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Chromatin remodelling at the topoisomerase II-beta promoter is associated with enhanced sensitivity to etoposide in human neuroblastoma cell lines

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ABSTRACT

Etoposide, an inhibitor of topoisomerase II, promotes DNA damage and apoptosis of cancer cells and is a component of standard therapy for neuroblastoma. Resistance to etoposide has been observed in neural tumour cells expressing lower levels of topoisomerase II. In the present study, we have examined the contribution of epigenetic modulation of gene expression in the potentiation of etoposide-mediated cytotoxicity in neuroblastoma cells. Specifically, we studied the effects of histone deacetylase inhibition with valproic acid on topoisomerase II gene expression and apoptosis in response to etoposide. Using human neuroblastoma cell lines SK-N-AS and SK-N-SH, we show that although the combination of valproic acid and etoposide promoted a reduction in growth compared to either drug alone in both cells, the effect was substantially enhanced in SK-N-AS compared to SK-N-SH cells. An increase in histone H3 acetylation and p21 expression was observed in both cell lines, however, upregulation of *topoisomerase II-beta* gene expression and an increase in PARP cleavage was observed in SK-N-AS cells only. Furthermore, chromatin immunoprecipitation assays revealed an increase in acetylation of histone H3 at the cognate *topoisomerase II-beta* gene after treatment with valproic acid in SK-N-AS cells. These results suggest a potential epigenetic mechanism of regulation of the *topoisomerase II-beta* gene and a possible role for its increased expression in the sensitivity of SK-N-AS neuroblastoma cells to etoposide.

1. Introduction

Neuroblastoma is the most common extracranial solid tumour of childhood, with an incidence of approximately 8 cases per million children. Children with disseminated, high-risk neuroblastoma have very poor long-term outcomes, even with intensive multimodal treatment strategies.[1] The poor outcome in response to current treatment strategies

Abbreviations: HDACI, histone deacetylase (inhibitor); PARP, poly-ADP ribose polymerase; VPA, Valproic acid; ChIP, chromatin immunoprecipitation; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A.

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clearly indicates a need for improved treatment strategies and new therapeutic combinations in order to improve long-term survival in children with neuroblastoma.

Etoposide is an inhibitor of topoisomerase II and a component of standard chemotherapeutic regimens used to treat neuroblastoma. Inhibition of topoisomerase II activity disrupts cell growth and DNA repair mechanisms, leading to DNA damage and cell-death. Etoposide has anti-tumour effects both as a single agent and as part of multi-drug regimens, but with side effects ranging from myelosuppression to increased risk of secondary myeloid leukaemias observed at high doses.[2,3] Resistance to etoposide has been observed in neural tumour cells expressing lower levels of topoisomerase II,[4] and increased levels of topoisomerase II have been associated with responses to etoposide in medulloblastoma tumour cells.[5] The mechanisms by which topoisomerase II gene expression is regulated in these cell types, however, are not fully understood.

A number of genes involved in cell cycle control (*p21/WAF1*, *gelsolin*, *p27Kip1* and *p16Ink4a*) and apoptosis (*BAD*, *TRAIL*, *DR5* and *FasL*) have been shown to be regulated by epigenetic modifications in many types of cancers.[6,7] These epigenetic modifications include DNA methylation, histone acetylation and histone methylation [reviewed in 8] Epigenetic modification of gene expression has been described in neuroblastoma tumour cells and primary tumour samples[9–13] and gene methylation in neuroblastoma tumours has been shown to be associated with poorer overall outcomes.[9,13] Histone deacetylase inhibitors (HDACIs) promote chromatin remodelling through decreased histone deacetylation, leading to changes in gene expression patterns, and inhibition of HDACs has been shown to be effective against neuroblastoma cells in preclinical models.[14–16]

Valproic acid (VPA) is a commonly used anti-convulsant and known to function as an HDACI.[17,18] VPA has been used with success in the treatment of children with neural tumours[19,20] and has also been shown to have efficacy against neuroblastoma tumour cells in preclinical models.[21,22] In the current study, we analysed the ability of VPA to potentiate the cytotoxic effects of etoposide on neuroblastoma cells. We show that the sensitisation of neuroblastoma cells to the effects of etoposide is associated with chromatin remodelling at the topoisomerase-II beta gene and an elevation in nuclear levels of topoisomerase II-beta protein.

2. Materials and methods

2.1. Cell lines and reagents

The characteristics of the neuroblastoma cell lines SK-N-AS and SK-N-SH used in this study have been previously described.[23,24] Neuroblastoma cell lines were grown at 37 °C in 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (USB, Minneapolis, MN), L-glutamine and penicillin, streptomycin and amphotericin (Sigma, St. Louis, MO). VPA (Sigma Aldrich, St. Louis, MO, USA) was prepared as a 1.2 M stock solution in DMSO. Etoposide solution (20 mg/ml) in 30% ethanol (Ben Venue Labs, Bedford OH, USA) and diluted with culture medium prior to use.

2.2. Drug treatment

For dose–response experiments, cell lines were seeded at a density of 3000 cells per well in 96 well tissue culture plates and incubated for 24 h at 37 °C and 5% CO_2 tension. Increasing concentrations of either VPA or etoposide or both were added to cells. Dilutions of VPA were made with growth media to obtain final concentrations ranging from 0.75 to 12 mM. Serial dilutions of the stock solution of etoposide were made to obtain final drug concentrations in the range of 0.001–100 mM. Cells were incubated in the presence of drug for 48, 72 or 96 h. In control experiments equal volumes of solvent (DMSO or ethanol) were added. Metabolic activity was measured using MTT assays as previously described[25] and absorbance was measured at 570 nm. For co-incubation experiments, 1.5 mM VPA and etoposide ranging from 0.001 μM to 100 μM were added to cells and incubations were carried out for 48, 72 or 96 h. Cell viability was measured by MTT assays. Each experiment was repeated four times and experimental variation for each measurement was found to be <10%.

2.3. Statistical analysis

IC50 values from the MTT assays were calculated using best-fit trendlines for each cell line and incubation time. Synergy calculations were performed using the Product Method of Webb as described in the text. The effect of drug synergy across a range of concentrations was assessed using Two-way Repeated Measures ANOVA. Statistical analysis was performed using Prism 5 for Mac OS X, version 5.0c (GraphPad Software, La Jolla, CA).

2.4. Cell cycle analysis

Neuroblastoma cells were seeded at a density of 100,000 cells per well of a six well plate and incubated for 24 h prior to addition of drugs. Cells were either untreated or treated with VPA alone (1.5 μM) or etoposide alone (0.1 μM) or co-incubated with 1.5 mM VPA and 0.1 μM etoposide for 72 h. Cells were trypsinised and harvested by centrifugation. Pellets were washed with 1 ml of phosphate buffered saline (PBS) and resuspended in 0.5 ml of 1 mg/ml sodium citrate, 0.1% Triton X-100 and 0.05 mg/ml propidium iodide, and stored overnight at 4 °C. Stained cells were analysed using a Becton Dickinson FacsScan (Franklin Lakes, NJ, USA) following manufacturer's instructions. The fraction of G0/G1, S, G2/M and sub-G1 cells in the populations was determined using CellQuest 3.2 software (Becton Dickinson Flowcytometry System).

2.5. Immunoblotting

Neuroblastoma cells were treated with VPA or etoposide for various time periods and lysed with Laemmli buffer.[25] Equal amounts of protein were subjected to SDS polyacrylamide gel electrophoresis, followed by western blotting using antibodies against topoisomerase II (Cell Signaling Technologies, Danvers, MA), topoisomerase II-beta (Abcam, Cambridge, MA), total histone H3 and acetyl-histone H3 and H4 (Millipore, Waltham, MA), cleaved poly-ADP ribose polymerase (PARP) (BD Pharmingen, San Jose, CA), p21 (Cell Signaling) and actin

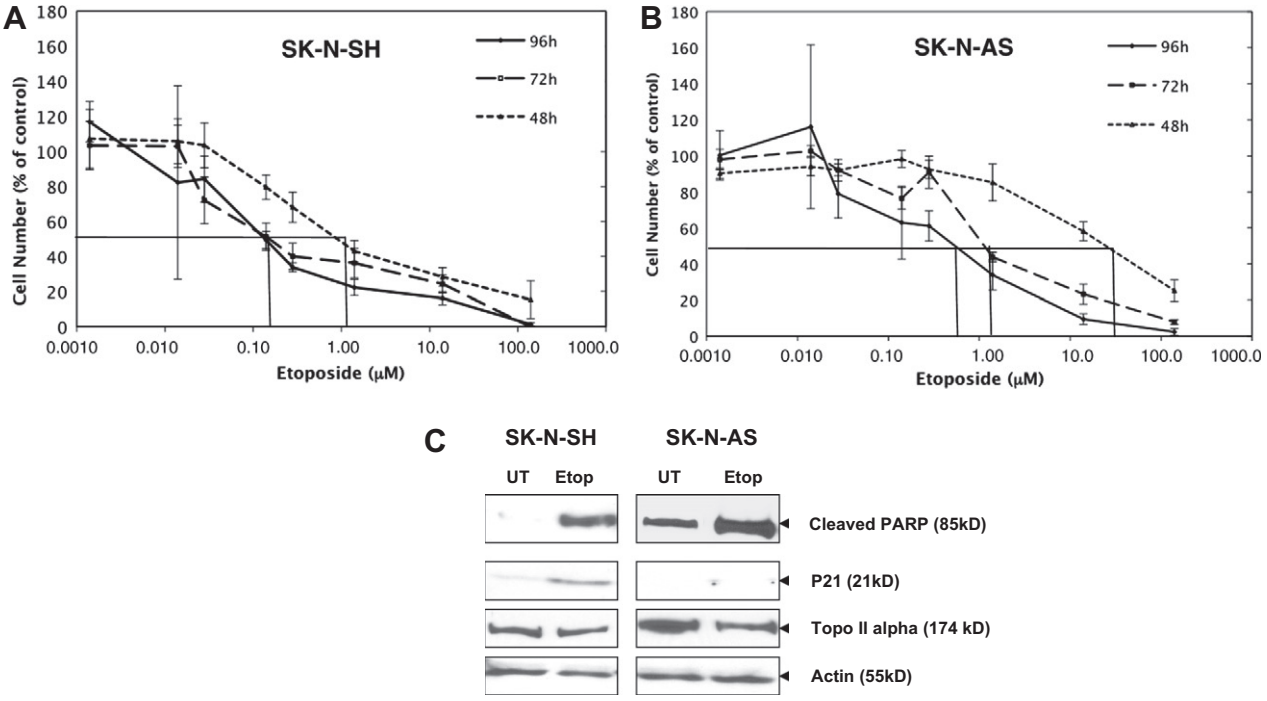

Fig. 1 – Sensitivity of neuroblastoma tumour cells to etoposide. (A) SK-N-SH and (B) SK-N-AS neuroblastoma tumour cells were exposed to etoposide at varying concentrations for 48, 72 and 96 h each. Cytotoxicity was determined by MTT assays. Cell numbers are represented as percentages normalised to untreated control cells. IC50 values are indicated by vertical lines. (C) SK-N-SH (left) and SK-N-AS (right) neuroblastoma tumour cells were treated with 0.1 μM etoposide (Etop) for 72 h or left untreated (UT), and cell lysates were collected and analysed by western blotting for cleaved PARP, p21, topoisomerase II-alpha and actin.

(Cell Signaling). Following incubation with HRP-conjugated secondary antibodies, the immunocomplexes were visualised using enhanced chemiluminescence assays (Pierce, Holmdel, NJ, USA).

2.6. *Chromatin immunoprecipitation (ChIP) assays*

SK-N-AS and SK-N-SH cells were treated with VPA (1.5 mM) for 72 h and analysed for changes in the acetylation of chromatin-associated histone H3 and H4 using antibodies specific to acetylated histone H3 and H4 (Millipore) or control IgG as previously described.[26] Immunoprecipitated DNA was purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA) following manufacturer's instructions and analysed by SYBR GREEN quantitative PCR analyses using primers specific for the transcription start site and 0.5 kb upstream of the start site in the human topoisomerase II-alpha and -beta gene promoters. Significance in DNA pull-down between VPA-treated and untreated samples was calculated using Statistica 6.0 software (Statsoft, Tulsa, OK).

3. Results

3.1. *Effects of etoposide on human neuroblastoma cells*

The effects of etoposide on SK-N-SH and SK-N-AS human neuroblastoma tumour cells were determined by treating the cells with different concentrations of etoposide for various time periods. IC50 values for SK-N-SH cells treated with etoposide ranged from 0.3 to 1 μM at 96, 72 and 48 h (Fig. 1A and Table 1). SK-N-AS cells were much less sensitive to etoposide, with IC50 values between 0.6 and 80 μM at 96, 72 and 48 h (Fig. 1B and Table 1).

To further investigate the mechanism underlying the response of neuroblastoma cells to etoposide, they were treated with 0.1 μM etoposide for 72 h and whole cell extracts were analysed by western blotting for changes in the levels of the 85 kD cleaved form of poly-ADP ribose polymerase (PARP), p21 and topoisomerase II-alpha. As expected, etoposide treatment resulted in an elevation in the 85 kD cleaved PARP band (Fig. 1C), suggesting that neuroblastoma cells underwent apoptosis in response to etoposide. However, while etoposide treatment resulted in an increase in p21 levels in SK-N-SH cells, a similar change in p21 expression in SK-N-AS cells

Table 1 – IC50 values for neuroblastoma cells treated with etoposide.

Cell line	Time (h)	IC50 (μM)
SK-N-SH	48	1
	72	0.5
	96	0.3
SK-N-AS	48	80
	72	1.8
	96	0.6

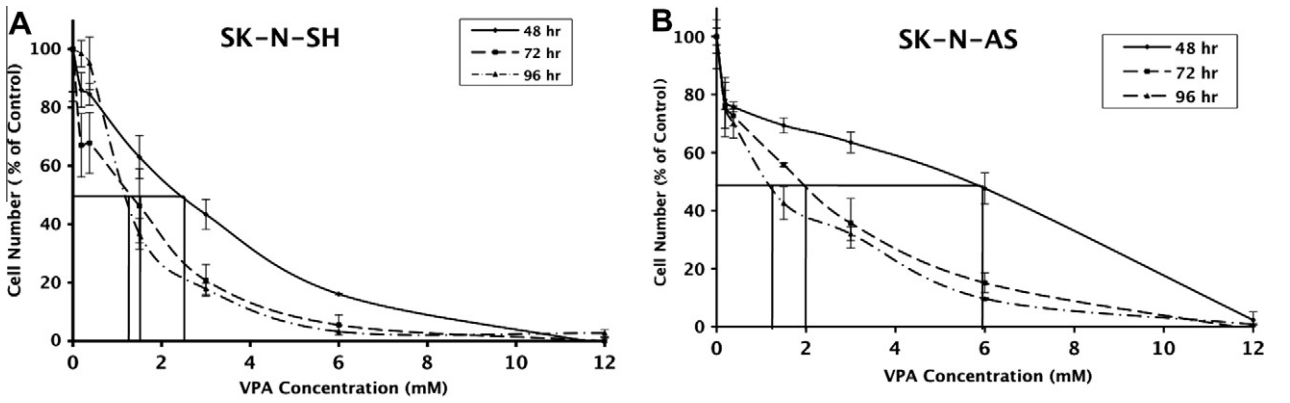

Fig. 2 – Sensitivity of neuroblastoma tumour cells to valproic acid. (A) SK-N-SH and (B) SK-N-AS neuroblastoma cells were exposed to valproic acid at varying concentrations for 48, 72 and 96 h each. Cytotoxicity was determined by MTT assays. Cell numbers are represented as percentages normalised to untreated control cells. IC50 values are indicated by vertical lines.

was not observed. Topoisomerase II-alpha levels remained essentially unchanged in both cell lines, whereas topoisomerase II-beta was not detected in either cell line (Fig. 1C and data not shown).

3.2. *Effects of valproic acid and etoposide on human neuroblastoma cells*

Since HDACIs are known to modulate gene expression and alter tumour cell response to chemotherapy, we evaluated the ability of VPA to change the response of SK-N-SH and SK-N-AS neuroblastoma cells to etoposide. SK-N-SH and SK-N-AS cells were treated with various concentrations of valproic acid for 48, 72 and 96 h time periods and the effects on cell growth were assessed by MTT assays. IC50 values for SK-N-SH cells treated with valproic acid ranged between 1 and 2.5 mM at 96, 72 and 48 h (Fig. 2A and Table 2). SK-N-AS cells were less sensitive to valproic acid, with IC50 values between 1.2 and 6.0 mM at 96, 72 and 48 h (Fig. 2B and Table 2).

We next asked if treatment with VPA altered the response of SK-N-SH and SK-N-AS tumour cells to etoposide. Cells were treated with 1.5 mM VPA and etoposide ranging in concentration from 0.001 μM to 100 μM for 48, 72 and 96 h. IC50 values for these cell lines in the presence of etoposide alone or etoposide and VPA together are shown in Tables 3A and C. We observed that VPA treatment caused a 8-fold increase and 10-fold decrease in IC50 values for SK-N-SH and SK-N-AS cells respectively at 48 h. The change in IC50 value upon co-treatment with VPA at 72 h was less than 2-fold for both cells, while VPA co-treatment for 96 h promoted a 3-fold and 10-fold decrease in IC50 values for SK-N-SH and SK-N-AS cells, respectively. These results suggest that while VPA antagonised the effects of etoposide on SK-N-SH cells at early time points, it sensitised both cells to etoposide at longer time-periods of incubation, although the extent of sensitisation was more significant for SK-N-AS cells (10-fold) at 96 h (see Fig. 3 and Table 3D).

To determine if the observed effects were synergistic, we applied the fractional product method of Webb as described previously.[25] This method uses the formula: $\gamma_{12} \leqslant$ or $\geqslant \gamma_1\gamma_2$, where γ_{12} is the fraction of cell surviving treatment with both drugs, γ_1 and γ_2 are the fraction of cells surviving treatment with VPA and etoposide alone, respectively, and $\gamma_1\gamma_2$ is the predicted survival in the presence of both drugs. Cell killing is considered synergistic if the predicted survival ($\gamma_1\gamma_2$) is greater than the observed survival γ_{12}, additive when $\gamma_1\gamma_2 = \gamma_{12}$, and antagonistic if $\gamma_1\gamma_2$ is less than γ_{12}. As seen in Table 3B, the predicted survival ($\gamma_1\gamma_2$) for SK-N-SH cells are 0.45, 0.31 and 0.24 for the combination of drugs at 48, 72 and 96 h which is less than the observed survival (γ_{12}) of 0.67, 0.38 and 0.27 at these time points. These numbers suggest clear antagonism at 48 h and an additive effect at 72 and 96 h for SK-N-SH cells. In contrast, the predicted survival ($\gamma_1\gamma_2$) of 0.6, 0.48 and 0.54 at 48, 72 and 96 h of treatment with both drugs for SK-N-AS cells was equal to or greater than the observed survival (0.59, 0.41 and 0.27) at these time points. These values indicate an additive effect at 48 h and modest to strong synergy at 72 and 96 h, respectively. Together, these data suggest that VPA caused a more significant decrease in the survival of SK-N-AS cells compared to SK-N-SH cells.

Table 2 – IC50 values for neuroblastoma cells treated with VPA.

Cell line	Time (h)	IC50 (mM)
SK-N-SH	48	2.5
	72	1.5
	96	1.0
SK-N-AS	48	6.0
	72	2.0
	96	1.25

Table 3A – SK-N-SH cells: IC50 values for etoposide treatment in the presence and absence of VPA.

Time (h)	–VPA (μM)	+VPA (μM)
48	1.0	8.0
72	0.12	0.075
96	0.12	0.045

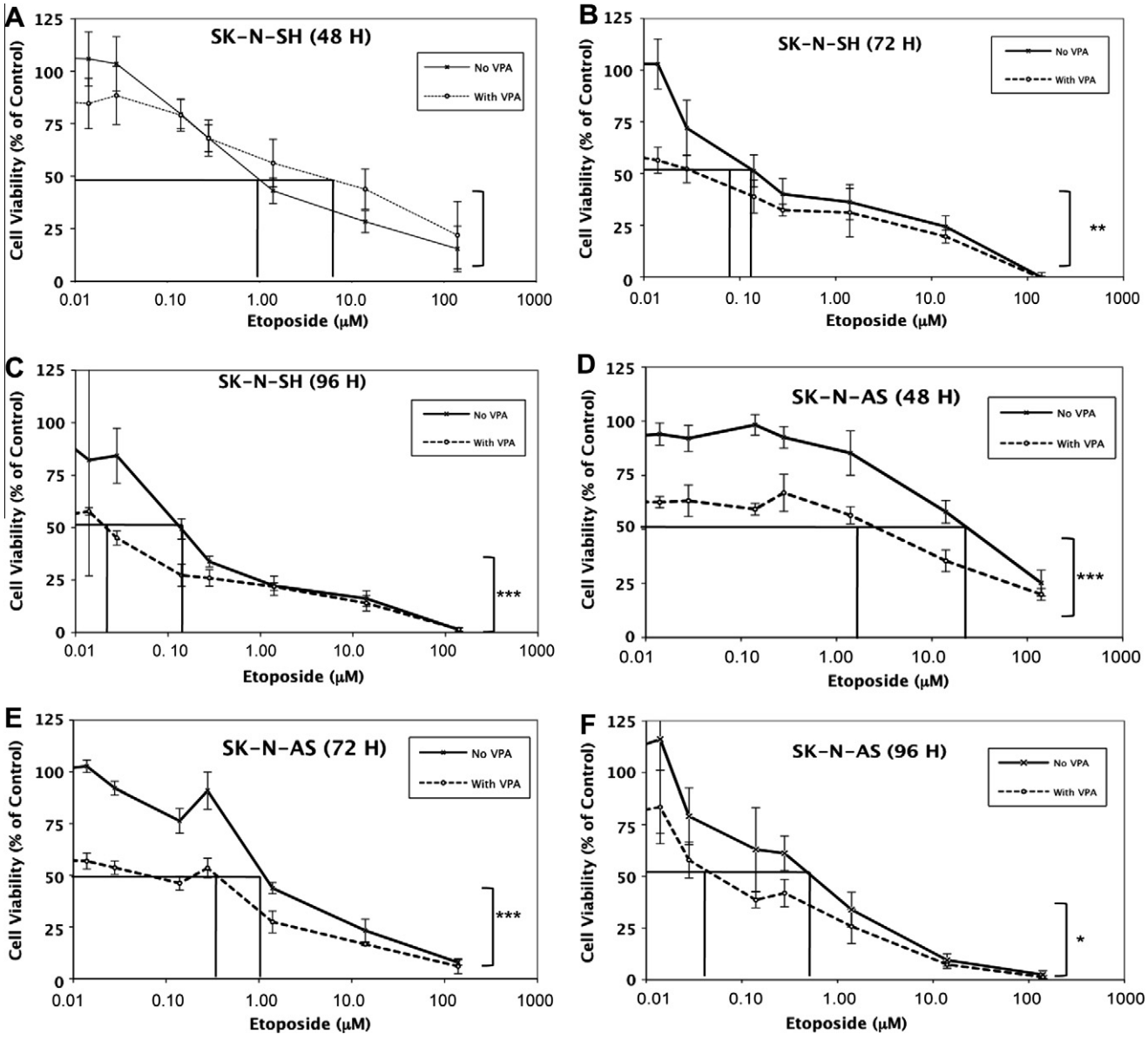

Fig. 3 – Sensitivity of neuroblastoma tumour cells to valproic acid and etoposide. SK-N-SH (A–C) and SK-N-AS (D–F) neuroblastoma tumour cells were exposed to 1.5 mM valproic acid and varying concentrations of etoposide for 48 (A, D), 72 (B, E) and 96 (C, F) h. Cytotoxicity was determined by MTT assays and cell numbers represented as percentages normalised to untreated control cells. IC50 values are indicated by vertical lines. The effect of drug synergy across a range of concentrations was assessed using Two-way Repeated Measures ANOVA. *P* values of 0.0003 (*), 0.0001 (**), or less than 0.0001 (***) are indicated.

Table 3B – Synergy calculations for SK-N-SH cells.

Time (h)	Survival (1.5 mM VPA) (γ_1)	Survival (1 μM Etop) (γ_2)	Predicted survival (VPA + Etop) ($\gamma_1\gamma_2$)	Observed survival (VPA + Etop) (γ_{12})
48	0.90	0.50	0.45	0.67
72	0.62	0.51	0.31	0.38
96	0.51	0.49	0.24	0.27

Additive: $\gamma_1\gamma_2 = \gamma_{12}$.
Synergy: $\gamma_1\gamma_2 \geqslant \gamma_{12}$.
Antagonism: $\gamma_1\gamma_2 \leqslant \gamma_{12}$.

Table 3C – SK-N-AS cells: IC50 values for etoposide treatment in the presence and absence of VPA.

Time (h)	–VPA (μM)	+VPA (μM)
48	13	1.25
72	1.0	0.6
96	0.7	0.07

Table 3D – Synergy calculations for SK-N-AS cells.

Time (h)	Survival (VPA) (γ_1)	Survival (Etop) (γ_2)	Predicted survival (VPA + Etop) ($\gamma_1\gamma_2$)	Observed survival (VPA + Etop) (γ_{12})
48	0.62	0.98	0.60	0.59
72	0.58	0.82	0.48	0.41
96	0.83	0.66	0.54	0.27

Additive: $\gamma_1\gamma_2 = \gamma_{12}$.
Synergy: $\gamma_1\gamma_2 \geqslant \gamma_{12}$.
Antagonism: $\gamma_1\gamma_2 \leqslant \gamma_{12}$.

Since maximal synergy in SK-N-AS cells was observed at 96 h, we performed our analyses described below after 72 h of treatment with VPA and etoposide so as to detect molecular events preceding maximal cell-death. To determine whether the reduction in cell growth after treatment with VPA and etoposide resulted from induction of apoptosis, we evaluated treated cells for the cleavage of PARP. As shown in Fig. 4, the 85 kD cleaved PARP band did not significantly increase after treatment with both agents compared to etoposide alone in SK-N-SH cells. However, a substantial enhancement in PARP cleavage was observed in SK-N-AS cells in the

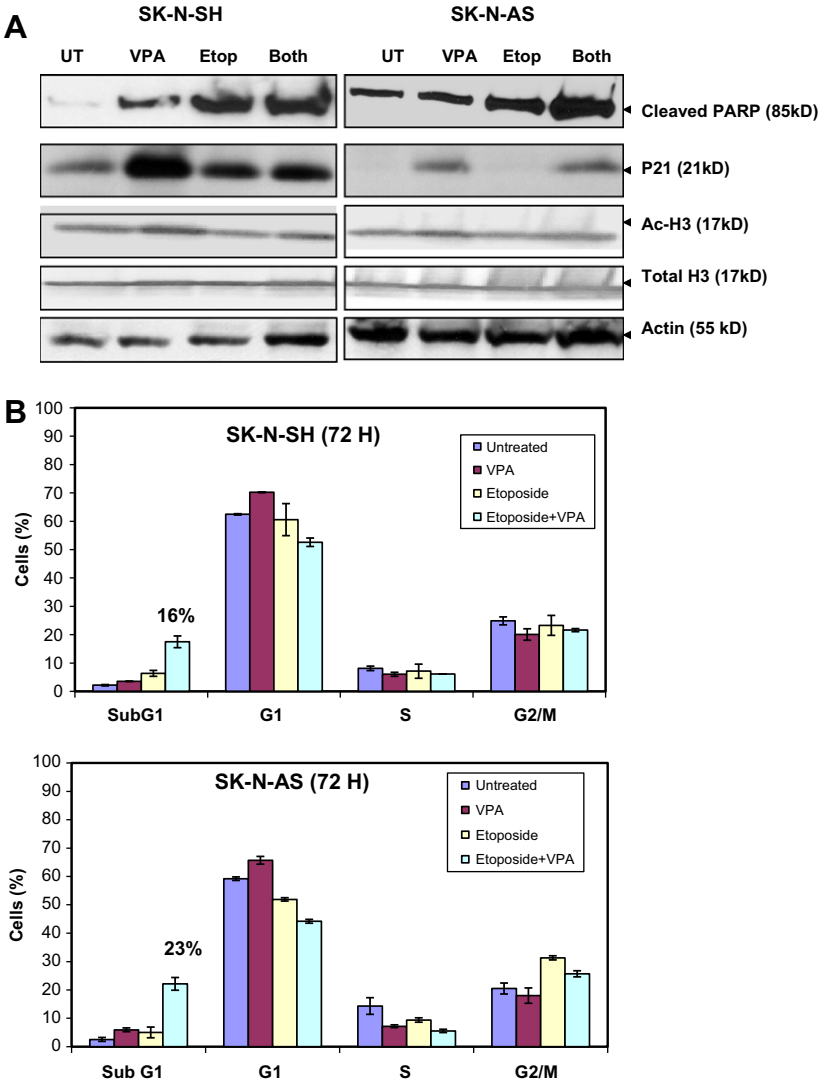

Fig. 4 – Measurement of apoptosis in the presence of VPA and etoposide. (A) SK-N-SH (left) and SK-N-AS (right) neuroblastoma tumour cells were treated with 1.5 mM VPA, 0.1 μM etoposide (Etop) or both VPA and etoposide (Both) for 72 h. Cell lysates were collected and studied by western blotting for cleaved PARP, p21, acetylated and total histone H3 and actin. (B) SK-N-SH (top) and SK-N-AS (bottom) cells were treated with 1 mM VPA, 0.1 μM etoposide or both VPA and etoposide for 72 h and analysed for cell cycle progression by flow cytometry.

presence of VPA and etoposide compared to either drug alone. Western blot analyses also revealed an increase in the acetylation of histone H3 and expression of p21 in the presence of VPA, while total histone H3 and actin levels remained constant under all conditions (Fig. 4A). Furthermore, cell cycle analysis demonstrated the treatment of SK-N-SH and SK-N-AS cells with VPA and etoposide resulted in an increase in the percentage of cells in the sub-G1 population (16% and 25%, respectively, at 72 h) (Fig. 4B).

3.3. *Effects of valproic acid on topoisomerase-II expression*

Etoposide is an inhibitor of topoisomerase II function, and increased levels of topoisomerase II have been associated with response to etoposide in neural tumour cells.[5] We therefore explored the effects of valproic acid on topoisomerase II expression levels in neuroblastoma tumour cells. SK-N-SH and SK-N-AS cells were treated with 1.5 mM VPA for 72 h and whole cell extracts were studied by western blotting for changes in the levels of topoisomerase II-alpha and -beta proteins. Topisomerase II-alpha was detected in both SK-N-SH and SK-N-AS cells and its levels were unaffected by VPA treatment. While topoisomerase-II beta was not detected in western blots of whole cell extracts in either cell type, nuclear preparations revealed low but detectable levels of the protein in SK-N-SH cells but not in SK-N-AS cells (Fig. 5A and B). VPA treatment caused perhaps a 2-fold upregulation of topoisomerase-II beta protein expression in SK-N-SH cells, whereas

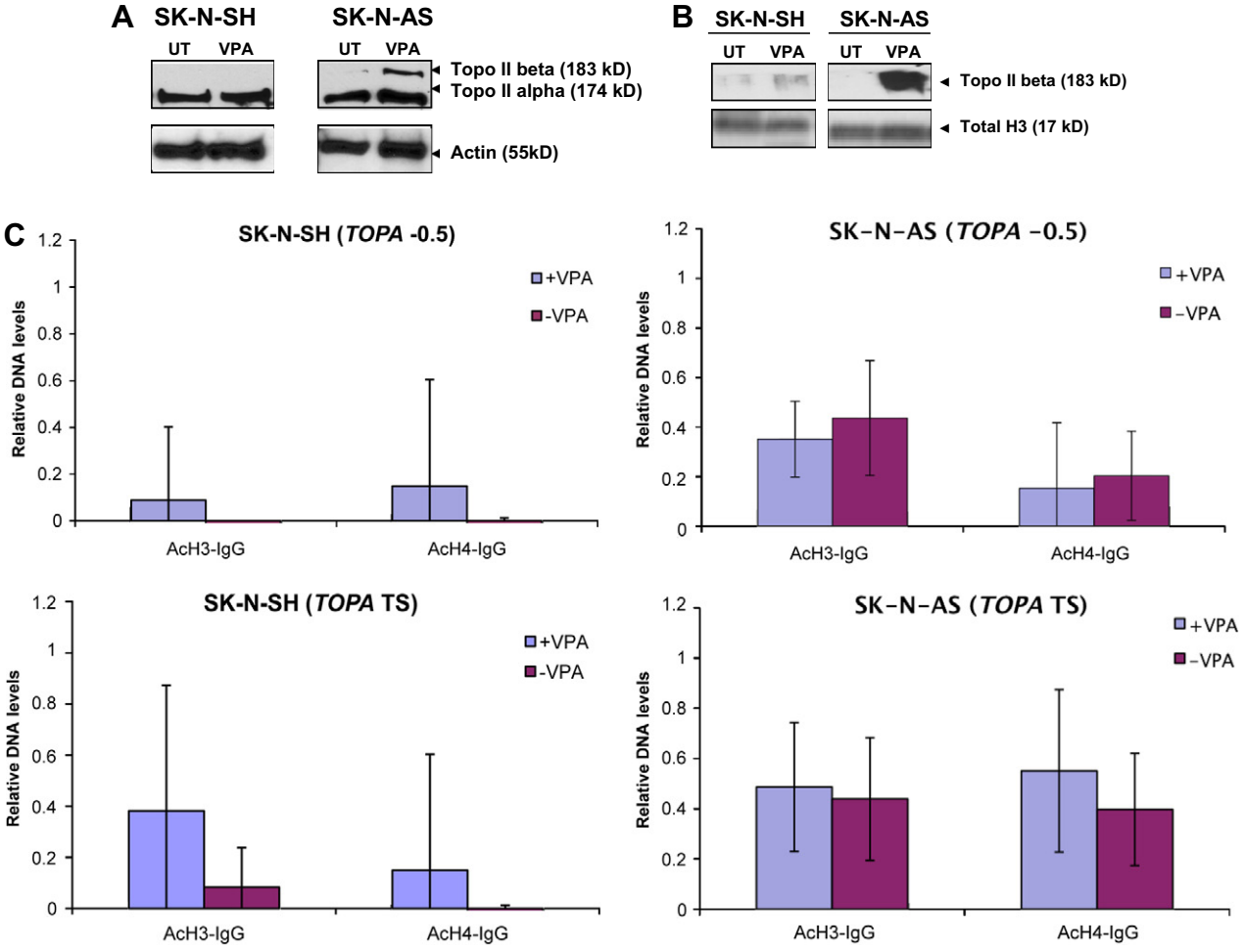

Fig. 5 – Valproic acid remodels chromatin and increases topoisomerase-II beta expression. (A) SK-N-SH (left) and SK-N-AS (right) neuroblastoma cells were treated with 1.5 mM VPA for 72 h and cell lysates were collected and analysed for topoisomerase II-alpha and -beta and actin. (B) SK-N-SH (left) and SK-N-AS (right) cells were treated with 1 mM VPA for 48 h, nuclear lysates were prepared and analysed for topoisomerase II-beta protein and total histone H3 (loading control). (C, D) Chromatin immunoprecipitation assay to study changes in the acetylation of histones H3 and H4 within the *Top2A* and *Top2B* promoters. SK-N-SH (left panels) and SK-N-AS (right panels) neuroblastoma cells were treated with 1 mM VPA for 48 h (+VPA) or left untreated (–VPA) and subjected to chromatin immunoprecipitation using anti-acetyl-histone H3 or H4 antibodies or control non-immune sera (IgG). SYBR GREEN PCR amplification of the transcription start site (TS) and 500 bp upstream (–0.5) of the *Top2A* (C) and *Top2B* (D) promoters was performed using primers flanking these regions. Relative binding of acetylated histones H3 and H4 to these regions was calculated by subtracting the signal obtained with control IgG from that obtained with specific antibodies following normalisation to input DNA. Significance was calculated using Statistica 6.0 software.

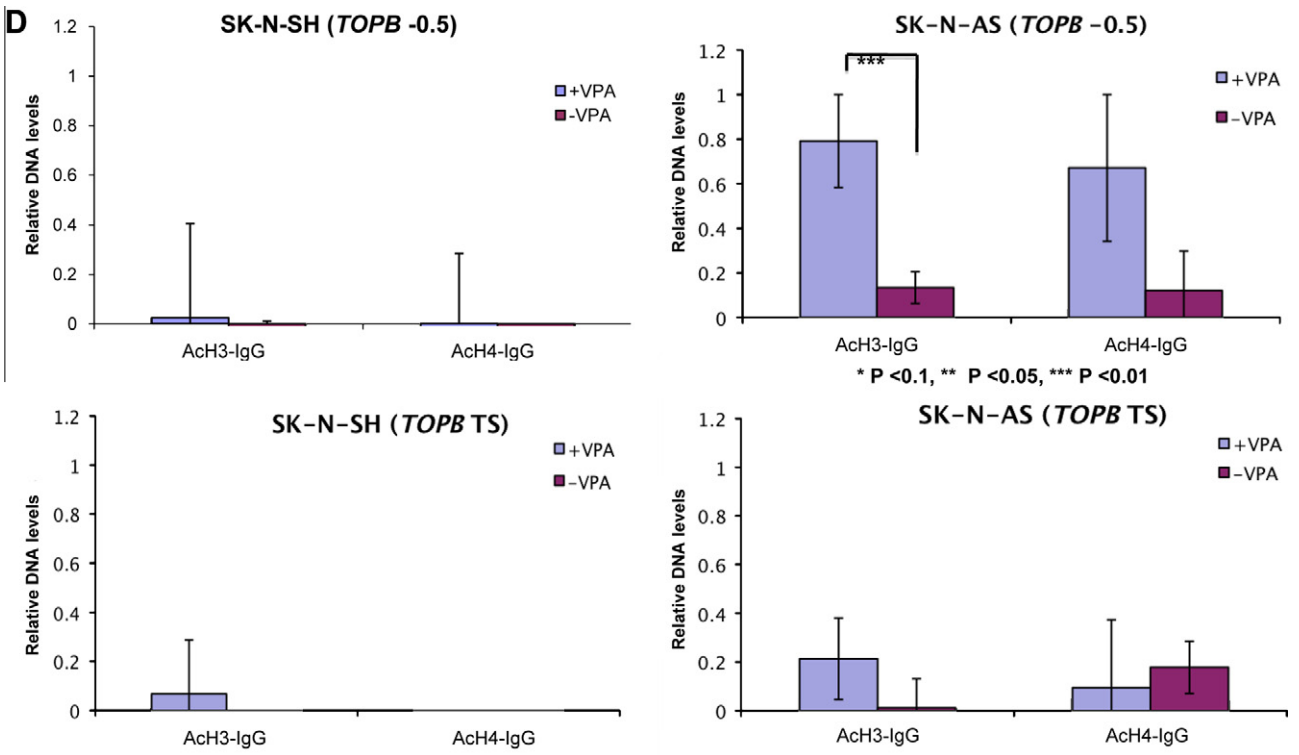

Fig 5. *(continued)*

a substantial increase in its levels were observed in SK-N-AS cells that was detectable in both whole cell and nuclear extracts (Fig. 5A and B).

To ask if VPA modulated topoisomerase II-beta expression through increased histone H3 and H4 acetylation and chromatin remodelling at the cognate promoter, we performed ChIP assays with untreated or VPA-treated SK-N-AS and SK-N-SH cells using anti-acetyl-histone H3 and H4 antibodies or control non-immune sera. The immunoprecipitated material was analysed by SYBR Green -PCR using primers specific to the transcription start site (TS) and 500 bp upstream of the TS (−0.5 kb) for each gene. The levels of DNA pulled down in each case were first normalised to input DNA. Then, levels of DNA associated with non-immune sera were subtracted from that associated with antibodies against acetylated histones H3 and H4 and plotted as relative DNA levels. VPA treatment produced no significant change in the levels of acetylated histones H3 and H4 at the TS or −0.5 kb region of the topoisomerase II-alpha gene promoter and the TS of topoisomerase II-beta gene promoter in both SK-N-SH or SK-N-AS cells (Fig. 5C and D). In contrast, a significant increase in the levels of acetylaed histones H3 but not H4 at the −0.5 kb region of topoisomerase II-beta gene promoter was observed in SK-N-AS (Fig. 5D). Similar changes were not observed in the topoisomerase II-beta gene promoter in SK-N-SH cells (Fig. 5D). These results suggest that VPA may potentiate the effect of etoposide in SK-N-AS cells through histone deacetylation at the topoisomerase II-beta gene promoter and subsequent upregulation of topoisomerase II-beta expression.

4. Discussion

Children with disseminated, high-risk neuroblastoma have very poor long-term outcomes, despite intensive multimodal treatment strategies.[1] The poor outcomes in response to current treatment strategies clearly indicate a need for novel therapies in order to improve long-term survival in children with neuroblastoma. A number of epigenetic changes in gene expression are associated with poor overall outcomes in children with neuroblastoma,[9,13] raising the possibility that reversal of these changes through epigenetic modifying agents/drugs may be effective for neuroblastoma therapy.

Acetylation of specific lysine residues on chromatin-associated histones H3 and H4 decreases chromatin compaction, making DNA more readily available to DNA damaging agents such as etoposide and modulating the expression of genes that can induce apoptosis in tumour cells. For example, the HDACIs VPA, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) reactivate the expression of aberrantly silenced genes involved in cell cycle control and apoptosis and exhibit anti-tumour activity in neuroblastoma preclinical models.[13–15]

In the present study we observed that SK-N-SH cells were more sensitive to treatment with VPA and etoposide alone compared to SK-N-AS cells. Although the combination of drugs modestly enhanced cell-death in SK-N-SH cells at longer incubation periods (72 and 96 h), it caused a decline in etoposide-mediated cell killing at shorter exposure times (48 h). The reason for this antagonistic effect is not clear at this time. In contrast, VPA synergistically increased the cytotoxicity of

etoposide in SK-N-AS cells *in vitro*. These findings are consistent with our prior work demonstrating enhanced etoposide-mediated cytotoxicity when combined with VPA against glioma cells *in vitro*,[25] and with other studies demonstrating increased sensitivity to etoposide in leukaemia cells treated with the HDACIs TSA or sodium butyrate.[27,28] SAHA has also been shown to potentiate DNA damage by etoposide in breast cancer lines in a synergistic manner, although this synergy was dependent upon the sequence of drug administration.[29] The HDACI, depsipeptide, was shown to sensitise neuroblastoma tumour cells to etoposide by Keshelava and colleagues, although the mechanism of sensitisation was not investigated.[30] Interestingly, the synergistic increase in cell-death of SK-N-AS cells was associated with a strong elevation in topoisomerase II beta expression in these cells. SK-N-SH cells expressed low basal levels of topoisomerase-II beta, which may account for its increased sensitivity to etoposide compared to SK-N-AS cells. The modest increase in topoisomerase-II beta expression upon VPA treatment may potentially explain the additive increase in cell-death in SK-N-SH cells in the presence of both agents.

Our demonstration that VPA promotes chromatin remodelling at the topoisomerase II-beta promoter is novel. Most studies to date have attributed genetic changes in the topoisomerase II-alpha gene as the reason for altered sensitivity of tumour cells to etoposide,[31,32] with limited evaluation of the role of topoisomerase II-beta. Our demonstration of HDACI-mediated elevation of topoisomerase II-beta levels agrees with previously described results in other model systems.[25,33] The increase in topoisomerase II-beta may enhance the sensitivity of SK-N-AS neuroblastoma cells to etoposide-mediated cytotoxicity as a result of increased target availability. Although elevated levels of topoisomerase II-beta protein may contribute to increased repair of etoposide-induced DNA damage, this seems unlikely in our studies since the upregulation in topoisomerase II-beta levels is associated with increased apoptosis in SK-N-AS cells. The observation that chromatin remodelling occurs at a region 500 base-pairs upstream of the transcription start site of the topoisomerase II beta promoter is intriguing and raises the possibility that VPA treatment may facilitate the binding of transcriptional activators to this region. In support of this possibility, a previous study attributed maximal (80%) promoter activity to the region between 500 and 481 base-pairs upstream of the transcription start site. Importantly, this region houses binding sites for the transcription factors nuclear factor-Y (NF-Y) and SP1, proteins known to be important for the transcriptional control of *Top2B* gene expression.[34] Further, the absence of chromatin remodelling at the transcription start site also supports this possibility and suggests that elevation in topoisomerase-II beta expression probably may not occur through enhanced RNA polymerase loading but may be the result of increased recruitment of co-activators such as NF-Y and SP1. Additional studies to determine changes in binding of these transcription factors to the *Top2B* promoter may yield more mechanistic information.

In summary, the combination of VPA and etoposide has anti-tumour effects in neuroblastoma tumour cells *in vitro*. Our observations suggest a potential epigenetic mechanism for modulation of topoisomerase II-beta gene expression and may explain the relative absence of mutations in this gene in human tumours. Our findings also raise the possibility of future studies evaluating the combination of VPA and etoposide and of HDACIs and topoisomerase-II inhibitors in general in the treatment of children with neuroblastoma.

Conflict of interest statement

None declared.

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