

Mutations in α - and β -Tubulin That Stabilize Microtubules and Confer Resistance to Colcemid and Vinblastine¹

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Abstract

Single-step selections were used to obtain Chinese hamster ovary cell lines resistant to Colcemid and vinblastine. Verapamil was included in the selections to circumvent the isolation of cells with P-glycoprotein-mediated multidrug resistance and thereby enrich for cells with tubulin alterations. The isolated cell lines were 2-fold resistant to the selecting drug, exhibited cross-resistance to other drugs that inhibit microtubule assembly, and had enhanced sensitivity to the microtubule-stabilizing drug paclitaxel. The concomitant resistance to microtubule-destabilizing drugs and enhanced sensitivity to paclitaxel suggested that these cell lines have changes in microtubule assembly. Consistent with this interpretation, drug-resistant cell lines exhibited altered α - or β -tubulin mobility on two-dimensional gels and higher levels (47–54%) of assembled tubulin compared with wild-type (39%) or paclitaxel-resistant cells (25%). Some drug-resistant cells also had bundled microtubules as judged by immunofluorescence. Genomic sequencing of 11 drug-resistant cell lines predicted five different alterations (D45Y, C211F, D224N, S234N, and K350N) in β -tubulin and four different alterations (H283Y, E55K, A383V, and R390C) in α -tubulin. The amino acid substitutions are dispersed on the primary and tertiary structures of tubulin and, together with the other mutant properties, argue against a mechanism involving changes in drug binding. Rather, we propose that the alterations in α - and β -tubulin increase microtubule stability by promoting longitudinal interdimer and intradimer interactions and/or lateral interactions between protofilaments. This enhanced stability of microtubules increases their resistance to drugs that inhibit assembly.

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Introduction

Colcemid and vinblastine are members of a large group of antimitotic drugs that inhibit microtubule assembly. Many of these drugs are successful cancer chemotherapeutic agents; e.g., *Vinca* alkaloids such as vinblastine, vincristine, and vinorelbine are used extensively to treat acute lymphocytic leukemia, Hodgkin's lymphoma, testicular carcinoma, and a variety of other malignant diseases. Other antimitotic drugs have found additional medical uses; e.g., colchicine is used to treat the inflammation associated with gout, griseofulvin is used to treat fungal infections, and mebendazole is used to treat helminthiasis (1). Although these drugs are successful therapeutic agents, drug resistance can limit their effectiveness.

Cell culture studies indicate that the most frequent mechanism of resistance to microtubule-destabilizing drugs such as Colcemid and vinblastine is P-glycoprotein-mediated *mdr*⁴ (2–4). Cells with *mdr* have decreased intracellular drug accumulation and exhibit cross-resistance to a large number of unrelated, mostly hydrophobic drugs (5). We reported previously (6) that *mdr* is responsible for approximately 80% of cell lines selected for resistance to colchicine (a Colcemid analogue) or vinblastine; the remaining cell lines had properties suggesting alterations in tubulin that affect the assembly and stability of microtubules. In the case of the microtubule-stabilizing drug paclitaxel, tubulin alterations represented the most common mechanism of resistance, and <10% of drug-resistant cells had properties suggestive of *mdr* (7). Additional mechanisms of resistance to microtubule-destabilizing drugs have been described previously (3, 8). These include changes in tubulin isotype expression (9, 10) and increases in microtubule-associated protein 4 expression (10, 11). The prevalence of these latter mechanisms has not yet been established, and we have not observed them in our selections.

Changes in the electrophoretic behavior of tubulin have also been described in cells selected for resistance to a variety of microtubule-disrupting drugs, and resistance has been attributed to changes in drug binding (12) or to altered microtubule assembly (6, 13–15). More recently, structural changes in β -tubulin have been reported in vincristine-resistant cells with altered microtubule stability (10) and in indanocine-resistant cells with altered drug binding (16). Here, we present molecular changes in both α - and β -tubulin that are found in Colcemid- and vinblastine-resistant CHO cells. These cells exhibit increased microtubule assembly and support a model in which tubulin mutations produce hyperstable microtubules and thereby confer resistance to microtubule-

⁴ The abbreviations used are: *mdr*, multidrug resistance; GST, glutathione S-transferase; CHO, Chinese hamster ovary; HA, hemagglutinin; pl, isoelectric point.

destabilizing drugs (8, 17). Location of the amino acid changes in the tertiary structure of tubulin is consistent with a role in modulating the assembly of microtubules.

Materials and Methods

Growth and Selection of Drug-resistant Cells. Wild-type CHO cells (13) and drug-resistant cell lines were maintained in α -MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were grown at 37°C in a humid chamber containing 5% CO₂. The Colcemid- and vinblastine-resistant cells were isolated as described previously (6). Briefly, 5×10^6 cells were UV-irradiated and allowed to recover for 3 days before plating them in media containing 5 μ g/ml verapamil and 15 ng/ml Colcemid or 2.5 ng/ml vinblastine in growth medium. Visible drug-resistant colonies were isolated after 8 days of incubation.

Two-dimensional Gel Electrophoresis. Samples for two-dimensional gel electrophoresis were prepared by incubating cells in methionine- and cysteine-free MEM supplemented with 20 μ Ci/ml [³⁵S]methionine and cysteine (Tran ³⁵S-label; ICN Biomedicals Inc., Irvine, CA) for 1 h at 37°C. Cells were lysed in SDS dissociation buffer (18), and proteins were precipitated with acetone and run on two-dimensional gels containing 0.5% (pH 3.5–10) and 1% (pH 5–6) ampholines (Crescent, Islandia, NY) as described previously (19). After electrophoresis the gel was stained with Coomassie Brilliant Blue R250, dried, and exposed to X-ray film.

Measurement of Drug Resistance. To determine the dose response to various drugs, an equal number of cells (~100–200) were plated into 6 wells of 24-well dishes containing increasing concentrations of paclitaxel (0–400 ng/ml), Colcemid (0–80 ng/ml), or vinblastine (0–60 ng/ml). After incubation for 7 days at 37°C, the medium was removed, and the cells were stained with 0.25% methylene blue in water as described previously (7). The plates were rinsed with water to remove excess stain and photographed with a Coolpix 990 digital camera (Nikon, Melville, NY).

Measurement of Tubulin Polymerization. Cells were grown in duplicate wells of 24-well dishes to 80% confluence, rinsed twice with PBS at room temperature, and then lysed with 100 μ l of microtubule buffer containing 20 mM Tris-HCl (pH 6.8), 0.5% NP40, 0.14 M NaCl, 1 mM MgCl₂, 2 mM EGTA, and 4 μ g/ml paclitaxel as described previously (20). The lysates were briefly mixed and centrifuged at $12,000 \times g$ for 10 min at 4°C. Supernatants containing soluble tubulin were transferred to separate tubes, and pellets containing polymerized tubulin were resuspended in water. An equal volume of bacterial extract containing GST was added to each fraction, the proteins were precipitated with 5 volumes of acetone, and the precipitates were resuspended in 50 μ l of SDS dissociation buffer (18).

For SDS-PAGE and Western blots, equal volumes of sample were run on 10% polyacrylamide SDS minigels (Bio-Rad, Hercules CA). Proteins in the gel were transferred to BA-85 nitrocellulose (Schleicher & Schuell, Keene, NH) as described previously (21), and the blots were incubated with a mixture of mouse monoclonal anti- α -tubulin antibody, (DM1A; 1:2000; Sigma-Aldrich), anti-actin antibody (C4; 1:3000;

Chemicon International, Inc., Temecula, CA), and anti-GST antibody (B11; 1:3000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoreactive protein bands were imaged using Cy5-conjugated goat antimouse IgG (Chemicon International Inc.), and fluorescence was detected in a STORM imager (Molecular Dynamics, Sunnyvale, CA). Quantification was carried out by analysis with Scion Image analysis software (Scion Corp., Frederick, MD) using the “gelplot 1” macro. The percentage of assembled tubulin was calculated by normalizing the tubulin in each lane to the amount of GST present in the same lane, dividing the normalized values for tubulin in the pellet by the sum of tubulin in the pellet and supernatant fractions, and multiplying the quotient by 100%.

Detection of Mutations in Drug-resistant Cell Lines.

Cells were grown to 80% confluence in 35-mm dishes, rinsed three times with PBS, and lysed with buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, and 0.5% Tween 20. The cellular residue was collected in a 1.5-ml microcentrifuge tube and treated with 0.1 mg/ml proteinase K (Sigma-Aldrich) at 55°C for 45 min followed by inactivation at 100°C for 10 min. This genomic DNA was used to PCR amplify exon regions of α 1- and/or β 1-tubulin genes using intron-specific primers derived from our published sequences of CHO α 1- or β 1-tubulin (GenBank accession numbers AY173017 and AF120325). The PCR-amplified products were sequenced by automated PCR sequencing (Seqwright, Houston, TX).

Site-directed Mutagenesis and Transfection. Specific mutations were introduced into C β 1, a CHO class I (see Ref. 22 for definition of tubulin isotypes) β -tubulin cDNA (GenBank accession number U08342) previously isolated in this laboratory (23). The tubulin encoded by this cDNA is identical to human, mouse, and other mammalian species. Class I is the most abundant β -tubulin isotype in CHO cells, where it makes up 70% of the total (24, 25), and it is the isotype most frequently altered in drug-resistant CHO (8) and human cell lines (10, 16, 26–28). The C β 1 cDNA was cloned into a pTOP vector to allow tetracycline-regulated expression and contained the sequences for a COOH-terminal HA tag to allow discrimination from endogenously encoded β -tubulin (29). Mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All mutant cDNAs were sequenced to ensure that they contained only the desired mutation.

LipofectAMINE (Invitrogen) was used to transfect mutant cDNA into CHO strain tTApur 6.6a, which stably expresses the tetracycline-regulated transactivator (29). Stable transfectants were selected using G418 (Invitrogen) and screened for expression by immunofluorescence using a monoclonal antibody specific for the HA tag (12CA5; Roche Applied Science, Indianapolis, IN) and by Western blot analysis using a monoclonal antibody that recognizes all β -tubulin (Tub 2.1; Sigma-Aldrich).

Immunofluorescence. Cells were grown on glass coverslips in α -MEM for 2 days, rinsed briefly in PBS, pre-extracted with microtubule buffer (without NaCl) for 2 min at 4°C, and fixed in methanol at –20°C for 20 min. The fixed cells were then stained with anti- α -tubulin antibody DM1A

Table 1 Properties of colcemid and vinblastine resistant cell lines

Cell line	Two-dimensional gel change (new spot)	Drug sensitivity		Tubulin assembly (% of total tubulin)
		Paclitaxel	Colcemid	
Wild-type	No change	S ^a	S	39
Tax 5-6	α 1 Basic shift	R	SS	25 ($P < 0.01$) ^b
Cmd4	β 1 Basic shift	SS ^c	R ^c	50 ^c
CV 1-1	β 1 Basic shift	SS	R	54 ($P < 0.01$)
CV 7-1	β 1 Lower molecular weight	S	R	47 ($P < 0.05$)
CV 4-4	β 1 Acidic shift	SS	R	50 ($P < 0.01$)
CV 8-1	No change	SS	R	48 ($P < 0.02$)
CV 1-5	α 1 Acidic shift	SS	R	51 ($P < 0.01$)
CV 2-8	α 1 Basic shift	SS	R	47 ($P < 0.02$)
CV 2-3	No change	SS	R	50 ($P < 0.01$)
VV 3-2	α 1 Acidic shift	SS	R	48 ($P < 0.01$)

^a S, same sensitivity as wild-type cells; R, 2-fold resistant; SS, 1.5–2-fold more sensitive.

^b Significance determined by Student's *t* test.

^c These data were reported previously (20).

(Sigma-Aldrich) followed by staining with fluorescein-conjugated goat antimouse IgG (Sigma-Aldrich). In the case of transfected cells, the procedure was the same, except that an antibody specific for the HA tag (12CA5; Roche Applied Science) was used in place of the DM1A. The microtubules were visualized using an Optiphot microscope equipped with epifluorescence and a $\times 60$ objective (Nikon). Images were captured using a Magnafire digital camera (Optronics, Goleta, CA).

Results

Resistance to Colcemid and Vinblastine Is Associated with Alterations in Tubulin. Our single-step selections of CHO cells resistant to Colcemid and vinblastine typically yield approximately 80% multidrug-resistant mutants (6, 13). To enrich for cells with tubulin alterations, we described a method to circumvent isolation of mdr mutants from selections for drug resistance in mammalian cells by inclusion of the P-glycoprotein-blocking drug, verapamil (6). Initial screening for resistance to puromycin and daunomycin, drugs that are also substrates for P-glycoprotein pump, showed that cells selected in this way remained sensitive to drugs unrelated to tubulin-binding agents and therefore did not exhibit the mdr phenotype. Resistance to tubulin-binding drugs and the absence of mdr suggested that these cell lines might have altered tubulin, and this was confirmed by labeling the cells with [³⁵S]methionine and analyzing them by two-dimensional gel electrophoresis. The mobility of either α - or β -tubulin was altered in 10 of the 12 cell lines we describe here (Table 1). Because CHO cells are diploid and express multiple tubulin genes (24, 25), mutation of a single allele alters the mobility of only a fraction of the total tubulin, causing a new spot to appear on the two-dimensional gels as demonstrated for both α - and β -tubulin in Fig. 1. In most cases, the altered tubulin migrated with a more basic or acidic pI, but CV 7-1 displayed a new β -tubulin spot with the same pI and faster migration in the SDS gel (Fig. 1C, arrow). It should also be noted that all α -tubulin species migrated as two spots with the same pI but different apparent molecular weights in two-dimensional gels. This anomalous migration does not appear to result from different gene products or

from posttranslational modifications of the α -tubulin.⁵ Others have noted the same phenomenon (30).

Cells Resistant to Colcemid or Vinblastine Exhibit Mutual Cross-Resistance. Cell lines selected for resistance to Colcemid or vinblastine all had normal growth rates. The cells were not resistant to unrelated drugs such as puromycin and daunomycin and therefore are unlikely to have the mdr phenotype (6). Instead, cross-resistance was limited to other drugs that inhibit microtubule assembly. Using a clonogenic assay that measures the ability of cells to grow and divide in varying concentrations of drug, for example, vinblastine-resistant VV 3-2 was found to be cross-resistant to Colcemid (Fig. 2, top), and conversely, Colcemid-resistant CV 4-4 was found to be cross-resistant to vinblastine (Fig. 2, bottom). Similar results were obtained for all of the other cell lines described here. Because Colcemid and vinblastine have distinct binding sites on tubulin (31), the reciprocal cross-resistance observed with these two drugs argues that resistance is not due to changes in the drug-binding sites. We have found previously that Colcemid-resistant cells with altered tubulin are also cross-resistant to estramustine (32), griseofulvin (13), maytansine (14), LY195448 (15), and other drugs that inhibit microtubule assembly.⁵

Colcemid- and Vinblastine-resistant Cells Are Frequently Supersensitive to Paclitaxel. We reported previously that paclitaxel-resistant cells with alterations in tubulin genes are often more sensitive to microtubule-depolymerizing drugs, and conversely, Colcemid- and vinblastine-resistant cells are frequently more sensitive to the microtubule-stabilizing drug paclitaxel (8, 17). To extend these observations, we tested the paclitaxel dose response of the present set of Colcemid- and vinblastine-resistant cells (Fig. 3). The results show that vinblastine-resistant cell line VV 3-2 and Colcemid-resistant cell line CV 2-8 are supersensitive to paclitaxel compared with wild-type cells (Fig. 3). Of all of the drug-resistant cell lines described here, only CV 7-1 failed to exhibit this behavior. To demonstrate that the converse is also true, we included data for Tax 5-6, a paclitaxel-resistant

⁵ F. Cabral, unpublished observations.

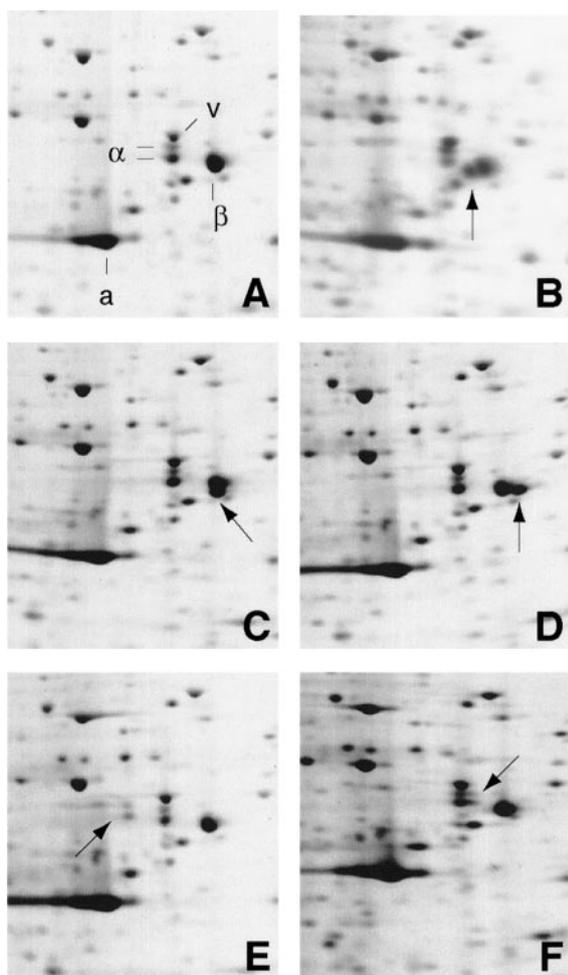


Fig. 1. Two-dimensional gel electrophoresis patterns of Colcemid- and vinblastine-resistant cells. Autoradiograms of the gels are oriented with the basic side to the left and the acidic side to the right. *A*, wild-type two-dimensional gel pattern shared by strains CV 8-1 and CV 2-3. *v*, vimentin; *a*, actin; α , α -tubulin; β , β -tubulin. Note that wild-type α -tubulin migrates as two spots with the same pI but different apparent molecular weights and that β -tubulin migrates as a single spot. *B*, a more basic β -tubulin spot (arrow) is seen in Cmid4, CV 1-1, and CV 1-9. *C*, β -tubulin with a lower apparent molecular weight (arrow) is seen in CV 7-1. *D*, β -tubulin with a more acidic pI (arrow) is seen in CV 4-4. *E*, two α -tubulin spots with a more basic pI (arrow) are seen in CV 2-8. *F*, two α -tubulin spots with a more acidic pI (arrow) are seen in CV 1-2, CV 1-5, CV 1-7, and VV 3-2. The identity of tubulin spots in these gels has been established by immunoreactivity with specific antibodies, peptide maps, and comigration with purified tubulin (13, 23, 24).

cell-line with an alteration in α -tubulin (7). This cell line was 4-fold resistant to paclitaxel (Fig. 3) and approximately 2–3-fold supersensitive to Colcemid (Fig. 2A). The results of drug sensitivity testing for each of the drug-resistant cell lines are summarized in Table 1. The enhanced sensitivity to paclitaxel seen in cell lines selected for resistance to Colcemid and vinblastine argues that the mechanism of resistance in these cells does not involve *mdr* or alterations in drug-binding sites.

Cells Resistant to Microtubule-destabilizing Drugs Have Increased Microtubule Assembly. We previously described a model suggesting that cells resistant to micro-

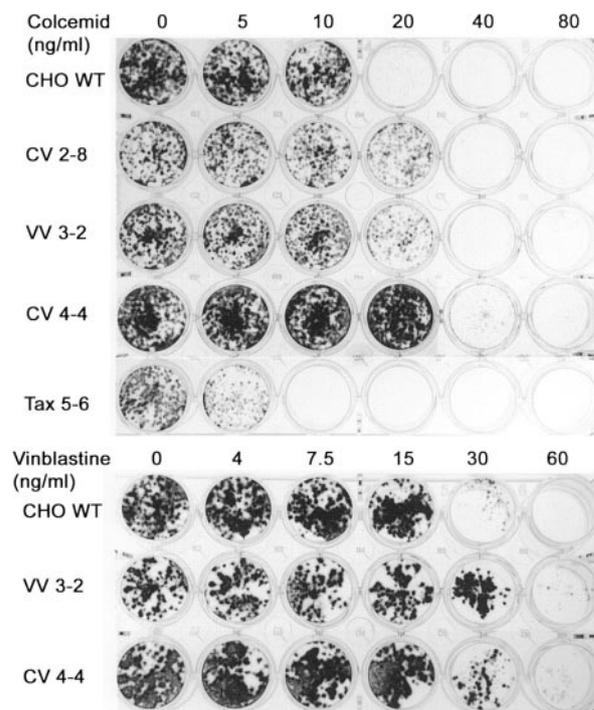


Fig. 2. Dose response of mutant CHO cells to Colcemid and vinblastine. Equal numbers of mutant or wild-type CHO cells (~100–200) were plated in each of 6 wells in α -MEM containing an increasing concentration of drugs as labeled. After 7 days of growth, the medium was removed, and the cells were stained with 0.25% methylene blue.

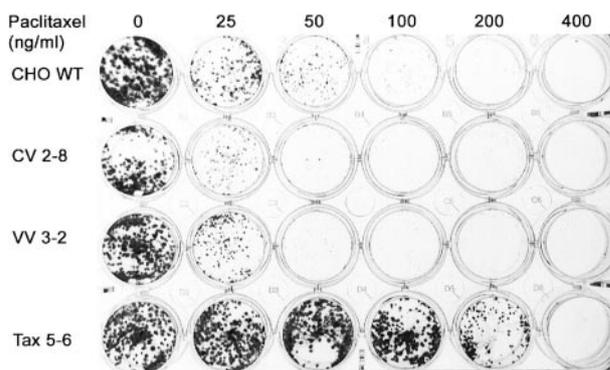


Fig. 3. Dose response of drug-resistant cells to paclitaxel. Equal numbers of mutant or wild-type CHO cells (~100–200) were plated in each of 6 wells in α -MEM containing an increasing concentration of paclitaxel as labeled. After 7 days of growth, the medium was removed, and the cells were stained with 0.25% methylene blue.

tubule-depolymerizing drugs have increased microtubule assembly and stability (17). These microtubule changes render cells more sensitive to the addition of paclitaxel, a drug that also increases microtubule assembly and stability. To test whether the current mutants actually have increased microtubule assembly, we used a procedure that allows the fraction of total tubulin that is assembled *in vivo* to be measured by lysing cells in microtubule-stabilizing buffer and separating soluble from polymerized tubulin by centrifugation (20).

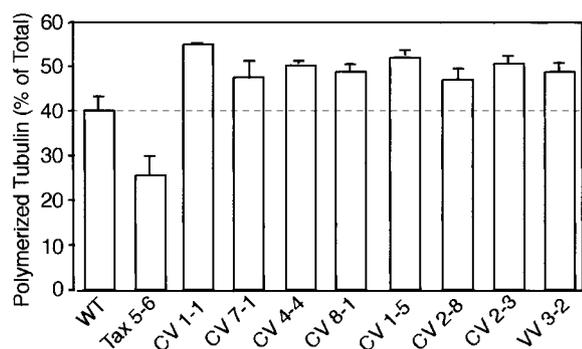


Fig. 4. Tubulin assembly in drug-resistant cells. Cells were lysed in microtubule-stabilizing buffer, soluble tubulin was separated from microtubules by centrifugation, and proteins in each fraction were separated by SDS-PAGE and transferred onto nitrocellulose. Tubulin in pellet (*P*) and supernatant (*S*) fractions was labeled with anti-tubulin antibodies followed by a Cy5-conjugated secondary antibody. The bands were detected by fluorescence using a STORM imager and quantified using Scion Image software. The amount of tubulin in the pellet was expressed as a percentage of total tubulin (*S* + *P*). The data shown represent averages from at least three independent experiments.

Tubulin in the pellet and supernatant fractions was then quantified. To simplify the original procedure, we used a modification that allows the use of one-dimensional SDS gels and immunodetection for the two-dimensional gels of radiolabeled cells used initially. The results show that wild-type CHO cells contain $39 \pm 2\%$ of their total tubulin in microtubules (Fig. 4). This value is consistent with data obtained previously in our laboratory by the two-dimensional gel procedure. The Colcemid- and vinblastine-resistant cell lines were all found to contain a higher proportion of tubulin in the microtubule fraction (Fig. 4), as our model predicted for cell lines resistant to drugs that destabilize microtubules. The data are summarized in Table 1. Also as predicted, paclitaxel-resistant mutant Tax 5-6 showed reduced assembled tubulin compared with wild-type CHO cells.

Drug Resistance Mutations Affect Both α 1- and β 1-Tubulin Genes. Altered tubulin mobility in two-dimensional gels suggested that the drug-resistant cell lines have mutated tubulin genes. To confirm this, we prepared genomic DNA from drug-resistant cells and amplified specific tubulin exons using primers to intron sequences as well as 5'- and 3'-untranslated regions. Mutations were found with no bias for α 1- or β 1-tubulin genes and resulted in amino acid substitutions consistent with the mobility shift of tubulin in two-dimensional gels (Table 2). The only anomalous behavior was the faster migration of β -tubulin containing a C211F mutation (strain CV 7-1, see Fig. 1C). We confirmed that the aberrant migration was caused by the C211F mutation by expressing β 1-tubulin cDNA containing a C211F mutation in CHO cells (data not shown).

Increased Microtubule Stability Can Give Rise to Microtubule Bundling. Analysis of various mutant cell lines indicates that the extent of microtubule assembly consistent with functional microtubules is 22–58% of total cellular tubulin (33). The mutants we describe here all fall into the upper end of that range. Their increased microtubule stability suggested the possibility that some of these cells could have an

Table 2 Mutations in colcemid- and vinblastine-resistant cell lines

Cell line	Wild-type sequence/mutant tubulin sequence	Amino acid change
Cmd4	CAG-CTG-GAC-CGA-ATC CAG-CTG- TAC -CGA-ATC ^a	β D45Y
CV 1-1	TAT-GGG-GAC-CTC-AAC	β D224N
CV 1-9	TAT-GGG- AAC -CTC-AAC	
CV 7-1	GAC-ATC-TGC-TTC-CGC GAC-ATC- TTC -TTC-CGC	β C211F
CV 4-4	AAC-GTC-AAG-ACC-GCC	β K350N
CV 8-1	AAC-GTC-AAT-ACC-GCC ACC-ATG-AGC-GGG-GTC ACC-ATG- AAC -GGG-GTC	β S234N
CV 1-2	GCC-TAC-CAT-GAG-CAG	α H283Y
CV 1-5, CV 1-7	GCC-TAC-TAT-GAG-CAG	
CV 2-8	TTC-AGT-GAG-ACA-GGC TTC-AGT- AAG -ACA-GGC	α E55K
CV 2-3	ACC-ACA-GCC-ATC-GCT ACC-ACA-GTC-ATC-GCT	α A383V
VV 3-2	TGG-GCT-CGC-CTA-GAT TGG-GCT-TGC-CTA-GAT	α R390C

^a Nucleotides in bold represent the mutations found in each of the mutant cell lines.

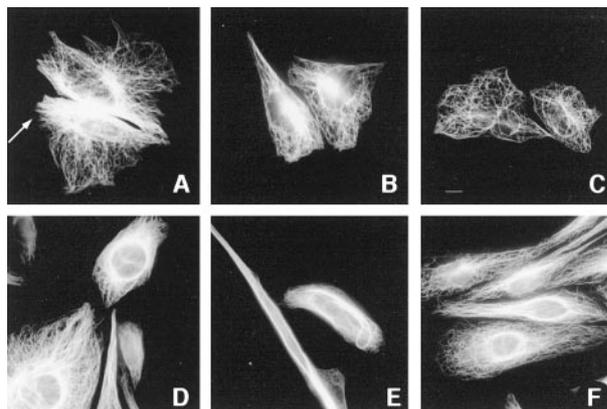


Fig. 5. Microtubule organization in drug-resistant CHO cells. Colcemid-resistant mutant CV 7-1 (A), wild-type CHO (B), or paclitaxel-resistant Tax 18 (C) or cells transfected with HA-tagged β 1 tubulin cDNA containing a C211F mutation (D and E) or a D45Y mutation (F) were grown on glass coverslips. Soluble tubulin was pre-extracted using microtubule-stabilizing buffer, cells were fixed in methanol, and microtubules were stained with anti- α -tubulin antibodies (A–C) or antibodies to a HA epitope tag (D–F). Note that CV 7-1 (A) has a high density of microtubules forming bundles near the nucleus and extending to the cell periphery (arrow). Bar in C is 10 μ m.

altered microtubule cytoskeleton. Although immunofluorescence examination of most of the Colcemid-resistant cell lines did not reveal obvious changes in microtubule organization, CV 7-1 often had bundles of cytoplasmic microtubules in the perinuclear area that extended to the cell periphery (Fig. 5A). This contrasted to wild-type cells (Fig. 5B), in which microtubule bundles were seldom seen. Moreover, Tax 18, a previously isolated paclitaxel-dependent cell line (34), clearly had fewer microtubules than wild-type cells (Fig. 5C). This latter result is consistent with previous biochemical measurements indicating that Tax 18 and other paclitaxel-

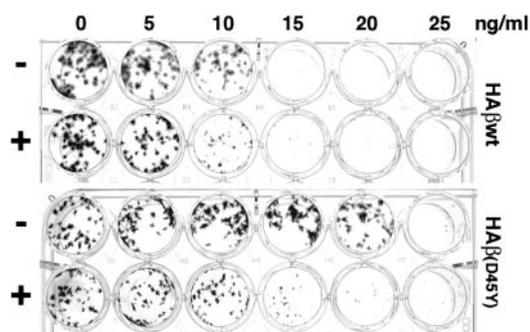


Fig. 6. Colcemid resistance of transfected cells. Approximately 50–100 stably transfected cells expressing wild-type ($HA\beta wt$) or mutant [$HA\beta(D45Y)$] tubulin were seeded into replicate wells of a 24-well dish containing the indicated concentrations of Colcemid. After 7 days of growth, the medium was removed, and the cells were stained with 0.25% methylene blue. Note that the assay was carried out in the presence (+) or absence (–) of 1 μ g/ml tetracycline to respectively inhibit or allow expression of the transgene.

resistant and -dependent cell lines have less assembled tubulin than wild-type CHO cells (20, 33).

To eliminate the possibility that microtubule bundles seen in CV 7-1 might be due to changes other than the altered β -tubulin, a C211F mutation was created in $HA\beta 1$ -tubulin cDNA by site-directed mutagenesis. Cells transfected with this mutant cDNA exhibited a dense microtubule network and microtubule bundles near the nucleus (Fig. 5D). At higher expression (as judged by brightness of immunofluorescence), cell elongation and occasional intracellular cables of microtubules were seen (Fig. 5E). A second mutation, D45Y, found in *Cmd4* was also tested by this method, and again evidence for microtubule bundling was seen (Fig. 5F). Additionally, the microtubules had an unusual “wavy” appearance, suggesting that incorporation of this particular altered tubulin might be altering the basic structure of the microtubule. The altered microtubule appearance and organization conferred by these two mutant tubulins further support the conclusion that the mutations we described affect drug resistance by altering microtubule assembly and stability rather than by altering the drug-binding site.

A D45Y Alteration Is Sufficient to Confer Colcemid Resistance. To demonstrate that tubulin mutations are indeed responsible for the Colcemid resistance phenotype, we introduced the D45Y mutation into CHO $HA\beta 1$ -tubulin cDNA cloned into a vector that allows tetracycline-regulated expression. CHO cells transfected with the wild-type cDNA or with the cDNA containing the D45Y mutation were then examined for their Colcemid sensitivities when the transgene is expressed (no tetracycline) or not expressed (with tetracycline). The results in Fig. 6 demonstrate that expression of the wild-type transgene had no effect on Colcemid sensitivity. In contrast, expression of the cDNA containing the D45Y mutation clearly increased the resistance of the transfected cells to Colcemid. It should be noted that we also reported previously that cDNAs containing paclitaxel resistance mutations confer resistance to paclitaxel when transfected into wild-type cells (29, 35).

Discussion

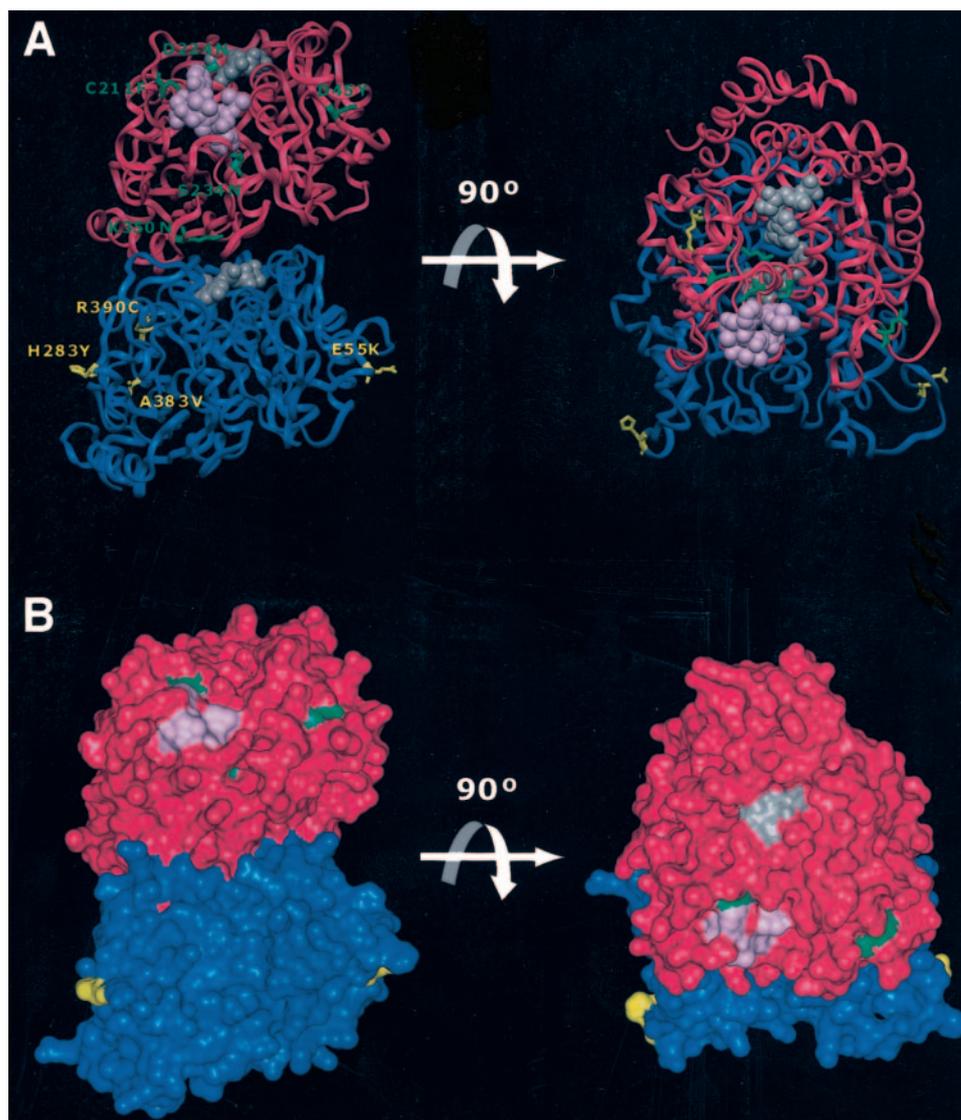
Tubulin-binding drugs such as Colcemid and vinblastine destabilize microtubules, inhibit mitotic spindle formation, and block cell division. Although most cells selected for resistance to these drugs have the *mdr* phenotype, a second major mechanism of resistance we and others have described involves mutations that alter the assembly characteristics of tubulin (7, 10, 15, 20, 28, 29, 33, 36). This latter mechanism can become the predominant form of resistance when verapamil is included to circumvent the isolation of multidrug-resistant cells (6). Using selections containing verapamil, we isolated cell lines with nine distinct alterations that confer resistance to Colcemid and vinblastine and are almost equally distributed between α - and β -tubulin. The locations of these alterations are shown in Fig. 7.

Diminished drug binding represents a third potential mechanism of resistance but would produce a recessive phenotype. Thus, lower eukaryotes with haploid genomes use altered drug binding as a common mechanism of resistance (37), but in mammalian cells, which are diploid and express multiple tubulin genes (38), this mechanism is much less apparent (8). The properties of the mutants described here strongly argue against alterations in drug binding as the cause of drug resistance. (a) The mutant cells are cross-resistant to Colcemid and vinblastine, drugs that have distinct binding sites on the tubulin heterodimer (31). (b) Cells selected for resistance to either vinblastine or Colcemid are more sensitive to paclitaxel, a drug that promotes rather than inhibits microtubule assembly and occupies yet another binding site (39). (c) Colcemid- and vinblastine-resistant cells consistently exhibit increased microtubule assembly in the absence of any drug treatment. (d) The mutational alterations are scattered between the α - and β -tubulin subunits, and it is unlikely they could all be acting by changing drug binding. These properties are more consistent with a mechanism in which the tubulin alterations stabilize microtubules and thereby make them less sensitive to drugs (e.g., Colcemid and vinblastine) that destabilize microtubule assembly (17).

The locations of the tubulin alterations are consistent with potential effects on microtubule assembly and stability. Amino acid 45 of β -tubulin, the residue altered in mutant *Cmd4*, for example, lies near the surface of a large H1/S2 loop that is believed to form lateral interactions with β -tubulin on the adjacent protofilament (40). Similarly, CV 2-8 has an alteration (E55K) in the same loop of α -tubulin. In both cases, the region believed to interact with this loop is the S7/H9 loop of the analogous tubulin subunit on the adjacent protofilament. This S7/H9 loop has been named the “M-loop” because it forms major lateral interactions in microtubule assembly (40). Interestingly, an amino acid substitution in the M-loop of α -tubulin (H283Y) was found in three drug-resistant mutants. Thus, several of the changes in our Colcemid-resistant mutants affect tubulin regions that are juxtaposed when forming lateral interactions during microtubule assembly.

Other amino acid substitutions appear to be more buried but may alter the conformation of regions affecting the assembly competence of the tubulin. Of particular interest are three β -tubulin alterations (C211F, D224N, and S234N) that

Fig. 7. Sites of amino acid substitutions that stabilize microtubule assembly. **A**, ribbon diagram of an $\alpha\beta$ -tubulin heterodimer with paclitaxel and GDP-bound β -subunit (red) and GTP-bound α -subunit (blue). Space-filling models of GDP and GTP are represented in gray, and paclitaxel is in lavender. The positions of altered residues are depicted as stick models in yellow (α -subunit) and green (β -subunit) and labeled with the amino acid substitution. **B**, atomic surface of a tubulin heterodimer illustrating the degree of exposure of altered residues to bulk solvent. Orientation of the molecule, coloring, and labels are as described for **A**. These representations show that in α -tubulin, Glu⁵⁵ and His²⁸³ are located on surface loops, whereas Ala³⁸³ and Arg³⁹⁰ (in helix 11) are buried. In the β -subunit Asp⁴⁵, Asp²²⁴, and Ser²³⁴ are partially solvent-exposed; Cys²¹¹ is buried near a lateral interface; and Lys³⁵⁰ is buried at the longitudinal intradimer interface. Residues Cys²¹¹, Asp²²⁴, and Ser²³⁴ are located within 8 Å of paclitaxel. Structural coordinates corresponding to the Protein Data Bank file 1TUB (42) were used to generate the model, and the illustration was prepared using InsightII software.



are located close to the paclitaxel-binding site and the M-loop. When paclitaxel binds tubulin, it stabilizes microtubules and prevents their depolymerization by a mechanism that is not yet understood. The β -tubulin subunit has a bound GTP that maintains the tubulin heterodimer in an assembly-competent state (41). It has been suggested that nucleotide hydrolysis produces a local conformational change that may move the core helix (H7) of the intermediate domain of β -tubulin and reposition the nearby M-loop to weaken lateral interactions between protofilaments. The action of paclitaxel may be to negate this conformational change and lock tubulin into its polymerization-favorable conformation (42, 43). Amino acid alterations in the area around the paclitaxel-binding site could be mimicking these effects. In support of this notion, mutant CV 7-1 with the C211F mutation exhibits evidence of microtubule bundling (Fig. 5A), and transfection of cDNA containing this and another Colcemid resistance mutation also produces clear microtubule bundling (Fig. 5, D–F), one of the hallmarks of cells treated with paclitaxel

(44). In contrast to our results, a C211A mutation in yeast produces hypersensitivity to benomyl, a drug whose action is similar to that of Colcemid (45). It would thus appear that different amino acid substitutions at this position can have opposing effects on drug sensitivity and perhaps microtubule assembly as well. This conclusion is consistent with the observation that the Cys²¹¹ side chain is within 8 Å of paclitaxel, but can also be cross-linked to GDP (46) and is close to a peptide that cross-links to colchicine (47), suggesting that it is located in a region of central importance in achieving an assembly competent conformation.

The significance of the region around the M-loop in microtubule assembly is highlighted not only by the fact that the paclitaxel- and colchicine-binding sites are nearby, but also by the fact that many mutations conferring resistance to drugs that promote or inhibit microtubule assembly are also located in this same area. These include the C211F, D224N, and S234N alterations that stabilize microtubules and confer resistance to Colcemid, as well as a series of Leu²¹⁵, Leu²¹⁷,

and Leu²²⁸ alterations in β -tubulin that we previously reported can destabilize microtubules and confer resistance to paclitaxel (29). Additional alterations in this area include F270V, T274I, A364T, and R282Q reported to affect the binding of paclitaxel and epothilones (26, 27) and Q292E associated with reduced stability of microtubules and epothilone resistance (28).

The most buried residue in our current studies is found in CV 4-4, which has a K350N β -tubulin alteration. This amino acid is located near the intradimer interface and is within 6–7 Å of the α -tubulin-bound GTP. This region has also been implicated in binding colchicine (48, 49). CV 4-4, however, is cross-resistant to vinblastine, exhibits increased sensitivity to paclitaxel, and has increased tubulin polymerization in the absence of any drug treatment; *i.e.*, it has properties similar to the other mutants we describe here, and there is no reason to suspect that altered drug binding accounts for its drug resistance. Rather, the alteration may affect the structure of the $\alpha\beta$ heterodimer and stabilize longitudinal interactions in microtubule assembly. The importance of this region in resistance to microtubule-inhibitory drugs is supported by the fact that K350M and K350E mutations have been associated with increased microtubule stability in colchicine-resistant *Chlamydomonas* (50), and K350N has recently been reported in human lymphoblastoid cells resistant to indanocene, another microtubule inhibitor that binds to the colchicine site (16). Moreover, a L240I β -tubulin alteration found in vincristine-resistant human T cells also maps to the $\alpha\beta$ heterodimer interface and is associated with increased microtubule assembly and stability (10).

Our studies have also identified two α -tubulin alterations (A383V and R390C) that are located in helix 11. Given the frequent occurrence of Val and Cys in α -helical secondary structures and their helix-forming propensities, these substitutions are not likely to destabilize the secondary structure. Helix 11 of α -tubulin is positioned on the outside surface of the microtubule (backside of surface shown in Fig. 7B) and appears to be involved in longitudinal interactions with the H10/S9 loop of β -tubulin (40). The amino acid substitutions could potentially change the orientation of the helix and thereby alter the heterodimer conformation. Alternatively, microtubule-associated proteins, kinesin-related proteins, and other potential regulators of microtubule assembly are known to bind the microtubule surface (51). It is possible that the A383V and R390C alterations affect one or more of these interactions and thereby change microtubule stability and sensitivity to microtubule-disrupting drugs.

It is interesting that alterations in some regions of tubulin can confer resistance to Colcemid or to paclitaxel. This is especially evident for amino acid substitutions C211F, D224N, and S234N, reported here to confer resistance to Colcemid. As already discussed, these are close to previously described alterations near the paclitaxel-binding site and the M-loop of β -tubulin. The observation that alterations in the same region of the protein can confer resistance to drugs with opposing effects on microtubule assembly again argues that the changes are affecting microtubule assembly and stability rather than drug binding. They also emphasize the importance of this region in microtubule assembly and

provide genetic confirmation for the microtubule structural model (40).

In summary, we have described a dispersed set of alterations in both α - and β -tubulin that stabilizes microtubules and confers resistance to drugs that inhibit their assembly. The mutant properties implicate altered microtubule assembly as the mechanism of resistance, and the locations of the mutations in the structure of the microtubule are consistent with this interpretation. Few tubulin mutations have been reported thus far in mammalian cells, but overlap of one of our mutations with previously reported mutations in human cells and in *Chlamydomonas* suggests that certain tubulin alterations may be very common in a cell culture model. The relevance of these model systems to clinical resistance, however, remains to be established. One recent study that attempted to find tubulin mutations in tumor samples from patients unresponsive to paclitaxel was unsuccessful (52). Whereas it is premature to draw firm conclusions from limited studies, it seems probable that additional mechanisms need to be considered in examining the responses of a patient to chemotherapy. For example, a drug may not achieve therapeutic concentrations in a tumor because of pharmacokinetic changes in metabolism, excretion, or organ distribution that are absent in cell culture models of resistance. As we learn to give drugs more effectively, however, it is anticipated that cellular-based mechanisms of resistance such as mdr and tubulin alterations will be seen more frequently in a clinical setting.

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Mutations in α - and β -Tubulin That Stabilize Microtubules and Confer Resistance to Colcemid and Vinblastine¹

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