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Multiple Microtubule Alterations Are Associated with *Vinca* Alkaloid Resistance in Human Leukemia Cells[1]

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ABSTRACT

***Vinca* alkaloids are used extensively in the treatment of childhood acute lymphoblastic leukemia (ALL) and despite their usefulness, drug resistance remains a serious clinical problem. *Vinca* alkaloids bind to the β-tubulin subunit of the α/β-tubulin heterodimer and inhibit polymerization of microtubules. Recent studies have implicated altered β-tubulin isotype expression and mutations in resistance to microtubule-stabilizing agents. Microtubule-associated protein (MAP) MAP4 binds to and stabilizes microtubules, and increased expression is associated with decreased sensitivity to microtubule-depolymerizing agents. To address the significance of β-tubulin and MAP4 alterations in childhood ALL, two CCRF-CEM-derived *Vinca* alkaloid resistant cell lines, VCR R (vincristine) and VLB100 (vinblastine), were examined. Decreased expression of class III β-tubulin was detected in both VCR R and VLB100 cells. VCR R cells and to a lesser extent VLB100 cells expressed increased levels of MAP4 protein. Increased microtubule stability was observed in these VCR R cells as identified by the high levels of polymerized tubulin (45.6 ± 2.6%; $P < 0.005$) compared with CEM and VLB100 cells (24.7 ± 3.3% and 24.7 ± 2.5%, respectively). Expression was associated with a single MAP4 isoform in the polymerized microtubule fraction in CEM and VCR cells. In contrast, VLB100 cells expressed a lower molecular weight isoform in the polymerized fraction. Two-dimensional-PAGE and immunoblotting revealed marked posttranslational changes in class I β-tubulin in VCR R cells not evident in CEM cells. Sequencing of the β-tubulin (HM40) gene identified a point mutation in VCR R cells in nucleotide 843 (CTC→ATC; Leu^{240}→Ile) that was not present in CEM or VLB100 cells. This mutation resides in a region of β-tubulin that lies in close proximity to the α/β tubulin interface. Multiple alterations related to normal microtubule function were identified in ALL cells selected for resistance to *Vinca* alkaloids, and these alterations may provide important insight into mechanisms mediating resistance to *Vinca* alkaloids.**

INTRODUCTION

ALL[3] is the most frequent childhood cancer. Whereas the majority of patients will be long-term survivors, almost one-third of patients will relapse and die because of the development of drug resistance. *Vinca* alkaloids are an important group of natural-product drugs used extensively in the treatment of ALL and other pediatric malignancies (1). The two most commonly used *Vinca* alkaloids, VCR and VLB, are structurally similar; however, they exhibit differences in toxicity and antiproliferative effects (2). Microtubules, which are composed of α/β tubulin heterodimers, are dynamic structures that are constantly growing and shortening (3). Microtubule dynamics and stability play an important role in many cellular events, particularly in cell division where vivid changes in microtubule architecture occur. This has resulted in the tubulin/microtubule system becoming an important target for cancer chemotherapy (4).

Vinca alkaloid resistance has been attributed to a number of mechanisms associated with the multidrug-resistance phenotype including overexpression of P-glycoprotein and the multidrug resistance-associated protein (5–7). We have described previously the selection of a highly VCR-resistant T-cell leukemia cell line, CEM/VCR R (8). Although these cells express P-glycoprotein, they are 22,600-fold resistant to the selecting agent, VCR, and display much lower levels of cross-resistance to other nonantimicrotubule multidrug-resistance drugs (range, 35–187-fold). Furthermore, verapamil, an agent that blocks the action of the P-glycoprotein-drug efflux pump, only partially reverses resistance to VCR in these cells (9). The multifactorial nature of drug resistance strongly suggests that other mechanisms distinct from P-glycoprotein are operating in these cells. We therefore postulated that high levels of resistance to the selecting agent may have been attributable to an alteration in the drug target.

Drugs such as the *Vinca* alkaloids disrupt microtubule dynamics by interacting with the β-tubulin subunit of the α/β tubulin heterodimer. At least six isotypes of both α- and β-tubulin have been identified and are encoded by a large, multigene family that has been highly conserved throughout evolution. Tubulin isotypes display tissue-specific expression, suggesting a possible functional role for individual isotypes. In recent years, interest has focused on the tubulin/microtubule system and its role in resistance to antimitotic drugs (4, 10, 11). Our own studies and those of others have described changes in the expression of specific α- and/or β-tubulin isotypes in cell lines resistant to the antimicrotubule agent Taxol (11–17). We also demonstrated altered expression of a neuronal-specific β-tubulin isotype in ovarian tumors that were clinically resistant to Taxol (15). A possible functional role in resistance to Taxol was demonstrated when down-regulation of class III β-tubulin expression by antisense oligonucleotides resulted in increased sensitivity to Taxol (18). Recently, mutations in the human β-tubulin gene-encoding class I β-tubulin, HM40, have been identified in human ovarian carcinoma cell lines selected for resistance to either Taxol or epothilone (19, 20). To date, studies examining altered tubulin and/or microtubule protein expression or mutations have been performed primarily on human cell lines resistant to agents that stabilize microtubules against depolymerization. The aim of our study was to determine whether selection of human ALL cells for resistance to *Vinca* alkaloids leads to alterations in microtubule proteins. Our findings strongly suggest that multiple microtubule changes are associated with resistance to *Vinca* alkaloids and that some of these lead to increased microtubule stability and, hence, counteract and reduce the effectiveness of *Vinca* alkaloids.

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[3] The abbreviations used are: ALL, acute lymphoblastic leukemia; VCR, vincristine; VLB, vinblastine; mAb, monoclonal antibody; 2DE, two-dimensional gel electrophoresis; IEF, isoelectric focusing.

MATERIALS AND METHODS

Maintenance of *Vinca* Alkaloid-resistant Cell Lines. Human T-cell ALL cells, CCRF-CEM, and drug-resistant sublines CEM/VCR R and CEM/VLB100, were maintained as suspension cultures in RPMI 1640 containing 10% FCS. As described in an earlier report, CEM/VCR R and CEM/VLB100 are 22,600- and 6,667-fold resistant to VCR, respectively (8).

Protein Analysis of Microtubule Proteins. Total cellular proteins (10 μg) were resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes using standard methods (21). Immunoblotting was performed using mAbs to α-tubulin, class III β-tubulin (Sigma Chemical Co.), and class I β-tubulin (1:5000; kindly provided by Dr. R. Luduena, University of Texas, San Antonio, TX), as described previously (15) with minor modifications. After incubation with an antimouse horseradish-peroxidase-linked IgG antibody (Amersham Pharmacia), membranes were developed using Supersignal (Pierce, Rockford, IL). Equal loadings and efficient transfer were confirmed by both Ponceau S staining the membrane and immunodetection with an antiactin antibody (Sigma Chemical Co.-Aldrich, St. Louis, MO). MAP4 immunoblotting was performed on proteins resolved on 4–15% SDS-PAGE (Ready Gels; Bio-Rad) as described previously using a rabbit polyclonal MAP4 antibody (1:1000; which was the kind gift of Dr. J. Olmsted, University of Rochester, Rochester, NY) and detected using antirabbit horseradish-peroxidase-linked IgG antibody (Amersham Pharmacia). Protein quantitation was determined by dividing the densitometric ratio of the test protein by that of the control protein (actin) and the results expressed as a normalized ratio to the parental CEM cells.

Tubulin Polymerization Assay. Levels of soluble (cytosolic) and insoluble (cytoskeletal) tubulin where determined in drug-resistant populations. Soluble and polymerized tubulin from cell lysates were separated by centrifugation as described previously (19, 22). Briefly, leukemia cell lines (1×10^6 cells) were washed twice with PBS before lysing at 37°C with 100 μl of hypotonic buffer {1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 10 μl Protease Inhibitor mixture (Sigma Chemical Co.-Aldrich), and 20 mM Tris-HCl, [pH 6.8]} in 1.5-ml microfuge tubes for 5 min in the dark. An additional 100-μl hypotonic buffer was added, the cell lysates were vortexed briefly, and the fractions separated by centrifugation at 14,000 rpm for 10 min at room temperature. The supernatant (200 μl) containing the soluble tubulin was transferred to another tube. The pelleted fraction containing the polymerized tubulin was then resuspended in 200 μl of hypotonic buffer. Proteins were solubilized in 4× sample buffer [45% glycerol, 20% mercaptoethanol, 9.2% SDS, 0.04% bromphenol blue, and 0.3 M Tris-HCl (pH 6.8)] and sonicated on ice before heating samples to 100°C for 5 min. Proteins (25 μl) were separated using 4–15% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with mAb to α-tubulin as described previously. Results were expressed as a percentage of polymerized tubulin by dividing the densitometric value of polymerized tubulin (insoluble) by the total tubulin content (sum of densitometric value of soluble and polymerized tubulin). To confirm MAP4 binding to polymerized microtubules, membranes containing soluble and insoluble fractions were probed with MAP4 antibody as described previously. All of the polymerization experiments were repeated between 4 and 6 times.

2DE. CEM and VCR R cells (1×10^7 cells) were solubilized in 1 ml of IEF buffer (5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 2% Sulfobetaine 3–10, 2 mM Tributyl phosphine, 40 mM Tris, 0.5% ampholytes) and 150 units of exonuclease (Sigma Chemical Co.). Samples were separated using IEF on pH 4–7 Immobiline Strips (Amersham Pharmacia). Second-dimension separation of proteins was achieved on 4–15% polyacrylamide gradient gels (Ready Gels; Bio-Rad) before electrotransfer and immunoblotting as described previously.

Sequencing of HM40 (Class I) β-Tubulin. Sequencing of the predominant β-tubulin isotype gene, HM40 (class I), in *Vinca* alkaloid-resistant cells was performed using overlapping sets of primers as described previously (19) with some modifications. Briefly, the following primers were used instead of, or in addition to, those published previously: -125F (5′UTR)- 5′-ACCTCGCTGCTCCAGCCTCT-3′; M40–803R, 5′-AAAGAAATGGAGACGT-3′; M40–3′UTR-4091R, 5′-ACTGAGAAGCCTGAGGTGAT-3′ (GenBank accession no. HM40 J00314). PCR-amplified cDNA was purified using the QIAfilter Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced using fluorescence-based cycle sequencing with BigDye terminators (PE Biosystems, Foster City, CA). Sequence analyses were performed by the Automated Sequencing Facility, Biological Sciences, University of New South Wales. The HM40 β-tubulin sequence of the resistant cell lines was compared with that of the parental CEM cell lines and the published sequence (23, 24). In addition, normal human heart and peripheral blood tissues were used to confirm the sequences.

Modeling of Tubulin Structure. Tubulin modeling was generated from the tubulin structure (Protein Data Base 1TUB) using the Insight II modeling program (Molecular Simulations Inc.) as described previously (25).

RESULTS

Class III β-Tubulin Protein Expression Is Altered in Resistant Cells. Mammalian cells selected for resistance to depolymerizing drugs have been reported previously to increase their total tubulin synthesis (22). To determine whether selection of *Vinca* alkaloid-resistant cell populations altered tubulin protein expression, CEM cells and their drug-resistant sublines, VCR R and VLB100, were examined by Western blotting using mAbs against α-tubulin, class I β-tubulin, and class III β-tubulin. Total tubulin, as reflected in the level of α-tubulin protein, was slightly increased in the VLB100 cells compared with CEM and VCR R cells (Fig. 1). Furthermore, levels of

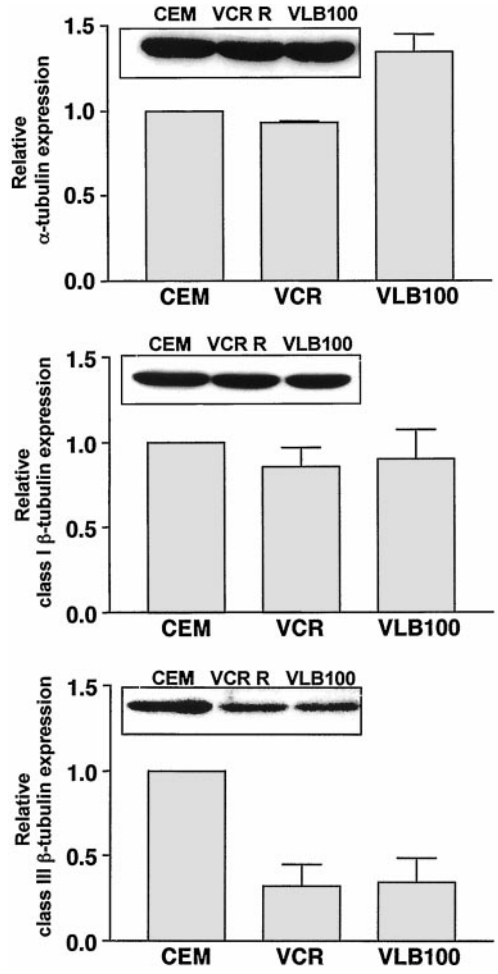

Fig. 1. Immunoblot analysis of tubulin in *Vinca* alkaloid-selected cells. Total cellular protein (10 μg) from CCRF-CEM (*Lane 1*), VCR R (*Lane 2*), and VLB100 (*Lane 3*) was resolved on 12% SDS-PAGE after electrotransfer onto nitrocellulose membranes and reacted with antibodies against α-tubulin, class I, and class III β-tubulin and visualized using horseradish peroxidase-linked secondary antibody and the Supersignal chemiluminescence procedure. Membranes were reprobed with actin to confirm loadings. Inset panel above each graph is a representative blot of three independent experiments. Protein quantitation was determined by dividing the densitometric volume of the tubulin by the densitometric volume of the actin control and the results expressed as a normalized ratio to the parental CEM cells. *Bars*, ± SE of three runs.

the constitutively expressed class I β-tubulin isotype were similar in parental and drug-resistant cell lines. In contrast, there was a significant decrease in the level of class III β-tubulin in both VCR R and VLB100 cells. Efficient electrotransfer of samples was confirmed by Ponceau S staining of the membrane before immunodetection (data not shown).

Altered Expression of MAP4 in *Vinca* Alkaloid-resistant Cells. MAPs bind to and stabilize microtubules. Modifying their expression can lead to the altered efficacy of antimicrotubule drugs (26, 27). To investigate whether the predominant non-neuronal MAP, MAP4, was altered in the *Vinca* alkaloid-resistant cells, protein expression levels were determined by Western blotting. A significant increase in MAP4 expression was detected in VCR R cells and to a lesser extent in the VLB100 cells compared with parental CEM cells (Fig. 2). This is consistent with the finding that MAP4 gene expression was significantly increased in the VCR R cells compared with either the CEM or VLB100 cells (28). MAP4 is a high molecular weight protein (M_r ~210,000) that can produce a number of alternate splice variants (29), and interestingly, the VLB100 cells expressed a lower molecular weight MAP4 species compared with either CEM or VCR R cells (Fig. 2).

Polymerized Tubulin Levels Are Increased in VCR-resistant Cells. To address the possibility that microtubule stability might be associated with the resistant phenotype, levels of polymerized and soluble tubulin were examined in CEM, VCR R, and VLB100 cells. Increased levels of polymerized tubulin were observed in VCR R cells compared with CEM and VLB100 cells (Fig. 3). When this data were expressed as percentage of polymerized tubulin, a significant increase was observed in VCR R (45.6 ± 2.6; $P < 0.005$) compared with CEM and VLB100 cells (24.7 ± 3.3 and 24.7 ± 2.5, respectively; Fig. 3).

Because MAP4 protein increased in VCR R cells (Fig. 2), it was important to determine whether it was indeed functional and binding to the microtubules. Western blotting was performed on soluble and polymerized cellular protein fractions, and the increased MAP4 expression strongly correlated with stable microtubules in VCR R cells (Fig. 4). However, in the VLB100 cells two prominent immunoreactive bands were detected in the soluble fraction. The upper band displayed the same electrophoretic mobility as the single band detected in CEM and VCR R cells. Consistent with the expression of a lower molecular weight MAP4 species in VLB100 cells (Fig. 2.), the polymerized fraction in VLB100 cells had an immunoreactive band that was a lower molecular weight than that of CEM or VCR R cells. In contrast with CEM and VCR R cells, the MAP4 associated with the polymerized fraction in VLB100 cells did not correspond in size to either of the two species detected in the soluble protein fraction in these cells (Fig. 4).

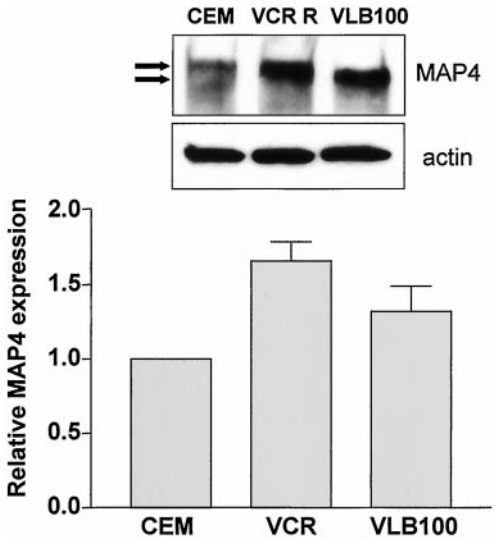

Fig. 2. MAP4 expression in *Vinca* alkaloid-resistant cells. Samples were probed with MAP4 antibody and immunoreactive bands detected as described in Fig. 1. CCRF-CEM (*Lane 1*), VCR R (*Lane 2*), and VLB100 (*Lane 3*). Proteins for immunoblotting were resolved on 4–15% SDS-PAGE. Inset panel is a representative blot of three independent experiments showing the MAP4 and actin immunoreactive bands. *Top arrow* for MAP4, migration of electrophoretic band of MAP4 in CEM and VCR R cells. *Bottom arrow*, migration in VLB100 cells. MAP4 protein levels were determined by dividing densitometric volume of MAP4 by densitometric volume of actin control and results expressed as a normalized ratio to the parental CEM cells. *Bars*, ± SE of three runs.

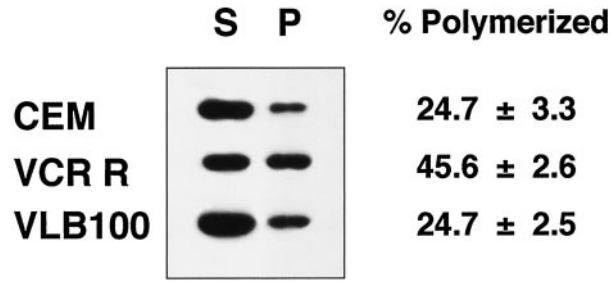

Fig. 3. Levels of polymerized tubulin were determined in drug-sensitive CEM and the *Vinca* alkaloid-selected VCR R and VLB100. Cells were lysed in hypotonic buffer for 5 min at 37°C followed by separation of the soluble (*S*) and polymerized (*P*) fractions by centrifugation. Protein fractions were separated and resolved by SDS-PAGE, transferred, and the membrane probed with antibody against α-tubulin. Fractionation of cells was performed on a minimum of four separate occasions, and percentage of polymerized tubulin was determined by dividing densitometric value of polymerized tubulin by total tubulin content (soluble + polymerized). *P*, polymerized; *S*, soluble; mean ± SE.

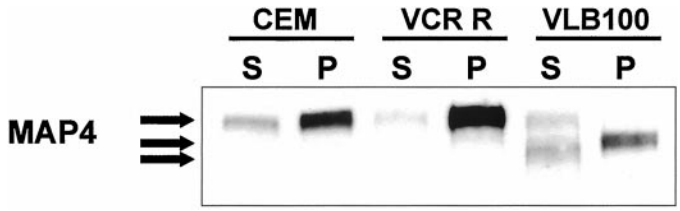

Fig. 4. MAP4 distribution in soluble and polymerized fractions. Protein fractions from CEM, VCR R, and VLB100 cells were prepared and separated as described in Fig. 2. Membranes were probed with MAP4 antibody. *Arrows*, position of MAP4 isoforms detected. *P*, polymerized; *S*, soluble.

2DE and Immunoblotting Identifies Posttranslational Changes in β-Tubulin (Class I). To identify whether the increased level of polymerized tubulin in the VCR R cells was associated with posttranslational changes or mutations, total cell lysates were separated by 2DE and electrotransferred proteins immunoblotted with the constitutively expressed class I β-tubulin mAb. VCR R cells exhibited a train of proteins that was not evident in the parental drug-sensitive CEM cells and was consistent with posttranslational modifications of this β-tubulin isotype (Fig. 5). The class I β-tubulin immunoblot profile of the VLB100 cells did not differ from that of the CCRF-CEM cells (data not shown).

A Mutation in HM40 (Class I) β-Tubulin Identified in VCR-resistant Cells. Mutations in HM40 (class I) β-tubulin have been associated previously with altered microtubule stability in the presence of drugs such as Taxol (19, 20). To establish whether a mutation was responsible for the altered levels of polymerized tubulin or resistance to *Vinca* alkaloids, sequencing of the HM40 gene was performed on cDNA from CEM, VCR R, and VLB100 cells. There was no difference in the nucleotide sequence between CEM and VLB100 cells (data not shown). In contrast, a heterozygous point mutation at nucleotide 843 (CTC→ATC; Leu240→Ile240) was identified in VCR R cells that was not present in CEM cells (data not shown). This heterozygous mutation was confirmed by sequencing the

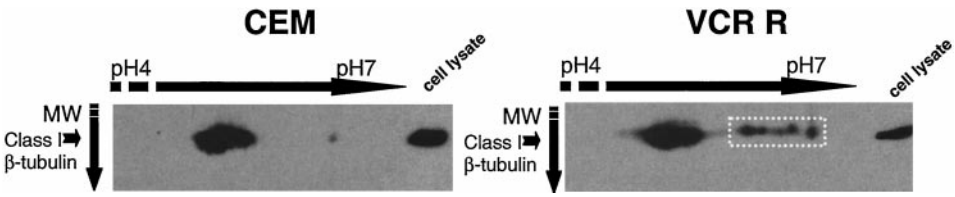

Fig. 5. Two-dimensional gel immunoblot was performed on drug-sensitive CEM (*left*) and VCR-selected VCR R (*right*) cells by IEF total cellular protein (~50 μg) at pH 4–7 before second-dimension separation on 4–15% SDS-PAGE. Proteins were transferred and membranes probed with class I β-tubulin antibody and visualized using horseradish peroxidase-linked secondary antibody and chemiluminescence. As a positive control for each immunoblot, *far right lane* on each gel represents total cell lysate (10 μg) of each sample separated according to molecular size.

forward and reverse strands of the entire HM40 gene. Modeling of the β-tubulin mutation revealed that the Leu240→Ile240 mutation was in close proximity to Asn247, Ala248, and Asp249, which are at the longitudinal interface between α- and β-tubulin monomers (Refs. 30, 31; Fig. 6*A*). The side chains of Leu240 are ~3Å from the side chain of Ala248. Leu240 is also at the COOH-terminus of the H7 helix, which is thought to play an important role in regulating microtubule stability (32). Amino acids 238–250 form a folded loop connecting the H7 and H8 helixes. The Leu240 to Ile240 mutation may change its interaction with Ala248 (Fig. 6*B*). However, it should be noted that the indicated distances between the side chains in Fig. 6*B* are approximate estimates only and were performed to visualize the mutation in relationship to its position to the other amino acids in β-tubulin and its possible effect on the conformation of the H7 and H8 helixes.

DISCUSSION

Drug resistance is a multifactorial process, and *Vinca* alkaloid-resistance has been associated with a number of mechanisms including increased levels of proteins associated with the multidrug-resistance phenotype (7, 33). Although both the *Vinca* alkaloid-resistant cell lines examined in this study express P-glycoprotein (8, 34), this did not negate the possibility that tubulin/microtubule changes may have occurred as an early event during the selection of these resistant cell populations. Indeed, tubulin changes in *Vinca* alkaloid-resistant cells expressing P-glycoprotein have been reported previously (16, 35–37) and are likely to have given these cells a selective advantage at an early stage during the drug selection process. In a recent study we found that alterations in β-tubulin occurred as an early event during the selection of resistance to Taxol and before P-glycoprotein expression being detected (15).

Class I β-tubulin is often the predominant isotype in cell lines of non-neuronal origin (38), and similarly, we detected strong expression of this isotype in CEM cells. Previous studies have reported altered levels of β-tubulin isotypes in human cells selected for resistance to Taxol (12, 14, 15). Similar studies on *Vinca* alkaloids have been limited. Recently, Sirotnak *et al.* (16), described increased expression of class I and IV β-tubulin in *Vinca* alkaloid-resistant Chinese hamster ovary cells that was associated with decreased binding of VCR to tubulin. In a lymphoma cell line selected for resistance to VCR and expressing P-glycoprotein, altered expression of β-tubulin isotypes was also associated with decreased VCR binding (37). In contrast, we have demonstrated that class I β-tubulin expression levels were unchanged in the *Vinca* alkaloid-resistant cell populations. Previously, we reported that Taxol-selected lung cancer cell lines and clinically resistant ovarian tumors expressed 2- and 3.6-fold increases in this isotype, respectively (15). In this same study, class III β-tubulin was increased in both Taxol-selected lung cancer cell lines and ovarian tumors clinically resistant to this agent. In contrast, both the VCR- and VLB-selected leukemia cells had decreased expression of class III β-tubulin. Moreover, we have reported previously decreased expression of the Hβ4 (class III) β-tubulin gene in VCR-resistant neuroblastoma cells (39). A functional role for this isotype in mediating resistance to an antimicrotubule drug was demonstrated when expression of class III β-tubulin was decreased using antisense oligonucleotides to Hβ4 (class III β-tubulin gene), and this lead to increased sensitivity to Taxol (18). On the basis of these findings we hypothesize that decreased expression of class III β-tubulin may favor either more stable microtubules or affect microtubule dynamics in such a way that the effect of *Vinca* alkaloids is diminished. This is supported by *in vitro* studies showing that class III β-tubulin-depleted microtubules undergo Taxol-induced polymerization much more readily than unfractionated tubulin (40). Differences between cells selected for resistance to either Taxol or VCR are not surprising, because *in vitro* studies suggest that β-tubulin isotype composition can regulate microtubule dynamics and that these drugs differ in their interaction with purified isotypes (41–43).

One approach to identify the role of specific tubulin isotypes in drug resistance has been to overexpress these proteins in cell lines. However, altering the expression of specific tubulin isotypes is far from straight forward because of the complexity of tubulin autoregulation (44). To date, increasing the expression of either class I, II, or IVb β-tubulin has failed to confer resistance in transfected cells (11, 45). Therefore, altered expression of particular isotypes may not be directly related to the resistance phenotype of the cells or, alternatively, transfection of a specific tubulin isotype alone does not reflect the complexity of other associated proteins or cofactors that interact with the specific tubulin isotype. The latter seems the most feasible, as it is likely that MAP interactions, microtubule dynamics, and stability influence the efficacy of antimicrotubule drugs in cells.

Minotti *et al.* (22) described increased levels of polymerized tubulin in Colcemid-resistant mutants of Chinese hamster ovary cells. Colcemid, like the *Vinca* alkaloids, destabilizes microtubules, and although the two drugs share the same cellular target, they differ in their binding sites on β-tubulin (46). Similarly, our studies demonstrated that VCR R cells, unlike VLB100 cells, maintained almost double the level of tubulin in the polymerized state than the parental CEM cells without an increase in their total tubulin protein. Increased stabilization of microtubules decreases the binding of depolymerizing agents to the tubulin heterodimer by decreasing access of the drug to the cellular target and ultimately reducing the efficacy of these drugs (47).

Stable microtubules often contain posttranslationally modified tubulin (48), but modifications such as acetylation and detyrosination may occur as a consequence rather than a trigger for increased microtubule stability (49). In support, elevated levels of acetylated α-tubulin have been reported previously in VCR R cells (50). The VCR R cells displayed a number of changes associated with increased microtubule stability including posttranslational modification of class I β-tubulin and increased MAP4 protein expression. Posttranslational modifications occur in the COOH-terminal domain of β-tubulin, and like a number of other isotypes, class I β-tubulin can undergo various

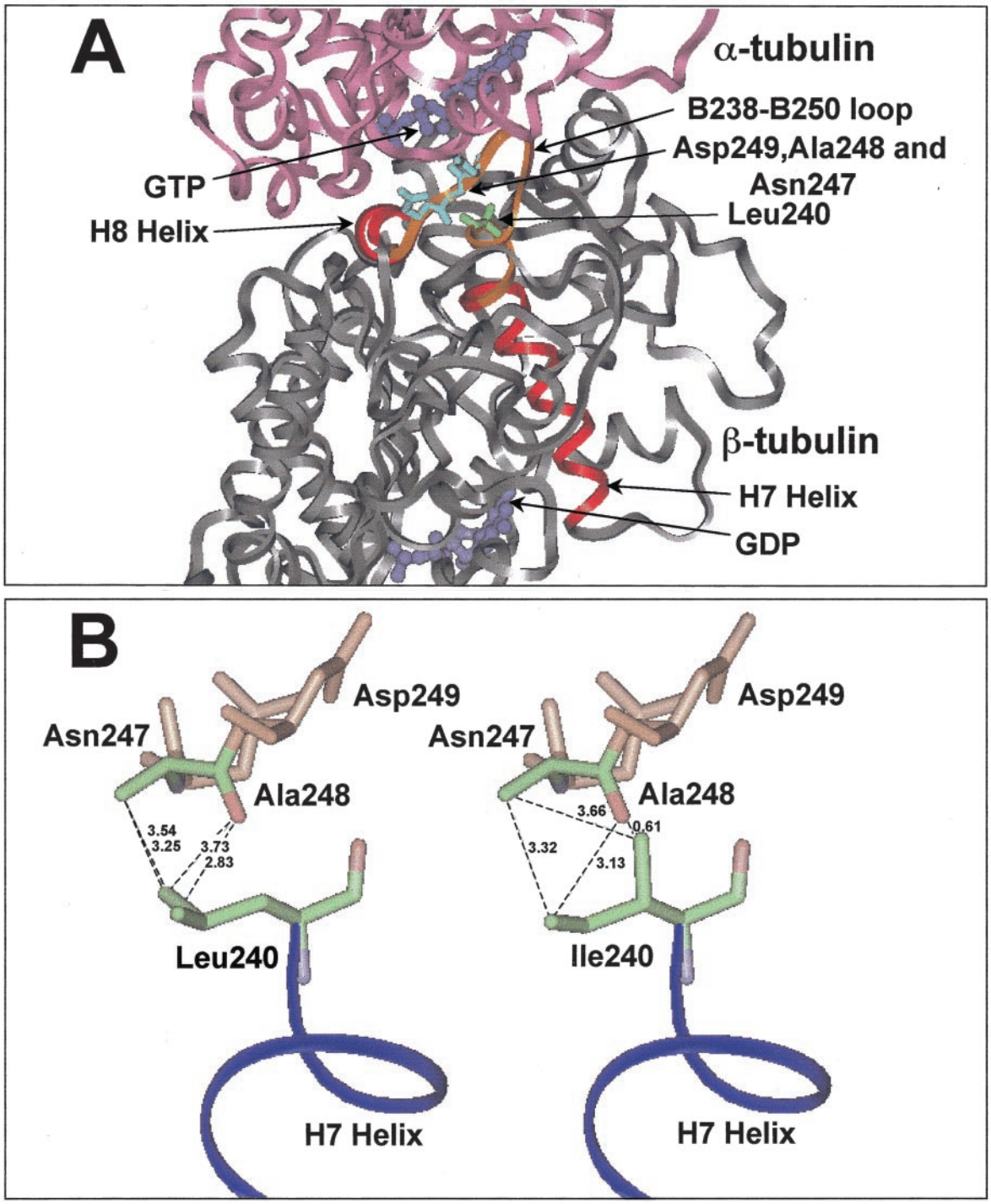

Fig. 6. Modeling of the Leu240 to Ile240 mutation in β-tubulin. *A*, Leu240 (*green*) is close to Asn247, Ala248, and Asp249 (*turquoise*) that are at the longitudinal interface between α-, β-tubulin monomers and located on the loop (B238-B250; *orange*) linking helixes H7 and H8 (*red*). *B*, the side chains of Leu240 are ~3Å from the side chains of Ala248. Mutation to Ile240 may affect the interaction between amino acid 240 and Ala248 and the conformation of the H7 helix. *Left*, Leu240; *Right*, Ile240. The indicated distances are an approximation and included to facilitate visualization of the distances between the side chains of the respective amino acids.

modifications including glutamylation (51). Whether these modifications are the predominant cause of the increased microtubule stability observed in the VCR R cells is not yet known. What is clear, however, is that increased stability of microtubules can counteract the effects of destabilizing drugs such as VCR (26, 27).

MAP4 is encoded by a single gene that can express multiple isoforms of the protein because of alternate RNA splicing (29). In contrast with CEM and VCR R cells, VLB100 cells expressed two isoforms of MAP4 in the soluble fraction and a single MAP4 isoform in the polymerized fraction. The molecular weight of the MAP4 associated with the polymerized fraction was lower than that observed in either the upper form in the soluble fraction or the CEM and VCR R cells. The role of alternate splicing of MAP4 and the possible role in VLB resistance is unclear. It has been demonstrated, however, that different MAP4 isoforms appear to display tissue and developmental-specific expression, and different isoforms may differentially affect microtubule dynamics or function (29). It is possible, therefore that microtubule dynamics are affected and, hence, the efficacy of VLB

reduced in these cells because of altered MAP4 expression and binding.

The highly resistant VCR R cells harbored a mutation in class I β-tubulin that changed Leu240 to Ile240. This residue is close to the intradimer surface of the α/β-heterodimer and is on the loop that links helixes H7 and H8 (Fig. 6*A*). Therefore, a mutation from Leu to Ile could affect the longitudinal interaction between α- and β-monomers and the conformation of the H7 and H8 helixes (Fig. 6*B*). It is likely that these changes may alter the stability of microtubules. This region falls outside the *Vinca* binding domain, which is thought to lie between amino acids 175 and 213 on β-tubulin (52). It is interesting to note that resistance to the microtubule depolymerizing plant herbicide, benzimidazole, has also been associated with a mutation at Leu240 (53). Furthermore, systematic analysis of yeast β-tubulin mutants between amino acids 249 and 252 generated a recessive-lethal phenotype (54). This strongly suggests that mutations near this region could lead to alterations in microtubule stability and may be contributing to the increase in stability of the microtubules in VCR R cells. The leucine at amino acid 240 has been highly conserved throughout evolution in both α- and β-tubulin, plus its close association with Asn247, Ala248, and Asp249 and its localization on the loop linking helixes H7 and H8 suggests that this region may play an important role in microtubule stability. It has recently been demonstrated that β-tubulin mutations in a cluster region affecting Leu^{215}, Leu^{217}, or Leu^{228} decreased the amount of polymerized tubulin and lead to reduced Taxol sensitivity (55). In contrast, the mutation identified in VCR R cells is also in a region rich in leucines (4 leucines of 14 amino acids), but is associated with increased levels of polymerized tubulin. Mutations in the class I β-tubulin gene have been described recently in human ovarian cancer cell lines selected for resistance to either Taxol (19) or epothilone (20). In contrast to our study, these mutations did not affect the ratio of soluble:polymerized tubulin in the resistant cells but did cause a defect in drug-driven polymerization. This implicated drug binding rather than altered microtubule stability as the primary defect in drug-driven microtubule polymerization (19, 20). Monzo *et al.* (56) recently reported that β-tubulin mutations correlated with Taxol resistance in primary advanced non-small cell lung cancer samples. Additional investigation into the role of β-tubulin mutations in resistance to antimicrotubule in clinical samples is required.

The important role of *Vinca* alkaloids in the treatment of childhood ALL requires that mechanisms responsible for resistance to this drug be elucidated. Changes in expression of a specific β-tubulin isotype and alterations in MAP4 were associated with resistance to *Vinca* alkaloids. Increased microtubule stability and, for the first time, a mutation in β-tubulin was associated with VCR resistance in ALL cells selected with this agent. The combined data strongly suggest that multiple microtubule changes may be contributing to the drug resistance phenotype of these cells. Moreover, the mechanism by which the β-tubulin mutation affects VCR resistance and microtubule stability is currently under investigation. Microtubule dynamics and stability are tightly regulated in cells, and any disruptions to this process are likely to affect the efficacy of antimicrotubule agents. It still remains to be determined whether microtubule changes identified in cancer cell lines are involved in clinical resistance to VCR in childhood ALL. To improve drug targeting and treatment strategies for drug refractory ALL, enhanced understanding of the effect that microtubule alterations have on the efficacy of *Vinca* alkaloids is required.

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