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Role of DNA Methylation in Acquired Drug Resistance in Neuroblastoma Tumours

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Changes in genomic DNA-methylation patterns are very important in the development of malignancies and acquired drug resistance. An association between increased levels of DNA (cytosine) methyltransferase (DNMT) activity, the enzyme that catalyzes DNA methylation at CpG sites in the promoter region of genes and tumour development, suggested a role for the DNMT in the initiation and progression of a variety of tumours (Belinsky et al. 1996). It has been reported that DNMT expression and aberrant DNA methylation increases during progressive stages in the development of human tumours (Toyota et al. 1999) and acquired drug resistance (Qiu et al. 2002). Data from our laboratory and others (Carpinelli et al. 1993) clearly demonstrate the role of DNA methylation in acquired drug resistance of neuroblastoma cells and its reversal following an alteration in DNA methylation status. Alteration in DNA methylation status holds a great promise toward providing an effective treatment to a variety of tumours in the future.

Key Words: DNA methyltransferases, Neuroblastoma tumours, Neuroblastoma, Drug resistance, DNA methylation, Murine and human neuroblastoma cells

Introduction

Neuroblastoma tumours, a neoplasm of neuroectodermal origin and tumour of sympathetic nervous system, is one of the most complex and challenging solid tumours of childhood. In the United States, neuroblastoma accounts for 8 to 10% of all tumours. According to the Surveillance, Epidemiology, and End Results (SEER) Program of the US National Cancer Institute, there are 550 new cases of neuroblastoma diagnosed every year in the United States. An incidence of 10.4 per million in white and 8.3 per million in black children by 15 years of age has been reported (Gurney et al. 1996). The tumour is slightly more common in boys than girls with a male: female ratio of 4.1 vs. 2.0. Prevalence (incidence rate per million) of neuroblastoma tumours in male versus female has also been reported in metropolitan cities of India such as Bangalore (3.2/1.9), Mumbai (3.5/3.0), Delhi (3.2/2.7), and Poona (3.7/1.7) for the period 1980-1992 (Perkin et al. 1998). The Pediatric Oncology Group (POG) has determined the median age of diagnosis of neuroblastoma in children to be 22 months (Brodeur 1991). The incidence of neuroblastoma is higher in North America, Europe, and Australia compared to Asia including India and China. Advanced stage neuroblastoma tumours associated with poor prognosis are very difficult to treat in spite of significant progress in chemotherapy due to development of resistance to drugs. The

Abbreviations:
Dnmt, DNA (cytosine-5) methyltransferase (murine); *DNMT*, DNA (cytosine-5) methyltransferase (human); *DMEM*, Dulbecco's modified eagle medium; *wMNB*, wild type murine neuroblastoma cells; *rMNB/MDL* (resistant murine neuroblastoma cells); *MDL 28,842* (Z-5'-fluoro-4', 5'-didehydro-5'deoxyadenosine); *RT-PCR*, reverse transcriptase polymerase chain reaction; IC_{50}, concentration of drug to kill 50% cell population

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underlying mechanism(s) leading to acquired drug resistance in neuroblastoma tumours are multifactorial such as alteration in DNA repair and detoxification pathways, apoptosis, and loss of tumour suppressor genes due to DNA methylation. This chapter will review the role of DNA methylation in acquired drug resistance mechanism of neuroblastoma tumours in addition to others.

Molecular & Genetic Aspects of Neuroblastoma

Recent studies on the biology of neuroblastoma have provided considerable insight into its genetic, biochemical, and molecular characteristics. Karyotype analysis revealed deletion of the short arm of chromosome 1 (1p) with loss of heterozygosity (LOH) on chromosomes 1p, 11q, 14q, 18q, trisomy of the long arm of chromosome 17 (17q), presence of double-minute chromosomes (dmns), and homogeneously staining regions (HSRs) in most tumours. Loss of heterozygosity for the deleted allele may ablate a tumour suppressor gene resulting in malignant transformation or progression of neuroblastoma tumours.

Mutations of p53, a known tumour suppressor gene, are generally uncommon in malignant solid tumours of childhood (Brodeur et al. 1992). However, in some childhood sarcomas, p53 mutations appear to have an important role in the development of these tumours (Kusafuka et al. 1997). The p53 gene is frequently affected by point mutations, rearrangements, or deletions that contribute to the genesis or progression of a wide variety of human adult solid tumours, however, alteration of the p53 gene has not been reported in neuroblastoma (Hosoi et al. 1994). Recent data obtained from melphalan (L-PAM) resistant neuroblastoma cell lines suggest the p53 mutations and/or loss of p53 function leading to acquired drug resistance in neuroblastoma tumours (Keshelava et al. 2001). These data also support development of p53-independent chemotherapies for recurrent neuroblastoma tumours. In a substantial number of untreated neuroblastoma patients (25%) with advanced stage of tumour and poor prognosis, amplification of the proto-oncogene n-Myc in the distal short arm of chromosome-2 has been demonstrated (Brodeur et al. 1992, Cohn et al. 2000). Amplification of n-Myc is predominantly associated with an advanced stage of tumour development. In a majority of tumours (91%), expression of the neurotrophin growth factor (NGF) receptor known as Trk-A (tropomyosin receptor kinase-A) has also been reported, however, the high level expression was found only in 82%. The expression of Trk-A has been correlated with a favourable outcome, while patients with amplification of n-Myc generally have a poor prognosis. The favourable outcome of Trk-A expression in neuroblastoma patients is believed to be associated with an enhanced response to NGF (Kogner et al. 1993).

Chemotherapy and Acquired Drug Resistance in Neuroblastoma

Some infants with metastatic disease may have a complete regression of tumour without any treatment, while others have very poor prognosis following therapy. Various chemotherapy regimens including cisplatin, cyclophosphamide, methotrexate, doxorubicin (adriamycin), etoposide and chlorambucil have been used in the treatment of metastatic neuroblastoma in children. Combination chemotherapy such as cyclophosphamide and doxorubicin, cisplatin and teniposide have also been used because of their synergistic effects and reduced toxicity (Brodeur et al. 1992); (Keshelava et al. 1998); (Cinatl et al. 1998). A major problem encountered in the chemotherapy of neuroblastoma, like many other neoplasms is intrinsic or acquired drug resistance (Brodeur et al. 1992); (Keshelava et al. 1998); (Cinatl et al. 1998). Drug resistance patterns expressed by neuroblastoma cell lines generally correlate with the drugs to which the patients were exposed. A cell line (LAN6) with the highest drug resistant phenotype was isolated from a patient who received multiple courses of chemotherapy containing cyclophosphamide, doxorubicine, teniposide, etoposide, vincristine, and dacarbazine due to progressive metastatic disease (Wada et al. 1993).

Multidrug Resistance (MDR) in Neuroblastoma

A process by which a number of structurally unrelated anticancer drugs exert their action involves the phenomenon of apoptosis or programmed cell death (Dive et al. 1992). The

ability of cancer cells to escape or bypass this pathway may account for the presence of drug resistance against a number of chemotherapeutic agents. Factors contributing toward the development of drug resistance in neuroblastoma include overexpression of the multidrug resistance gene (MDR1) which encodes for P-glycoprotein (P-gp) (Volm 1998) multidrug-resistance-associated protein gene (MRP), (Norris et al. 1996) which may occur in association with n-Myc oncogene amplification. The MDR phenotype has been shown to develop in the course of chemotherapy, and presents a major obstacle to successful treatment of tumours. P-gps are members of the family of ABC (Adenosine triphosphate Binding cassette) transmembrane proteins (Allikmets et al. 1996). P-gp transports agents such as anthracyclines, vinca alkaloids, and epidophyllotoxins through an energy dependent drug-efflux pump.

In humans, two genes encoding P-gps (MDR1 and MDR3) have been identified while in mice there are three P-gp genes, Mdr1a (also called Mdr3), Mdr1b (Mdr1) and Mdr2 (Borst & Schinkel 1997). Cytotoxic drugs being used for treatment are extruded from the cells by the transport mechanism of P-gps, and cells develop the MDR phenotype (Germann 1996). The role of P-gp associated multidrug resistance in neuroblastoma patients is very controversial (Kutlik et al. 2002). Using the sensitive immunosandwich technique, Kurowski and Berthold (1998) have demonstrated that P-gp associated multidrug resistance was present only in the SK-N-SH human neuroblastoma cell line but not in 11 other neuroblastoma cell lines that were resistant to vincristine and adriamycin. P-gp expression was detected in only 22.6% of neuroblastoma tumours, and no correlation was found between the stage of disease and n-Myc amplification. The study illustrates that P-gp expression, as evaluated by the MDR phenotype, does not provide much assistance in understanding the complex phenomenon of drug resistance. Recent data from our laboratory do confirm the nonparticipation of MDR genes in acquired drug resistance of murine neuroblastoma cells (rMNB/MDL) made resistant to MDL, a nucleoside analogue inhibitor of S-Adenosylhomocystein (AdoHcy) hydrolase (Dwivedi et al. 1999, Wang et al. 2001). Neuroblastoma tumours can also become resistant to drugs without substantially affecting the MDR phenotype (Kurowski & Berthold 1998) suggesting the presence of additional mechanism(s) of drug resistance other than MDR in some types of neuroblastoma tumours (McGrath & Center 1987); (Murren & Hait 1992).

Multidrug Resistance Associated Protein Expression in Neuroblastoma

The Multidrug Resistance Associated Protein (MRP) genes, members of the ABC super family, are involved in a non P-gp mediated mechanism of drug resistance which initiated the search for additional transporters (Cole et al. 1992). By searching the EST (Expressed Sequence Tag) database, Allikmets et al. (1996) have identified 21 ABC transporter genes with known and unknown functions. Transmembrane proteins P-gp, MRP, and multispecific organic anion transporter (cMOAT) are thought to be involved in the normal excretion of xenobiotics as well as drug resistance. cMOAT as measured by quantitative RNA-polymerase chain reaction (PCR) is rarely expressed in neuroblastoma tumours (Matsunaga et al. 1998). Levels of MRP gene expression were significantly higher in neuroblastoma tumours with n-Myc amplification and poor outcome indicating its association with reduced survival rate (Norris et al. 1996). Transfection of full length n-Myc cDNA into SH-EP human neuroblastoma cells resulted in an increased MRP expression and significantly increased resistance to vincristine and doxorubicin (Haber et al. 1999). This suggests that MRP gene confers resistance to a similar, but not identical, spectrum of drugs as MDR. A poor response was noticed with taxol and cisplatin in these transfection experiments. An investigation has recently demonstrated that a number of human neuroblastoma cell lines with resistance to mitoxantrone, exhibit multidrug resistance phenotype without the expression of P-gp or MRP (Miyake et al. 1999).

Other Types of Drug Resistance in Neuroblastoma

Expression of non-Pgp lung resistance protein (LRP) identified in non-small cell lung carcinoma (Scheper et al. 1993), DNA topoisomerase II alpha (TOPO IIa)

that alters the topological state of DNA (Alton & Harris 1993), Bcl-2 oncoprotein (Hanada et al. 1993), wild-type p53 gene (Ronca et al. 1999), and glutathione S-transferases - GST pi (Piredda et al. 1999; Kutlik et al. 2002), play a significant role in the development of acquired drug resistance (Kellen 1994) in a variety of tumours are very poorly understood in neuroblastoma (Bader et al. 1999). Overexpression of metallothionine against alkylating agents in Chinese hamster ovary K1-2 cells (Kaina et al. 1990) and in some cis-diamminedichloroplatinum (CDDP)-resistant cells also reportedly associated with a drug resistance phenotype. Topoisomerase-IIα (TOPO-IIα) gene expression is associated with cellular proliferation and DNA replication. Decreased levels of TOPO-IIα as a result of down-regulation of its expression provide a level of drug resistance against a variety of drugs (Alton & Harris 1993). A very low level of TOPO-IIα expression has been reported in neuroblastoma cell lines (Yanagisawa et al. 1999).

Glutathione S-transferases (GSTs) are believed to mediate a variety of cellular detoxication reactions, which belong to a multigene family of enzymes (Dwivedi et al. 1993). A genetic variant in GSTp which is associated with preneoplastic and neoplastic changes is encoded by the GST-P1 gene and confers an increased risk of cancer. The 5' region of GSTP-1 contains a CpG island, and the loss of its expression as a result of hypermethylation has been reported in human prostrate carcinomas. Seven of eight neuroblastoma patients with resistant disease have shown the presence of GSTπ (Hall et al. 1994). The overexpression of apoptosis modulating genes such as Bcl-2 and Bcl-$_{xL}$ is associated with pleiotropic drug resistance in neuroblastoma. Since acquired drug resistance is a multifactorial phenomenon, it seems likely that there are other potential mechanisms of drug resistance in operation during the treatment of neuroblastoma and other childhood tumours. One such mechanism involves the hypermethylation of CpG islands in promoter regions of tumour related genes (Nyce et al. 1993).

DNA Methylation and Drug Resistance in Neuroblastoma

Drug-induced DNA hypermethylation may be capable of creating drug-resistant phenotypes by inactivating genes that generate products which are required for drug cytotoxicity. Paradoxically, drug-induced DNA hypermethylation may also produce a second class of drug-resistant tumour cells, characterized by overexpression of particular gene products, which potentiate the process of gene amplification (Nyce 1989). In a model system employing Chinese hamster V-79 cells, the DNA synthesis inhibitor 3'-azido-3'-deoxythymidine (BWA509U, AZT) was shown to induce genome-wide DNA hypermethylation, low-frequency silencing of thymidine kinase (TK; EC 2.7.1.21) gene expression, and resistance to AZT (Nyce et al. 1993).

Drug resistance against zidovudine (AZT) in Jurkat-T cells has been associated with decreased expression of the thymidine kinase (TK) gene and hypermethylation of the 5' end of the human TK gene (Wu et al. 1995). DNA methylation of the CpG-rich 5' region of the deoxycytidine kinase (dCK) gene is potentially involved in the silencing of the gene and acquired resistance of tumour cells to arabinosylcytosine (ara-C). 2-chlorodeoxyadenosine (cladribine, CdA) and 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine (CAFdA) which are phosphorylated (Leegwater et al. 1998) for their activity. Despite growing interest in the methylation-mediated silencing/inactivation of tumour related genes in the neoplastic process (Zochbaer-Muller et al. 2001, Conway et al. 2000, Eads et al. 2000) the signaling mechanism for silencing the genes involved during acquired drug resistance remains largely unknown.

Experimental evidence from several studies has identified a link between DNA mismatch repair (MMR) deficiency and cytotoxic drug resistance. Selection of cisplatin resistance in the human ovarian cancer cell line A2780, results in loss of expression of the mismatch repair protein hMLH1, in most (90%) of the resultant cisplatin-resistant cell lines (Strathdee et al. 1999). DNA analysis of the hMLH1 gene suggests that hypermethylation of the hMLH1 promoter is a common mechanism for loss of hMLH1 expression and cisplatin-resistance in these cancers (Plumb et al. 2000). Treatment of tumour bearing mice with the demethylating agent 2'-deoxy-5-azacytidine (DAC) at nontoxic dose induces hMLH1 expression, and sensitizes the

xenografts to cisplatin, carboplatin, temozolomide, and epirubicin treatment.

These experiments further suggest that another potential mechanism of drug resistance could be drug-induced DNA hypermethylation and resulting transcriptional inactivation of cellular genes (switching on/off) whose products are required for drug sensitivity. DNA sensitization to chemotherapeutic agents could be accomplished by transfecting the hMLH1 gene into hMLH1 null mouse cells. Restriction landmark genome scanning (RLGS) and virtual genome scan (VGS) analyses which identify amplification, deletion or methylation changes in CpG islands have recently reported the absence of a NotI-EcoRV fragment of the ALX3 gene and its suppression in neuroblastoma due to hypermethylation. ALX3 is a human orthologue of the hamster homeobox gene Alx3. Hypermethylation associated silencing and inactivation of the caspase-8 gene has also been reported in neuroblastoma (Teitz et al. 2000). Disruption of apoptotic pathways (caspase inactivation) and resistance to TNF-related apoptosis-inducing ligand (TRAIL) in neuroblastoma cells suggest the involvement of gene hypermethylation in these events. Loss of heterozygosity (LOH) on chromosomes 2q, 9p and 18q due to hypermethylation has frequently been observed in neuroblastoma. Loss of chromosome 9q34 investigation has further indicated the deletion of p16 (CDKN2A), a tumour suppressor gene in neuroblastoma due to p16 gene hypermethylation and inactivation (Takita et al. 1997).

DNA(cytosine)methyltransferase (DNMT) Protein Overexpression in Drug Resistant Neuroblastoma cells and reversal of acquired drug resistance following 2'-deoxy-5-azacytidine (DAC) Treatment.

Recent data from our laboratory have demonstrated a ~2- fold increase ($p<0.001$) in the expression of DNA (cytosine) methyltransferase (DNMT) protein in drug resistant murine neuroblastoma cells (rMNB-MDL) compared to wild type cells, as determined by Western blot analysis (figure 1). Murine erythrocyte leukemia (MEL) cell lysate was used as a positive control to examine the expression of DNMT protein in resistant cells. A~10 fold increase in DNA

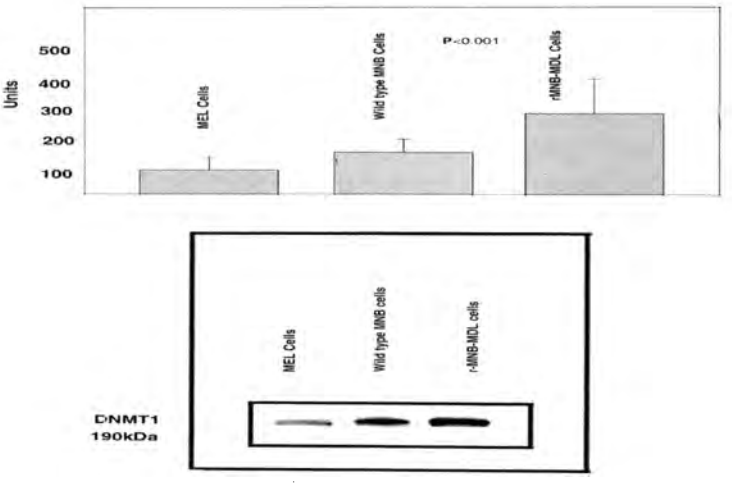

Figure 1 Immunoblot analysis of DNA (cytosine)-5-methltransferase (DNMT1) in wild type murine neuroblastoma (MNB) and resistant r-MNB-MDL cells. The bottom panel shows cell lysates fractionated on 7.5% SDS-polyacrylamide gel and probed with DNM1specific antibody to N-terminal region from amino acid 137 to 635 of the DNA methyl transfferase enzyme (Li et al. 1992). The blot was developed using ECL Immunodetection Kit. Murine erythroleukemia (MEL) cells were used as positive control for DNMT1 expression. A 190 kDa protein was detected in wild type MNB and nucleoxide resistant r-MNB-MDL cells. The upper panel represents a densitometric analysis of immunoreactive bands in the lower panel.

methyltransferase (Dnmt3b) expression was reported in drug resistant murine neuroblastoma cell line compared to wild type using Real Time quantitative RT PCR analysis (Qiu et al. 2002). A 2-fold ($P< 0.001$) increase in Dnmt1 expression in drug resistant murine neuroblastoma cells compared to wild type cells was also observed using the Real Time RT/PCR analysis (Qiu et al. 2002). Expression of β-actin, used as an internal control did not change between the wild type and drug resistant murine neuroblastoma cells. The increase in Dnmt mRNA expression in drug resistant cells compared to wild type cells, supports our earlier findings in which a significant increase in the methylation index of drug resistant cells was reported (Hamre et al. 1995).

Alterations in DNMT expression levels, mutation in the DNMT genes, and changes in global DNA methylation could influence the expression of tumour suppressor genes in tumours. DNMT expression is often elevated in transformed cell lines when compared to wild type cells. Considerable overexpression of DNMT mRNA was found in colorectal tumours (Schmutte et al. 1995). The mechanism(s) by

which DNA methylation contributes to the development of a variety of human cancers is extremely complex. Methylation of cytosine to 5-methylcytosine leads to a higher rate of C to T mutations. Mice deficient in DNMTs activity have a decreased rate of polyp formation. Schmutte et al. (1995) have clearly demonstrated the role of DNA methylation and progressive increments in the expression of DNA-methyltransferase protein during tumour development. Using RT-PCR methods, Schmutte et al. (1996) have also shown that average DNAMT1 expression levels were elevated to approximately 3.7- fold in human colon tumour samples compared with surrounding normal mucosa from the same patient.

Several hundred fold higher expression of DNMTs mRNA was detected in human colon cancer tissues than in tissue samples from patients with benign disorders (Schmutte et al. 1996). A common pattern of functional inactivation of the tumour suppressor genes, p16 (or MTS1 or CDKN2), p18 (INK4C) and p73 (a novel member of the p53 family), has been reported to be specific in the development and progression of human cancers including neuroblastoma tumours (Castresana et al. 1997). DNA methyltransferases, could critically control the methylation pattern of genomic DNA in tumours and the transcriptional activity of tumour suppressor genes in addition to other regulatory genes critical to cellular proliferation and drug resistance. A significant increase in DNMT activity, global methylation (figure 2), and DNA methyltransferase (Dnmt3b) gene expression (figure 3) may contribute to the functional inactivation or silencing of tumour suppressor genes (Gonzalgo & Jones 1997) in drug resistant tumour cells as a result of increased DNA hypermethylation through epigenetic mechanism(s). Recent IC_{50} (concentration of drug to kill 50% cell population) data from our laboratory have demonstrated that 2'-deoxy-5-azacytidine (DAC: a DNMT inhibitor) treatment to cisplatin resistant human neuroblastoma cell line (rSH-SY5Y/CisPt) increases the sensitivity of these cells to cisplatin (table 1). Reversal of acquired drug resistance and increased sensitivity

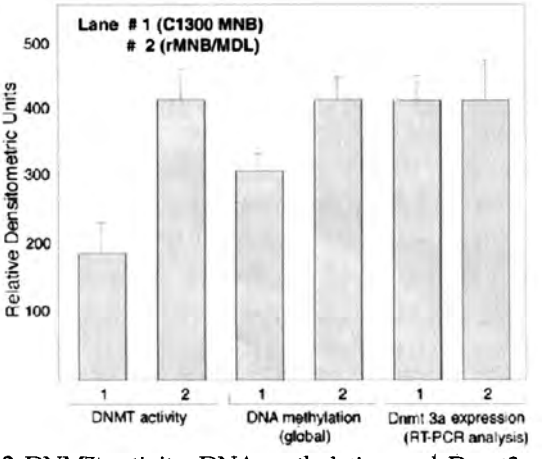

Figure 2 DNMT activity, DNA methylation and Dnmt3a expression in wild type(1) and drug resistant(2) murine neuroblastoma cells, DNMT activity and DNA methylation are significantly increased in drug resistant cells.

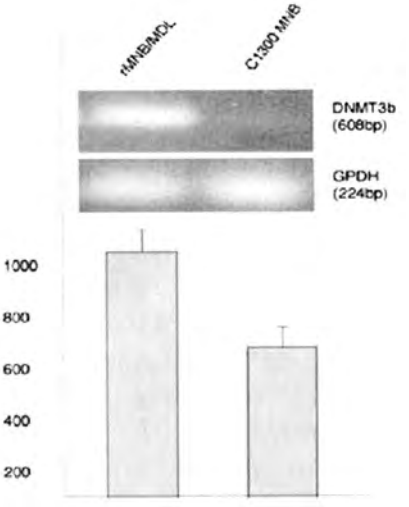

Figure 3 RT-PCT analysis of Dnmt3b expression demonstrates a significant increase in drug resistant murine neuroblastoma cells compared to wild type cells.

Table 1 *IC_{50} values of Cisplatin (CisPt) inhibiting the rate of human Neuroblastoma (SH-SY5Y HNB) cell proliferation.*

Cell lines	IC_{50} values for Cis-Pt
H-SY5Y wild type (HNB)	$1.83X10^{-7}$ M*
rSH-SY5Y CisPt resistant	$7.02X10^{-6}$ M*
rSH-SY5Y CisPt resistant +DAC treatment (96 hrs)	$1.28X10^{-7}$ M*

**Statistical significance was determined as P<0.001. Results are ± SEM of three sets of experiments performed in triplicates. DAC (2'-deoxy-5-azacytidine) treatment (5 mM, a non-toxic dose) for 96 hrs. to CisPt resistant (rSH-SY5Y/CisPt) cells decreased the IC_{50} (concentration of drug to kill 50% of cell population) dose of cisplatin indicating the increased sensitivity of resistant cells towards cisplatin treatment. SH-SY5Y human neuroblastoma cells were made resistant to cisplatin by continuous incubation and selection of resistant clones to cisplatin (10-6 M) concentration. IC_{50} values were determined using the GraphPad Software (GraphPad Software Inc. San Diego, CA).*

of drug resistant cells as a result of an alteration of DNA methylation (figure 4) status holds a great promise toward providing an effective treatment of a variety of tumours in future.

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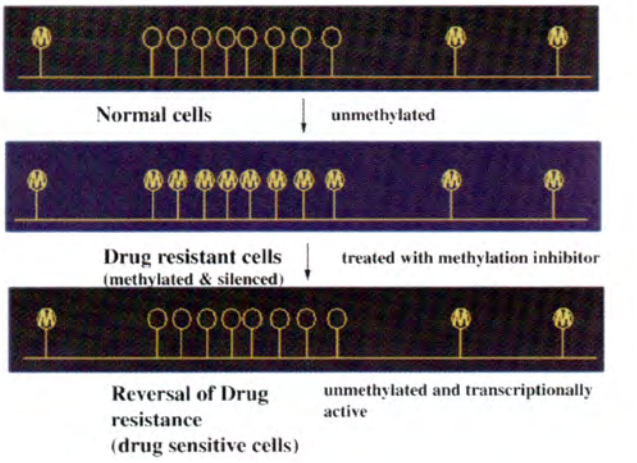

Figure 4 Reversal of acquired drug resistance in drug resistant neuroblastoma cell lines due to an alteration in DNA methylation.

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