Neuronal-associated microtubule proteins class III β-tubulin and MAP2c in neuroblastoma: Role in resistance to microtubule-targeted drugs

Sima Don,¹ Nicole M. Verrills,¹ ² Tracy Y.E. Liaw,¹ Marjorie L.M. Liu,¹ Murray D. Norris,¹ Michelle Haber,¹ and Maria Kavallaris¹

¹Children’s Cancer Institute Australia for Medical Research, Randwick, New South Wales, Australia and ²Australian Proteome Analysis Facility, Macquarie University, Sydney, New South Wales, Australia

Abstract
Advanced stage neuroblastoma has a poor clinical outcome and microtubule-destabilizing agents, such as the Vinca alkaloids, are an important component in the treatment of this childhood cancer. Vinca alkaloids bind to β-tubulin on the α/β-tubulin heterodimer and disrupt microtubule dynamics, leading to cell death. To date, studies examining the contribution of microtubules and associated proteins to the efficacy of microtubule destabilizing agents in neuroblastoma have been limited. In this study, BE(2)-C neuroblastoma cells previously selected for resistance to either vincristine (BE/VCR10) or colchicine (BE/CHC80.2) were found to display significant decreases in neuronal-specific class III β-tubulin. Interestingly, vincristine-selected cells exhibited increased levels of polymerized tubulin that were not due to α-tubulin and class I, II, or III β-tubulin mutations. Expression levels of the microtubule-depolymerizing protein stathmin were significantly increased in BE/VCR10 cells. In contrast, levels of MAP2a and MAP2b were relatively unaltered. A marked decrease in the neuronal protein, MAP2c, was identified in the vincristine-selected cells and, to a lesser extent, in the colchicine-selected cells. This is the first report describing specific microtubule alterations in neuroblastoma cells resistant to tubulin-targeted agents. The results indicate a need to identify the factors responsible for resistance to tubulin-targeted agents in neuroblastoma so that improved and novel treatment strategies can be developed for this drug refractory disease. [Mol Cancer Ther 2004;3(9):1137–46]

Introduction
Neuroblastoma is the most common solid tumor of infancy, and despite the increased survival rates achieved for many other childhood cancers using combination chemotherapy, advanced stage disease often remains refractory to treatment. Resistance to chemotherapeutic agents is one of the primary causes of treatment failure in neuroblastoma. A poor prognosis for neuroblastoma is associated with amplification or overexpression of the MYCN oncogene (1), which we have shown to be associated with expression of the multidrug resistance–associated protein (MRP1; refs. 2, 3).

Neuroblastoma treatment usually involves the use of surgery and combination chemotherapy. An important component in this chemotherapeutic approach are the Vinca alkaloids, particularly in the treatment of advanced stage disease (4). Vinca alkaloids, such as vincristine and vinblastine, are a group of natural product antimitotic drugs that disrupt microtubule stability and induce mitotic arrest and apoptosis (5). The cellular target of the antimicrotubule drugs is the β-tubulin subunit of the α/β-tubulin heterodimer. Tubulin heterodimers assemble to form microtubules, which are dynamic structures that are constantly growing and shortening. Seven β-tubulin isoforms have been identified in mammalian cells, encoded by different genes and with a distinct pattern of tissue expression (6). Several studies have provided strong evidence for a functional role of the tissue-specific expression of individual β-tubulin isoforms (7–9). Several of these isoforms, specifically class II β-tubulin (encoded by the Hβ9 gene), class III β-tubulin (encoded by the Hβ4 gene), and class IVa β-tubulin (encoded by the Hβ5 gene), are highly expressed in cells of neuronal origin (6). Neuroblastoma is believed to arise in the embryonal neural crest, and as a result, microtubules in neuroblastoma cells consist of high levels of neuronal-specific tubulin isoforms and microtubule-associated proteins.

Resistance to Vinca alkaloids has been associated with increased expression of P-glycoprotein (P-gp) in neuroblastoma cell lines, yet its role in clinical resistance to this childhood malignancy remains controversial, with studies providing evidence both for and against its value as a prognostic indicator (10). Previous reports have shown
that alterations in the cellular target of antimicrotubule drugs may play an important role in the early development of resistance. Specifically, altered expression of specific α-tubulin and β-tubulin isotypes and tubulin mutations have been reported in association with resistance to microtubule-stabilizing drugs such as the taxanes and epothilones (reviewed in refs. 11–13). Our earlier studies showed increased expression of the neuronal-specific class III β-tubulin isoform in paclitaxel-resistant lung cancer cell lines and in clinically resistant ovarian tumors (14). Increased expression of class III β-tubulin associated with decreased clinical response to the taxanes in breast cancer has also been reported recently (15). Studies involving microtubule alterations in drug-resistant cells described thus far have focused mainly on malignancies of epithelial and hematopoietic origin. We recently reported several microtubule protein alterations in \( Vinca \) alkaloid–resistant leukemia cells that included decreased expression of class III β-tubulin, a neuronal protein aberrantly expressed in these cells (16). Because altered expression of class III β-tubulin and several other neuronal-specific isotypes has been strongly associated with resistance to antimicrotubule agents (reviewed in refs. 12, 17), we hypothesized that altered expression or modifications of these proteins may influence the differential sensitivity of neuroblastoma cells to antimicrotubule agents. Further basis for this hypothesis is that not all antimicrotubule agents have been effective in neuroblastoma. For example, the administration of paclitaxel was unsuccessful in the treatment of neuroblastoma xenografts with amplified \( \text{N-myc} \) (18), and clinical use of these agents has not been adequately investigated.

The aim of this study was to determine whether alterations in neuronal-specific microtubule proteins have occurred and are involved in resistance to microtubule-destabilizing agents. We have identified distinct and novel alterations in microtubule proteins that are associated with increased microtubule stability and reduced efficacy of microtubule-destabilizing agents in neuroblastoma cells.

Materials and Methods

Cell Culture

Human neuroblastoma cells, BE(2)-C, and drug-resistant sublines, BE/CHCb0.2 (selected in 0.2 μg/mL colchicine) and BE/VCR10 (selected in 10 μg/mL vincristine), were generously provided by Drs. J. Biedler and B. Spengler (Fordham University, New York, NY) and maintained as monolayer cultures in DMEM containing 10% FCS or 20% FCS (BE/VCR10 cells only). BE/CHCb0.2 cells are 54-fold resistant to colchicine and BE/VCR10 cells are 567-fold resistant to vincristine, compared with parental cells, and both express P-gp (20).

Immunofluorescence Staining and Microscopy

BE(2)-C cells and drug-resistant sublines were grown to 80% confluency on chamber slides previously coated overnight with laminin (Sigma-Aldrich Chemical Co., St. Louis, MO). The slides were rinsed briefly with PBS, fixed in ice-cold methanol for 12 minutes at −20°C, and blocked in 10% FCS/PBS for 10 minutes at room temperature. The slides were incubated with antibody to class III β-tubulin (1:500 in 5% FCS/PBS, Covance, Richmond, CA) at 37°C for 30 minutes in a humidified chamber. Following three 10-minute washes in 0.1% Tween 20/PBS at room temperature, the slides were incubated with Cy3 anti-mouse fluorescent-tagged antibody (1:1,000 in 5% FCS/PBS). This incubation step was done for 40 minutes in a humidified chamber in the dark at room temperature. Following incubation, the slides were washed again three times for 10 minutes in 0.1% Tween 20/PBS at room temperature and rinsed briefly in deionized H2O to remove salts. The chambers were removed from the slides and the slides were mounted on a coverslip using ProLong Antifade kit (Molecular Probes, Eugene, OR). Images were captured using a Leica DM IRB inverted microscope and confocal imaging system, with Leica confocal software (Mannheim, Germany).

Protein Analysis of Microtubule Proteins

Total cellular proteins from parental and drug-resistant cells were separated on 4% to 15% SDS-PAGE (Ready gels, Bio-Rad, Hercules, CA) and electrotransferred to nitrocellulose membranes using standard methods (21). Immunoblotting was done using monoclonal antibodies to α-tubulin (1:5,000, Sigma-Aldrich Chemical), class III β-tubulin (1:1,000, Covance), class II β-tubulin (1:500, Sigma-Aldrich Chemical), and class I β-tubulin (1:5,000, a kind gift from Dr. R. Luduena, University of Texas, San Antonio, TX) as described previously with minor modifications (19). MAP4 and stathmin immunoblotting was done using rabbit polyclonal MAP4 antibody (1:1,000, kindly provided by Dr. J. Olmsted, University of Rochester, Rochester, NY) and stathmin monoclonal antibody (1:500, BD Transduction Labs, Lexington, KY). Antibodies directed against high molecular weight isofoms MAP2a and MAP2b (clone AP-20, 1:500, BD Transduction Labs) and MAP2a, MAP2b, and MAP2c isofoms (clone HM-2, 1:1,000, Sigma-Aldrich Chemical) were used for MAP2 detection. Primary antibodies were detected using horseradish peroxidase–linked secondary antibody (Amersham Pharmacia Biotech, Uppsal, Sweden) and membranes were developed using SuperSignal (Pierce Chemical Co., Rockford, IL) or Enhanced Chemiluminescence Plus (Amersham Pharmacia Biotech). Equal loadings and efficient transfer were confirmed by Ponceau S staining and reprobing of membranes for expression of a house keeping protein, glyceraldehyde-3-phosphate dehydrogenase (Abcam Ltd., Cambridgeshire, United Kingdom). Densitometry was done using a Phospholager system and Multi-Analyst software 1.02 (Bio-Rad) and results were expressed as a normalized ratio to the parental BE(2)-C cells.

Tubulin Polymerization Assay

Levels of soluble (cytosolic) and insoluble (cytoskeletal) tubulin were determined in parental and resistant cells as...
described previously (22) with minor modifications. Briefly, the cells were grown in six-well plates to 80% confluency, washed twice with PBS prior to lysing in 400 μL hypotonic buffer [1 mmol/L MgCl2, 2 mmol/L EGTA, 0.5% NP40, 10 μL protease inhibitor cocktail (Sigma-Aldrich Chemical), 20 mmol/L Tris (pH 6.8)], with or without paclitaxel (4 μg/mL; Bristol-Myers Squibb Co., Evansville, IN), and incubated at 37°C for 5 minutes in the dark. A further 200 μL hypotonic buffer was added and fractions were separated by centrifugation at 14,000 g for 10 minutes at room temperature. The supernatant (600 μL) containing the polymerized tubulin was resuspended in 600 μL hypotonic buffer. Proteins were solubilized in 4x sample buffer [4% glycerol, 20% mercaptoethanol, 9.2% SDS, 0.04% bromophenol blue, 0.3 mol/L Tris-HCl (pH 6.8)] and equal volumes of each fraction were separated by SDS-PAGE and immunoblotted with monoclonal antibody to α-tubulin as described above. All polymerization experiments were repeated at least three times.

**Sequencing of HM40 (Class I) β-Tubulin, H9 (Class II) β-Tubulin, H4 (Class III) α-Tubulin, Kα1-Tubulin, and Stathmin**

Sequencing of the HM40 (class I) β-tubulin gene, Kα1-tubulin, and stathmin in BE(2)-C and resistant cell lines was done using overlapping sets of primers as described previously (16, 23, 24). In addition, PCR amplification and sequencing primers were designed to cover the coding sequence of the H9 (class II) and H4 (class III) β-tubulin gene. The following primers were used: H9-5′-UTR-4F, 5′-ACCATGCCGAGATCGTCG-3′; H9-598R, 5′-AGGTTTCATCTCGTGTTTCCACC-3′; H9-544F, 5′-CCCTACAACGCCCACCTTCCTC-3′; H9-1099R, 5′-AGGGTGCCGACATCTTCCAGG-3′; H9-1325R, 5′-TCGCCCCCTCCTCCTCCTGA-3′; H9-929F, 5′-ACCTGACGGTGTTGCTGAC-3′; H9-1399R, 5′-TTTAAAGCCTGTTCCCTGCC-3′; H4-14F, 5′-ATGCGGAGATCGTCGAC-3′; H4-258R, 5′-CCTGGAAGATGTCCAAAGGC-3′; H4-258F, 5′-CCTGGAAGATGTCCAAAGGC-3′; H4-628R, 5′-TTGAGGTTGCGGAACCAGAT-3′; H4-491F, 5′-TGAAACACCCTT-CAGCGTCTGTG-3′; H4-1037R, 5′-CCACCTTACAGTTGTG-TGG-3′; H4-1051F, 5′-GTGGGCGGTTGTCGACATCC-3′; H4-1415R, 5′-TTTAGACACTGGTGCTGCCGC-3′; primers were designed based on the published sequence (Genbank accession nos. H9 BC018780 and H4 AF247941). QIAfilter gel extraction kit (Qiagen, Hilden, Germany) was used to purify PCR amplified cDNA, which was sequenced using fluorescence-based cycle sequencing with BigDye terminators (PE Applied Biosystems, Foster City, CA). Sequence analyses were done by the Automated Sequencing Facility, Department of Biological Sciences, University of New South Wales (Sydney, New South Wales, Australia) or the Sydney University Prince Alfred Macromolecular Analysis Center (Sydney, New South Wales, Australia). The β-tubulin sequences of the resistant cell lines were compared with that of the parental BE(2)-C cell line and the published sequence.

**Results**

**Drug-Resistant Neuroblastoma Cells Have Altered Cellular Morphology**

The morphology of the BE(2)-C and drug-resistant derivatives was examined by both phase-contrast microscopy and confocal microscopy (Fig. 1). BE/CHCb0.2 cells exhibited a similar, although slightly flatter and less rounded, morphology to BE(2)-C cells by both phase-contrast microscopy (Fig. 1A) and staining of the microtubule network (Fig. 1B). In contrast, BE/VCR10 cells exhibited a less adherent, rounder, and clumpy phenotype compared with parental BE(2)-C cells (Fig. 1A). In addition, staining of the microtubule network revealed a dense cytoplasmic staining pattern and rounded cells compared with BE(2)-C and BE/CHCb0.2 cells (Fig. 1B), suggestive of cytoskeletal alterations.

**Altered Expression of Neuronal-Specific Tubulin Isoforms in Resistant Sublines**

To determine whether neuroblastoma cells selected for resistance to either vincristine or colchicine display altered expression of tubulin proteins, immunoblotting against α-tubulin and class I, II, and III β-tubulin was done on parental BE(2)-C cells and drug-resistant sublines BE/CHCb0.2 and BE/VCR10. Levels of total α-tubulin in vincristine-resistant or colchicine-resistant cells were not significantly different compared with parental cells (P = 0.697 and 0.065, respectively; Fig. 2). Similarly, the levels of constitutively expressed class I β-tubulin were not significantly altered in either drug-resistant subline (P = 0.479 and 0.511, respectively; Fig. 2). In contrast, we observed a
small decrease in class II β-tubulin expression in BE/CHCb0.2 cells (P < 0.005; Fig. 2). Interestingly, a significant decrease in neuronal-specific class III β-tubulin expression was observed in both BE/VCR10 cells (P < 0.05) and BE/CHCb0.2 cells (P < 0.005; Fig. 3).

Increased Levels of Polymerized Tubulin in Neuroblastoma Cells Resistant to Vincristine

Resistance to microtubule-destabilizing agents has been associated previously with increased microtubule stability in cells of non-neuronal origin (16, 26). To assess the stability of microtubules in resistant neuroblastoma cells, the levels of polymerized and soluble tubulin were determined. A significant increase in the percentage of polymerized tubulin was detected in BE/VCR10 cells (26.28 ± 3.86%; P < 0.005) compared with either BE(2)-C cells or BE/CHCb0.2 cells (9.69 ± 1.88% and 8.69 ± 1.32%, respectively; Fig. 4; Table 1).

To determine the effect of altered expression of class II β-tubulin (BE/CHCb0.2 subline) and class III β-tubulin (BE/VCR10 and BE/CHCb0.2 sublines) and increased polymerized tubulin (BE/VCR10 subline) on drug-induced microtubule polymerization, the resistant cells were lysed in the presence of 4 μg/mL paclitaxel. Tubulin in all the cell lines examined was able to polymerize in the presence of paclitaxel (Fig. 4; Table 1). Having started with increased intrinsic levels of polymerized tubulin, microtubules in BE/VCR10 cells polymerized to a greater extent (59.30%) at 4 μg/mL paclitaxel than microtubules in BE(2)-C cells or BE/CHCb0.2 cells (34.86% and 27.20%, respectively; Table 1). However, when compared with drug-free controls, BE/VCR10 cells had a 2.3-fold increase in the levels of polymerized tubulin at 4 μg/mL paclitaxel. The parental cells had a 3.6-fold increase in polymer at 4 μg/mL paclitaxel compared with drug-free controls and BE/CHCb0.2 cells had a 3.1-fold increase at the same concentration of the drug (Table 1).

Absence of HM40 (Class I), Hβ9 (Class II), and Hβ4 (Class III) β-Tubulin Mutations in BE(2)-C-Resistant Sublines

Neuroblastoma BE(2)-C cells express class I, II, and III β-tubulin, and to examine whether mutations in HM40 (class I), Hβ9 (class II), or Hβ4 (class III) β-tubulin isotype genes were associated with altered microtubule stability or resistance, overlapping primers were used to sequence these genes in resistant cells. No difference in the nucleotide coding sequence between BE(2)-C, the resistant cells, and the published sequence was found for the HM40, Hβ9, and Hβ4 genes (data not shown). These results suggested that factors other than mutations in these three β-tubulin genes were contributing to resistance and altered microtubule stability.

Two-Dimensional PAGE Immunoblots of Tubulins in Resistant Sublines

To investigate whether protein modifications in α-tubulin and class I, II, or III β-tubulin were responsible for changes in microtubule stability or reduced drug efficacy in resistant cells, total cell lysates were separated by two-dimensional PAGE and immunoblotted with antibodies to these proteins. The electrophoretic mobility of protein spots identified as isoforms of α-tubulin (Fig. 5A), class I β-tubulin (Fig. 5B), class II β-tubulin (Fig. 5C), and class III β-tubulin (Fig. 5D) were not altered between parental and resistant sublines,
confirming the absence of mutations that could carry a charge change and/or of post-translational modifications. Both isoforms of class III β-tubulin were markedly reduced in resistant cells (Fig. 5D), in agreement with total cell lysate Western blotting data (Fig. 2). The ratio between the two isoforms in BE/CHCb0.2 cells was altered, with the more acidic isoform being reduced to a greater extent (Fig. 5D).

Expression of Stathmin and MAP4 Proteins in Resistant Cells

A recent study has reported an association between levels of the microtubule-destabilizing protein stathmin and altered antimicrotubule drug efficacy (27). To determine whether stathmin played a role in the resistance phenotype of the BE(2)-C sublines, protein expression was determined by Western blotting. Interestingly, stathmin was significantly increased in BE/VCR10 cells compared with either BE(2)-C or BE/CHCb0.2 cells (P < 0.05; Fig. 6A).

Figure 2. Tubulin protein expression in resistant neuroblastoma cells. Total cell lysate (7.5 μg) from BE(2)-C (lane 1), BE/VCR10 (lane 2), and BE/CHCb0.2 (lane 3) cells was resolved on 4–15% SDS-PAGE and transferred onto nitrocellulose membranes. Following electrotransfer, the membranes were probed with antibodies against α-tubulin (top), class I β-tubulin (middle), and class II β-tubulin (bottom) and visualized using horseradish peroxidase–linked secondary antibody and the SuperSignal chemiluminescence procedure. Equal loadings and efficient transfer were confirmed by Ponceau S staining of the membrane and reprobing with the housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Inset, representative blots of four independent experiments. Columns, mean of four independent runs relative to parental cell line BE(2)-C; bars, SE. *P < 0.05; **P < 0.005.

Figure 3. Class III β-tubulin expression in resistant neuroblastoma cells. Immunoblot analysis of class III β-tubulin expression was done as described in Fig. 1. Equal loadings and efficient transfer were confirmed by Ponceau S staining of the membrane and reprobing with the housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Inset, representative blots of four independent experiments. Columns, mean of four independent runs relative to parental cell line BE(2)-C; bars, SE. *P < 0.05; **P < 0.005.

Figure 4. Levels of polymerized tubulin. Sensitive BE(2)-C (top) and resistant BE/VCR10 (middle) and BE/CHCb0.2 (bottom) cells were lysed in hypotonic buffer with or without 4 μg/mL paclitaxel for 5 minutes at 37°C. Soluble (S) and polymerized (P) fractions were separated by centrifugation, resolved by SDS-PAGE, electrotransferred, and probed with antibody against α-tubulin. At least four separate experiments were done with each cell line and a representative immunoblot is shown.
Resistance to Antimicrotubule Drugs in Neuroblastoma

Table 1. Paclitaxel-induced microtubule polymerization

<table>
<thead>
<tr>
<th>Cells</th>
<th>Paclitaxel (µg/mL)</th>
<th>0</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE(2)-C</td>
<td>9.69 ± 1.88</td>
<td>34.86 ± 1.30 (3.6)</td>
<td></td>
</tr>
<tr>
<td>BE/VCR10</td>
<td>26.28 ± 3.86*</td>
<td>59.30 ± 2.49 (2.3)*</td>
<td></td>
</tr>
<tr>
<td>BE/CHCb0.2</td>
<td>8.69 ± 1.32</td>
<td>27.20 ± 4.22 (3.1)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Numbers represent % polymerized tubulin as determined by dividing the densitometric value of polymerized tubulin by the total tubulin content ± SEM of four independent experiments. Numbers in brackets represent the fold increase in % polymerized tubulin as determined by dividing % polymerized tubulin at 4 µg/mL paclitaxel by % polymerized tubulin of a drug-free control for each cell line.

*P < 0.005.

Because overexpression of a microtubule-stabilizing protein in the BE/VCR10 cells was unexpected, we sought to determine whether a mutation was responsible for this increase. The entire stathmin gene coding sequence was analyzed and no mutations in this gene were found in the BE(2)-C or resistant sublines (data not shown). To further try and identify a cause for the increased stathmin levels, we sequenced the K1-tubulin because stathmin binds to the α-tubulin component of the α/β-tubulin heterodimer (28). No nucleotide sequence alterations were found in K1-tubulin between parental and resistant cells (data not shown).

An association between the expression of microtubule-stabilizing protein MAP4 and drug resistance has been shown previously (16, 29). MAP4 is a ubiquitously expressed microtubule-associated protein and levels of this protein were not altered between the BE(2)-C cells and the resistant sublines (Fig. 6B). Multiple isoforms of MAP4 can result from the alternate splicing of RNA before transcription (30) or protein phosphorylation. BE/CHCb0.2 cells were found to have a slightly higher molecular weight MAP4 species compared with either BE(2)-C or BE/VCR10 cells (Fig. 6B).

Significant Decrease in MAP2c Protein in Resistant Cells

The microtubule-stabilizing protein MAP2 is expressed predominantly in neurons and has high molecular weight isoforms (MAP2a and MAP2b) and low molecular weight isoforms (MAP2c and MAP2d) generated by alternate splicing (31). Antibodies recognizing isoforms of MAP2 were used to examine expression levels of these proteins in BE(2)-C cells and drug-resistant sublines. Investigation of the expression levels of MAP2 isoforms in resistant cells showed no significant differences in the expression of MAP2a and MAP2b (Fig. 7A). In contrast, a dramatic decrease in the expression of MAP2c in vincristine-resistant cells (P < 0.005) and a decrease in colchicine-resistant cells were observed (P < 0.05; Fig. 7B).

Discussion

Antimicrotubule drugs are highly effective at inducing mitotic arrest and are used in the treatment of a range of human cancers, yet their use in the treatment of the childhood cancer neuroblastoma is often negated by resistance or toxicity problems. The molecular mechanisms responsible for the limited efficacy of antimicrotubule agents in the treatment of this childhood cancer have not been defined. To date, multiple factors have been found to contribute to drug resistance to antimicrotubule drugs including increased expression of drug transporters and/or tubulin/microtubule changes (12, 32). Specifically, we have previously reported microtubule alterations in high P-gp-expressing leukemia cells resistant to Vinca alkaloids (16), suggesting that these changes can occur at the early stages of drug resistance development prior to P-gp induction. Neuroblastoma cells BE(2)-C and their vincristine-resistant and colchicine-resistant sublines were originally characterized in terms of their MDR phenotype and found to express high levels of P-gp (20). However, a later study, while confirming original P-gp measurements using a...
self-competitive binding assay, also revealed that P-gp concentrations in neuroblastoma resistant lines did not display a direct correlation with relative resistance levels to the P-gp substrates (33), thus supporting the view that P-gp is not the only variable responsible for the drug resistance phenotype in these cells.

Using phase-contrast and confocal microscopy, we observed differences in morphology and microtubule structure between BE(2)-C and drug-resistant sublines, suggesting that changes in the tubulin/microtubule system may have occurred in these cell lines. Indeed, our current study of these drug-resistant sublines has identified distinct alterations in both the cellular target of antimicrotubule drugs and in proteins that regulate microtubule dynamics and stability.

Several β-tubulin isotypes are expressed at high levels in neuronal cells, including constitutively expressed class I β-tubulin and neuronal-specific class II and III β-tubulin. Increased expression of class I β-tubulin was described in paclitaxel-resistant non-small cell lung cancer cell lines and ovarian tumors (14). In contrast, the levels of class I β-tubulin were unchanged in either colchicine-resistant or vincristine-resistant neuroblastoma cells, suggesting that altered expression of this isotype is unlikely to be contributing to resistance. Unlike several previous studies describing resistance to antimicrotubule drugs (16, 22, 25, 34, 35), no mutations in class I β-tubulin were identified in the drug-resistant neuroblastoma cells examined in this study. There has been one report on β-tubulin mutations in clinical samples (36). However, subsequent reports have been unable to substantiate these findings (reviewed in ref. 37), and if they do occur, they are likely to be a rare event.

Both vincristine-resistant and colchicine-resistant neuroblastoma cell lines displayed significant decreases in expression of class III β-tubulin. Class III β-tubulin is predominantly expressed in neuronal cells, although increased expression of this isotype has been detected in several non-neuronal malignant cell types (reviewed in ref. 38). We recently reported aberrant expression of class III β-tubulin in a T-cell leukemia cell line, CCRF-CEM, and Vinca alkaloid–resistant sublines derived from these cells had significantly reduced levels of this tubulin protein (16). Importantly, this current study now provides independent evidence in neuronal cells that class III β-tubulin down-regulation is associated with resistance to microtubule-stabilizing drugs. This is the opposite effect to what has been observed with microtubule-stabilizing agents such as paclitaxel and the epothilones, in which increased levels of class III β-tubulin have been associated with resistance in cell lines and clinical samples (14, 15, 25). Taken collectively, these data support the hypothesis that cancer cells expressing class III β-tubulin are modulating their levels of this isotype as an adaptive strategy to circumvent the...
effects of microtubule-targeted drugs. Moreover, tubulin depleted of class III isotype assembled faster compared with unfractionated tubulin in the presence of microtubule-associated proteins (39) or paclitaxel (40), whereas microtubules assembled from this isotype were found to be more dynamic (41) and less sensitive to paclitaxel (42). Similar studies on microtubule-destabilizing agents have been limited. It is highly likely therefore that decreased levels of class III β-tubulin in resistant neuroblastoma cells lead to subtle changes in microtubule dynamics so that the effect of microtubule-destabilizing drugs is diminished. A recent study reported that increasing the exogenous levels of class III β-tubulin led to a decrease in microtubule assembly and decreased sensitivity to paclitaxel (43). Interestingly, decreased expression of class III β-tubulin in our study was only associated with changes in microtubule stability in vincristine-resistant neuroblastoma cells and not in the colchicine-resistant cells, suggesting that these BE/VCR10 cells may have accumulated different changes that help them to counteract the effects of the drug. Similarly, we have shown previously that changes in class III β-tubulin in vinblastine-resistant leukemia were not associated with increased microtubule polymer levels (16).

The decrease in class II β-tubulin observed in colchicine-resistant neuroblastoma cells is also of interest. This protein is one of the major β-tubulin isotypes expressed in neurons and has been found at increased levels in some malignant cell types (44). Previously, increased levels of this isotype were found in association with paclitaxel resistance in a murine macrophage-like cell line (45) and in a non-small cell lung cancer cell lines (14). These findings suggest that changes in class II β-tubulin may be involved in resistance to both microtubule-stabilizing and microtubule-destabilizing drugs depending on whether expression is increased or decreased. However, overexpression of this isotype by transfection has failed to show a role in paclitaxel resistance (11, 46). One alternate explanation is that exogenous expression of class II β-tubulin isotype alone might be insufficient to confer resistance or a combination of factors is necessary. This was exemplified in a comprehensive proteomic analysis of Vinca alkaloid drug response and resistance in leukemia cells, wherein we recently highlighted the complex interplay between cytoskeletal proteins (47). In recent studies, compensatory microtubule changes have been shown to occur in drug-resistant cell lines to counterbalance tubulin mutations (23, 25). Because drug-resistant neuroblastoma cells displayed altered expression of predominantly neuronal class II and III β-tubulin but not of constitutive class I β-tubulin, the changes in the tubulin/microtubule system in these cells could reflect a specific response to the action of antimicrotubule agents rather than a global change in microtubule proteins.

Increased polymerized tubulin has been associated with tubulin mutations and resistance to antimitotic drugs (16, 26). Similarly, we identified a significant increase in polymerized tubulin in BE/VCR10 cells compared with BE(2)-C and BE/CHCb0.2 cells. Microtubules in these cells retained their ability to undergo paclitaxel-induced polymerization, albeit to slightly variable extents. We recently described increased levels of polymerized tubulin associated with a mutation in HM40 (class I) β-tubulin gene in vincristine-resistant leukemia cells, CEM/VCR R (16). In contrast, BE/VCR10 cells have increased levels of polymerized tubulin in the absence of a class I β-tubulin mutation. Moreover, mutations were also absent in H19 (class II) and Hj4 (class III) β-tubulin isotype genes. It was clear therefore that mutations in class I, II, or III β-tubulin were not involved in resistance or changes in microtubule stability in the neuroblastoma drug-resistant sublines. This was further supported by the fact that there was no isolectric shift in the protein position on two-dimensional immunoblots. Increased polymerized tubulin identified in the vincristine-resistant cells may reflect increased microtubule stability, which in turn leads to the reduction of the microtubule-destabilizing effects of vincristine. During the selection of vincristine-resistant cells, prior to P-gp induction, this alteration in microtubule stability would have given these cells a distinct survival advantage in the presence of drug.

A somewhat unexpected result was the significant increase in the expression of the microtubule-destabilizing protein, stathmin, in BE/VCR10 cells. Stathmin (also known as Op18, metablastin) is a small cytoplasmic protein overexpressed in several tumor types (48, 49), including aggressive neuroblastoma (50). Stathmin is thought to act by two mechanisms—as a tubulin-sequestering protein and as a catastrophe promoter (51, 52)—and its activity is regulated by phosphorylation at multiple sites (53–55). Recently, a mutation in this protein has been identified in human esophageal carcinoma cells (24). We reasoned that a mutation in stathmin could potentially result in altered stathmin binding to tubulin and consequently affect microtubule stability and lead to drug resistance in drug-resistant neuroblastoma cells. However, no stathmin mutations were identified in the BE(2)-C resistant sublines. In addition, the phosphorylation status of stathmin isoforms, as determined by two-dimensional PAGE, was not altered (data not shown). Another possibility for altered microtubule stability is a mutation in α-tubulin. Martello et al. (23) recently showed increases in the active nonphosphorylated forms of stathmin and decreased microtubule stability associated with a K41-tubulin mutation. In contrast, resistant neuroblastoma cells characterized in our study did not harbor mutations in K41-tubulin. Taken together, overexpression of the destabilizing protein stathmin in BE/VCR10 cells is most likely compensating for the increased microtubule stability. Because the microtubule turnover in dividing cells is ~20 times faster compared with interphase cells (56), microtubule dynamics is crucial for cell division. We propose that increased expression of stathmin allows successful completion of mitosis in vincristine-resistant cells, which have more stable microtubules but are not dependent on the drug for survival. However, at this stage,
we cannot exclude the possibility that a mutation in another component of the tubulin/microtubule system is involved in the altered microtubule stability in BE/VCR10 cells.

Microtubule stability is also regulated by microtubule-stabilizing proteins, such as MAP4, which bind to the microtubule wall and stabilize microtubules (57). Unlike our recent findings of increased expression of MAP4 in vincristine-resistant and vinblastine-resistant leukemia cells (16), expression levels of this microtubule-associated protein were not altered in the resistant neuroblastoma cells. Interestingly, colchicine-resistant cells expressed a higher molecular weight MAP4 isoform compared with BE(2)-C and BE/VCR10 cells, which may represent an alternate splice variant or a different phosphoisoform of MAP4. Whether alternative splicing of MAP4 has a role in resistance is not known, but it has been shown that different isoforms have different effects on microtubule dynamics and function (30). Phosphorylation of MAP4 without a change in expression levels has been associated with sensitivity to antimicrotubule agents (58).

Because neuronal cells express other microtubule-associated proteins such as MAP2 that can also affect microtubule stability (59), we examined the expression of this protein in the resistant cells. MAP2 functions primarily in neuronal morphogenesis and regulation of cytoskeletal dynamics. This protein is able to bind both tubulin and actin through its tubulin binding domain and modify stability of microtubules and microfilaments (60, 61). High molecular weight isoforms MAP2a and MAP2b and low molecular weight isoforms MAP2c and MAP2d are expressed at different stages of development and differentiation, with MAP2c appearing in more primitive, undifferentiated cells compared with high molecular weight MAP2 isoforms (62). Several studies have suggested that increased expression of MAP2c in BE/VCR10 cells is likely to be contributing to hyperstable microtubules by shifting the equilibrium toward MAP2 isoforms such as MAP2a and MAP2b that are stronger promoters of microtubule stability than MAP2c. Decreased expression of MAP2a was also observed in BE/CHCbo.2 cells but to a much lesser extent than BE/VCR10 cells. Decreased MAP2c in BE/CHCbo.2 cells may play a subtle role in counteracting the destabilizing effects of colchicine. To date, studies into the role of MAP2 isoforms in drug resistance in neuroblastoma have been limited. This is the first report of altered expression of a MAP2 isoform in association with antimicrotubule drug resistance in neuroblastoma and further investigation of the significance of MAP2c in resistance to antimicrotubule agents is warranted.

Elucidation of drug resistance mechanisms and improved therapeutic targeting in childhood neuroblastoma is crucial to improve the long-term survival of patients with advanced stage disease. We have identified distinct alterations in expression of neuronal-specific tubulin/microtubule proteins associated with resistance to microtubule-destabilizing drugs in neuroblastoma. These findings provide suggestive evidence that microtubule protein changes in neuroblastoma may play a role in resistance to antimicrotubule agents. The finding of decreased class III β-tubulin and MAP2c expression in neuroblastoma cells is novel and further studies investigating these changes in drug refractory tumors are necessary to determine the clinical significance of these data. Delineating mechanisms of resistance to microtubule-targeted agents in neuroblastoma and other cancers will allow rational drug design and better treatment strategies to be implemented.

Acknowledgments
We thank Drs. June Biedler and Barbara Spengler for providing the drug-resistant neuroblastoma cell lines and Claudia Flemming for helpful suggestions and reading of the article.

References
Resistance to Antimicrotubule Drugs in Neuroblastoma


Molecular Cancer Therapeutics

Neuronal-associated microtubule proteins class III β-tubulin and MAP2c in neuroblastoma: Role in resistance to microtubule-targeted drugs

Sima Don, Nicole M. Verrills, Tracy Y.E. Liaw, et al.


Updated version  Access the most recent version of this article at: http://mct.aacrjournals.org/content/3/9/1137

Cited articles  This article cites 56 articles, 20 of which you can access for free at: http://mct.aacrjournals.org/content/3/9/1137.full.html#ref-list-1

Citing articles  This article has been cited by 13 HighWire-hosted articles. Access the articles at: /content/3/9/1137.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.