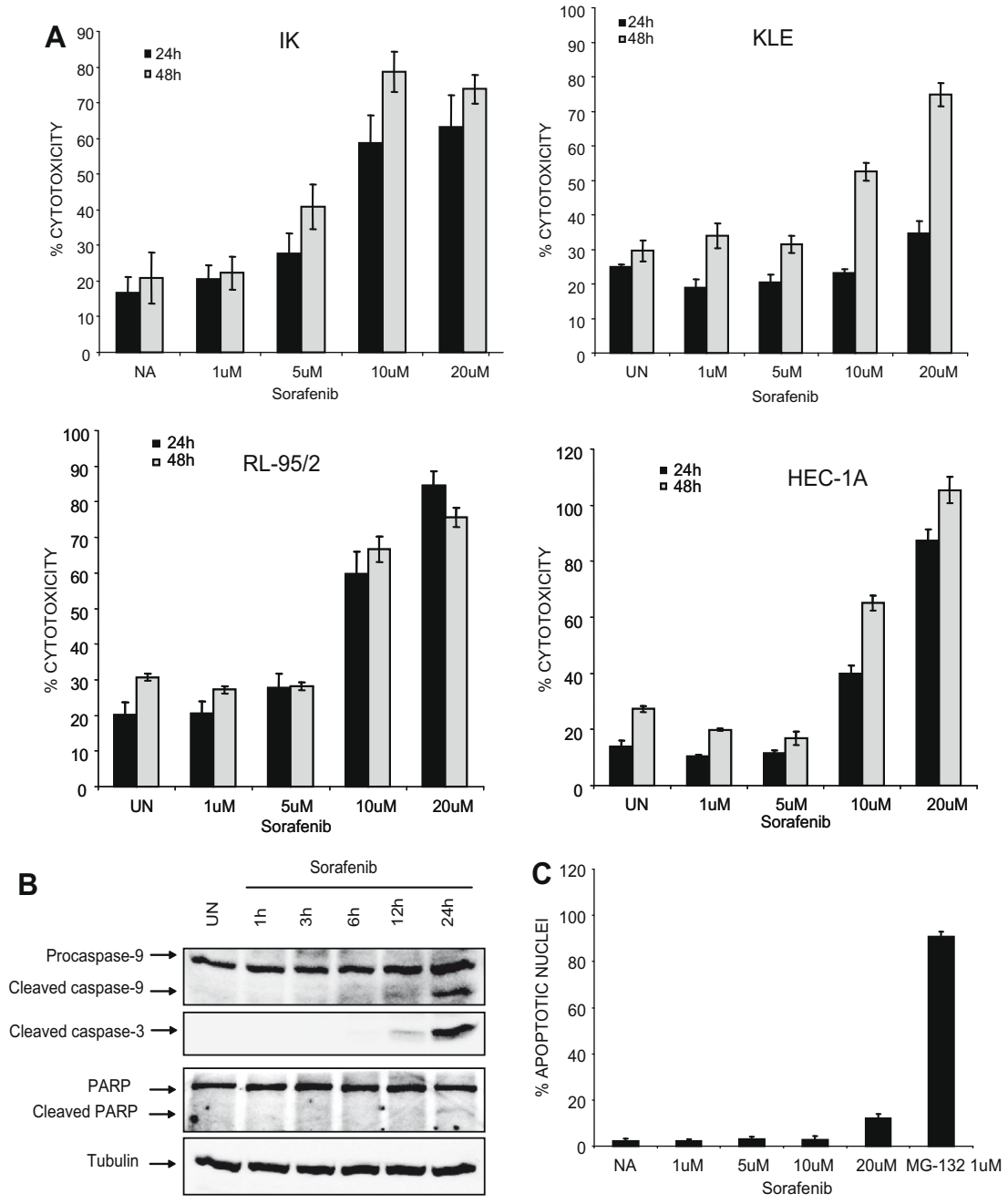

Fig. 1 – Sorafenib induces apoptosis in endometrial cancer cells. (A) IK, KLE, HEC-1A and RL-95/2 cells were exposed to increasing concentrations of Sorafenib for 24 or 48 h. Supernatant lactate dehydrogenase (LDH) was quantified to measure cell death. (B) IK cells were treated for the indicated times with Sorafenib. Western blots to active caspase-9, -3 and PARP cleavage were performed to characterise an apoptotic response. Immunodetection of tubulin was performed to ensure equal protein amounts. (C) IK cells were treated with the indicated doses of Sorafenib for 8 h and the number of apoptotic nuclei were visualised by Hoechst staining and quantified. As a control, parallel cultures were treated with MG-132 which effectively induces apoptosis after 8 h of treatment.

Both Mcl-1[19–22] and FLIP[23–25] protein levels are also regulated by ubiquitin-proteasome-mediated degradation. To determine whether proteasomal degradation was also involved in downregulation of Mcl-1 and FLIP after Sorafenib treatment, we treated IK cells with Sorafenib in the presence or absence of the proteasome inhibitor MG-132. As shown in Fig. 3, addition of MG-132 completely inhibited the reduction in FLIP and Mcl-1 protein (Fig. 4D) caused by Sorafenib. These results suggest that Sorafenib triggers Mcl-1 and FLIP degradation through the proteasome.

3.5. *Expression of Mcl-1 but not FLIP prevents Sorafenib-induced apoptosis*

Next, we evaluated the contribution of FLIP and Mcl-1 downregulation in apoptosis induced by Sorafenib alone. For this

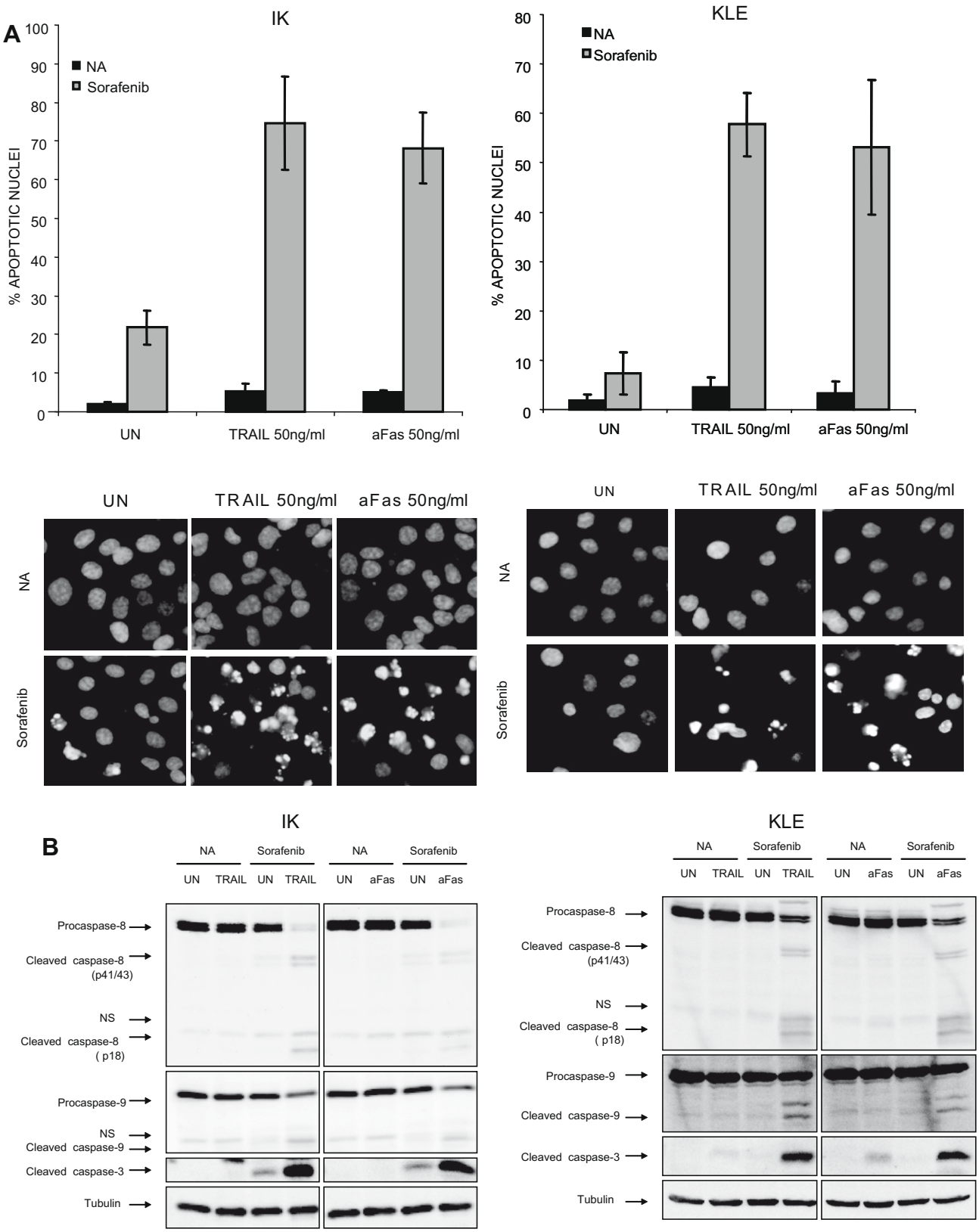

Fig. 2 – Sorafenib sensitises ECC to apoptosis triggered by TRAIL and aFas. IK and KLE cells were treated with Sorafenib or with no additives (NA) in the presence or absence of TRAIL (UN). (A) Quantification (top) and representative images (bottom) of cells IK and KLE displaying nuclei with apoptotic morphology after Hoechst staining. (B) Western blot of IK and KLE cells showing activation of caspases-8, -9 and -3 after TRAIL plus Sorafenib treatment. Immunodetection of tubulin was performed to ensure equal protein amounts.

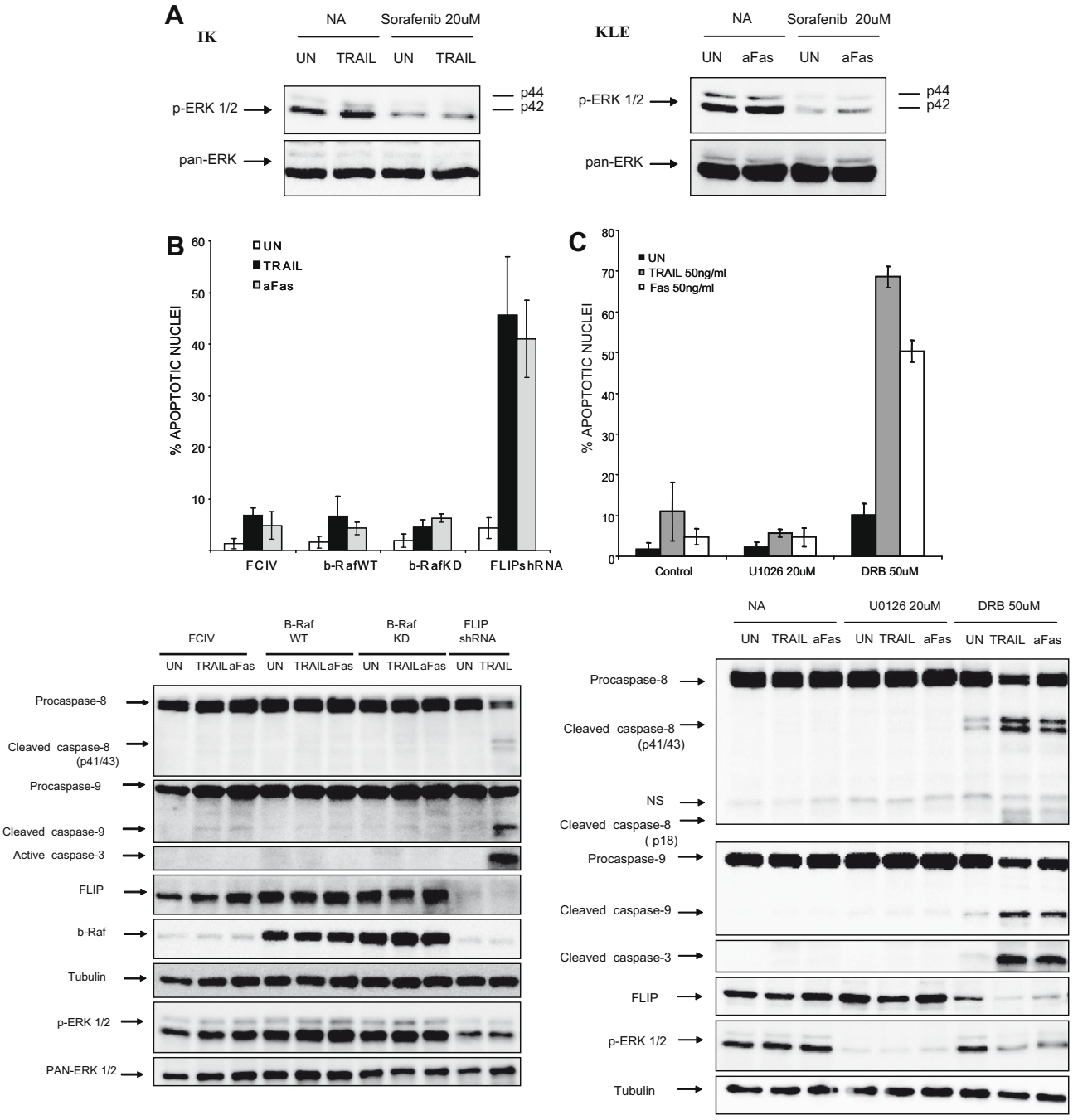

Fig. 3 – Sensisitation of ECCs to TRAIL and aFas by Sorafenib is independent of ERK/MAPKs. (A) Western blot showing that Sorafenib alone or in combination with TRAIL or aFas for 8 h inhibits ERK phosphorylation in IK (left) and KLE (right) cells. (B) IK cells were infected with lentiviruses carrying either an empty vector (FCIV), a wild type form of B-Raf (B-Raf WT) or an inactive form – kinase death of B-Raf (B-Raf KD). As a control, parallel cultures were infected with FLIP shRNA lentiviruses. After 48 h to allow expression of constructs, TRAIL or aFas were added to culture media for 12 h. Cells were stained with Hoechst and the number of nuclei displaying apoptotic morphology was quantified (top). Parallel IK cell cultures were lysed and caspase activation, ERK phosphorylation, B-Raf and FLIP levels were analysed by Western blot (bottom). (C) IK cells were pre-treated for 30 min with MEK1 inhibitor U0126 20 μM or DRB (used as positive control). TRAIL or aFas were added to culture media for 12 h. Cells were stained with Hoechst and the number of nuclei displaying apoptotic morphology was quantified (top). Parallel IK cell cultures were lysed and caspase activation, ERK phosphorylation, and FLIP levels were analysed by Western blot (bottom).

purpose, we either infected IK cells with lentiviruses carrying a plasmid encoding Flag-tagged FLIP or transfected IK cells with pcDNA3 plasmid expressing Mcl-1. After 3 days, to allow FLIP or Mcl-1 expression, cells were treated with Sorafenib for 48 h. Subsequently, Western blot assays were performed to determine caspase activation and nuclei

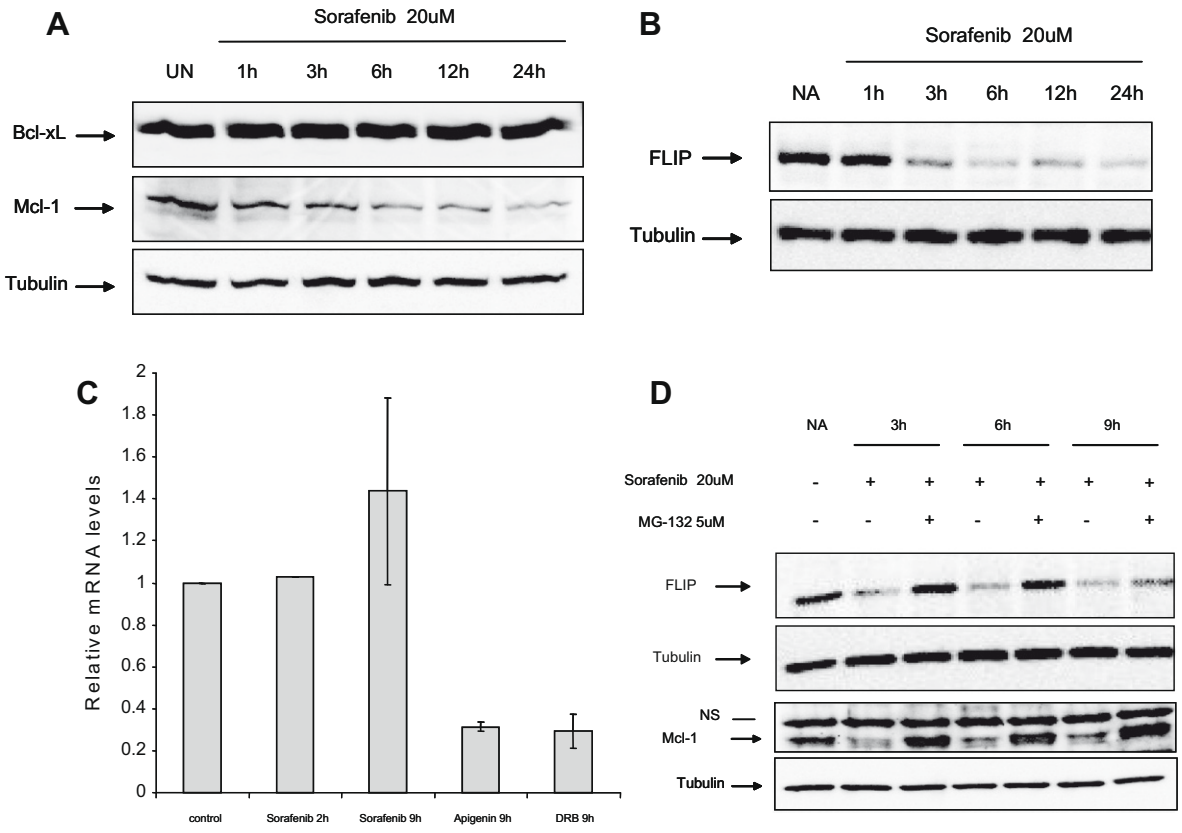

Fig. 4 – Soranenib causes a proteasome-mediated downregulation of Mcl-1 and FLIP. (A) Western blot showing Mcl-1 and Bcl-XL expression in IK cells exposed to Sorafenib 20 μM for the indicated times. (B) IK cells were exposed to Sorafenib 20 μM for the indicated times. Cells were lysed and protein extracts were analysed for the expression of FLIP protein. (C) Real-time PCR was performed to examine FLIP mRNA levels of IK cells treated with Sorafenib 20 μM for 2 and 9 h, respectively. (D) Proteasome inhibition restores FLIP and Mcl-1 protein levels in IK cells treated with Sorafenib. IK cells were pre-treated with MG-132 5 μM for 30 min and thereafter cells were treated with Sorafenib 20 μM for the indicated periods of time. FLIP and Mcl-1 protein levels were detected by Western blot.

displaying apoptotic morphology were quantified. FLIP ectopic expression did not inhibit Sorafenib-induced apoptosis determined by caspase processing and activation through Western blot analysis (Fig. 5A). In contrast to FLIP, Mcl-1 overexpression significantly impaired processing and activation of caspases and the cleavage of caspase substrate PARP (Fig. 4B). However, ectopic expression of Mcl-1 did not restore FLIP levels (Fig. 5B). Furthermore, to study the involvement of endogenous Mcl-1 levels in Sorafenib-induced apoptosis, we took advantage of the fact that KLE cells display a delayed apoptotic response after Sorafenib treatment. Hence, we decided to infect KLE cells with lentiviruses carrying shRNA to block endogenous Mcl-1 expression. Two shRNAs, 1.1 and 2.3, were designed and tested for its effectiveness. Subsequent Western blot analysis determined shRNA 1.1 to be the most effective one (Fig. 5C). Results indicate that knockdown of Mcl-1 sensitises KLE cells to Sorafenib-induced apoptosis as assessed by immunodetection of processed caspases as well as nuclei displaying apoptotic morphology (Fig. 5C and D). These results suggest that Mcl-1, but not FLIP, downregulation is involved in apoptosis triggered by Sorafenib.

3.6. *Expression of FLIP but not Mcl-1 restores TRAIL and aFas resistance*

Both FLIP and Mcl-1 have been involved in the regulation of TRAIL sensitivity of cancer cells. We examined the contribution of each of these proteins in Sorafenib-induced sensitisation to TRAIL. To ascertain whether downregulation of endogenous FLIP triggered by Sorafenib was responsible for TRAIL-induced apoptosis, we infected IK cells with lentiviruses carrying a plasmid encoding FLIP cDNA. After 3 days, to allow FLIP expression, cells were treated with TRAIL in the presence or absence of Sorafenib. Apoptotic nuclei were then visualised by Hoechst staining and caspase processing by Western blotting. As shown in Fig. 6A, overexpression of FLIP resulted in a significant reduction of apoptotic nuclei caused by Sorafenib plus either TRAIL or aFas. Consistent with this observation, FLIP overexpression inhibited processing of the caspases-8, -9 and -3 caused by TRAIL or aFas in the presence of Sorafenib (Fig. 6B).

In contrast to FLIP, expression of Mcl-1 did not prevent apoptosis triggered by treatment of ECCs with Sorafenib plus TRAIL as assessed by LDH cytotoxicity assay, Hoechst staining of apoptotic nuclei (Fig. 7A) or caspase activation (Fig. 7B).

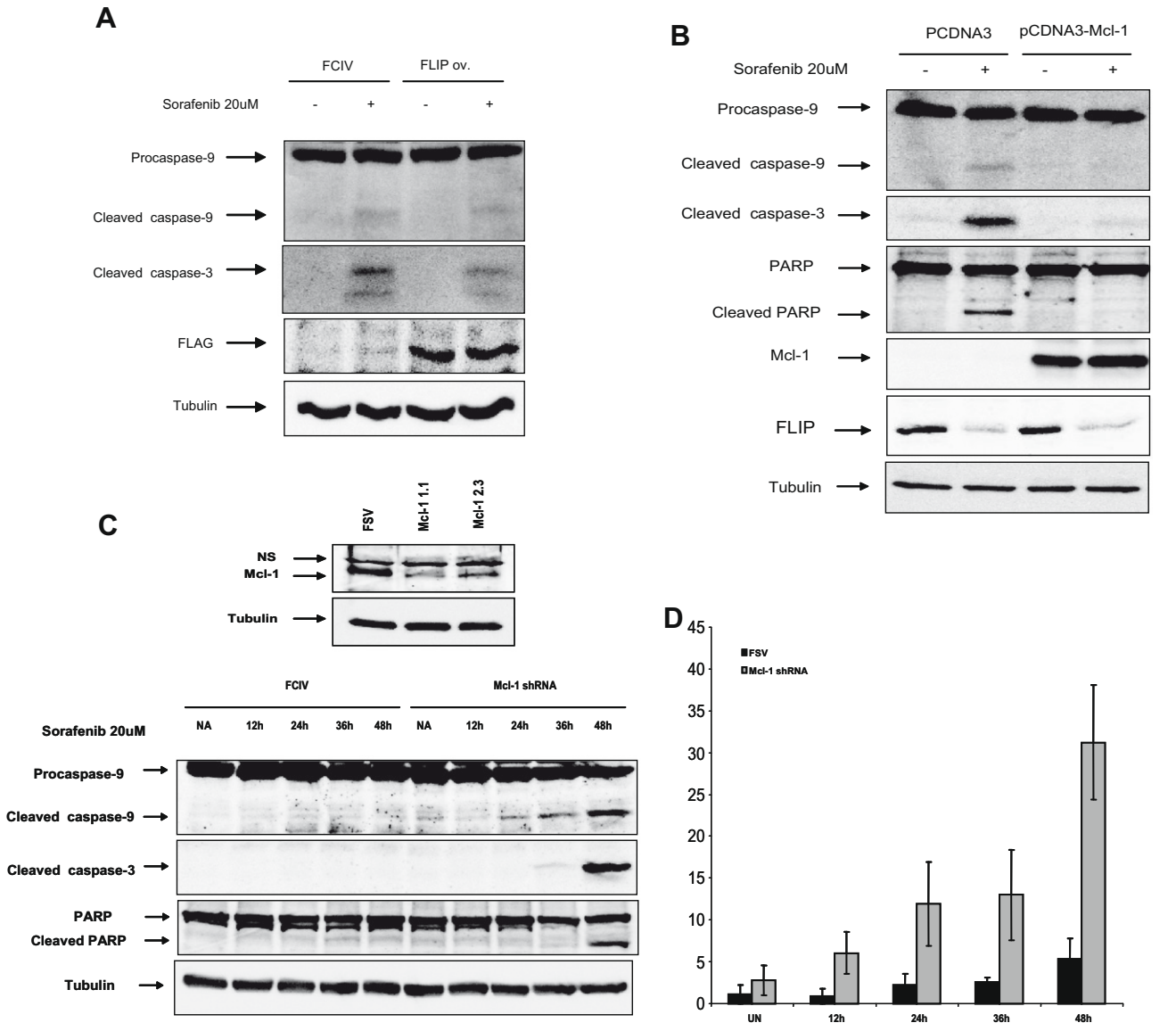

Fig. 5 – Mcl-1 but not FLIP overexpression protects from Sorafenib-induced apoptosis. (A) IK cells were infected with lentiviruses containing either an empty vector (FCIV) or a vector encoding full length mouse Flag-tagged FLIP (FLIP ovex). After 3 days cells were left untreated (UN) or treated for 48 h with Sorafenib 20 μM. Cleavage and activation of indicated caspases were assessed by Western blot. Flag immunostaining was performed to ensure correct FLIP overexpression. (B) Western blot showing that Mcl-1 overexpression inhibits caspase activation and PARP cleavage but not FLIP downregulation after Sorafenib 20 μM exposure for 48 h. (D) (top) two shRNA, 1.1 and 2.3, to block endogenous Mcl-1 were designed. (bottom) Mcl-1 shRNA 1.1 sensitises resistant KLE cells to Sorafenib-induced cell death as measured by cleaved caspases and cleaved PARP immunodetection by Western blot. (E) Control infected (FSV) and Mcl-1 knockdown KLE cells (Mcl-1 shRNA) were treated for the indicated times with Sorafenib 20 μM and apoptotic nuclei were quantified by Hoechst staining. Representative microphotographs are shown.

Interestingly, expression of FLIP restored TRAIL and aFas resistance in the presence of Sorafenib but the levels of Mcl-1 remained low (Fig. 7C).

The evidence that TRAIL plus Sorafenib-induced apoptosis was independent on Mcl-1levels suggested that mitochondrial independence of apoptosis triggered this co-treatment. To further demonstrate that Sorafenib sensitisation to TRAIL does not require mitochondrial amplification, we treated IK cells with TRAIL plus Sorafenib in the presence or absence of the specific Bid inhibitor BI-6C9.[26] Consistently with the results observed with Mcl-1, addition of BI-6C9 did not prevent caspase-3 activation triggered by TRAIL plus Sorafenib co-treatment (Fig. 7D). These results indicate that Sorafenib plus TRAIL-induced apoptosis do not require mitochondrial amplification.

3.7. *Sorafenib sensitises primary endometrial carcinoma explants to TRAIL-induced apoptosis*

TRAIL is a potential anti-cancer agent because of its ability to trigger apoptosis in cancer cells without affecting

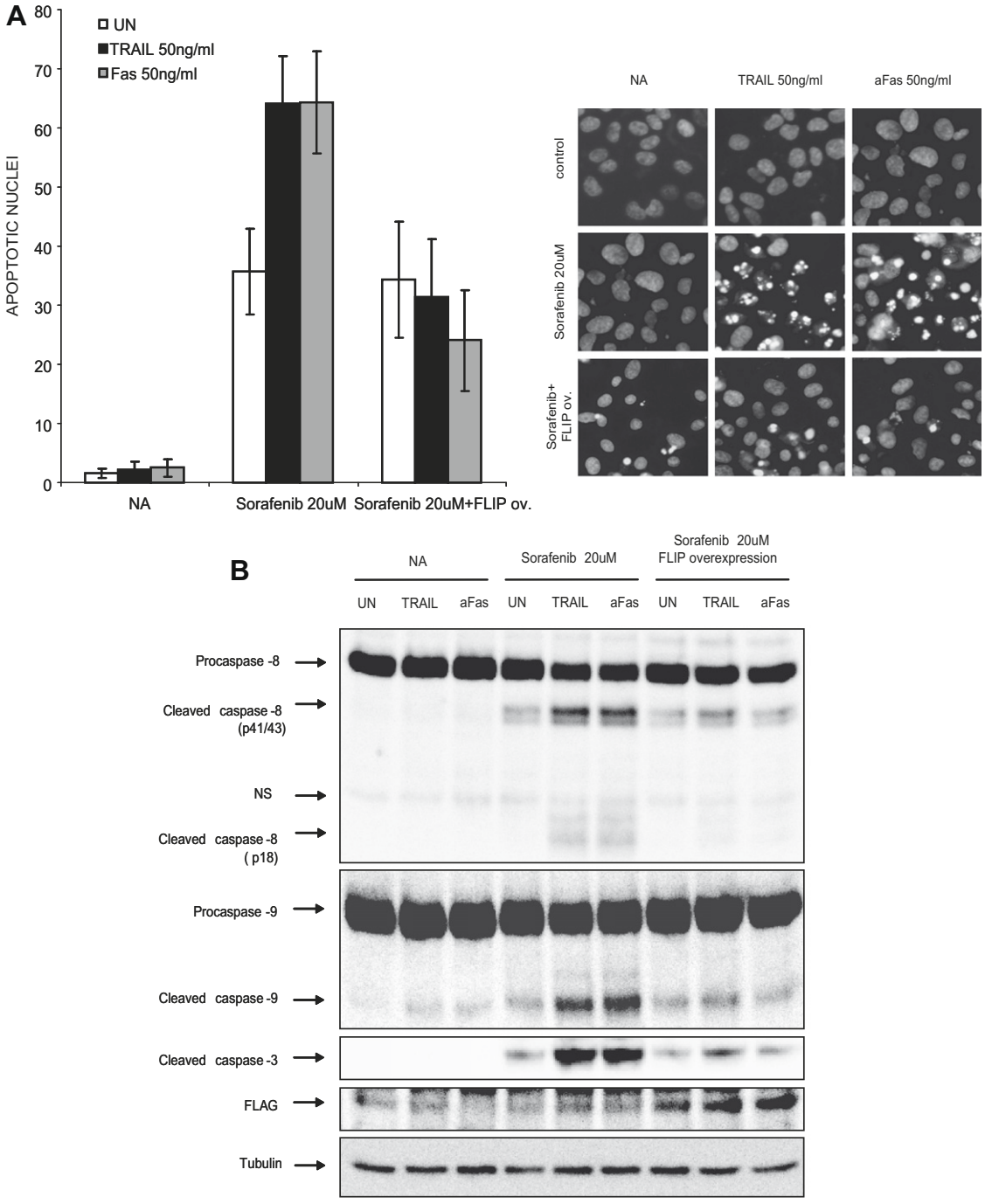

Fig. 6 – FLIP protects from apoptosis triggered by Sorafenib plus TRAIL and aFas. (A) IK cells were infected with lentiviruses containing either an empty vector (FCIV) or a vector encoding full length mouse Flag-tagged FLIP (FLIP ovex). After 3 days, cells were exposed to medium without additives (NA) or medium containing Sorafenib 20 μM alone or in combination with TRAIL 50 ng/ml or aFas 50 ng/ml or left untreated (UN) for 8 h. Top, quantification of nuclei displaying apoptotic cell death visualised by Hoechst staining. Bottom, representative microphotographs of cultures stained with Hoechst. (B) Parallel IK cultures were lysed and resulting protein extracts were analysed by Western blot to the indicated caspases. Immunodetection of FLAG epitope reveals correct FLIP overexpression. Membranes were reprobed with tubulin to ensure equal protein loading.

normal cells. Humanised anti-DR4 and anti-DR5 are currently in advanced clinical trials.[5,27] However, an increasing number of tumoural cells display mechanisms of TRAIL resistance to apoptosis. Such resistance has increased the interest of combinatorial therapies.[4,5] We decided to test whether Sorafenib could be effective in killing primary endometrial carcinoma explants treated with TRAIL. We cultured different endometrial carcinoma explants obtained

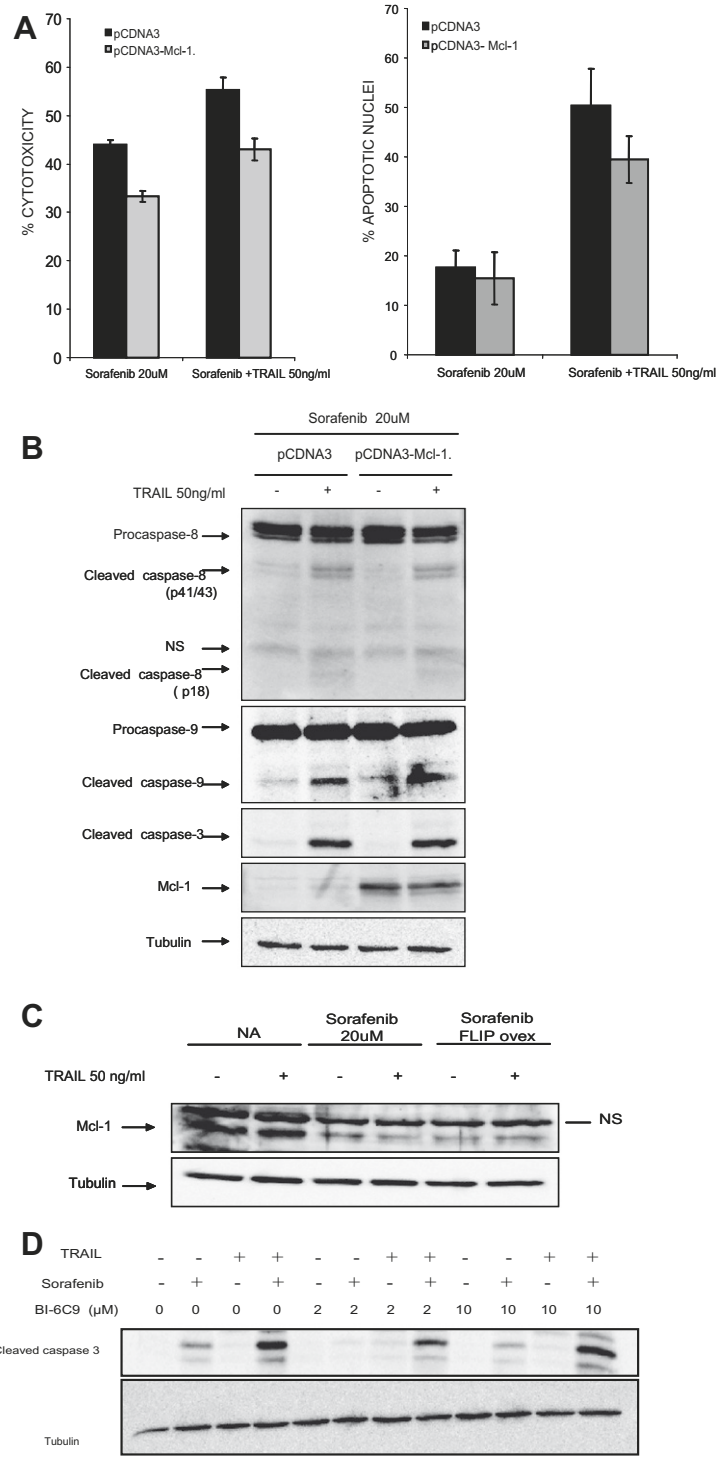

Fig. 7 – Mcl-1 overexpression does not inhibit Sorafenib sensitisation to TRAIL. (A) Overexpression of Mcl-1 does not block cell death triggered by a combination of Sorafenib plus TRAIL. IK cells were transfected with either an empty PCDNA3 vector or a PCDNA3 vector coding for Mcl-1. After 2 days, post-transfection cells were exposed to Sorafenib for 8 h and cytotoxicity was assessed by LDH (left) or by quantification of apoptotic nuclei visualised by Hoechst staining (right). (B) Parallel cultures were lysed and caspase activation was analysed by western blot. Membranes were reprobed with Mcl-1 antibody to ensure overexpression and with tubulin to monitor equal protein loading. (C) FLIP overexpressing cells display equal Mcl-1 protein decrease compared to control infected IK cells exposed to Sorafenib 20 μM. (D) Western blot showing that addition of 2 or 10 mM of the Bid inhibitor BI-6C9 did not prevent activation of caspase-3 triggered by Sorafenib plus TRAIL treatment.

from biopsies of patients with endometrial carcinoma. We have previously characterised these explants to be of epithelial origin by means of cytokeratin and β-catenin expression.[28]

First, we analysed the levels of phosphorylated ERK by Western blot in three different primary explants treated with or without Sorafenib. As we observed for endometrial cancer cell lines, we found that Sorafenib reduced ERK phosphorylation (Fig. 8A). In agreement with the results observed in endometrial carcinoma cell lines, treatment of parallel primary culture explants with Sorafenib caused a marked downregulation of both FLIP and Mcl-1 protein levels (Fig. 8A). Moreover, Sorafenib alone caused activation of caspase-3 which was further increased after addition of TRAIL or aFas (Fig. 8B). Accordingly, treatment of parallel explants with Sorafenib plus either TRAIL or aFas caused an increase in cytotoxicity and nuclei displaying apoptotic morphology (Fig. 8C). Moreover, Sorafenib plus TRAIL treatment activated capases-8, -9, and -3 (Fig. 8D).

All the above results suggest that co-treatment with TRAIL and Sorafenib may be a useful strategy to induce apoptosis of endometrial cancer cells.

4. Discussion

In the present study we have assessed the effects of the multikinase inhibitor Sorafenib on endometrial carcinoma cell lines and primary cultures. We provide evidence of the differential mechanisms underlying Sorafenib-induced apoptosis from those involved in sensitisation or enhancement of TRAIL-induced apoptosis.

First, we have demonstrated that Sorafenib causes a dose-dependent killing of endometrial carcinoma cells. Such cell death displayed features of apoptosis as cells had typical apoptotic morphology and activation of caspases-3 and -9. Moreover, we showed that at time points where Sorafenib alone did not induce apoptosis, it sensitised ECCs to apoptosis induced by TRAIL. TRAIL plus Sorafenib treatment resulted in activation of the extrinsic apoptotic pathway with concomitant caspase-8 processing.

Among other kinases, B-Raf is a target of kinase inhibitory activity of Sorafenib. Raf isoforms are top of Raf/MEK/ERK signalling and activation of serine/threonine kinase of Raf results in phosphorylation of MEK which in turn phosphorylates ERK/MAPKs. Inhibition of ERK by specific inhibitors sensitises or enhances TRAIL-induced apoptosis of melanoma or breast cancer cells.[29–33] In contrast, others have shown that ERK inhibition does not change the apoptotic response of TRAIL resistant cells[34] or have questioned whether ERK2 activation is even required for induction of apoptosis by TRAIL.[35] Because of this duality about the role of ERKs in TRAIL apoptosis, our first question was whether sensitisation to TRAIL by Sorafenib could be caused by inhibition of the MAPK pathway. We have found that inhibition of the ERK/MAPK signalling pathway does not result in sensitisation to TRAIL, suggesting that the inhibitory effects of Sorafenib on ERK/MAPK activity were not responsible for sensitisation to TRAIL. Recent reports have demonstrated that Sorafenib enhances TRAIL-induced apoptosis in other cell types.[15–17] However, it is not fully demonstrated whether inhibition of kinase activity of B-Raf by Sorafenib is involved in the sensitisation of cancer cells to TRAIL or FasL-apoptosis. In line with the results obtained with U0126-mediated inhibition of ERK, we have found that expression of the kinase inactive form of B-Raf did not sensitise ECCs to TRAIL apoptosis. These results demonstrate that Sorafenib sensitises ECCs to TRAIL and aFas apoptosis by a Raf/MEK/ERK independent mechanism. To this regard, increasing evidences support the hypothesis that Raf isoforms may promote survival independent of MAPK signalling.[36,37] Moreover, mice lacking Raf-1 are embryonically lethal, but mice expressing the kinase inactive form display a normal phenotype which strongly suggests kinase-independent effects of Raf proteins.[38] Interestingly, Raf-1 can control pro-apoptotic proteins such as MST2 independently of its MEK kinase activity[39,40]

Next, we found that apoptosis triggered by Sorafenib correlated with downregulation of both Mcl-1 and FLIP. Both Mcl-1 and FLIP have been associated with Sorafenib-induced cytotoxicity. To date, Sorafenib induces downregulation of Mcl-1 though inhibition of its translation[41] or Mcl-1 stabilisation.[42,43] Recent findings also demonstrate that Sorafenib in combination with Vorinostat induces autonomous cell death by decreasing FLIP levels and increasing CD95 activation.[44] We have found that overexpression of Mcl-1 but not FLIP reduces apoptosis triggered by Sorafenib. Of note, although we were able to inhibit Sorafenib-induced apoptosis by overexpressing Mcl-1, FLIP protein remained at low levels. Previous works from our laboratory demonstrated that FLIP protein levels are the main regulator of live/dead decisions of ECCs after exposure to TRAIL and aFas.[12,18] If FLIP levels are really important, Sorafenib treated cells should undergo apoptosis after TRAIL treatment even in cells with overexpressed Mcl-1. Indeed, we have found that Mcl-1 overexpression did not protect from Sorafenib plus TRAIL-induced apoptosis. In contrast, FLIP overexpression restored TRAIL resistance in the presence of Sorafenib. The fact that Mcl-1 protein was kept at low levels when FLIP was ectopically expressed, reinforces the hypothesis that downregulation of Mcl-1 is not responsible for sensitisation to TRAIL caused by Sorafenib.

The role of FLIP in cancer has been widely demonstrated. FLIP is constitutively or highly expressed in different types of malignancies such as prostate cancer,[45] Hodgkin lymphoma,[46] gastric cancer,[47] bladder carcinoma,[48] malignant mesothelial cell lines[49] and endometrial carcinoma.[12] We previously demonstrated that siRNA to FLIP is enough to sensitise IK cells to TRAIL-induced apoptosis,[12] suggesting that FLIP levels are critical in sensitisation to TRAIL-induced apoptosis. We also explored the mechanism by which Sorafenib may regulate FLIP protein levels. Recent findings have demonstrated that Sorafenib regulates FLIP by inhibition of translation.[17] Our results suggest that Sorafenib induces downregulation of FLIP by inducing its ubiquitin-proteasome degradation, without changing FLIP mRNA levels. The FLIP amount of protein can be controlled at different points. FLIP can be transcriptionally downregulated by some anti-neoplasic drugs such as 5-fluorouracil, oxaliplatin and irinotecan in colon carcinoma cells.[50] Such FLIP mRNA downregulation has been shown to sensitise these cells to TRAIL-induced apoptosis. FLIP levels are also regulated by ubiquitin-proteasome-mediated degradation.[23–25] In fact, some anticancer drugs such as the cyclooxygenase-2

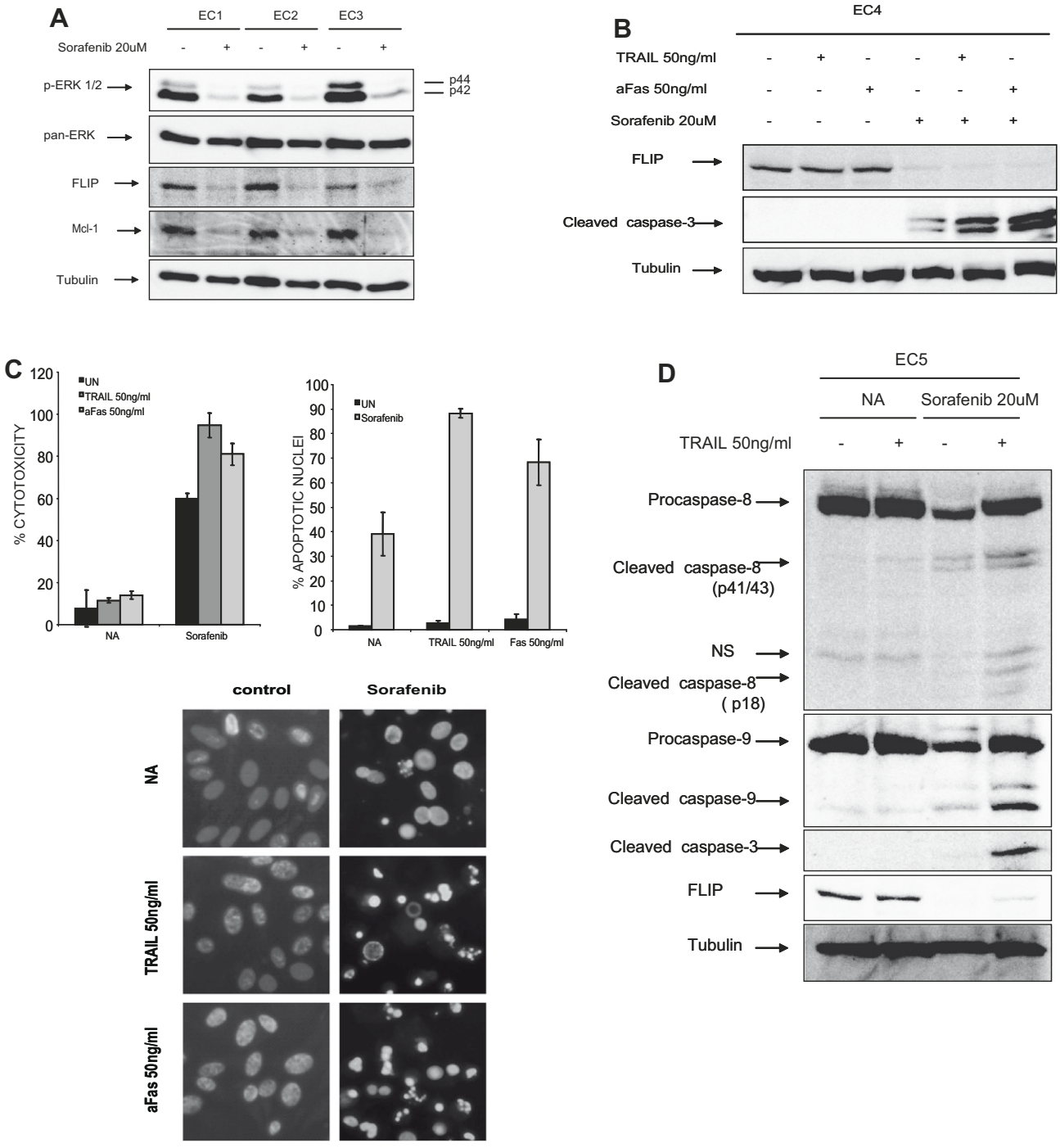

Fig. 8 – Sorafenib exposure renders endometrium adenocarcinoma primary explants sensitive to TRAIL-induced apoptosis. (A) Western blot showing p-ERK1/2, ERK, Mcl-1 and FLIP immunoblots of three different endometrial adenocarcinoma explants (EC1, EC2, EC3) cultured for 2 days and treated with Sorafenib 20 μM for 12 h. (B) EC4 endometrial carcinoma explant was pre-treated with Sorafenib for 30 min. Thereafter, TRAIL and aFas were added to culture media to a final concentration of 150 ng/ml. After 12 h, active caspase-3 or FLIP were assessed. (C) EC4 culture explant was treated as in C and LDH (left) and percentage of apoptotic nuclei (right) were quantified. Bottom, representative microphotographs of Hoechst stained culture explant. (D) Western blot showing activation of indicated caspases and reduction of FLIP protein levels of EC5 explant treated with medium without additives (NA) or medium containing Sorafenib in the presence or absence of TRAIL (UN).

inhibitor celecoxib[51] or the flavonoids[52] and flavopiridol[53] can sensitise cancer cells to TRAIL-induced apoptosis by inducing a proteasome-mediated degradation of FLIP. Moreover, we have recently found that in endometrial cancer cells, FLIP levels can be regulated both transcriptionally and through its degradation by the ubiquitin-proteasome system.[18]

Finally, we demonstrated that Sorafenib sensitised primary endometrial carcinoma explants to TRAIL-induced apoptosis. Recombinant TRAIL or agonistic anti-TRAIL receptor antibodies are in current clinical trials for treatment of both solid and haematological malignancies.[5] Although these agents show some anti-tumoural activity as a monotherapy, increasing evidences demonstrate that these agents may be more effective used in combination with other anti-cancer treatments. These observations may be because there are many tumoural cell types that display resistance to apoptosis after TRAIL exposure. Our previous data support the hypothesis that most endometrial cancer cell lines and primary cultures are insensitive to TRAIL.[12,18] Therefore, a combination of treatments may be a useful tool to sensitise ECCs to TRAIL. Here, the data obtained in both cell lines and primary explants suggest that treatments with TRAIL together with Sorafenib may be interesting for combinatorial therapies for endometrial carcinomas.

In summary, our results show that the mechanistic effectors of apoptosis triggered by Sorafenib or by a combination of Sorafenib with TRAIL or aFas are different. Whereas Mcl-1 is important for Sorafenib-induced apoptosis, FLIP but not Mcl-1 is involved in sensitisation to TRAIL- or aFas-induced apoptosis by Sorafenib. Such molecular duality may be useful to induce apoptosis in cancer cells displaying resistance to apoptosis. That is, if a cancer cell type displays resistance to Sorafenib treatment due to increased Mcl-1 expression, a combination of TRAIL plus Sorafenib can be useful to reduce FLIP levels and sensitise these cells to apoptosis triggered by TRAIL. On the other hand, FLIP is constitutively expressed in many tumours, conferring to these cells resistance to death receptor-induced apoptosis. In this scenario, Sorafenib treatment can bypass apoptosis resistance by reducing Mcl-1 levels.

Conflict of interest statement

None declared.

Acknowledgements

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REFERENCES

1. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999;**104**:155–62.
2. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999;**5**:157–63.
3. Srivastava RK. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* 2001;**3**:535–46.
4. Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. *Oncogene* 2007;**26**:3745–57.
5. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* 2008;**8**:782–98.
6. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* 2003;**10**:66–75.
7. Kischkel FC, Lawrence DA, Chuntharapai A, et al. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* 2000;**12**:611–20.
8. Bodmer JL, Holler N, Reynard S, et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* 2000;**2**:241–3.
9. Sprick MR, Weigand MA, Rieser E, et al. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* 2000;**12**:599–609.
10. Irmler M, Steiner V, Ruegg C, Wajant H, Tschopp J. Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* 2000;**468**:129–33.
11. Thome M, Schneider P, Hofmann K, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997;**386**:517–21.
12. Dolcet X, Llobet D, Pallares J, et al. FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Lab Invest* 2005;**85**:885–94.
13. Flaherty KT. Sorafenib: delivering a targeted drug to the right targets. *Expert Rev Anticancer Ther* 2007;**7**:617–26.
14. Wilhelm S, Carter C, Lynch M, et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 2006;**5**:835–44.
15. Meng XW, Lee SH, Dai H, et al. Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43–9006)-induced TRAIL sensitization. *J Biol Chem* 2007;**282**:29831–46.
16. Ricci MS, Kim SH, Ogi K, et al. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer cell* 2007;**12**:66–80.
17. Rosato RR, Almenara JA, Coe S, Grant S. The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res* 2007;**67**:9490–500.
18. Llobet D, Eritja N, Encinas M, et al. CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells. *Oncogene* 2008;**27**:2513–24.
19. Liu H, Peng HW, Cheng YS, Yuan HS, Yang-Yen HF. Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. *Mol Cell Biol* 2005;**25**:3117–26.
20. Shmueli A, Oren M. Life, death, and ubiquitin: taming the mule. *Cell* 2005;**121**:963–5.
21. Warr MR, Acoca S, Liu Z, et al. BH3-ligand regulates access of MCL-1 to its E3 ligase. *FEBS Lett* 2005;**579**:5603–8.
22. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 2005;**121**:1085–95.
23. Kim Y, Suh N, Sporn M, Reed JC. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* 2002;**277**:22320–3329.

24. Poukkula M, Kaunisto A, Hietakangas V, et al. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* 2005;**280**:27345–55.
25. Chang L, Kamata H, Solinas G, et al. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* 2006;**124**:601–13.
26. Becattini B, Culmsee C, Leone M, et al. Structure-activity relationships by interligand NOE-based design and synthesis of antiapoptotic compounds targeting Bid. *Proc Natl Acad Sci USA* 2006;**103**:12602–6.
27. Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol* 2006;**3**:388–98.
28. Dolcet X, Llobet D, Encinas M, et al. Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* 2006;**281**:22118–30.
29. Grosse-Wilde A, Voloshanenko O, Bailey SL, et al. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* 2008;**118**:100–10.
30. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene* 2003;**22**:2869–81.
31. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* 2001;**276**:16484–90.
32. Lee TJ, Lee JT, Park JW, Kwon TK. Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. *Biochem Biophys Res Commun* 2006;**351**:1024–30.
33. Ortiz-Ferron G, Tait SW, Robledo G, et al. The mitogen-activated protein kinase pathway can inhibit TRAIL-induced apoptosis by prohibiting association of truncated Bid with mitochondria. *Cell Death Differ* 2006;**13**:1857–65.
34. Vaculova A, Hofmanova J, Soucek K, Kozubik A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. *FEBS Lett* 2006;**580**:6565–9.
35. Frese S, Pirnia F, Miescher D, et al. PG490-mediated sensitization of lung cancer cells to Apo2L/TRAIL-induced apoptosis requires activation of ERK2. *Oncogene* 2003;**22**:5427–35.
36. Hindley A, Kolch W. Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases. *J Cell Sci* 2002;**115**:1575–81.
37. Kolch W, Kotwaliwale A, Vass K, Janosch P. The role of Raf kinases in malignant transformation. *Expert Rev Mol Med* 2002;**4**:1–18.
38. Huser M, Luckett J, Chiloeches A, et al. MEK kinase activity is not necessary for Raf-1 function. *Embo J* 2001;**20**:1940–51.
39. O'Neill E, Kolch W. Taming the Hippo: Raf-1 controls apoptosis by suppressing MST2/Hippo. *Cell Cycle* 2005;**4**:365–7.
40. O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science* 2004;**306**:2267–70.
41. Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem* 2005;**280**:35217–27.
42. Ding Q, Huo L, Yang JY, et al. Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Res* 2008;**68**:6109–17.
43. Yu C, Bruzek LM, Meng XW, et al. The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43–9006. *Oncogene* 2005;**24**:6861–9.
44. Zhang G, Park MA, Mitchell C, et al. Vorinostat and sorafenib synergistically kill tumor cells via FLIP suppression and CD95 activation. *Clin Cancer Res* 2008;**14**:5385–99.
45. Zhang X, Jin TG, Yang H, et al. Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* 2004;**64**:7086–91.
46. Dutton A, O'Neil JD, Milner AE, et al. Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci USA* 2004;**101**:6611–6.
47. Lee SH, Kim HS, Kim SY, et al. Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. *Apmis* 2003;**111**:309–14.
48. Korkolopoulou P, Goudopoulou A, Voutsinas G, et al. C-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. *Urology* 2004;**63**:1198–204.
49. Rippo MR, Moretti S, Vescovi S, et al. FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. *Oncogene* 2004;**23**:7753–60.
50. Galligan L, Longley DB, McEwan M, et al. Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* 2005;**4**:2026–36.
51. Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* 2006;**66**:11115–9.
52. Son YG, Kim EH, Kim JY, et al. Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* 2007;**67**:8274–84.
53. Palacios C, Yerbes R, Lopez-Rivas A. Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. *Cancer Res* 2006;**66**:8858–69.