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DNA demethylation increases sensitivity of neuroblastoma cells to chemotherapeutic drugs

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ABSTRACT

Neuroblastoma is a common embryonal malignancy in which high-stage cases have a poor prognosis, often associated with resistance to chemotherapeutic drugs. DNA methylation alterations are frequent in neuroblastoma and can modulate sensitivity to chemotherapeutic drugs in other cancers, suggesting that manipulation of epigenetic modifications could provide novel treatment strategies for neuroblastoma. We evaluated neuroblastoma cell lines for DNA demethylation induced by 5-Aza-2′-deoxycytidine, using genome-wide and gene-specific assays. Cytotoxic effects of chemotherapeutic agents (cisplatin, doxorubicin and etoposide), with and without 5-Aza-2′-deoxycytidine, were determined by morphological and biochemical apoptosis assays. We observed that the extent of genome-wide DNA demethylation induced by 5-Aza-2′-deoxycytidine varied between cell lines and was associated with expression differences of genes involved in the uptake and metabolism of 5-Aza-2′-deoxycytidine. Treatment of neuroblastoma cells with a combination of chemotherapeutic drugs and 5-Aza-2′-deoxycytidine significantly increased the levels of apoptosis induced by cisplatin, doxorubicin and etoposide, compared to treatment with chemotherapeutic drugs alone. The variable demethylation of cell lines in response to 5-Aza-2′-deoxycytidine suggests that epigenetic modifiers need to be targeted to suitably susceptible tumours for maximum therapeutic benefit. Epigenetic modifiers, such as 5-Aza-2′-deoxycytidine, could be used in combination with chemotherapeutic drugs to enhance their cytotoxicity, providing more effective treatment options for chemoresistant neuroblastomas.

1. Introduction

Neuroblastoma (NBL) is one of the commonest solid childhood cancers, which arises from neural crest cells of the sympathetic nervous system and causes about 15% of all paediatric oncology deaths [1]. NBLs diagnosed antenatally or in the newborn period have a good prognosis, unlike in older children, who have a poor outcome [2].

High-risk tumours with disseminated disease (stages 3–4) often bear *MYCN* amplification [3] and are mostly fatal [4]. Other genetic alterations in NBL include loss of chromosome 1p, loss of 11q and gain of 17q, which are independent of *MYCN* status [1,2]. Genes found mutated in NBL include the tumour suppressors *PHOX2B*, mutated in a few cases of inherited NBL [5], *NF1*, mutated in 6% of primary NBLs [6] and p53, which is mutated in 2% of NBLs, however other functional defects in the p53 pathway such as *MDM2* amplification are found in NBL [7]. The proto-oncogene *ALK* is frequently mutated in familial NBL [8] and in about 10% of sporadic cases, where it is associated with poor prognosis [9].

In addition to genetic abnormalities, epigenetic deregulation plays an important role in NBL pathogenesis, including aberrant promoter DNA hypermethylation of tumour suppressor genes such as *RASSF1A*, *CASP8* and *DCR2* [10–13]. DNA hypermethylation of individual genes e.g. *CASP8* may be associated with poor outcome in NBL [11,14,15] but methylation at multiple CpG islands may be more closely associated with poor prognosis [16]. A recent genome-wide analysis of DNA methylation in NBL has identified large-scale genomic alterations [17], as we have described in Wilms' tumour [18], suggesting that both large-scale and gene-specific epigenetic changes contribute to the pathogenesis of embryonal tumours.

One important phenotypic consequence of DNA hypermethylation events may be resistance to chemotherapeutic drugs [19], leading to treatment failure as observed in relapsed NBLs [20,21], in which apoptosis genes may be epigenetically silenced [22]. Inhibitors of DNA methyltransferases, such as 5-azacytidine and 5-Aza-2′-deoxycytidine (5-Aza-dC), have been used to successfully re-sensitise cancer cells to chemotherapeutic drugs [23–27],

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suggesting that this could be a beneficial strategy for therapy-resistant NBL. Clinical chemotherapy resistance in NBL has previously been shown to be reflected in neuroblastoma cell cultures [21], demonstrating that neuroblastoma cell lines provide relevant in vitro models for studying mechanisms of drug resistance and the re-establishment of drug sensitivity. We therefore asked whether inhibition of DNA methylation by 5-Aza-dC in human NBL cell lines could increase their sensitivity to clinically relevant cytotoxic drugs.

Here we show, for the first time, that pre-treatment of NBL cell lines with 5-Aza-dC significantly increases their sensitivity to cisplatin, etoposide and doxorubicin. Interestingly, NBL cell lines vary in their extent of DNA demethylation in response to 5-Aza-dC, associated with altered expression of transporters and enzymes involved in the metabolism of 5-Aza-dC. These results reveal a possible novel epigenetic strategy to fight high-stage, aggressive and chemoresistant NBLs, whilst highlighting the necessity to target treatment to those tumours that are most responsive to demethylating agents.

2. Materials and methods

2.1. Cell culture and treatments

The NBL cell lines BE(2)-M17, SK-N-AS and SHSY-5Y were from our collaborator Dr C. McConville, University of Birmingham, UK, who obtained them directly from ECACC (Porton Down, Salisbury, UK). All cell lines originated from patients that had undergone chemotherapy [28]. Simple tandem repeat (STR) fingerprints (D13S317, D16S539 and D5S818) were initially tested in the McConville lab and further verified at 2 loci (D16S539 and TH01) in the Brown lab. All STR results matched those in the Cell Line Integrated Molecular Authentication database (bioinformatics.ist-ge.it/clima/). Additionally, all lines were karyotyped in the McConville lab and assayed by qPCR for *MYCN* amplification in the Brown lab and results were completely consistent with published results.

NBL cell lines were cultured in DMEM/F12-HAM medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Prior to treatment, cells were seeded for 24 h at 10^5 cells per well in 6-well dishes. Cisplatin was obtained from Teva pharmaceuticals (Platosin®, Bucharest, Romania) at a stock concentration of 1 mg/ml. Doxorubicin, etoposide and 5-Aza-dC (5-Aza-2′-deoxycytidine) were purchased from Sigma and were at stock concentrations of 1 mM in H_2O, 50 mM in DMSO and 10 mM in DMSO respectively. Single drug treatments were for 24 h; combinatorial treatments were performed by pre-treatment with 5-Aza-dC at 2 μM for 3 days and then the chemotherapeutic drug was added on the fourth day for 24 h.

2.2. Morphological determination of apoptosis

1 μg/ml Hoechst 33342 (Sigma) was directly added to the cells and cultures were incubated for 10–15 min at 37 °C, then 1 μg/ml propidium iodide (Sigma) was added and cells were analysed on a fluorescence microscope for viable, apoptotic, necrotic and secondary necrotic cells by observing cell colour and nuclear morphology. Each field of view contained approximately a hundred cells that were counted by eye.

2.3. Statistical analysis

The Chi^2 test was used to determine whether there was a significant increase in apoptosis for the combination treatments (5-Aza-dC plus chemotherapeutic drugs) compared to separate treatments.

2.4. Whole genome DNA methylation analysis

Genomic DNA was extracted from cells with the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Methylation sensitive restriction analysis (MSRA) was used to investigate genomic DNA methylation in the cell lines. Briefly, 1 μg DNA was digested with either HpaII or MspI (New England Biolabs) and digests were run on an agarose gel [27]. Percentage methylation loss was determined using ImageJ software (rsbweb.nih.gov/ij/) by comparing the intensity of the high molecular weight band in the HpaII lane to the undigested control lane.

2.5. Methylation-specific PCR (MSP)

Bisulphite conversion of genomic DNA was performed with the MethylDetector kit (Active Motif) according to manufacturer's protocol. Bisulphite converted DNA was amplified with gene specific primers (Table 1) for M (methylated) and UM (unmethylated) DNA by end-point PCR, using HotStarTaq DNA Polymerase (Qiagen) according to manufacturer's protocol. PCR amplicons were then run on a non-denaturing polyacrylamide gel.

2.6. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted with the RNeasy elution kit (Qiagen) according to the manufacturer's protocol. RNA was DNase treated with the TURBO DNA-free kit (Applied Biosystems) and cDNA was synthesised using the Thermoscript RT-PCR system (Invitrogen). Gene-specific primers (Table 1) were used to measure mRNA levels with the SYBRGreen kit (Invitrogen) on an MX3000P real-time PCR machine (Stratagene). The amount of target gene was normalised to the endogenous level of *TBP*.

Table 1
Primers used in qRT-PCR and MSP. Sequences of primers used in PCR; M = MSP primers specific for methylated DNA, UM = MSP primers specific for unmethylated DNA.

Method	Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
qRT-PCR	*RASSF1A*	TGCGACCTCTGTGGCGACTTCAT	TAGTGGCAGGTGAACTTGCAATGCG
	DNMT1	TCAGCAAGATTGTGGTGGAG	CAAGTTGAGGCCAGAAGGAG
	DNMT3A	TGCCAAAACTGCAAGAACTG	CAGCAGATGGTGCAGTAGGA
	DNMT3B	TTTGGCCACCTTCAATAAGC	GGTCCTCCAATGAGTCTCCA
	TBP	GCCCGAAACGCCGAATAT	CCGTGGTTCGTGGCTCTCT
	CDA	TGCCCCTACAGTCACTTTCC	CGGGTAGCAGGCATTTTCTA
	DCK	TCTCCATCGAAGGGAACATC	TCAGGAACCACTTCCCAATC
	ENT1	TCTTCTTCATGGCTGCCTTT	CCTCAGCTGGCTTCACTTTC
	ENT2	TCCTCATGTCCATCGTGTGT	AGCTCAGCTTTGGTCTCCAG
MSP	*RASSF1A* (M)	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA
	RASSF1A (UM)	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA

2.7. Western blot analysis

The total population of cells was harvested by scraping them off in their medium and proteins were extracted with the M-PER® reagent (ThermoFisher) supplemented with complete mini inhibitors (Roche) and phosphatase inhibitors (Roche) according to manufacturer's protocol. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred by electroblotting onto PVDF membrane (Millipore). Primary antibodies were rabbit polyclonal against caspase 9 (cell signalling; 1:1000 dilution), mouse monoclonal against caspase 8 (cell signalling; 1:1000 dilution), rabbit polyclonal against PARP-1 (Santa Cruz Biotechnology; 1:2000 dilution) and mouse monoclonal against β-actin (Sigma; 1:20,000 dilution), followed by secondary HRP-conjugated anti-mouse or anti-rabbit IgG (Sigma; 1:5000 dilution). ECL+ (GE Healthcare) was used for detection and β-actin was used as a loading control.

3. Results

3.1. Sensitivity of NBL cells to cytotoxic chemotherapy

In order to examine the role of DNA hypermethylation in chemoresistance in NBL, we conducted our study on cell lines derived from aggressive, high-stage (4) NBL [28], combining the demethylating agent 5-Aza-dC with cytotoxic drugs currently in use clinically for the treatment of NBL [1]. First, the chemotherapeutic drugs doxorubicin, etoposide and cisplatin were tested at increasing concentrations on *MYCN* amplified BE(2)-M17 cells and *MYCN* non-amplified SK-N-AS and SHSY-5Y cells (Fig. 1). BE(2)-M17 and SK-N-AS cells were very resistant to both doxorubicin and etoposide; even at the highest concentrations of 1 μM doxorubicin, 96% BE(2)-M17 and 91% SK-N-AS cells remained viable and at 100 μM etoposide, 91% BE(2)-M17 and 89% SK-N-AS were viable. SHSY-5Y cells were more sensitive to these two drugs, with doxorubicin and etoposide decreasing the fraction of viable cells to 60% and 41% respectively at the highest doses (Fig. 1A and B). BE(2)-M17 cells were also the most resistant to cisplatin treatment, with SHSY-5Y cells showing the lowest cell viability after cisplatin treatment and SK-N-AS cells having an intermediate sensitivity (Fig. 1C).

Thus BE(2)-M17 cells were the most resistant to all three drug treatments and SHSY-5Y cells were the most sensitive (Fig. 1A–C).

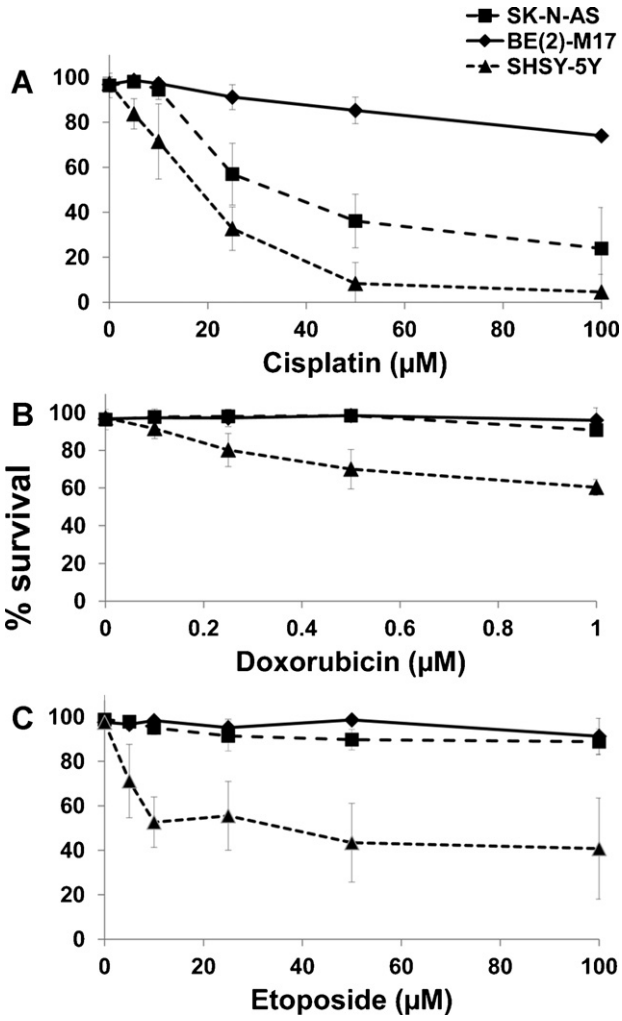

Fig. 1. Dose response curves for chemotherapeutic drug treatments of three different NBL cell lines. Each treatment lasted 24 h at the indicated concentrations. Graphs show the percentage of viable cells for each treatment as assessed by Hoechst/PI staining. Error bars indicate the standard deviation of three independent experiments, each performed in duplicate. (A) Cisplatin treatment, (B) doxorubicin treatment and (C) etoposide treatment.

3.2. DNA demethylation induced by 5-Aza-dC

We investigated the ability of 5-Aza-dC to induce DNA demethylation in the NBL cell lines, to determine which cells would be most suitable for testing the combinatorial effects of 5-Aza-dC and chemotherapeutic drugs (Fig. 2). Firstly, the whole genome methylation status of the cell lines was determined by methylation sensitive restriction analysis, in which methylated DNA is uncut by HpaII and shows as a high molecular weight band at the top of the gel, whilst unmethylated DNA is cut by HpaII, resulting in a long smear of smaller DNA fragments on the gel. MSRA revealed high genomic DNA methylation for all three cell lines (Fig. 2A). Interestingly, the most resistant cell lines to the three chemotherapeutic drugs, BE(2)-M17 and SK-N-AS, were the most sensitive to the DNA demethylating agent, losing 63 ± 4% and 70 ± 3%, respectively of their DNA methylation after 4 days of 5-Aza-dC treatment. In contrast, the most chemosensitive cell line (SHSY-5Y) showed less demethylation (45 ± 9%) (Fig. 2A).

Secondly, we examined the DNA methylation status of *RASSF1A* and *CASP8* to test the efficiency of gene-specific reactivation after treatment with 5-Aza-dC (Fig. 2B and C). *RASSF1A* and *CASP8* are tumour suppressor genes inactivated by promoter hypermethylation in most NBLs and NBL-derived cell lines [10]. Four days of treatment with 5-Aza-dC resulted in demethylation of the promoter of *RASSF1A* in both BE(2)-M17 and SK-N-AS cells (Fig. 2B), with an accompanying 71-fold increase in *RASSF1A* RNA expression in BE(2)-M17 cells and a 110-fold increase in SK-N-AS cells compared to untreated cells (Fig. 2C). The *CASP8* promoter did not demethylate as well as the *RASSF1A* and only resulted in a 4-fold expression increase in BE(2)-M17 cells, whilst SK-N-AS cells were not methylated at *CASP8* (Fig. 2B and C). These experiments show 5-Aza-dC treatment induces DNA demethylation in NBL cell lines but that the extent of demethylation varies between different cell lines and genes.

3.3. DNMT expression in NBL cell lines

To investigate whether the differences in demethylation induced by 5-Aza-dC could result from altered expression of DNA methyltransferases, *DNMT1*, *3A* and *3B* levels were assessed by real-time PCR (Fig. 3). Although BE(2)-M17, which showed extensive demethylation by 5-Aza-dC (Fig. 2A), expressed the highest levels of the three *DNMT* genes, SHSY-5Y and SK-N-AS cells, which had divergent 5-Aza-dC-induced demethylation (Fig. 2A), both had similar, lower mRNA levels for the three DNMT enzymes

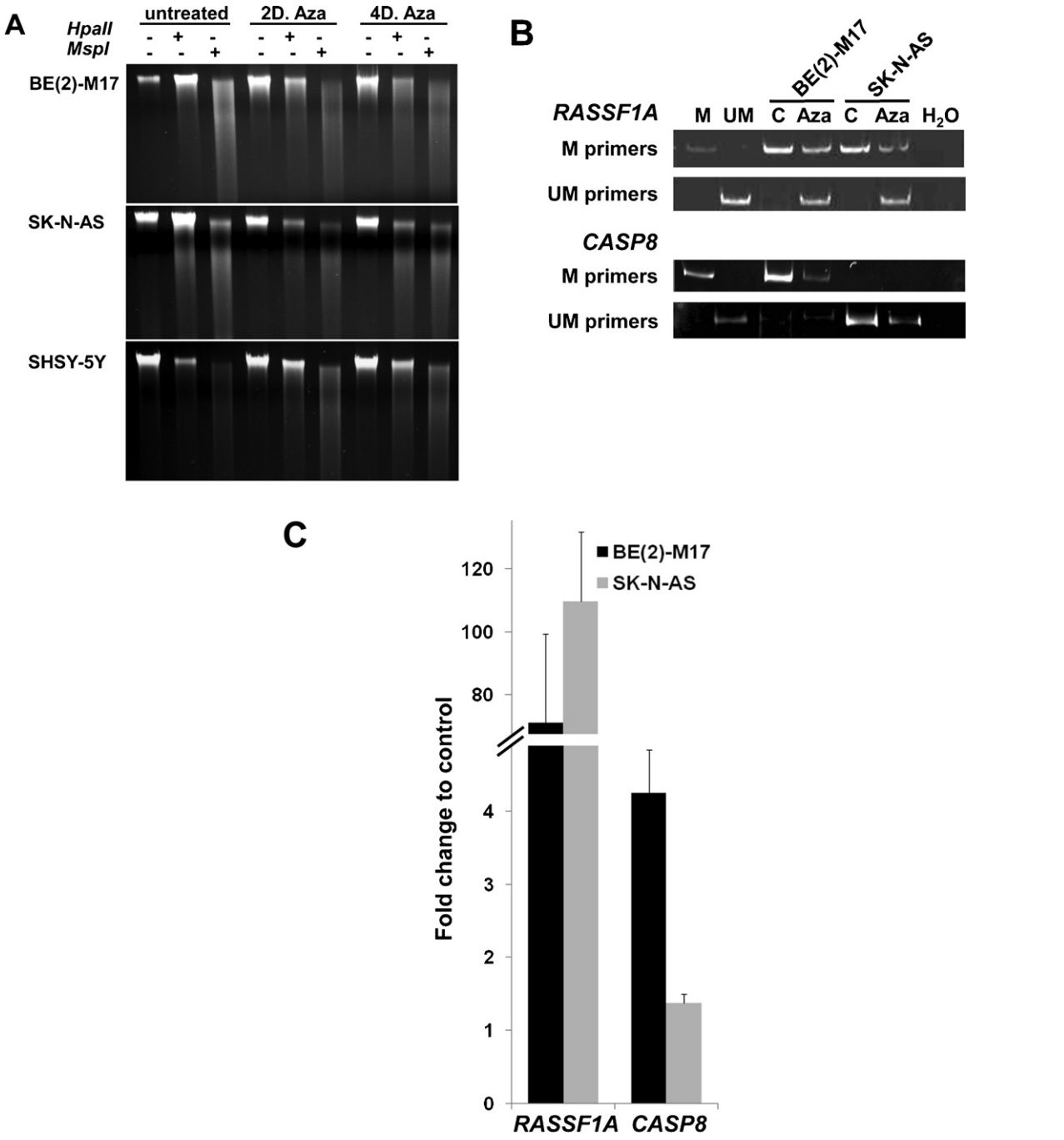

Fig. 2. Demethylation induced by 5-Aza-dC. (A) Methylation sensitive restriction analysis (MSRA) showing the whole genome methylation in NBL cell lines. Cells were treated with 5-Aza-dC at 2 μM for 2 and 4 days (D.) with daily medium changes. Undigested genomic DNA and DNA digested with *HpaII* or *MspI* from untreated and treated cells was separated on an agarose gel. *HpaII* digests only unmethylated sequences; therefore methylated DNA remains at the top of the gel whereas unmethylated DNA results in a smear. *MspI* serves as a control enzyme, digesting both methylated and unmethylated DNA. (B and C) *RASSF1A* and *CASP8* methylation and expression after 5-Aza-dC treatment. SK-N-AS and BE(2)-M17 cells were treated for four days with 2 μM 5-Aza-dC. (B) *RASSF1A* and *CASP8* methylation analysed by MSP. UM; unmethylated DNA, M; fully methylated DNA, M primers; primers specific for methylated DNA, UM primers; primers specific for unmethylated DNA, C; untreated controls, Aza; 5-Aza-dC treated. (C) RASSF1A and CASP8 expression as assessed by qRT-PCR, relative to the control sample at 4 days after 5-Aza-dC treatment.

(Fig. 3). Thus there did not appear to be a direct relationship between *DNMT* expression and sensitivity to 5-Aza-dC.

3.4. 5-Aza-dC induced demethylation and expression of metabolic genes

To further investigate why SHSY-5Y cells appeared to be relatively resistant to 5-Aza-dC-induced demethylation, we analysed the RNA expression level of genes involved in the metabolism of 5-Aza-dC [29]. Compared to BE(2)-M17 cells, which had extensive demethylation by 5-Aza-dC (Fig. 2A), SHSY-5Y cells expressed reduced levels of the *ENT* nucleoside transporters that are involved in the uptake of 5-Aza-dC (*ENT1*, *ENT2*; Fig. 4A and B) and reduced levels of the 5-Aza-dC-activating kinase *DCK* (Fig. 4C). These results are similar to what has been reported for other human cancer cell lines that are relatively resistant to 5-Aza-dC-induced demethylation [30]. Thus the relative resistance of SHSY-5Y to 5-Aza-dC-induced demethylation may result from reduced expression of genes involved in the uptake and activation of 5-Aza-dC.

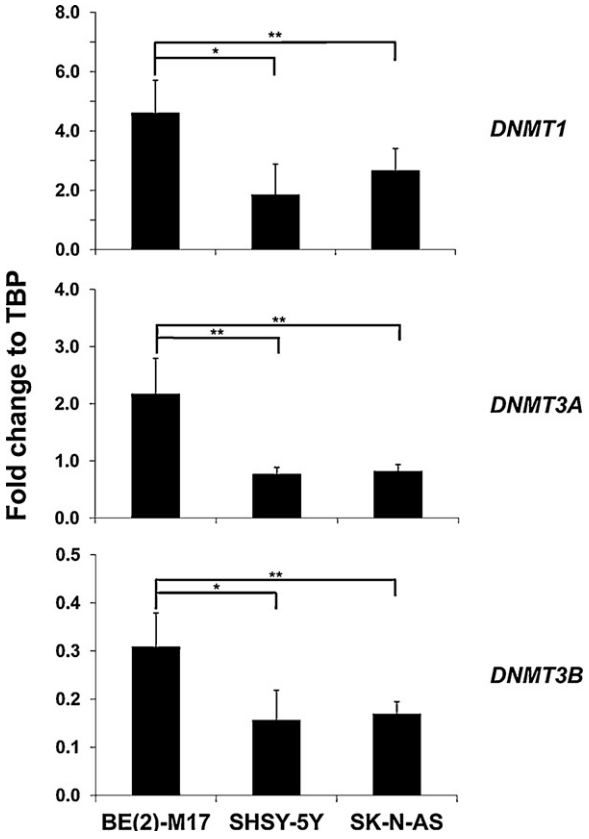

Fig. 3. DNMT expression in NBL cell lines. Levels of expression of DNMT1, DNMT3A and DNMT3B were analysed by qRT-PCR in BE(2)-M17, SHSY-5Y and SK-N-AS cell lines. Error bars indicate standard deviations of three independent experiments performed as duplicates. DNMT1 had the highest expression levels in BE(2)-M17 cells whilst SHSY-5Y had on average the lowest. Student's *T*-test was performed for detection of significant differences between cell lines ($^{*}p < 0.05$; $^{**}p < 0.01$).

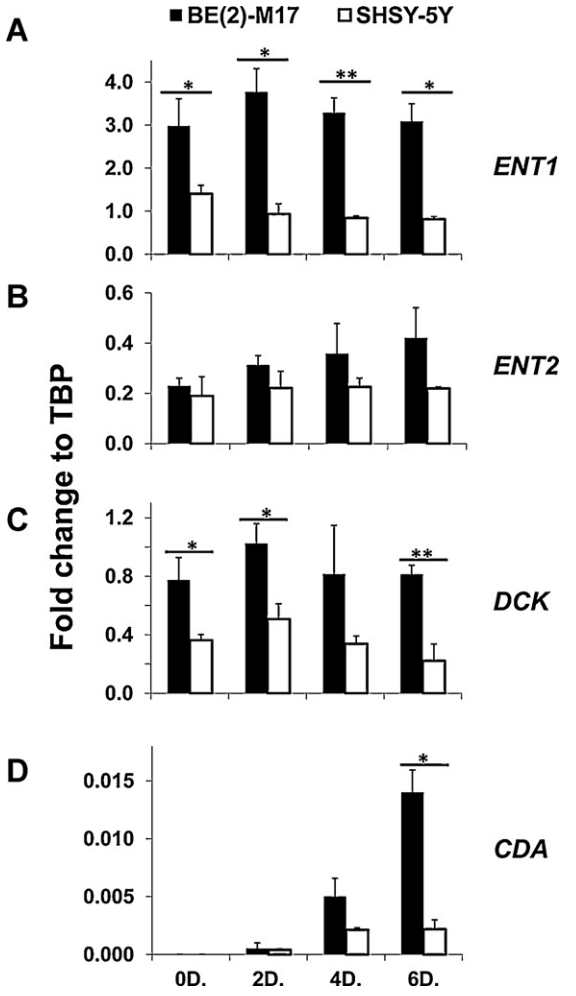

Fig. 4. ENT1, ENT2, DCK and CDA expression in 5-Aza-dC treated NBL cell lines. QRT-PCR results are shown in response to 5-Aza-dC treatment over 6 days. Error bars indicate standard deviations of three independent experiments. (A) *ENT1* and (B) *ENT2* encode membrane transporters that were more highly expressed in the sensitive BE(2)-M17 cell line compared to SHSY-5Y, contributing to the influx of Aza into the cell. (C) *DCK* was constantly more highly expressed in BE(2)-M17 than in SHSY-5Y during the six days of treatment. Its role consists of phosphorylating and thereby activating deoxycytidines. (D) *CDA* is involved in the inactivation of Aza; its expression increased during Aza treatment and was higher in BE(2)-M17 than in SHSY-5Y. Student's *T*-test was performed for detection of significant differences between days of 5-Aza-dC treatment ($^{*}p < 0.05$; $^{**}p < 0.01$).

Interestingly, the expression of cytidine deaminase (*CDA*), which deaminates deoxycytidines in order to catabolise them, was induced by 5-Aza-dC in both cell lines, although BE(2)-M17 had a much higher expression than SHSY-5Y cells (Fig. 4D). This could reflect the higher uptake and activation of 5-Aza-dC in BE(2)-M17 cells, which is predicted from their increased expression of *ENT1*, *ENT2* and *DCK* (Fig. 4A–C). The dose of 5-Aza-dC used in our experiments (2 μM) was similar to the maximum plasma levels of 5-Aza-dC attained with the highest clinically relevant doses [31,32]. However, we only observed increased *CDA* expression after several days' exposure to 5-Aza-dC (Fig. 4). Thus, our findings might be relevant to some of the differences in clinical response that have resulted from changes in the dose and timing of 5-Aza-dC administration to patients [33,34].

3.5. Combinatorial treatments of 5-Aza-dC and chemotherapeutic drugs

Having determined that BE(2)-M17 and SK-N-AS showed the most extensive 5-Aza-dC-induced demethylation, we focussed on these two cell lines to investigate the effect of 5-Aza-dC on the response of NBL cells to chemotherapeutic drugs. BE(2)-M17 and SK-N-AS cells were pre-treated with 5-Aza-dC for three days before adding doxorubicin, cisplatin or etoposide. For each treatment, a low and a high dose were chosen from the concentration panel that had been used to investigate the dose response (Fig. 1). All the combined treatments with chemotherapeutic drugs and 5-Aza-dC resulted in enhanced cell apoptosis, compared to the 5-Aza-dC and drug treatments alone (Fig. 5). We calculated the Chi^2 value of the observed apoptosis with combination treatments, versus the amount of apoptosis for the separate 5-Aza-dC and drug treatments added together (Fig. 5). All combinatorial treatments gave significantly higher apoptosis ($p < 0.01$ to $p < 0.001$), except for 100 μM cisplatin and etoposide with 5-Aza-dC in SK-N-AS cells (Fig. 5A and C). A very pronounced effect was seen with doxorubicin and etoposide in combination with 5-Aza-dC, where the percentage of apoptotic cells in the cells treated with 5-Aza-dC plus 0.25 μM doxorubicin or 25 μM etoposide, was approximately double the amount of apoptosis achieved with 1 μM or 100 μM of the drugs on their own (Fig. 5B and C). Interestingly, for cisplatin combinations, the level of apoptosis induced by 5-Aza-dC with 25 μM of the drug was almost the same in both cell lines as the level induced by cisplatin treatment alone at 100 μM (Fig. 5A).

Further analysis of apoptosis in the treated cells was investigated by studying the cleavage of caspases and PARP-1 by Western

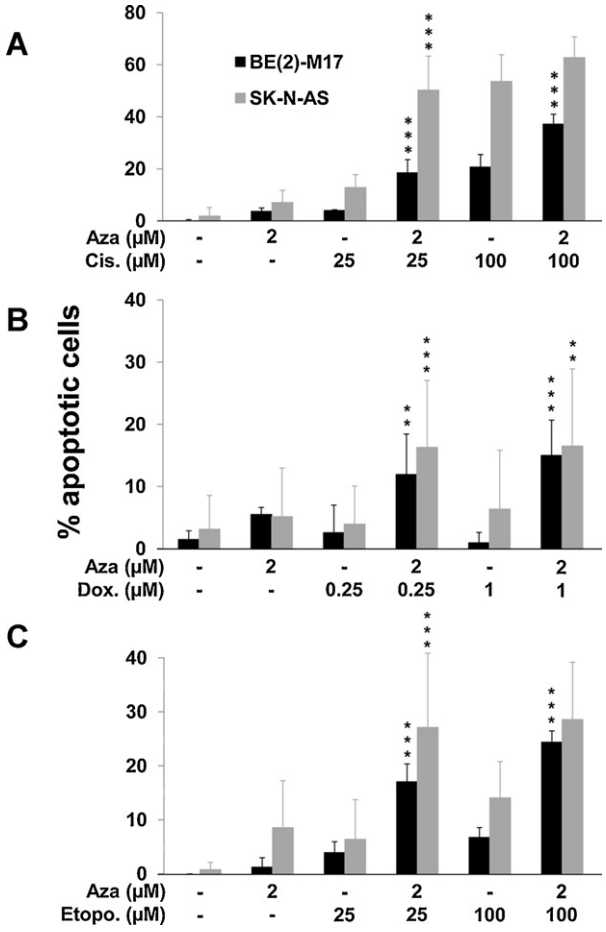

Fig. 5. Effect of combined cytotoxic drug and 5-Aza-dC treatment in BE(2)-M17 and SK-N-AS cells. Cells were pre-treated with 5-Aza-dC at 2 μM for 3 days and then the chemotherapeutic drugs were added on the fourth day for 24 h. Apoptotic cells were determined by Hoechst/PI staining in the total cell population; error bars indicate the standard deviation of three independent replicates. The Chi^2 test was used to test for significant differences between combined treatments and individual treatments added together. Cis.; cisplatin, Dox.; doxorubicin, Etop.; etoposide, $^{**}p < 0.01$; $^{***}p < 0.001$.

Blotting in SK-N-AS and BE(2)-M17 cells after 5-Aza-dC treatment with cisplatin (Fig. 6A), doxorubicin (Fig. 6B) or etoposide (Fig. 6C). Cleavage of CASP8, CASP9 and PARP-1 was detected in SK-N-AS cells, although interestingly, there was a large increase in cleavage in the cells treated with cisplatin and 5-Aza-dC in combination, compared to cisplatin and 5-Aza-dC on their own (Fig. 6A). This is exemplified by the finding that CASP8 and CASP9 were cleaved in SK-N-AS cells treated with 25 μM cisplatin plus 5-Aza-dC, whereas an almost complete lack of cleavage was seen in cells treated with 25 μM cisplatin alone (Fig. 6A). Similar increases in CASP8, CASP9 and PARP-1 cleavage were detected in SK-N-AS cells as a result of combining 5-Aza-dC with doxorubicin (Fig. 6B) or etoposide (Fig. 6C). BE(2)-M17 cells showed no cleavage of the effector caspases 8 and 9 but PARP-1 was cleaved in all three drug treatments (Fig. 6A–C) but to a lower extent than in SK-N-AS cells. Similarly to SK-N-AS, cleavage of PARP-1 was only detectable in BE(2)-M17 cells treated with cisplatin (Fig. 6A), doxorubicin (Fig. 6B) or etoposide (Fig. 6C) plus 5-Aza-dC and not with chemotherapeutic drugs alone at the same concentration.

In summary, these results suggest that 5-Aza-dC can potentiate the cytotoxic effects of the three chemotherapeutic drugs as demonstrated by cell staining/nuclear morphology analysis and cleavage of apoptotic proteins.

4. Discussion

Drug resistance in primary tumours or acquired during treatment causes a major problem in all kinds of cancers when treated with established therapies [19]. One striking example is provided by high-stage NBL, in which chemotherapeutic resistance is an important clinical problem, leading to frequent treatment failure [20]. The development of drug resistance in NBL during therapy is well reflected in the increased chemoresistance of cell lines derived from patients that had already undergone treatment [21], demonstrating that NBL cell culture systems can be effective tools for investigating chemoresistance. One mechanism for the acquisition of chemoresistance in cancer may be epigenetic alterations [19]. DNA hypermethylation has been frequently reported in NBL, inactivating genes which could play a role in chemoresistance, such as *CASP8*, an important initiator of apoptosis [22] and *RASSF1A* [13], a regulator of cell cycle arrest, mitotic arrest and apoptosis [35,36]. We therefore investigated the potential of the DNA demethylating agent 5-Aza-dC to augment the cytotoxic effects of currently used chemotherapeutic drugs on NBL cells in culture. Our results clearly showed that 5-Aza-dC significantly increased apoptosis induced by cisplatin, doxorubicin and etoposide, suggesting that this approach could provide novel treatment strategies for therapy-resistant NBLs.

In this study, three NBL cell lines were first tested for their sensitivity to cisplatin, etoposide and doxorubicin (Fig. 1). The *MYCN* amplified BE(2)-M17 cell line was most resistant to all drug treatments whilst the SHSY-5Y non-amplified cells were most sensitive. The same cell lines were also tested to determine how effectively 5-Aza-dC induced DNA demethylation (Fig. 2A), to identify which cell lines were the most suitable to investigate the effects of DNA demethylation on chemosensitivity. All three cell lines had a relatively high overall DNA methylation but the BE(2)-M17 and SK-N-AS cell lines showed more DNA demethylation after 5-Aza-dC treatment in comparison to SHSY-5Y cells. The relative resistance of SHSY-5Y cells to demethylation by 5-Aza-dC, compared to BE(2)-M17 cells, might be explained by decreased expression levels of nucleoside transporters and kinases that are involved in the metabolism of 5-Aza-dC [29] (Fig. 4). We therefore focussed the combinatorial treatments on BE(2)-M17 and SK-N-AS, which were the cell lines that showed the highest genomic DNA demethylation in response to 5-Aza-dC. The combined treatments with 5-Aza-dC and chemotherapeutic drugs led to a marked increase in apoptosis, compared to the individual treatments, as demonstrated by morphological and biochemical criteria (Figs. 5 and 6).

Although 5-Aza-dC has also been successfully used to re-sensitise other resistant human cancer cell lines to chemotherapeutic drugs [24,25], to our knowledge this is the first time that the demethylating agent 5-Aza-dC has been used in combination with established chemotherapeutic drugs on human NBL cell lines. Previously, Qiu et al. [37] showed that cisplatin-resistant murine NBL cells had increased enzymatic activity of DNMT3A and DNMT3B, as well as an acquired resistance to cisplatin in clones overexpressing DNMT3A and DNMT3B. 5-Aza-dC treatment reversed this resistance in cells by re-sensitising them up to 10-fold to cisplatin [38]. These results support our contention that DNA demethylation is a potentially viable strategy for re-sensitising chemoresistant human neuroblastoma cells to chemotherapeutic drugs.

A recent phase I trial was performed on children with refractory NBL, in which they were treated with 5-Aza-dC in combination with doxorubicin and cyclophosphamide [39]. Low doses of 5-Aza-dC were tolerated by children and there was evidence of DNA demethylation of marker genes in some patients. Although there

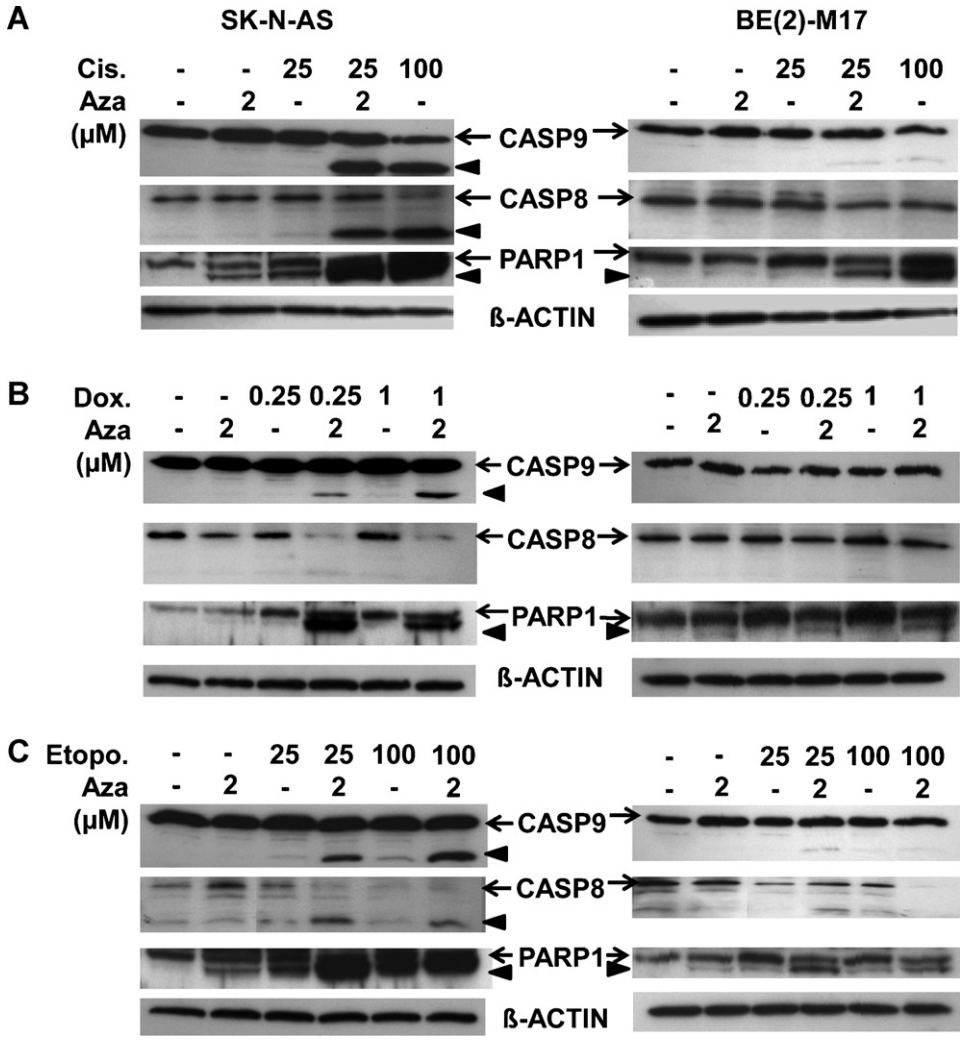

Fig. 6. CASP8, CASP9 and PARP-1 cleavage. Western blotting for CASP8, CASP9 and PARP-1 cleavage after 5-Aza-dC and/or chemotherapy treatments on BE(2)-M17 and SK-N-AS cell lines Drug combinations and concentrations are indicated above the gel lanes. Arrows indicate full length proteins and arrowheads indicate cleaved proteins.

was no significant therapeutic benefit in this group of patients, the children in this trial had already been heavily pre-treated, which the authors suggested may have limited the ability to escalate the dose of 5-Aza-dC to a level sufficient to induce a biological and clinical response [39]. Our in vitro study showed that 5-Aza-dC potentiated the cytotoxic effects of cisplatin, etoposide and doxorubicin, although the degree of benefit varied between the three drugs. Further investigations using NBL cell lines could thus shed new light on the use of concentrations, treatment periods and type of cytotoxic drugs that could be used in combination with 5-Aza-dC for possible future clinical trials.

Epigenetic alterations are now recognised as being fundamental to the development of cancer [40]. and have been previously postulated to have a role in chemoresistance [19]. Recent evidence suggests that chromatin changes may provide a dynamic strategy by which cancer cells can achieve a reversible drug-resistant state [41,42]. Our results show that chemotherapeutic drug sensitivity may be at least partly epigenetically regulated in NBL cells. We have shown here evidence of DNA demethylation by 5-Aza-dC in NBL at both the genomic (Fig. 2A) and individual gene level i.e. *RASSF1A* (Fig. 2B). Reactivation of genes involved in tumour suppression and/or apoptotic pathway activation [35] could contribute to the drug-sensitising effects of 5-Aza-dC that we have demonstrated. However, it is most likely that many genes contribute to this effect, so in future studies it will therefore be important to combine functional genomic approaches with DNA demethylation, in order to identify epigenetic changes that drive chemotherapeutic drug resistance.

We also found that the ability of 5-Aza-dC to induce DNA demethylation varied between NBL cell lines; BE(2)-M17 and SK-N-AS exhibited extensive DNA demethylation in response to 5-Aza-dC, whereas SHSY-5Y showed much less demethylation (Fig. 2). In the recent phase I trial where children with NBL were treated with 5-Aza-dC in combination with chemotherapeutic drugs, the biological effects of 5-Aza-dC in promoting marker gene demethylation and re-expression varied from patient to patient [39]. Our results suggest that there may be inherent differences in tumour cell DNA demethylation induced by 5-Aza-dC, which could arise as a result of altered expression of transporters and enzymes that regulate the uptake and activation of 5-Aza-dC (Fig. 4). Taken together, these results suggest that the use of DNA demethylating agents to enhance chemosensitivity would need to be targeted to those tumours in which epigenetic drugs exhibit maximal biological activity.

In summary therefore, we have shown in this paper that the use of a DNA demethylation agent can markedly enhance the sensitivity of highly chemoresistant NBL cells to chemotherapeutic drugs, suggesting that the clinical use of epigenetic modulators to enhance drug sensitivity may be a promising approach in neuroblastoma therapy.

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