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# Vorinostat/SAHA-induced apoptosis in malignant mesothelioma is FLIP/caspase 8-dependent and HR23B-independent

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ABSTRACT

*Introduction:* Malignant pleural mesothelioma (MPM) is a rapidly fatal malignancy that is increasing in incidence. The caspase 8 inhibitor FLIP is an anti-apoptotic protein over-expressed in several cancer types including MPM. The histone deacetylase (HDAC) inhibitor Vorinostat (SAHA) is currently being evaluated in relapsed mesothelioma. We examined the roles of FLIP and caspase 8 in regulating SAHA-induced apoptosis in MPM.

*Methods:* The mechanism of SAHA-induced apoptosis was assessed in 7 MPM cell lines and in a multicellular spheroid model. SiRNA and overexpression approaches were used, and cell death was assessed by flow cytometry, Western blotting and clonogenic assays.

*Results:* RNAi-mediated FLIP silencing resulted in caspase 8-dependent apoptosis in MPM cell line models. SAHA potently down-regulated FLIP protein expression in all 7 MPM cell lines and in a multicellular spheroid model of MPM. In 6/7 MPM cell lines, SAHA treatment resulted in significant levels of apoptosis induction. Moreover, this apoptosis was caspase 8-dependent in all six sensitive cell lines. SAHA-induced apoptosis was also inhibited by stable FLIP overexpression. In contrast, down-regulation of HR23B, a candidate predictive biomarker for HDAC inhibitors, significantly inhibited SAHA-induced apoptosis in only 1/6 SAHA-sensitive MPM cell lines. Analysis of MPM patient samples demonstrated significant inter-patient variations in FLIP and caspase 8 expressions. In addition, SAHA enhanced cisplatin-induced apoptosis in a FLIP-dependent manner.

*Conclusions:* These results indicate that FLIP is a major target for SAHA in MPM and identifies FLIP, caspase 8 and associated signalling molecules as candidate biomarkers for SAHA in this disease.



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## 1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive cancer associated with exposure to asbestos. The global incidence is increasing in Europe and other parts of the world in line with the prior history of asbestos exposure.[1] The majority of patients present in the more advanced stages for which palliative systemic chemotherapy is usually the mainstay of initial treatment. Cisplatin/pemetrexed is now the standard-of-care in the first-line setting and offers a small benefit in terms of survival, as well as for symptom palliation. Several novel agents are currently in early phase clinical trials, but have yet to make a significant impact on outcome for these patients.[2,3] Importantly, there is currently no standard of care in patients who have relapsed following pemetrexed/cisplatin. Prognosis therefore remains poor with a median survival time of less than 12 months. Improvements in the effective use of chemotherapy and targeted agents, and the development of reliable predictive biomarkers of response to treatment are urgently needed. There is emerging pre-clinical and clinical evidence that histone deacetylase inhibitors (HDACIs) and, in particular, Vorinostat/SAHA (suberoylanilide hydroxamic acid), are promising therapeutic options for mesothelioma.[4] The phase I trials of Vorinostat in advanced malignancies showed sufficient activity[5,6] to warrant the initiation of the Vantage 014 phase III randomised, placebo-controlled trial of Vorinostat plus best supportive care (BSC) versus BSC alone for patients with relapsed disease (NCT00128102).[4]

Tumour response as well as clinicopathological factors are essential predictors of survival in mesothelioma.[7] Tumour response is determined by susceptibility to apoptosis.[8] Death receptors such as Fas and the TRAIL (TNF-related apoptosis-inducing ligand) death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) trigger death signals when bound by their natural ligands.[9] Ligand binding to the death receptors leads to the recruitment of Fas-associated death domain protein (FADD), which in turn recruits caspase 8 (FLICE, FADD-like interleukin-1β-converting enzyme) to form death-inducing signalling complexes (DISCs). Caspase 8 is converted from its pro-form (procaspase 8) to its active form at the DISC and subsequently activates pro-apoptotic downstream molecules such as caspases 3 and 7.[9] Death receptors have been investigated as novel targets for cancer therapy, and several clinical trials are currently evaluating the effectiveness of various TRAIL agonists.[10] Clinical efficacy of TRAIL agonists however has been limited, potentially due to high levels of the death receptor signalling pathway inhibitor FLIP (FLICE-inhibitory protein) in cancer. FLIP blocks caspase 8 activation at the DISCs formed by Fas and the TRAIL receptors.[11] Differential splicing gives rise to long ($FLIP_L$) and short ($FLIP_S$) forms of FLIP, both of which bind to FADD and block caspase 8 activation. Significantly, FLIP has been found to be overexpressed in numerous cancers, including mesothelioma,[12] suggesting that it may play an important role during malignant transformation. We and others have found that FLIP is a key regulator of cell viability and chemotherapy- and TRAIL-induced apoptosis in several cancers.[13,14]

HDACIs are a novel class of agents which exert their anticancer effects by altering gene expression and the function of a wide range of proteins and cellular pathways regulating cell proliferation, differentiation and cell death.[15] There have been a number of *in vitro* studies showing FLIP down-regulation by HDACIs, including SAHA, in a range of malignancies.[16–19] In this study, we demonstrate the therapeutic potential of targeting FLIP in mesothelioma. We show here that SAHA is active in mesothelioma cell line models and induces apoptosis mediated by down-regulation of FLIP with subsequent activation of caspase 8. In contrast, HR23B, a putative HDACI predictive biomarker[19] does not appear to play a major role in mediating the cytotoxic effects of HDACI in mesothelioma. Moreover, analysis of patient samples revealed variable levels of FLIP and procaspase 8 expression levels in mesothelioma. These studies identify FLIP and procaspase 8 (but not HR23B) as potential biomarkers of response to SAHA in this disease.

## 2. Materials and methods

### 2.1. Compounds

SAHA was purchased from Selleck Chemicals (Houston, TX), cisplatin (Hospira UK) was obtained from the Belfast City Hospital Pharmacy. Recombinant human TRAIL and Z-VAD-fmk were purchased from Calbiochem (Gibbstown, NJ), and MG-132 (Z-Leu-Leu-Leu-al) from Sigma–Aldrich (St. Louis, MO).

### 2.2. Antibodies

FLIP (NF-6) and caspase-8 (12F5) specific antibodies were from Alexis Biochemicals (San Diego, CA), PARP antibody was from eBioscience (San Diego, CA), β-actin was from Sigma–Aldrich. Antibodies specific for Bak, Bax, Bid, XIAP, Bcl-2, Bcl-X, Mcl-1 and caspase-3 were from Cell Signaling Technology (Danvers, MA). Anti-HR23B was from Bethyl Laboratories (Montgomery, TX), and anti-FADD from BD Biosciences (Oxford, United Kingdom). Phycoerythrin-conjugated monoclonal antibodies specific for DR4, DR5, Fas and DcR2 were purchased from eBioscience and TNFR1 antibody was from R&D Systems (Minneapolis, MN).

### 2.3. Cell lines and cell culture

The Ren cell line was obtained from Prof. Steven M. Albelda, University of Pennsylvania Medical Center, PA, United States of America. NCI-H28, ONE58, MSTO-211H, H2461 and H2591 cells were donated by Dr. Peter Szlosarek from Queen Mary University of London. MM98 cell line was kindly given by Dr. Stefano Biffo from San Raffaele Scientific Institute, Milan, Italy. Ren cells were maintained in F-12 (Ham) medium supplemented with 10% foetal calf serum, 50 μg/ml penicillin/streptomycin and 10% non-essential amino acids (all from Invitrogen, Paisley, UK). The remaining cell lines were maintained in RPMI medium supplemented with 10% foetal calf serum and 50 μg/ml penicillin/streptomycin (all from Invitrogen). All cell

lines were maintained in 5% $CO_2$ at 37 °C and regularly screened for the presence of mycoplasma using the Myco-Alert® Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

### 2.4. Stable cell line generation

Ren cells were transfected with expression vectors encoding $FLIP_L$ or $FLIP_S$ as previously described[20] using GeneJuice® (Novagen, Nottingham, UK). Positive clones were selected and further maintained in a medium containing 600 μg/ml G418 (Gibco, Paisley, UK).

### 2.5. Generation and treatment of spheroids

Multicellular spheroids were generated in non-adsorbent round-bottomed 96-well plates, as previously described.[21] Before treatment, 18–20 multicellular spheroids were transferred to each well of a polyHEMA-coated 24-well plate. The spheroids were treated as described in full DMEM for 24 h.

### 2.6. Patient samples

Mesothelial tumour samples were collected at the time of debulking surgery at Glenfield Hospital, Leicester and stored in The Leicestershire Mesothelioma Tissue Bank (Lecicestershire Research Ethics Committee reference number 6742). Anonymised specimens from 20 patients who have not received preoperative treatment were transferred to St. James' Hospital, Dublin. Samples were homogenised in TriReagent (MRCgene) using the Qiagen TissueLyser, and proteins isolated using the manufacturer's protocol (MRCgene). Proteins were dissolved in a 1% SDS solution and tributylphosphine (2.5% of final solution volume) and subjected to Western blotting. Studies were approved by St. James' Hospital and The Adelaide & Meath Hospital incorporating the National Children's Hospital, Research Ethics Committee (2008/07/AMDL).

### 2.7. Clonogenic assay

Ren cells were seeded at 200 cells per well in 6 well plates. The next day cells were treated with 5 or 10 μM Vorinostat. After 24 h, the media was changed and the cells were allowed to grow until colonies were formed. The cells were stained with crystal violet solution, and the number of colonies counted.

### 2.8. Western blotting

Western blotting was carried out as previously described.[13] Primary antibodies were used in conjunction with a horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibody (Amersham, Buckinghamshire, UK).

### 2.9. Flow cytometry

Samples were analysed on a BD FACS Calibur flow cytometer machine, using Cell Quest Pro software (Becton Dickinson, Franklin Lakes, NJ). Cell death was determined using propidium iodide (Sigma–Aldrich) staining to evaluate the percentage of cells with sub-diploidal DNA content as previously described.[13] Surface expression of death receptors was assessed following live cell staining with one of the specific antibodies. In all experiments, isotype control (mouse $IgG_1$) was used.

### 2.10. siRNA transfections

The non-silencing control (SC) and c-FLIP targeting siRNAs were obtained from Dharmacon (Chicago, IL) and previously described.[20] siRNAs specific for caspase 8, FADD and HR23B were from Qiagen (Crawley, UK). siRNA transfections were carried out using OligofectAMINE reagent (Invitrogen) according to the manufacturer's instructions.

### 2.11. Quantitative PCR

Total RNA was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, UK). Reverse transcription of 2 μg of RNA was carried out using a Moloney Murine Leukaemia Virus-based Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed in a final volume of 15 μL, containing Taqman® Universal PCR master mix, 20× specific TaqMan probe (FLIP (Hs01116280_m1) or GAPDH (Hs99999905_m1), Applied Biosystems, Carlsbad, CA), and 50 ng of cDNA using an Opticon DNA Engine Thermal Cycler (Bio-Rad Laboratories Inc., Waltham, MA), with the cycling conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative total FLIP mRNA expression was determined using the $\Delta\Delta C_t$ method.[22]

### 2.12. Statistical analysis

Results were expressed as mean value ± SEM. Differences between groups were evaluated using two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered to indicate statistical significance. Statistical analyses were conducted using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. FLIP and procaspase 8 expression in MPM

FLIP has been reported to be overexpressed in primary MPM cells compared to normal mesothelial cells.[12] In another study, caspase 8 was detected by IHC in 16/37 (43%) of mesothelioma patient specimens.[29] Using Western blot analysis, we compared expression of FLIP and procaspase 8 in a panel of benign mesothelial samples ($n = 5$) with expression in biphasic ($n = 5$), sarcomatoid ($n = 4$) and epithelial ($n = 6$) MPM patient samples. Overall, both FLIP and procaspase 8 expressions were higher in the MPM patient samples than in the benign tissue samples, however expression was variable among the malignant tissues (Fig. 1A). Of note, samples with the highest levels of procaspase 8 also had high levels of $FLIP_L$ and/or $FLIP_S$ (or expression of a third FLIP splice variant, $FLIP_R$[30]; this splice variant is functionally equivalent to $FLIP_S$).

Baseline expression of key components of the extrinsic apoptotic pathway was determined in a panel of seven MPM cell lines. Western blot analysis indicated that FADD, $FLIP_L$, $FLIP_S$ and procaspase 8 were all expressed in MPM cell lines

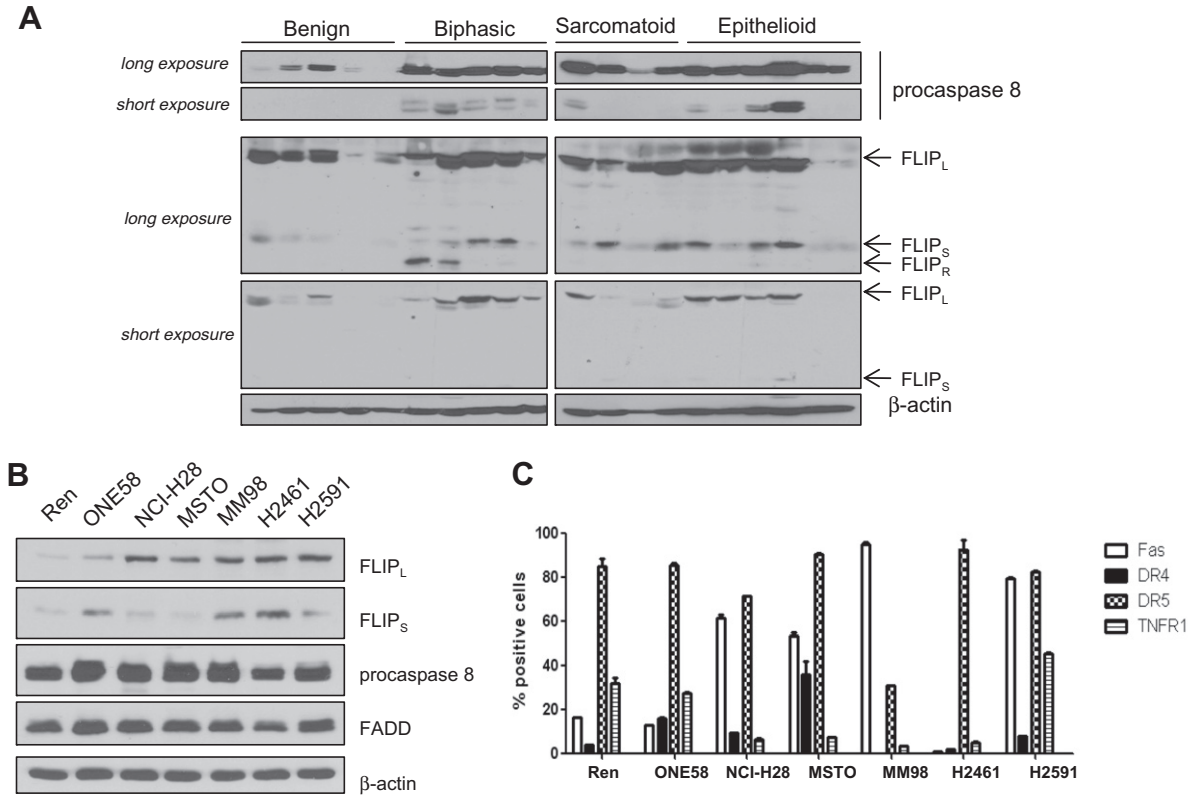


**Fig. 1 – (A) Western blot analysis of FLIP and procaspase 8 expression in a panel of benign mesothelial tissue samples ($n$ = 5) and biphasic ($n$ = 5), sarcomatoid ($n$ = 4), and epithelial ($n$ = 6) malignant pleural mesothelioma (MPM) patient samples. Short (1 min) and long (5 min) exposure times were applied in order to better visualise the differences in protein expression between the samples. (B) The expression of FLIP, Fas-associated death domain protein (FADD) and procaspase 8 in a panel of MPM cell lines detected by Western blotting. (C) Cell surface expression of death receptors in MPM cell lines as measured by flow cytometry.**

(Fig. 1B). FADD expression was similar across the cell line panel, whereas expression of FLIP and procaspase 8 was more variable. Cell surface analysis of death receptor expression indicated that DR5 was the most widely and highly expressed, with at least 70% positive cells in six out of seven cell lines (Fig. 1C). Thus, the key cytoplasmic and cell surface signalling molecules of the extrinsic apoptotic pathway are expressed in these MPM models.

### 3.2. *Mesothelioma cell survival is dependent on maintaining FLIP expression*

FLIP down-regulation leads to spontaneous cell death in several malignancies.[13,20] To assess whether MPM is also FLIP-addicted, Ren, ONE58 and NCI-H28 cells were transfected with an siRNA targeting all FLIP splice forms (FT). Western blot analysis indicated that FLIP silencing resulted in caspase 8 activation and PARP cleavage (a hallmark of apoptosis) in all three cell lines (Fig. 2A). The induction of apoptosis in FLIP-silenced cells was confirmed by flow cytometry (data not shown). PARP cleavage following FLIP silencing could be detected to varying degrees in four other MPM cell lines, with the MSTO cell line being particularly sensitive (Fig. 2B).

We next assessed the importance of each of the two main FLIP splice variants individually using splice form-specific siRNAs: FLIP$_L$ (FL) and FLIP$_S$ (FS). Notably, transfection of Ren cells with either FL or FS siRNA alone failed to result in caspase 8 activation or PARP cleavage (Fig. 2C), indicating that expression of either FLIP splice form is sufficient to maintain MPM cell survival. Because silencing both FLIP splice forms resulted in caspase 8 activation, we determined whether activity of caspase 8 was necessary for apoptosis induction. In cells in which procaspase 8 expression was down-regulated by siRNA, the apoptosis induced by FLIP silencing was completely attenuated (Fig. 2D, Supplementary Fig. S1 and data not shown). Thus, these results indicate that inhibition of FLIP expression initiates caspase 8-dependent apoptosis in MPM cells, indicating a critical role for FLIP in regulating cell viability in MPM cell lines and suggesting that FLIP is a potential therapeutic target in this disease.

### 3.3. *SAHA inhibits FLIP expression*

It has been previously reported that HDACIs can induce FLIP down-regulation in human cancer cell lines.[16–18] For example, Vorinostat/SAHA has been shown to down-regulate FLIP in multiple myeloma, hepatocellular carcinoma and thyroid cancer cell lines.[23–25] Given the promising clinical activity of SAHA in mesothelioma and its evaluation in a phase III clinical trial due to report this year,[4] we assessed whether FLIP is

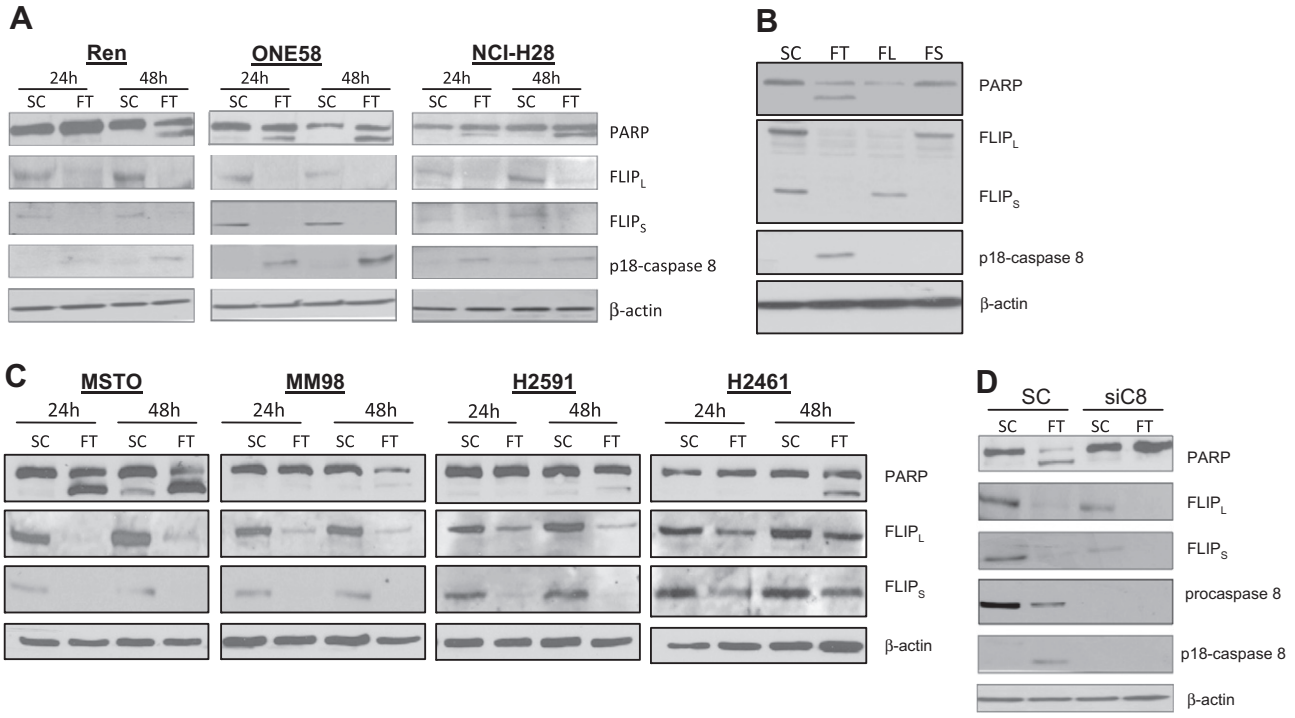


**Fig. 2 – (A) Silencing FLIP is sufficient to induce apoptosis in MPM cells. Ren, ONE58 and NCI-H28 cells were transfected with 10 nM control (SC) or FLIP-targeting (FT) siRNA for 24 and 48 h. Apoptosis induction was assessed by immuno-detection of caspase 8 (p18-subunit) and PARP cleavage. (B) MSTO, MM98, H2461 and H2591 cells were transfected with control (SC) or FLIP siRNA (FT) for 24 and 48 h. FLIP expression and PARP cleavage were assessed by Western blot analyses. (C) Silencing of both splice variants of FLIP is required for apoptosis induction. Ren cells were transfected with 10 nM control (SC), FLIP-targeting (FT) siRNA or siRNA specific for either short (FS) or long (FL) FLIP splice form for 48 h and analysed by Western blotting. (D) Apoptosis induced by FLIP silencing is caspase 8-dependent. Ren cells were transfected with 10 nM of control (SC) or caspase 8 (siC8) targeting siRNA. After 36 h, the cells were transfected with SC or FT siRNA for 48 h and analysed by Western blotting.**

a target for this agent in MPM. In Ren, ONE58 and NCI-H28 cells, SAHA down-regulated both splice forms of FLIP at ~$IC_{50}$ concentrations (1–5 μM), and this correlated with caspase 8 activation (as indicated by detection of the p18 subunit) and PARP cleavage (Fig. 3A). Similarly, $FLIP_L$ and $FLIP_S$ were potently down-regulated in MSTO, MM98 and H2461 cells (Fig. 3B). Apoptosis induction by SAHA was also assessed by flow cytometry after 24 h treatment. Five cell lines (Ren, ONE58, NCI-H28, MSTO and H2461) were found to be sensitive to SAHA-induced apoptosis, one cell line (MM98) was less sensitive and one (H2591) was resistant (Fig. 3C). Of note, the resistance to SAHA in H2591 cells, and to a lesser extent in MM98 cells, correlated with a relative lack of PARP cleavage after FLIP silencing in these cell lines (Fig. 2), whereas the cell lines which were more sensitive to SAHA were also more sensitive to RNAi-mediated FLIP down-regulation.

Collectively, the above results suggested FLIP as a target for SAHA in MPM. To confirm this in a further disease-relevant model, we assessed the effect of SAHA on FLIP expression in 3-dimensional (3D) spheroid models.[21] As shown in Fig. 3D, FLIP was down-regulated and caspase 3 was activated in Ren MPM spheroids treated with SAHA for 24 h, in a dose-dependent manner. Importantly, FLIP down-regulation and apoptosis activation were observed at the same concentrations as in the adherent cell line model (Fig. 3A). Thus, SAHA potently down-regulates FLIP protein in both 2D and 3D MPM models.

Further analyses revealed that down-regulation of FLIP protein was a relatively early event (detected at 6 h) that preceded SAHA-induced apoptosis as identified by caspase 8 activation and PARP cleavage (which were detected no earlier than 24 h after treatment; Supplementary Fig. S2B). In the ONE58 cell line, down-regulation of FLIP mRNA preceded that of FLIP protein (Supplementary Fig. S2A), suggesting repression of FLIP expression occurs at the transcriptional level in this model. However, in NCI-H28 cells, down-regulation of FLIP protein preceded that of FLIP mRNA. In Ren MPM cells, FLIP protein and mRNA levels were down-regulated concomitantly; although there was still significant FLIP mRNA expression at 12 h, when protein expression was barely detectable. Overall, these results suggest that both transcriptional and post-transcriptional events can regulate FLIP protein down-regulation in MPM cells in response to SAHA. The involvement of post-transcriptional events was confirmed in Ren cells in which proteasome inhibition blocked SAHA-induced FLIP down-regulation (Supplementary Fig. S2C). Importantly, caspase inhibition failed to prevent SAHA-mediated FLIP down-regulation, further indicating that FLIP down-regulation is not a secondary result of caspase activation and apoptosis induction (Supplementary Fig. S2C).

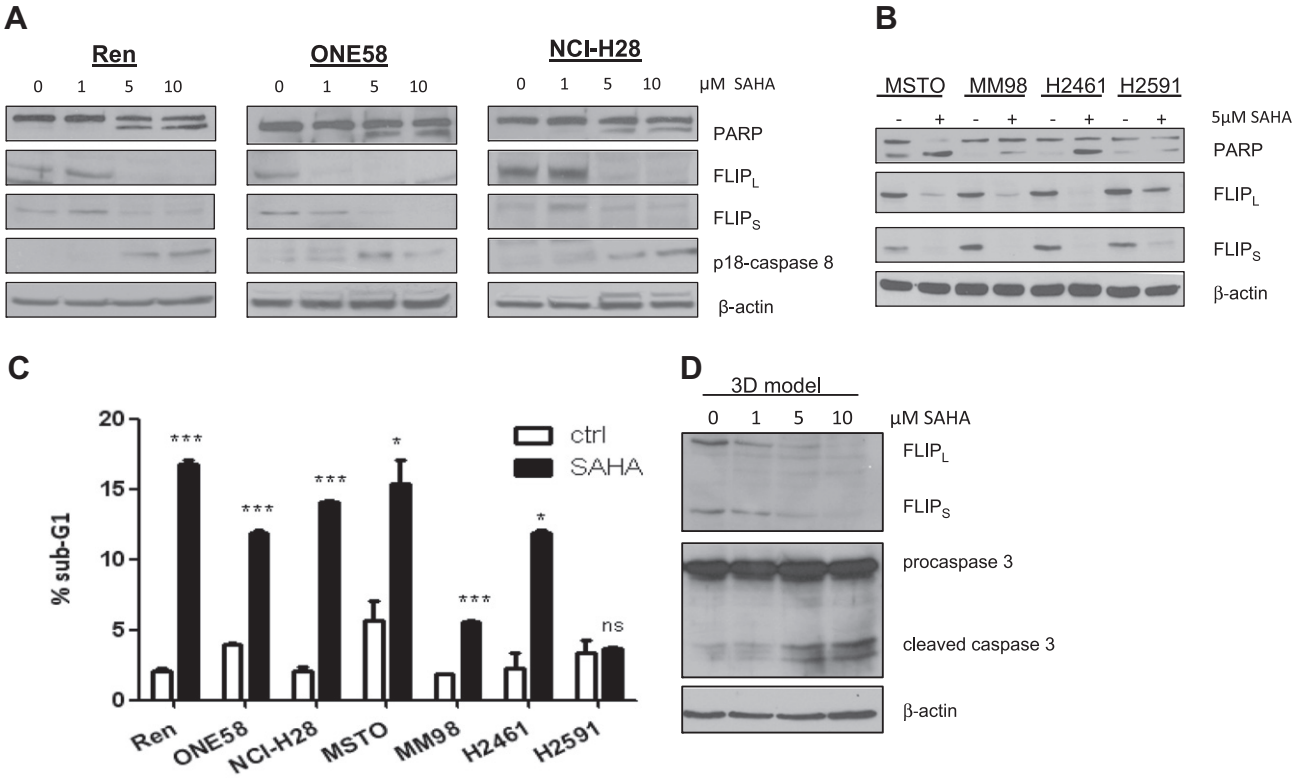


**Fig. 3 – SAHA induces FLIP down-regulation and apoptosis in MPM cell lines. (A) Ren, ONE58 and NCI-H28 cell lines were treated with 1, 5 or 10 μM SAHA for 24 h. Immunoblot analysis revealed FLIP down-regulation, caspase 8 activation and PARP cleavage at 5 and 10 μM SAHA. (B) MSTO, MM98, H2461 and H2591 cell lines were treated with 5 μM SAHA for 24 h. Western blot analysis shows FLIP down-regulation and PARP cleavage. (C) Flow cytometry analysis of apoptosis induction after 24 h treatment with 5 μM SAHA in panel of seven MPM cell lines (***$P < 0.001$; *$P < 0.05$; ns, not significant; compared to control; Student's t-test). (D) Ren cell line-derived spheroids were treated with 1, 5 or 10 μM SAHA for 24 h. Immunoblot showing FLIP down-regulation and caspase 3 activation.**

### 3.4. *FLIP over-expression attenuates apoptosis induced by SAHA*

Our results suggested that FLIP down-regulation is a critical event for apoptosis induction by SAHA in a significant number of MPM cell lines. In order to confirm this, we generated Ren cell lines stably over-expressing either $FLIP_L$ (FL4a and FL4b) or $FLIP_S$ (FS1b and FS1f). A cell line transfected with empty vector (EV1a) was used as a control. In EV1a cells, SAHA induced FLIP downregulation, caspase 8 activation and PARP cleavage, as expected (Fig. 4A). However, in FLIP-overexpressing clones, apoptosis was significantly inhibited as shown by a reduction in caspase 8 activation and PARP cleavage (Fig. 4A) and confirmed by caspase-activity assays (data not shown) and flow cytometry analysis (Fig. 4B). Thus, over-expression of either FLIP splice form was sufficient to inhibit caspase 8 activation and apoptosis induction following SAHA treatment. This is in agreement with the observation that silencing of both splice forms is required to induce apoptosis in MPM cells (Fig. 2C). The effect of FLIP overexpression was confirmed by clonogenic assay (Fig. 4C). In untreated cells, more colonies were observed in either $FLIP_L$- or $FLIP_S$-over-expressing cells compared to EV cells, suggesting that FLIP over-expression results in greater constitutive clonogenicity. Importantly, in SAHA-treated $FLIP_L$ or $FLIP_S$ over-expressing cells, significantly more colonies were observed compared to the SAHA-treated control cells (Fig. 4C and D). Collectively, these results provide further evidence to support the significance of FLIP as a target for apoptosis induction in response to SAHA in MPM cells.

### 3.5. *SAHA-induced apoptosis of MPM cells is caspase 8-dependent*

Having observed that SAHA induces FLIP down-regulation prior to activation of caspase 8 in three MPM cell lines, we investigated whether caspase 8 activation is required for SAHA-induced apoptosis in mesothelioma. Procaspase 8 was down-regulated in 6 MPM cell lines using RNAi prior to treatment with SAHA. Down-regulation of procaspase 8 was confirmed by Western blot, and apoptosis was assessed by flow cytometry (Fig. 5). As was the case following siRNA-mediated FLIP silencing, procaspase 8 silencing significantly reduced SAHA-induced cell death in MPM cell lines ($P < 0.01$: Ren, ONE58, NCI-H28; $P < 0.05$: MSTO, MM98, H2461; H2591 cells are SAHA resistant). In addition, FADD silencing also significantly reduced SAHA-induced apoptosis in MPM cell lines (data not shown). These results demonstrate the importance of caspase 8 activation in mediating the apoptotic response of MPM cells to SAHA.

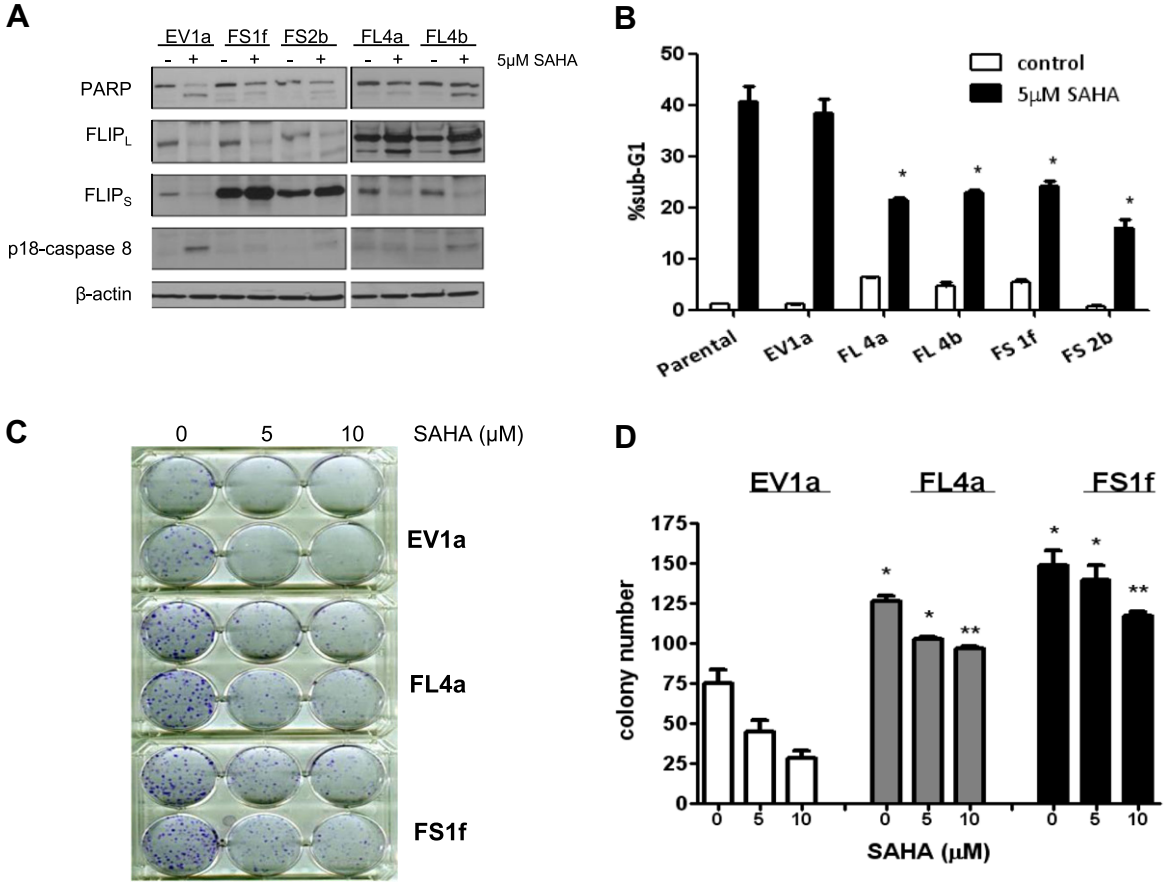


**Fig. 4 – FLIP over-expression reduces sensitivity to SAHA and enhances clonogenicity of MPM cells. FLIP$_L$ (FL) or FLIP$_S$ (FS) over-expressing cells were derived from the Ren cell line. (A) FLIP-overexpressing and empty vector-transfected (EV1a) cells were treated with 5 μM SAHA for 24 h and subjected to Western blot analysis of FLIP expression levels, caspase 8 activation (p18-subunit) and PARP cleavage. (B) Flow cytometry analysis showing apoptosis induction in the parental, EV and FLIP over-expressing Ren cells treated with 5 μM SAHA for 48 h. (C) Empty vector and FLIP over-expressing clones were seeded at low density and treated with 5 or 10 μM SAHA for 24 h, each well in duplicate. The media was then changed and cells were allowed to grow until colonies were visible. The colonies were then stained with crystal violet and counted. (D) A graphical representation of the absolute number of colonies ($^{**}P < 0.01$; $^{*}P < 0.05$; compared to EV cells; Student's *t*-test).**

### 3.6. *SAHA-induced apoptosis is independent of HR23B*

Fotheringham et al. carried out a genome-wide loss-of-function screen using shRNA targeted against 8000 genes and identified a group of genes necessary for HDAC inhibitor-induced apoptosis.[19] One of these genes, HR23B, has been validated as a sensitivity determinant for HDACI-induced apoptosis using cutaneous T-cell lymphoma (CTCL) biopsies obtained from a phase II clinical trial.[26] HR23B is involved in nucleotide excision repair and also plays a role in trafficking ubiquitinated proteins to the proteasome for degradation.[27,28] HR23B was expressed in each MPM cell line at a similar level (Fig. 6A). In order to investigate the potential role of HR23B as a determinant of SAHA activity in MPM, we silenced its expression in the six SAHA-sensitive cell lines. Surprisingly, SAHA-induced apoptosis was significantly attenuated in only one cell line (Ren) following HR23B silencing (Fig. 6B). Moreover, in all cell lines SAHA-induced FLIP down-regulation was unaffected in the absence of HR23B (Supplemental Fig. S3 and data not shown). These results indicate that HR23B is not involved in FLIP protein proteosomal degradation in MPM cells and is not a critical determinant of SAHA-induced apoptosis in the majority of MPM cell lines.

### 3.7. *SAHA enhances effect of chemotherapy and TRAIL*

Since MPM is a highly drug-resistant cancer, new drug combinations are desirable to improve response to treatment. Cisplatin-based chemotherapy is the current 1st line treatment for mesothelioma,[1,2] so the effect of SAHA in combination with cisplatin was assessed. In each cell line, the combination of SAHA and cisplatin resulted in a significant increase in apoptosis compared to either agent alone (Fig. 7). Moreover, even in the cell lines resistant to SAHA alone (MM98 and H2591), we observed significant sensitisation to cisplatin. These results indicate that the use of SAHA in combination with cisplatin may be an effective way to combat chemo-resistance in MPM. Of note, overexpression of either FLIP splice variant significantly inhibited apoptosis induction in response to SAHA and cisplatin (Fig. 7B). TRAIL receptor-targeted therapeutics are novel

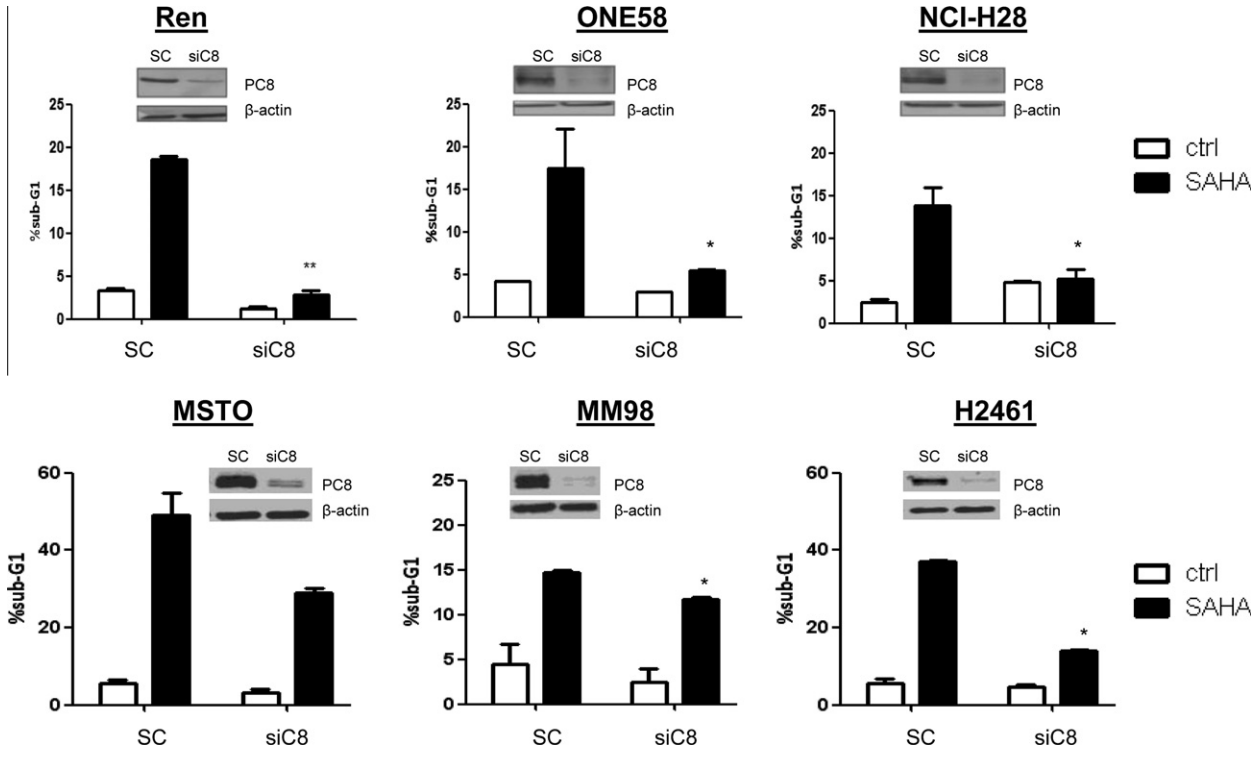


**Fig. 5 – SAHA-induced apoptosis is caspase 8-dependent. Six MPM cell lines were transfected with control (SC) siRNA or caspase 8-targeting siRNA (siC8). After 36 h, the cells were treated with 5 μM SAHA for 30 h (Ren, ONE58, NCI-H28) or 48 h (MSTO, MM98, H2461). Procaspase 8 expression (PC8) was assessed by Western blotting and apoptosis induction by flow cytometry (**P < 0.01; *P < 0.05; compared to SC; Student's t-test).**

apoptosis-inducing agents that are being clinically investigated in a number of cancers.[31–33] Similar to its effect on cisplatin, we found that SAHA synergised with rTRAIL in a FLIP-dependent manner (Supplemental Fig. S4). Thus, FLIP is not only a determinant of response to SAHA monotherapy, but also to combined therapies of SAHA with either cisplatin or rTRAIL.

## 4. Discussion

The global burden of mesothelioma is increasing and is set to continue, especially in the developing world, due to continued importation and use of asbestos. There has been slow progress in the development of effective therapy for mesothelioma, in part due to the significant problem of drug resistance. This is often due to dysfunctional cancer cell death, and MPM is recognised as a particularly drug-resistant cancer.[8] Mutations or alterations in the expression of proteins regulating apoptosis often arise during carcinogenesis, and therapies that specifically target these proteins may therefore be able to overcome drug resistance. One of these proteins is FLIP, which is over-expressed in several cancer types, including mesothelioma,[12] and has been linked to drug resistance and poor prognosis.[13,29,34–36] FLIP down-regulation leads to cell death and increased drug sensitivity in several malignancies.[13,20] In the present study, FLIP down-regulation induced spontaneous apoptosis in MPM cell line models in a caspase 8-dependent manner. This effect was only observed when both $FLIP_L$ and $FLIP_S$ were co-silenced and suggests that FLIP plays an important role in regulating survival in MPM and that expression of either of the main FLIP splice variants is sufficient to maintain cell viability. FLIP is therefore an attractive therapeutic target in this disease setting. However, to date there are no existing treatments in routine clinical use that specifically target FLIP.

Histone deacetylase inhibitors (HDACIs) are a novel class of agents that exert their anti-cancer effects by altering gene expression and the function of a wide range of proteins and cellular pathways regulating cell proliferation, differentiation and cell death.[37] There are many HDACIs at various stages of clinical development and, to date, Vorinostat/SAHA and Istodax/romidepsin have been approved by the Federal Drugs Administration for use in cutaneous T-cell lymphoma (CTCL).[38] Although all the biological effects of HDACIs are not fully understood at present, there has been pre-clinical and clinical evidence of their effectiveness in MPM.

A number of *in vitro* studies in a range of malignancies have shown FLIP down-regulation in response to HDACIs, including SAHA,[16–18] however to our knowledge, no previous studies have directly examined whether FLIP down-regulation is a sufficient death signal for apoptosis induction in response to these agents. We show for the first time that SAHA potently down-regulates both $FLIP_L$ and $FLIP_S$ in MPM and that this is a major mechanism of apoptosis induction in response to this agent in this disease. Other groups have demonstrated that changes in expression of other proteins involved in regulating apoptosis, such as XIAP, BCL-2 and BCL-$X_L$, are important for HDACI-induced cell death.[39–41] However, we did not observe

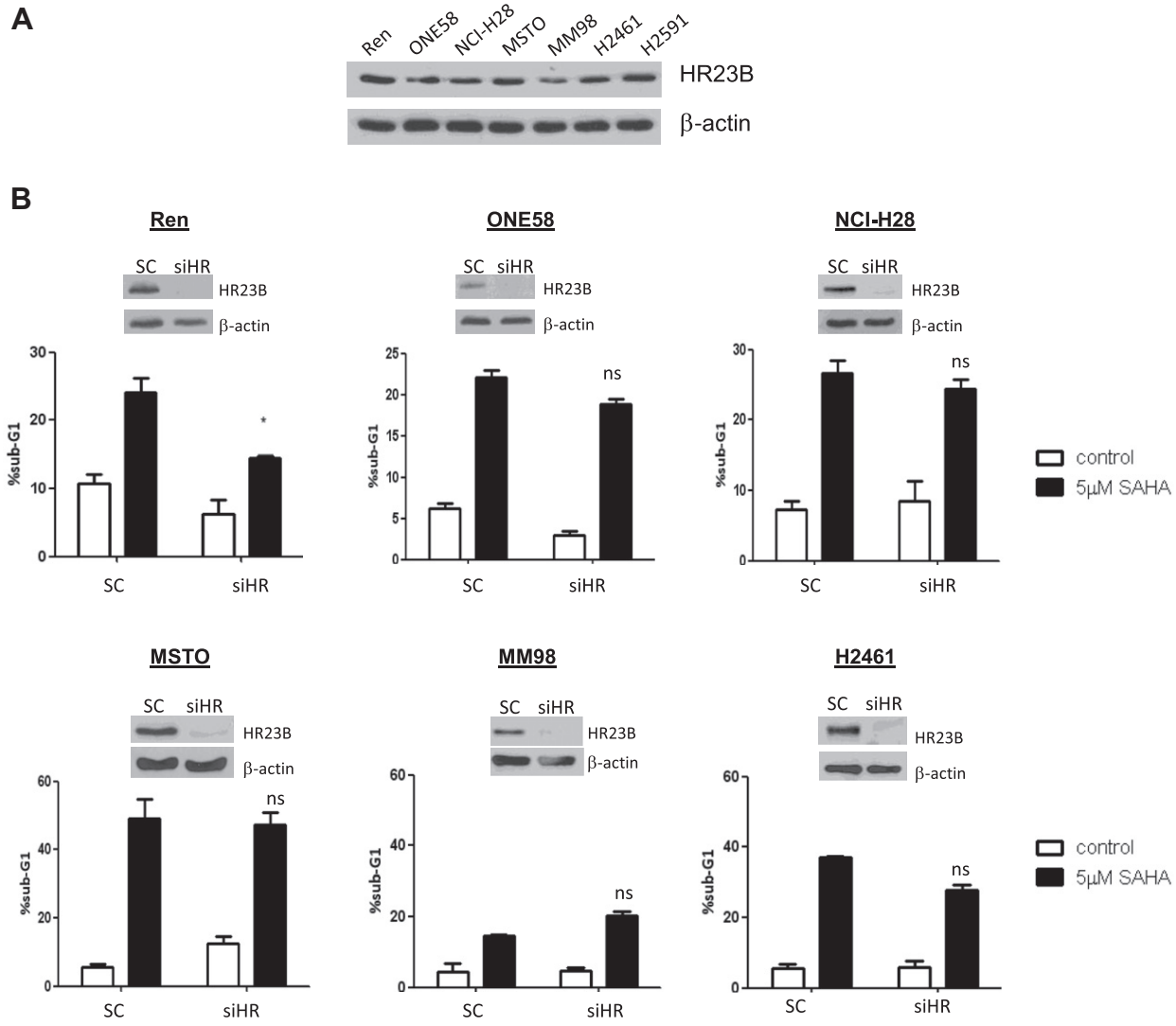


**Fig. 6 – SAHA-induced apoptosis is HR23B-independent. (A) HR23B expression in seven MPM cell lines assessed by Western blot. (B) Effect of HR23B silencing on SAHA-induced apoptosis in MPM cells. Six MPM cell lines were transfected with 20 nM control (SC) or siRNA specific for HR23B (siHR) and 36 h later treated with 5 μM SAHA for 48 h. Western blot confirmed HR23B down-regulation and apoptosis was assessed by flow cytometry analysis (*$P < 0.05$; ns, not significant; compared to SC; Student's *t*-test).**

any changes in expression of these and several other proteins involved in regulating apoptosis following SAHA treatment (Supplemental Fig. S5); this suggested to us that FLIP down-regulation is an important event in mediating SAHA-induced cell death in MPM. This hypothesis was supported by our finding that SAHA-induced apoptosis is highly caspase 8-dependent in MPM cells lines and by the fact that SAHA-induced apoptosis was significantly inhibited in FLIP-overexpressing models. Furthermore, silencing the key DISC adapter molecule FADD also attenuated SAHA-induced apoptosis in MPM cells (data not shown). These results are consistent with FLIP down-regulation followed by caspase 8 activation and apoptosis induction being a major cytotoxic mechanism of action of SAHA in MPM. Moreover, FLIP down-regulation and activation of caspase 3 indicative of apoptosis was observed following SAHA treatment in a 3D model of MPM, a model of acquired multicellular resistance.[21]

Cisplatin, in combination with pemetrexed, is currently the first line standard of care for patients with MPM. We observed however, high levels of resistance to clinically relevant doses of cisplatin in our MPM cell lines. Notably, co-treatment with SAHA significantly enhanced cisplatin-induced apoptosis in MPM cell lines. Importantly, the increased apoptosis observed when SAHA and cisplatin were combined was significantly attenuated by FLIP over-expression, again indicating the importance of FLIP down-regulation for the anti-cancer activity of SAHA in MPM. There is already pre-clinical and clinical evidence for combining platinum drugs with HDACIs. Down-regulation of FLIP has previously been shown to increase sensitivity to cisplatin in ovarian and cervical cell lines.[42,43] Also, SAHA in combination with cisplatin in oral squamous cell carcinoma or with cisplatin/pemetrexed in mesothelioma cell lines resulted in synergistic increases in apoptosis.[41,44] In the clinical setting, the phase II trial of

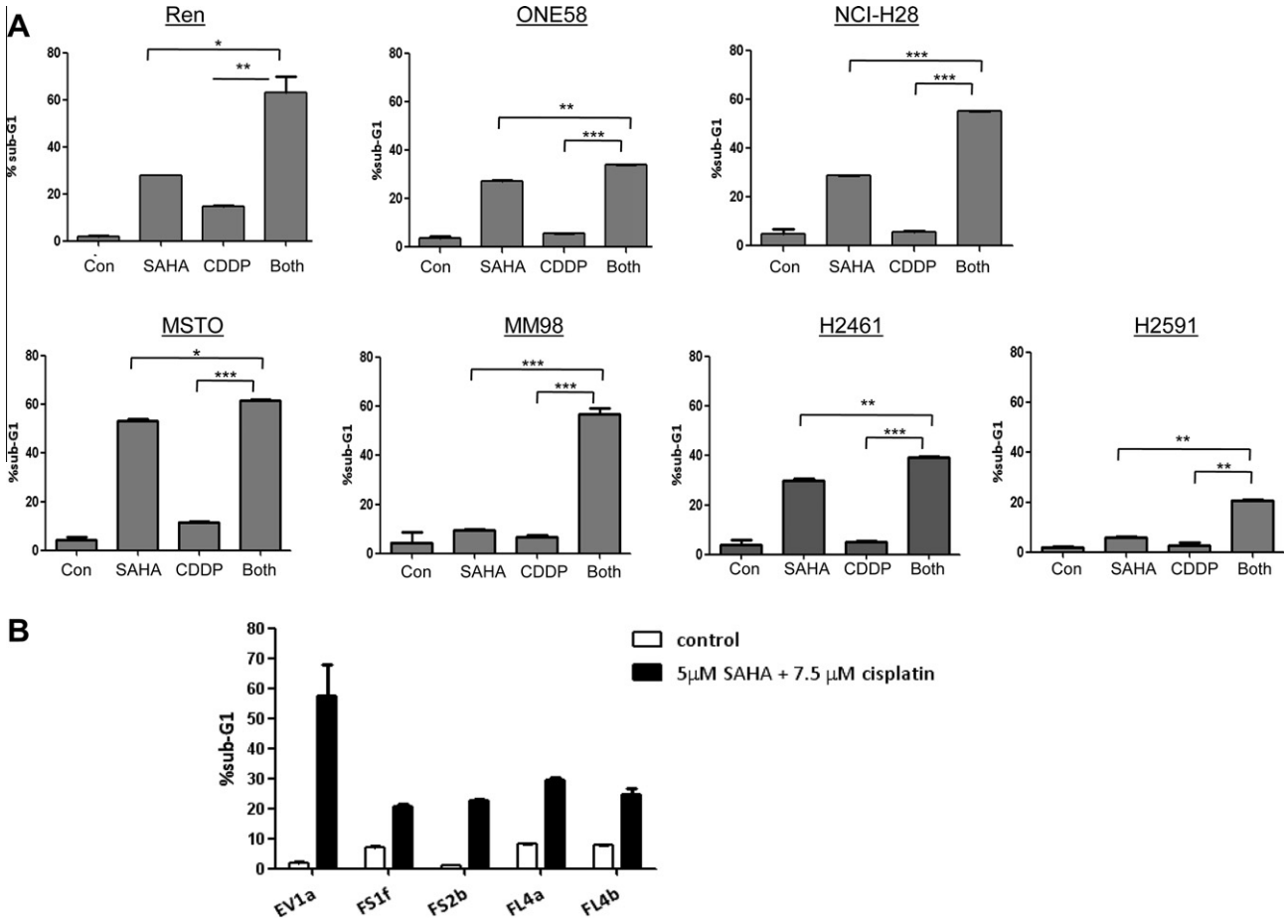


**Fig. 7 – SAHA enhances the effect of cisplatin treatment in a FLIP-dependent manner. (A) Seven MPM cell lines were treated for 72 h with either 5 μM SAHA alone, 7.5 μM cisplatin alone, or with both agents simultaneously. Apoptosis was measured by flow cytometry (**$P < 0.001$; *$P < 0.05$; ns, not significant, Student's *t*-test). (B) Empty vector (EV) and FLIP over-expressing Ren clones were co-treated for 72 h with 5 μM SAHA plus 7.5 μM cisplatin and subjected to flow cytometry analysis.**

carboplatin/paclitaxel with or without SAHA in NSCLC reported significantly improved response rates and a trend towards improved survival for SAHA versus placebo.[45] We found that MPM cell lines exhibited marked resistance to rTRAIL, however, co-treatment with SAHA sensitised MPM cell lines to TRAIL in a FLIP-dependent manner. This result is in agreement with those from other groups. For example, Frew et al. reported that SAHA acted synergistically with MDS-1 (a TRAIL-R agonistic monoclonal antibody) in a panel of cancer cell lines and caused tumour regression in a mouse breast cancer model.[46] Also, Nuezil et al. demonstrated increased TRAIL sensitivity with the addition of SAHA in mesothelioma cell lines, although they related this to down-regulation of BCL-$X_L$.[40] We postulate that resistance to TRAIL agonists is frequently mediated by high levels of FLIP expression in cancer cells and may therefore be overcome by HDACI-mediated FLIP down-regulation.

Development of personalised therapy is a major goal in cancer therapeutic drug development. Use of histone acetylation in peripheral blood mononuclear cells as a surrogate marker is one method that has been used in pre-clinical and clinical studies as a marker of biological activity of HDACIs, but does not appear to reflect tumour response accurately.[47] Several groups have attempted to correlate gene expression levels of potential HDACI biomarkers with response to HDACIs in cancer cell lines,[48,49] however these studies will require clinical validation. In an alternative strategy, Fotheringham et al. carried out a genome-wide loss-of-function screen using shRNA of 8000 genes and identified a group of genes that when silenced prevented HDACI-induced apoptosis.[19] One of these genes, HR23B, has been validated as a sensitivity determinant for HDACI-induced apoptosis using CTCL biopsies obtained from a phase II clinical trial.[26] However, we found only one MPM cell line in which HR23B silencing attenuated apoptosis induction following treatment with SAHA. In contrast, caspase 8 silencing very significantly attenuated SAHA-induced apoptosis in all six SAHA-sensitive MPM cell lines. Importantly, we and others[12,29] have detected variable levels of FLIP and procaspase 8 expression among samples derived from MPM patients. As FLIP and caspase 8 appear to be important determinants of SAHA-induced cell death in MPM, this suggests that a subset of patients may be more responsive than others to SAHA-based therapy.

During the review of this paper, the Vantage 014 phase III randomised, placebo-controlled trial of SAHA plus best supportive care (BSC) versus BSC alone for patients with relapsed

MPM was reported (NCT00128102).[50] Disappointingly, no significant difference was observed in overall survival nor progression-free survival in the two arms. Moreover, preplanned analyses did not identify any subgroups that may have benefitted, and the response rate was low. Importantly, however, no molecular stratification was conducted in this clinical trial. Our data show that caspase 8 expression may be a critical requirement for efficacy. However, as much as 57% of mesotheliomas may be deficient in caspase 8 expression.[29] In addition, the identity of the critical HDAC targets for Vorinostat and their expression in mesothelioma is currently not known. Accordingly, a significant proportion of mesotheliomas may exhibit *de novo* resistance. As recently shown in non-small cell lung cancer,[51] molecular stratification is critical for the success of targeted therapy. Based on this, a predictive biomarker study examining the impact of caspase 8 deficiency in the Vantage trial is warranted. Furthermore, our pre-clinical data suggest that Vorinostat is likely to be more effective when combined with cisplatin than when used as a single agent.

In conclusion, the data presented in this manuscript suggest that FLIP and caspase 8 are key determinants of sensitivity to Vorinostat/SAHA in mesothelioma and may thus be useful biomarkers of response to this agent in this disease.

## Conflict of interest statement

D.A. Fennell has accepted honorarium for speaking, as a member of an Advisory Board related to Vorinostat in mesothelioma. All other authors of this manuscript have no conflicts of interest to declare.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.11.009.

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