

## **β-Tubulin Mutations Are Associated with Resistance to 2-Methoxyestradiol in MDA-MB-435 Cancer Cells**

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### **Abstract**

**2-Methoxyestradiol is an estradiol metabolite with significant antiproliferative and antiangiogenic activity independent of estrogen receptor status. To identify a molecular basis for acquired 2-methoxyestradiol resistance, we generated a stable 2-methoxyestradiol-resistant (2ME2R) MDA-MB-435 human cancer cell line by stepwise exposure to increasing 2-methoxyestradiol concentrations. 2ME2R cells maintained in the presence of the drug and W435 cells maintained in the absence of the drug showed 32.34- to 40.07-fold resistance to 2-methoxyestradiol. Cross-resistance was observed to Vinca alkaloids, including vincristine, vinorelbine, and vinblastine (4.29- to 6.40-fold), but minimal resistance was seen to colchicine-binding agents including colchicine, colcemid, and AVE8062A (1.72- to 2.86-fold). No resistance was observed to paclitaxel and epothilone B, polymerizing agents (0.89- to 1.14-fold). Genomic sequencing identified two different heterozygous point mutations in the class I (M40) isotype of β-tubulin at amino acids 197 (Dβ197N) and 350 (Kβ350N) in 2ME2R cells. Tandem mass spectrometry confirmed the presence of both wild-type and the mutant β-tubulin in 2ME2R cells at the protein level. Consistently, treatment of parental P435 cells with 2-methoxyestradiol resulted in a dose-dependent depolymerization of microtubules, whereas 2ME2R cells remained unaffected. In contrast, paclitaxel affected both cell lines. In the absence of 2-methoxyestradiol, 2ME2R cells were characterized by an elevated level of detyrosination. Upon 2-methoxyestradiol treatment, levels of acetylated and detyrosinated tubulins decreased in P435 cells, while remaining constant in 2ME2R cells. These results, together with our structure-based modeling, show a tight correlation between the antitubulin and antiproliferative effects of 2-methoxyestradiol, consistent with acquired tubulin mutations contributing to 2-methoxyestradiol resistance.** (Cancer Res 2005; 65(20): 9406-14)

### **Introduction**

2-Methoxyestradiol is a potent anticancer agent known to have both antiproliferative and antiangiogenic activity in a variety of *in vitro* and *in vivo* models (1, 2) and is currently in phase I/II clinical trials (3). 2-Methoxyestradiol is an endogenous metabolite of 17β-estradiol formed by sequential hydroxylation and methylation at

the 2-position. The antiproliferative activity of 2-methoxyestradiol is independent of estrogen receptors α and β (4). 2-Methoxyestradiol exhibits its antiproliferative and antiangiogenic activity through several mechanisms including disruption of microtubule dynamics by its binding ability to colchicine site (5, 6), regulation of cell cycle kinases and arrest (7-9), effect on superoxide dismutase (10, 11), apoptotic activity in various tumor cell lines (2), up-regulation of p53 (12, 13), death receptor 5 (14), and dysregulation of hypoxia-inducible factor-1 (HIF-1; ref. 15).

2-Methoxyestradiol, like colchicine and vinblastine, depolymerizes microtubules by binding to tubulin dimers (16). Whereas 2-methoxyestradiol competes for colchicine binding to tubulin and disrupts interphase microtubules causing inhibition of cell growth in cancer cells (5, 6), vinblastine binds to a different region on the tubulin named as *Vinca* domain site (16, 17). Disruption of microtubules is also critical for the dysregulation of HIF-1 by 2-methoxyestradiol and inhibition of angiogenesis (15). At low concentrations, however, 2-methoxyestradiol arrests cells in mitosis without depolymerizing tubulin (9). Nearly all of the microtubule-targeted drugs inhibit microtubule dynamics at their lower concentrations, which is correlated with cell cycle arrest at G<sub>2</sub>-M and subsequent cell death (16).

Tumor cell drug resistance, intrinsic or acquired, is the major cause for the failure of antineoplastic agents. Overexpression of multidrug resistance transporters (MDR) is one mechanism of resistance to the microtubule agents (18). Other mechanisms involve the alterations in tubulin/microtubule system (16, 18-20). Mutations in human class I (M40) β-tubulin gene, the predominant isotype, have been reported in several cell lines resistant to both microtubule-destabilizing and microtubule-stabilizing agents (21-30). These mutations can alter the microtubule polymer levels and dynamics and may contribute as mechanisms of resistance to microtubule-targeting agents. In addition, altered expression levels of tubulin isotypes (18, 19, 31) and changes in microtubule-associated protein 4 (27, 32) have also been associated with resistance.

In this study, we developed a stable 2-methoxyestradiol-resistant cell line that exhibits modest cross-resistance to *Vinca* alkaloids rather than colchicine-binding site agents. Immunofluorescence and *in vitro* polymerization assays showed that 2-methoxyestradiol-driven tubulin depolymerization is impaired in 2ME2R cells. We identified two acquired point mutations in the class I (M40) β-tubulin both at the DNA and protein levels. We provide a structure-based model suggesting an explanation for these findings.

### **Materials and Methods**

**Generation of 2-methoxyestradiol-resistant MDA-MB-435 (2ME2R) cell line.** Parental MDA-MB-435 (P435) and 2-methoxyestradiol-resistant cell

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lines (2ME2R and W435) were grown in MEM supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 1 mmol/L MEM nonessential amino acids and vitamins, and penicillin/streptomycin. The reported IC<sub>50</sub> for MDA-MB-435 cells is 0.08 to 0.61 μmol/L 2-methoxyestradiol (1). The 2ME2R cell line was developed through a stepwise increase of 2-methoxyestradiol (EntreMed, Rockville, MD) drug concentration from 0.2 to 10 μmol/L. Over 75 weeks after initial treatment, cells continued to proliferate in the presence of 2-methoxyestradiol and were used for the experiments described below. To assess the stability of 2-methoxyestradiol resistance, cells were also grown in 2-methoxyestradiol-free medium for 5 months and tested for 2-methoxyestradiol sensitivity (2-methoxyestradiol-withdrawn cells, W435). 2ME2R cells were maintained in the presence of 2-methoxyestradiol, whereas W435 cells after acquiring resistance to 2-methoxyestradiol were maintained in the absence of 2-methoxyestradiol.

**Bromodeoxyuridine proliferation assay.** Cell survival was assessed by bromodeoxyuridine (BrdUrd) proliferation assay. Briefly, P435, 2ME2R, and W435 cells were plated at 2,500 cells per well in a 96-well plate in the absence of 2-methoxyestradiol, allowed to attach overnight, and then exposed to serial dilutions of each compound for 48 hours. Resistance profiles for each compound were measured by use of BrdUrd cell colorimetric ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. IC<sub>50</sub> values were determined from dose-response curves using GraphPad Prism 4 (San Diego, CA). AVE8062A was a kind gift from Aventis Pharmaceuticals (Bridgewater, NJ). Epothilone B was from Calbiochem (San Diego, CA). Other drugs were from Sigma (St. Louis, MO).

**PCR and sequencing of class I (M40) β-tubulin from P435 and 2ME2R cells.** PCR amplification and sequencing of the class I (M40)  $\beta$ -tubulin gene from parental and resistant cells were done as previously described (21).

**Isolation and separation of tubulins for matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis.** Microtubule pellets were isolated from cytosolic extracts using the method of Vallee (33) as described previously (34). The purity and enrichment of tubulins were confirmed by Coomassie stain and by Western blotting with an antibody against  $\beta$ -tubulin (Sigma; data not shown). Isoelectric focusing was done as described previously (34). Briefly, microtubule pellets (150 μg) were resuspended in 200 μL rehydration buffer [8 mol/L urea, 2% CHAPS, 50 mmol/L DTT, 0.2% ampholytes (pH 3-10), 0.1% ampholytes (pH 4-6)]. The Immobilized-pH Gradient (IPG) strips [Immobiline DryStrips from Amersham Biosciences (pH 4.5-5.5), 240 × 3 × 0.5 mm, gel matrix of 4% polyacrylamide T, 3% polyacrylamide C] were rehydrated with samples at room temperature for 24 hours and isoelectrically focused for 100,000 V hour at 20°C using a Bio-Rad PROTEAN IEF Cell. Coomassie blue stained protein bands were directly excised from the IPG strips and destained with a 50% acetonitrile/50 mmol/L ammonium bicarbonate solution. The proteins were reduced with a 10 mmol/L DTT in 10 mmol/L ammonium bicarbonate solution (Sigma) and alkylated with a 55 mmol/L iodoacetamide in 10 mmol/L ammonium bicarbonate solution. After the proteins were digested with bovine chymotrypsin (Princeton Separations, 0.3 μg/sample in 10 mmol/L ammonium bicarbonate) overnight at 30°C, the solutions were resuspended in a 3% acetonitrile, 96.9% water, and 0.1% formic acid solution compatible for reverse-phase liquid chromatography.

One microliter of the peptide resuspension solution was spotted on a matrix-assisted laser desorption/ionization (MALDI) target plate with 1 μL  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile/49.9% water/0.1% trifluoroacetic acid matrix solution. MALDI time-of-flight mass spectrometry (MALDI-TOF-MS) was done to confirm the identity of tubulin proteins within the sample. Mass spectra were recorded in positive ion mode of a MALDI-TOF mass spectrometer (Micromass, Manchester, United Kingdom). The mass to charge ratios (*m/z*) of sample ions were measured using the following variables: 3,250 V pulse voltage, 15,000 V source voltage, 500 V reflectron voltage, 1,950 V MCP voltage, and low mass gate of 400 Da. For high accuracy mass measurement, the instrument was tuned to a resolution of 5,000.

**Nanoflow electrospray ionization tandem mass spectrometric analysis using a quadrupole time-of-flight mass spectrometer.** Liquid chromatography electrospray tandem MS (LC-ESI-MS/MS) analysis of the digested proteins was done using a CapLC system coupled to a quadrupole TOF mass spectrometer (Micromass, Manchester, United Kingdom) fitted

with a Z-spray ion source. Samples were desalted and concentrated using an on-line precolumn (C18, 0.3-mm inner diameter, 5-mm length from LC Packings, Sunnyvale, CA). Separation of the peptides was carried out on a reverse-phase capillary column (self-packed C18, 100 μm inner diameter, 12-cm length) running with a 300 nL/min flow rate. The gradient profile consisted a linear gradient from 97% solution A/(0.1% formic acid/3% acetonitrile/96.9% H<sub>2</sub>O, v/v) to 40% solution B (0.1% formic acid/2.9% H<sub>2</sub>O/97% acetonitrile, v/v) in 30 minutes followed by a linear gradient up to 50% B in 4 minutes. Mass spectra were recorded in positive ion mode. MS to MS/MS switch criteria detection window was set at 2 Da.

**Immunofluorescence and confocal microscopy.** Microtubules were visualized as described previously (15, 35) and immunostained with mouse anti- $\alpha$ -tubulin antibody (clone DM1A, Sigma) followed by a secondary Alexa Fluor 568 goat anti-mouse antibody (Molecular Probes, Eugene, OR). DNA was counterstained with Sytox Green (Molecular Probes) following manufacturer's instructions.

**Tubulin polymerization assay.** The percent of polymerized tubulin from the P435 and 2ME2R cell lines was assessed as previously described (21). Antibodies against  $\alpha$ -tubulin (clone DM1A) and acetylated  $\alpha$ -tubulin (clone 6-11B-1) were from Sigma. Antibody against detyrosinated tubulin was obtained from Chemicon International, Inc. (Temecula, CA). Quantification of band densities was done using the public domain NIH Image (version 1.61). The percentage of polymerized tubulin (% P) was determined by dividing the densitometric value of polymerized tubulin by the total tubulin content.

## Results

**Characterization of MDA-MB-435 cancer cells selected with 2-methoxyestradiol.** The sensitivity to 2-methoxyestradiol in parental (P435) and resistant (2ME2R and W435) cells was determined by measuring the survival rates. IC<sub>50</sub> values, the concentrations of 2-methoxyestradiol that are necessary to kill 50% of the cells, were 0.38 μmol/L for P435, 12.29 μmol/L for W435, and 15.23 μmol/L for 2ME2R cells (Table 1). These results represent a 32.34- to 40.07-fold increase in resistance to 2-methoxyestradiol compared with parental cells.

Estrogen and its metabolites including 2-methoxyestradiol are not substrates for MDR. Consistent with this fact, overexpression of MDR1 did not confer resistance to 2-methoxyestradiol.<sup>8,9</sup> The sensitivity of the resistant cells to 2-methoxyestradiol was also not altered in the presence of verapamil (36), an inhibitor of MDR transporters (data not shown). To establish whether 2-methoxyestradiol-resistant cells also exhibit cross-resistance to other microtubule targeting agents, P435, W435, and 2ME2R cells were treated with the indicated drugs in Table 1, and proliferation rates were measured. 2-Methoxyestradiol-resistant cells showed (4.29- to 6.40-fold) modest cross-resistance to vincristine, vinorelbine, and vinblastine compared with P435 cells (Table 1), whereas the cells were minimally resistant to colchicine, colcemid, and AVE8062 (1.72- to 2.86-fold). In contrast, parental and resistant cells exhibited similar sensitivity to paclitaxel and epothilone B (0.89- to 1.14-fold). No cross-resistance was observed for cisplatin, Adriamycin, etoposide, and 5-fluorouracil (data not shown).

**Two mutations in  $\beta$ -tubulin M40 isotype are identified in 2-methoxyestradiol-resistant cells.** We have previously shown that mutations in the  $\beta$ -tubulin gene are one of the mechanisms responsible for acquired resistance to different microtubule-targeting agents including paclitaxel (21, 22) and epothilones A and B (23-26). Most of these acquired mutations were identified in

<sup>8</sup> T.M. LaVallee et al. Proc AACR, 45: #4085, March 2004.

<sup>9</sup> T.M. LaVallee, personal communication.

**Table 1.** Resistance profile of cells selected with 2-methoxyestradiol

Compound	IC <sub>50</sub> * (95% confidence interval)			Relative resistance <sup>†</sup>	
	P435	2ME2R	W435	2ME2R	W435
Colchicine site					
2-Methoxyestradiol	0.38 (0.30-0.48)	15.23 (13.79-16.83)	12.29 (10.74-14.05)	40.07	32.34
Colchicine	6.64 (5.79-7.62)	18.99 (17.19-20.98)	18.29 (15.44-21.67)	2.86	2.76
Colcemid	21.07 (18.61-23.86)	44.26 (38.93-50.32)	42.65 (36.66-49.62)	2.10	2.02
AVE8602A	2.64 (2.46-2.82)	4.53 (4.08-5.03)	4.90 (4.25-5.66)	1.72	1.86
Vinca domain					
Vincristine	2.35 (2.03-2.71)	10.24 (9.12-11.49)	10.08 (8.90-11.40)	4.36	4.29
Vinorelbine	0.61 (0.55-0.68)	3.03 (2.67-3.45)	3.23 (2.90-3.59)	4.96	5.30
Vinblastine	0.25 (0.21-0.28)	1.56 (1.41-1.74)	1.60 (1.43-1.80)	6.24	6.40
Polymerizing agents					
Paclitaxel	4.23 (3.90-4.60)	4.83 (4.30-5.41)	4.72 (4.26-5.23)	1.14	1.11
Epothilone B	0.48 (0.41-0.56)	0.43 (0.36-0.51)	0.51 (0.43-0.59)	0.89	1.06

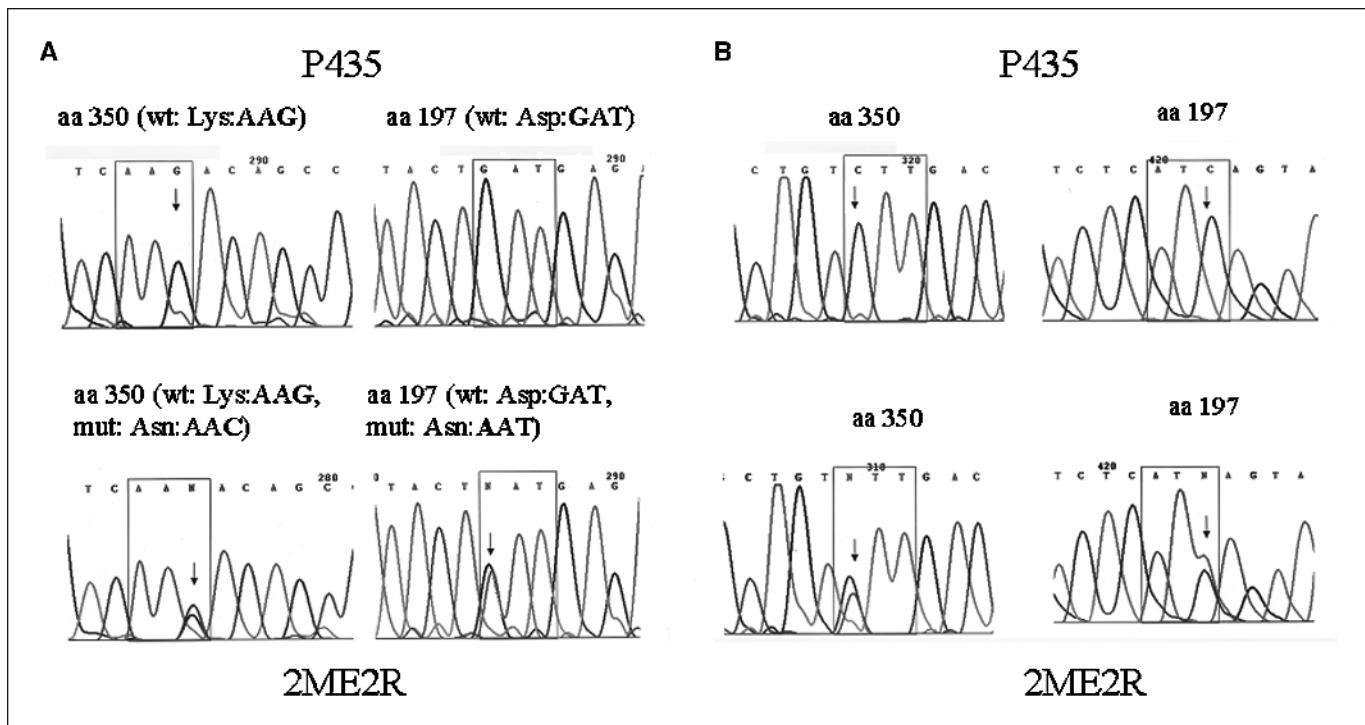
NOTE: All IC<sub>50</sub>s are in nmol/L, with the exception of which are in μmol/L 2-methoxyestradiol.

\*Values are mean IC<sub>50</sub>s from three independent experiments with quadruplets.

<sup>†</sup>Relative resistance, ratio of IC<sub>50</sub> of the resistant cells to IC<sub>50</sub> of parental cells.

the predominant β-tubulin isotype (gene M40/protein class βI), which accounts for >85% of total β-tubulin mRNA (37). Molecular characterization of 2ME2R revealed the presence of two distinct β-tubulin point mutations in the M40 tubulin isotype, in which both Asp<sup>197</sup> and Lys<sup>350</sup> amino acids were converted to asparagines (Fig. 1). These mutations are acquired, because the P435 cells harbor the wild-type amino acids at the above locations. W435 cell line also harbors the same heterozygous mutations (data not shown).

**Tandem mass spectrometry spectra confirmed the presence of the point mutations at amino acid sites 197 and 350 in 2ME2R cells.** To determine the presence of the mutant tubulin at the protein level, we isolated tubulins from both P435 and 2ME2R cells and separated them using isoelectric focusing as described previously (34). To confirm the identity of the tubulin band changes observed in the IPG strips (data not shown), we did LC-ESI-MS/MS analysis for each band. After digestion with chymotrypsin, peptides



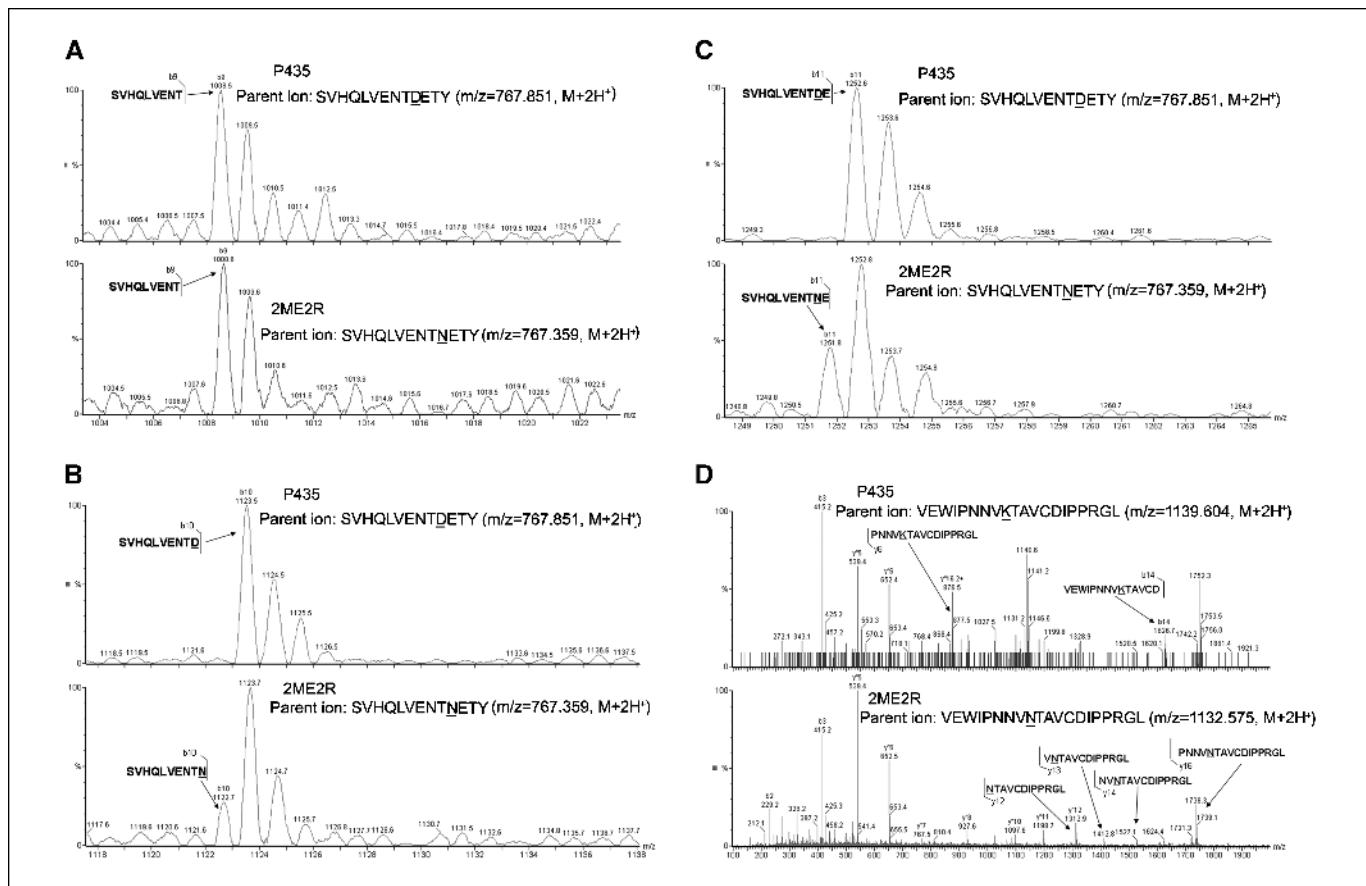
**Figure 1.** β-Tubulin mutations in 2ME2R cells identified by genomic sequencing of the β-tubulin isotype M40. The sequence of the class I β-tubulin isotype M40 was obtained using both (A) forward and (B) reverse primers. Arrows, nucleotide position that is different between the parental (P435) and 2-methoxyestradiol-resistant (2ME2R) cells.

were separated by LC, peptide masses were obtained from an ESI mass spectrum, and selected peptide parent ions were sequenced using MS/MS fragmentation to identify the nature and location of amino acid changes. Figure 2A-C shows selected ions of the MS/MS fragmentation spectra of the peptides involving amino acid 197; Fig. 2D shows peptide fragment ions diagnostic of the mutation at amino acid 350. The mutation at amino acid 197 results in a 1-amu change in mass of the peptide 188–200 (D197N). The wild-type and mutated  $M^{+2}H^+$  ions have 0.5-amu difference and cannot be separated to obtain MS/MS spectra on pure parent ions. Therefore, the predicted 1-amu change in fragment ion masses was expected to be identified as a shift in the apparent isotope distribution pattern of diagnostic fragment ions. Figure 2A shows that the mutation in peptide 188 to 200 does not lie in the nine NH<sub>2</sub>-terminal amino acids; that is, the isotype mass distributions of the b9 fragment ions are identical in tubulins isolated from P435 and 2ME2R. However, Fig. 2B and C clearly indicate the presence of mutant peptide <sup>188</sup>SVHQLVENTN<sup>200</sup>. In comparison with Fig. 2A, the isotopic distribution patterns of the b10 and b11 fragment ions in Fig. 2B and C indicate heterogeneity of the peptides and thereby show the presence of the D197N mutation. In Fig. 2D, the parent ion mass of peptide 342 to 361 changes from  $M^{+2}H^+ = 1,139.604$  (P435) to  $M^{+2}H^+ = 1,132.575$  (2ME2R), consistent with the mass change from a lysine

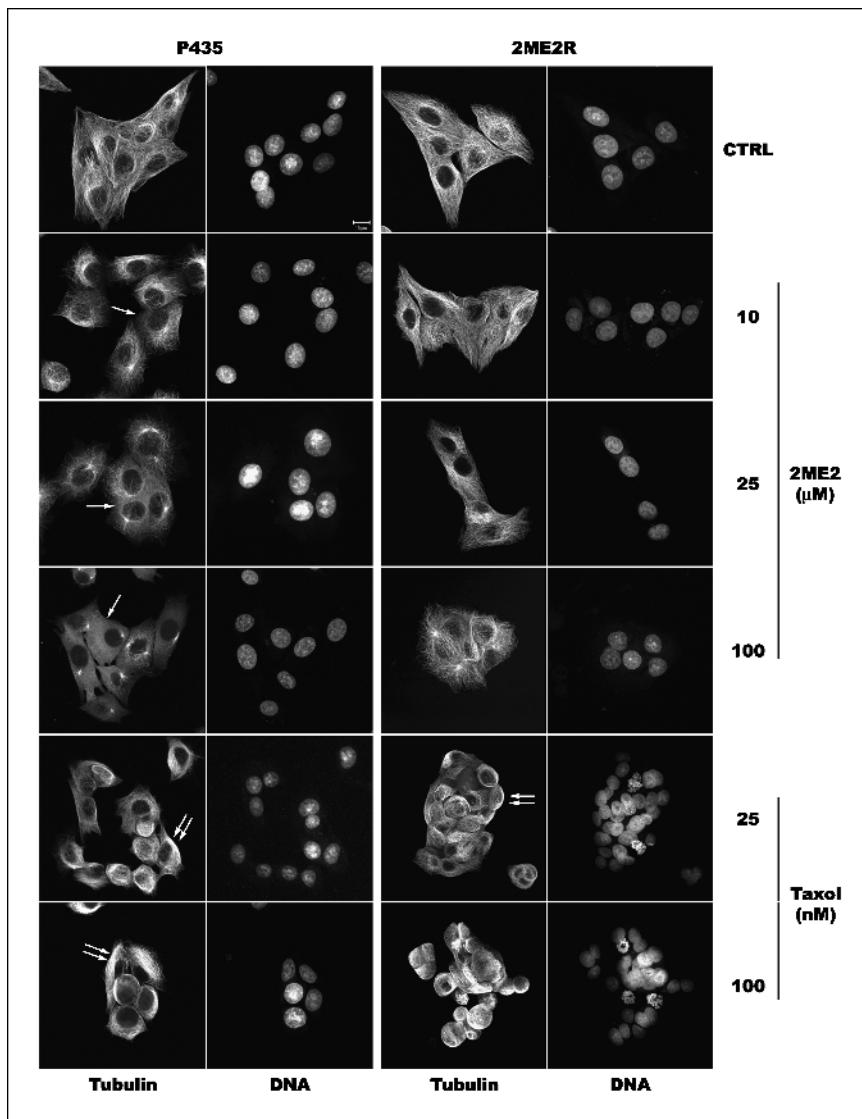
to an asparagine residue. These parent ions were selected and used to generate MS/MS fragmentation patterns. The series of "b" and "y" fragment ions shown in Fig. 2D confirmed the location of the mutation to amino acid 350.

**The ability of 2-methoxyestradiol to disrupt microtubules is impaired in 2ME2R cells.** The residues harboring these mutations are located at the colchicine-binding pocket on  $\beta$ -tubulin (38, 39). To determine the effect of these acquired mutations on 2-methoxyestradiol resistance, P435 and 2ME2R cells were treated with the indicated concentrations of 2-methoxyestradiol or paclitaxel for 6 hours and microtubules were immunostained with an antibody against  $\alpha$ -tubulin and analyzed by laser scanning confocal microscopy (Fig. 3). Treatment of P435 cells with 2-methoxyestradiol resulted in a dose-dependent depolymerization of microtubules, as shown by the disruption of the fine and intricate microtubule network at 25  $\mu$ mol/L of the drug, whereas at 100  $\mu$ mol/L a complete loss of the microtubule cytoskeleton was observed. In contrast, the microtubule cytoskeleton of 2ME2R cells remained almost unaffected even when 100  $\mu$ mol/L 2-methoxyestradiol was used. Paclitaxel treatment led to the formation of distinct microtubule bundles in both cell lines at the same concentrations, consistent with the cytotoxicity profile of P435 and 2ME2R cells to this drug.

**2ME2R cells do not exhibit 2-methoxyestradiol-driven tubulin depolymerization.** To further analyze the effects of



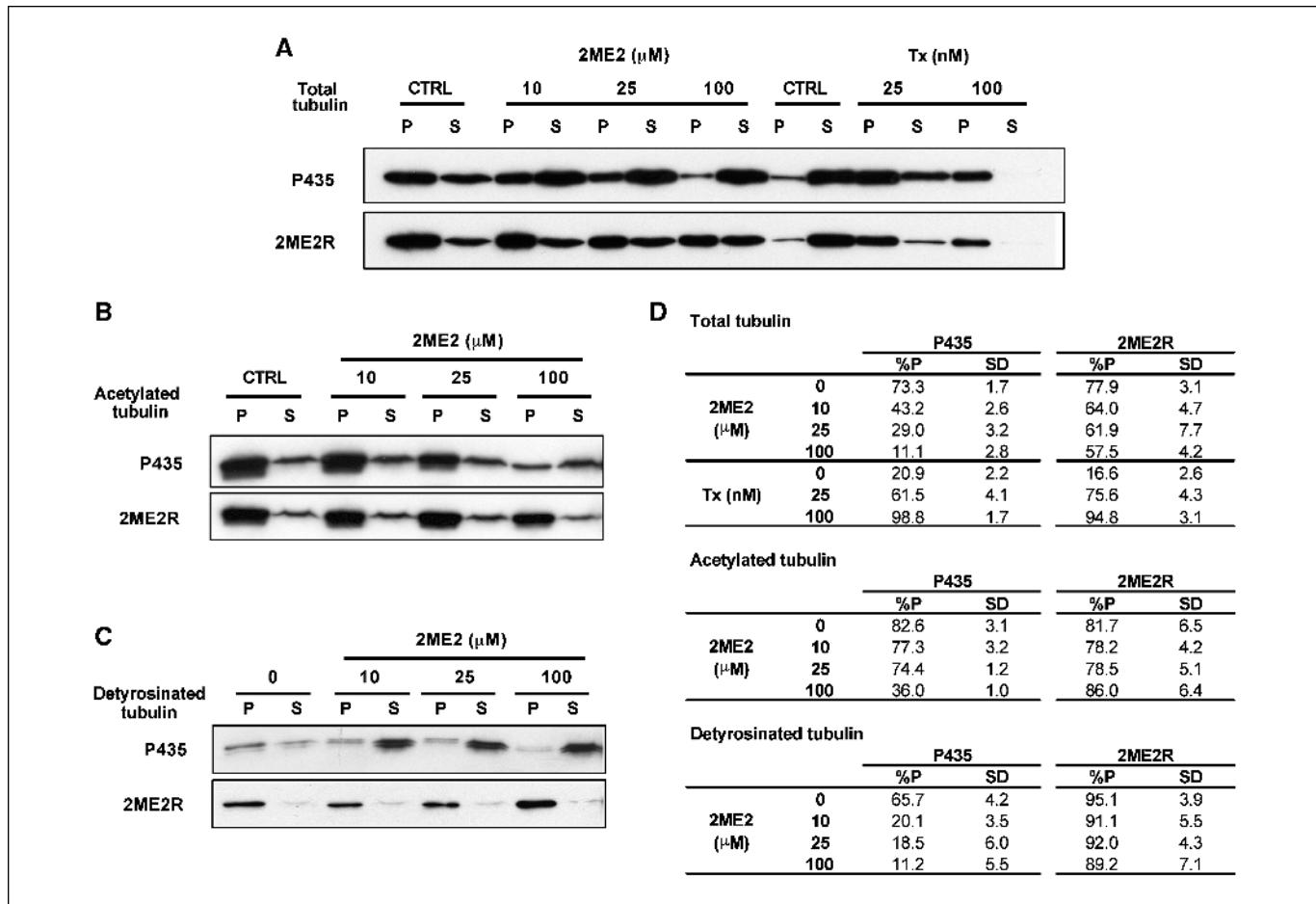
**Figure 2.** MS/MS spectra of peptide ions of  $\beta$ -tubulin involving amino acid residues 197 and 350. *Top*, wild-type form (P435 cells); *bottom*, mutant form (2ME2R cells). *A*, amino acid site preceding the point mutation at 197 (b9 ion); *B*, amino acid site 197 (b10 ion); *C*, amino acid site after 197 (b11 ion). *D*, fragmentation ions (b and y ions) are compared between the wild-type control peptide containing lysine (K) at site 350 and the mutated peptide containing asparagine (N) at the same site. Cysteine residues were modified by iodoacetamide ( $\Delta M = 57.02$  Da) during the same preparation step.



**Figure 3.** Effect of 2-methoxyestradiol (2ME2) on microtubule cytoskeleton in P435 and 2ME2R cells. P435 and 2ME2R cell lines were treated with the indicated concentrations of 2-methoxyestradiol or paclitaxel (taxol) for 6 hours. Cells were fixed with PHEMO buffer [0.068 mol/L PIPES, 0.025 mol/L HEPES, 0.015 mol/L EGTA, 0.003 mol/L MgCl<sub>2</sub>, 10% DMSO (pH 6.8)] containing 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 for 10 minutes at room temperature. After blocking with 10% goat serum/PBS, microtubules were immunostained with an anti  $\alpha$ -tubulin antibody and DNA was counterstained with Sytox Green. Staining was analyzed by confocal laser-scanning microscopy. Single arrows, cells with depolymerized microtubules; double arrows, cells with hyperstabilized microtubules forming bundles. Bar, 10  $\mu$ m.

2-methoxyestradiol on microtubules in P435 and 2ME2R cells, we did a quantitative tubulin polymerization assay (Fig. 4A). This quantitative assay is based on the fact that stabilized microtubule polymers remain detergent insoluble when extracted in a microtubule-stabilizing buffer and therefore remain in the pellet (*lanes P*) after centrifugation (21). Conversely, drugs that depolymerize microtubules cause a shift towards the pool of soluble tubulin dimers that remain in the supernatant (*lanes S*). Representative blot from three experiments was shown in Fig. 4A. In the absence of drug, the majority of the tubulin was found in the polymerized fraction in both cells, under our experimental conditions. Treatment of the parental P435 cells with 2-methoxyestradiol depolymerized microtubules in a dose-dependent manner, as evidenced by the decrease in tubulin polymerization from % *P* = 73.3  $\pm$  1.7 in untreated cells to % *P* = 11.1  $\pm$  2.8 in cells treated with 100  $\mu$ mol/L of 2-methoxyestradiol (Fig. 4A and D). Treatment of the 2ME2R cells with 2-methoxyestradiol failed to depolymerize microtubules as seen by the lack of shift of the total tubulin mass from the polymerized (*P*) to the soluble pool (*S*) fractions. In contrast, paclitaxel treatment equally affected both cells as evidenced by the robust accumulation of tubulin in the polymerized fraction (Fig. 4A and D).

Acetylation and detyrosination of  $\alpha$ -tubulin are two posttranslational modifications that are associated with stable microtubules (40). Tubulin acetylation occurs at the conserved lysine residue at position 40 in the NH<sub>2</sub> terminus of the  $\alpha$ -tubulin. Drugs that hyperstabilize microtubules, such as the taxanes, enhance tubulin acetylation, whereas drugs that depolymerize microtubules decrease acetylation. The detyrosinated tubulin exposes a COOH-terminal glutamic acid and is therefore referred to as Glu-tubulin. This detyrosination is specific to  $\alpha$ -tubulin in polymerized microtubules (40). To further characterize the effects of 2-methoxyestradiol on microtubule stability in the P435 and 2ME2R cells, we reprobed the same blot with either antibody against acetylated  $\alpha$ -tubulin or antibody against detyrosinated  $\alpha$ -tubulin. Representative blots from three experiments for acetylated and detyrosinated tubulin were shown in Fig. 4B and C, respectively. In the absence of drug, we observed similar acetylated  $\alpha$ -tubulin in parental and resistant cells (parental P435 % *P* = 82.6  $\pm$  3.1; 2ME2R % *P* = 81.7  $\pm$  6.5) but elevated levels of detyrosination in resistant cells compared with parental cells (parental P435 % *P* = 65.7  $\pm$  4.2; 2ME2R % *P* = 95.1  $\pm$  3.9; Fig. 4B-D). Levels of acetylated tubulin in the P435 dropped in a dose-dependent



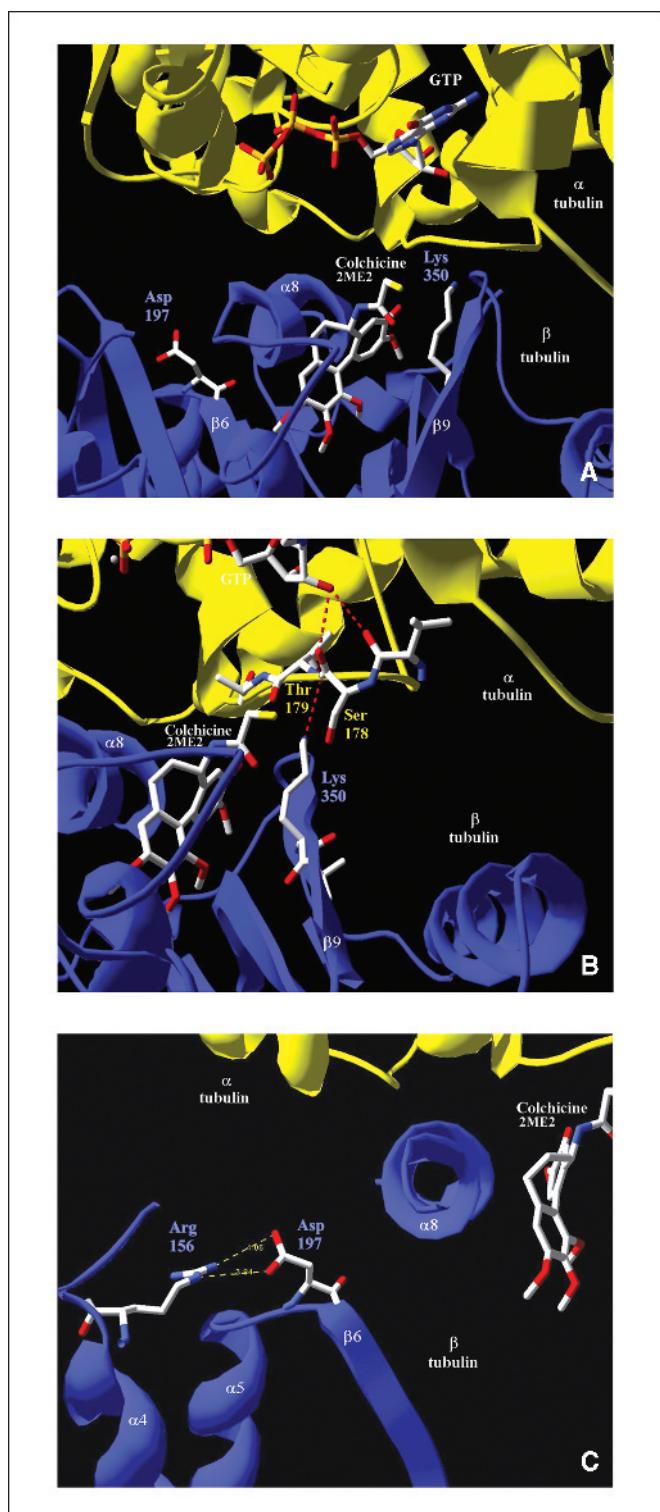
**Figure 4.** Effect of 2-methoxyestradiol (2ME2) on tubulin polymerization in P435 and 2ME2R cells. *A*, P435 and 2ME2R cell lines were treated with the indicated concentrations of 2ME2 and Taxol (Tx) for 6 hours. Control and 2-methoxyestradiol-treated cells (lanes 1–8) were lysed with a microtubule-stabilizing buffer (21) and cells treated with Taxol (lanes 9–14) were lysed using a low salt buffer (21) for 10 minutes at 37°C. Following cell lysis the polymerized (P) and the soluble (S) tubulin fractions were separated by centrifugation (21,000 × g), and each fraction was resolved on adjacent lanes by electrophoresis. After transfer, the filter was probed with an antibody against α-tubulin (Sigma, clone DM1A). The resistant cells were maintained in drug-free medium for 1 week before the experiments. The same samples were probed with antibodies against (*B*) acetylated α-tubulin (Sigma, clone 6-11B-1) and (*C*) detyrosinated tubulin (Glu-tubulin, Chemicon International, Inc., Temecula, CA). *A–C*, representative blots from three experiments. *D*, the percentage of polymerized tubulin (%P) was determined by dividing the densitometric value of polymerized tubulin by the total tubulin content. The %P values (SD) for total and acetylated tubulin represent the median values of three different experiments.

manner from %P = 82.6 ± 3.1 in the nontreated cells to %P = 36 ± 1.0 in cells treated with 100 μmol/L of 2-methoxyestradiol, whereas levels of the soluble fraction seemed unaltered indicating that acetylation occurs solely in the polymerized form of microtubules (Fig. 4*B* and *D*). As expected, no changes were observed in the levels of acetylated tubulin in the 2ME2R cells, consistent with the lack of 2-methoxyestradiol activity on tubulin depolymerization. In addition, levels of detyrosinated tubulin in the P435 cells also decreased in a dose-dependent manner from %P = 65.7 ± 4.2 in the nontreated cells to %P = 11.2 ± 5.5 in 100 μmol/L 2-methoxyestradiol-treated cells, whereas no effect of 2-methoxyestradiol treatment was detected on the detyrosination levels in 2ME2R cells (Fig. 4*C* and *D*). These results support the role of altered microtubule stability as a cause of drug resistance in 2ME2R cells.

**Structure-based hypotheses for Dβ197N and Kβ350N in 2-methoxyestradiol resistance.** The relative location of the two mutations (Dβ197N and Kβ350N) is shown in Fig. 5*A*. Structure-based hypotheses of resistance to 2-methoxyestradiol by mutations at these sites are addressed in detail in the Discussion (Fig. 5*B* and *C*).

## Discussion

Drug resistance is a multifactorial phenomenon where several resistance mechanisms can be active at the same time. Elucidating possible mechanisms of resistance is important for understanding the mechanism of action of drugs as well as to provide information for analogue design. To this end, we generated a 2-methoxyestradiol-resistant (2ME2R) MDA-MB-435 human cancer cell line. 2-Methoxyestradiol, an orally available and well-tolerated small molecule with antitumor and antiangiogenic activity, binds tubulin at the colchicine site and depolymerizes cellular microtubules. Several studies indicate that development of resistance to microtubule-targeting agents occurs through multiple mechanisms including alterations in the drug target, tubulin, and microtubule regulatory proteins (18). Single point mutations in tubulin are among the changes that are associated with resistance to other antitubulin agents (21–30). In the present study, we characterize a novel resistant cancer cell line to 2-methoxyestradiol that express two heterozygous point mutations in the class I β-tubulin (Dβ197N and Kβ350N) both at the DNA and protein levels. Tubulin mutations can be associated



**Figure 5.** Location of the residues mutated in the  $\alpha,\beta$ -tubulin heterodimer in 2ME2R cells. *A*, the relative location of the two mutations ( $D\beta 197N$  and  $K\beta 350N$ ). The coordinates for the models were obtained from pdb id 1sa0 (39). *A-C*,  $\beta$ -tubulin (bottom, blue). Additionally, all labeled secondary structural elements are components of  $\beta$ -tubulin. Throughout the three panels both colchicine and  $\beta$ -tubulin helix 8 is shown to aid in orienting the three views. *B* and *C*, several of the interactions for  $Lys^{350}$  and  $Asp^{197}$ , respectively, and illustrate a possible structural basis of the resistance to 2-methoxyestradiol when these residues are mutated to asparagines. A significant portion of  $\beta$ -tubulin has been removed from (*C*) to provide a clear view of the interaction between  $Asp^{197}$  and  $Arg^{156}$  of  $\beta$ -tubulin.

with either altered drug binding site or changes in microtubule stability. Our cross-resistance data argue against an altered drug-binding site, as 2-methoxyestradiol-resistant cells exhibit a higher cross-resistance to *Vinca* domain compounds compared with the colchicine-binding agents.

In contrast, our data support altered microtubule stability in 2ME2R cells. 2-Methoxyestradiol's ability to destabilize microtubules was impaired in 2-methoxyestradiol-resistant cells. Both immunofluorescent microscopy and *in vitro* polymerization assays revealed that tubulin polymers in P435 cells exhibited 2-methoxyestradiol-driven, dose-dependent depolymerization, whereas 2ME2R cells failed to respond. Similarly, 2-methoxyestradiol had no effect on tubulin acetylation and detyrosination in 2ME2R cells, whereas it decreased acetylation and detyrosination in a dose-dependent manner in P435 cells. Collectively, these data suggest that drug-induced microtubule destabilization is compromised in 2-methoxyestradiol-resistant cells.

Location of amino acid changes in the tertiary structure of tubulin is correlated with the alterations of microtubule polymer levels and stability. In 2ME2R cells, the K350N and D197N residues are proximal to the colchicine-binding pocket on  $\beta$ -tubulin (38, 39). Using recent structural models (39, 41, 42), we determined the positions of the mutant residues in the three-dimensional tubulin structure (Fig. 5*A*). Both of these residues are located at the interface between the plus side surface of  $\alpha$  tubulin and the minus side surface of  $\beta$ -tubulin. This places them close to the colchicine-binding site (38, 39) but distant from the *Vinca* binding site (17), which resides at the plus side surface of  $\beta$ -tubulin. Mutation at K350N site is associated with resistance to other microtubule-destabilizing agents like indanocine (28) and colcemid (29). Furthermore, K350M and K350E mutations have been associated with increased microtubule stability in colchicine- and *Vinca*-resistant *Chlamydomonas* (43, 44). 2-Methoxyestradiol binds to the colchicine site, and the K350 residue is located at the colchicine-binding pocket on  $\beta$ -tubulin. In our model, (Fig. 5*B*), although Lys350 is in Van der Waals contact with colchicine, no sterical clashes seem to be created with the mutation to asparagine at this site. There are no indications of neighboring residues restricting this region and causing a tight packing between the lysine and colchicine. However, a hypothesis for why mutating Lys<sup>350</sup> to asparagine would affect colchicine binding is derived from comparing and contrasting the straight/polymerizing conformation of tubulin (41, 42) with the curved/depolymerizing conformation of tubulin (39). Lys<sup>350</sup> seems to play a minor role in stabilizing the  $\alpha$  phosphate moiety of  $\alpha$ -tubulin bound GTP in the straight/polymerizing conformation. In the curved/depolymerizing conformation of tubulin, Lys<sup>350</sup> directly hydrogen bonds with either Ser<sup>178</sup> (pdb id 1sa1) or Thr<sup>179</sup> (pdb id 1sa0), these residues are located in the loop connecting  $\beta$  strand 5 to helix 5 of  $\alpha$ -tubulin. The side chain of Lys<sup>350</sup> is the only residue within hydrogen bonding distance of this loop and seems an important stabilizing factor for the curved/depolymerizing conformation of tubulin, which is also the energetically preferred binding state for colchicine or vinblastine. The side chain for asparagine in this position is too distant to hydrogen bond with this loop in  $\alpha$ -tubulin. Thus, mutating Lys<sup>350</sup> to asparagine would destabilize the curved/depolymerizing conformation of tubulin and disfavor the binding of colchicine or vinblastine.

We report a D197N  $\beta$ -tubulin mutation for the first time in this study. Unlike Lys<sup>350</sup>, which forms bonds with portions of  $\alpha$ -tubulin, all of the local interactions for Asp<sup>197</sup> reside within  $\beta$ -tubulin. Asp<sup>197</sup>, which resides just before the amino terminal end of  $\beta$  strand 6, has a series of hydrogen bonds that are identical in both the straight/polymerizing and the curved/depolymerizing conformations of tubulin. Additionally, none of these hydrogen bonds would be disrupted by the mutation of Asp<sup>197</sup> to asparagine. However, Asp<sup>197</sup> forms a salt bond with Arg<sup>156</sup>, of helix 4. This charge to charge interaction would be negated by the mutation of negatively charged Asp<sup>197</sup> to the neutral charge of asparagine. This salt bond is the sole interaction for anchoring the COOH-terminal end of helix 4. Removing this anchor would alter the mobility of this helix and could readily alter the propagation of conformational change within  $\beta$ -tubulin. Additionally, the NH<sub>2</sub>-terminal end of helix 4 forms part of the plus side surface of  $\beta$ -tubulin and thus may alter the binding surface for the *Vinca* alkaloids in addition to affecting any conformational changes within  $\beta$ -tubulin required for drug binding.

In summary, our findings based on the cross-resistance profile and structural hypotheses for these mutations suggest that tubulin mutations are involved in the altered phenotype of 2ME2R cells and consequently resistance to 2-methoxyestradiol. Due to the lack of apparent steric restraints around K350 to alter drug binding, we favor the model where the mutations alter the conformational change of tubulin. Unlike the tubulin mutations providing resistance to other depolymerizing drugs (29, 30), we do not see an increase in the overall stability of the microtubules as shown by an increase in the portion in microtubules in the pellet

or an increase in acetylation. However, this no apparent change in microtubule stability was also observed in the work of Hua et al. (28), wherein they also identified a mutation in K350. Such differences are readily reconciled by the numerous additional factors that can be involved in altering microtubule dynamics, (e.g., changes in the level or activity of microtubule stabilizing proteins, destabilizing proteins, or tubulin modifying enzymes). For example, vincristine-resistant T-cell leukemia cell line (CEM/VCR R) showed not only increased microtubule stability but also increased levels of MAP4, a microtubule-stabilizing protein (27). It is intriguing that our cell line shows elevated levels of detyrosination, generally associated with increased microtubule stability, without increase in microtubule acetylation or a greater portion of tubulin present in the microtubule pellet. All of this implies that the drug resistance for this cell line proceeds by a different mechanism from those reported previously (27, 29, 30). Characterization of  $\beta$ -tubulin mutations and its microtubule-associated proteins should contribute to our understanding of drug target interactions and help to reveal the resistance mechanisms to microtubule-targeting agents.

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