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Mutations at Leucine 215 of β-Tubulin Affect Paclitaxel Sensitivity by Two Distinct Mechanisms†

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ABSTRACT: Paclitaxel resistance mutations in Chinese hamster ovary cells frequently alter a cluster of leucine residues in the H6−H7 loop region of β-tubulin. To gain further insight into the role of this region in microtubule assembly and drug resistance, site-directed mutagenesis was used to systematically change amino acid L215. The mutated genes were cloned into a tetracycline-regulated expression vector and transfected into wild-type cells. Most of the mutations destabilized microtubule assembly, causing a decreased fraction of tubulin to appear in the microtubule cytoskeleton. In each case, the decreased level of assembly was associated with paclitaxel resistance and increased colcemid sensitivity. In two cases, however, the alteration did not significantly perturb the level of assembled tubulin or confer resistance to paclitaxel. One of these, L215V, produced little or no detectable phenotype, while the other, L215I, conferred increased sensitivity to paclitaxel. The increased drug sensitivity did not extend to epothilone A, a drug that binds to the same site and has a mechanism of action similar to that of paclitaxel, or colcemid, a drug with an opposing mechanism of action and a distinct binding site. Moreover, L215I conferred enhanced paclitaxel sensitivity at very low levels of expression, and sensitivity was not further enhanced in cells with higher levels of expression, implying that paclitaxel acts substoichiometrically. These properties, along with the proximity of L215 to the drug binding site, suggests that the L215I substitution may enhance the binding or effectiveness of paclitaxel. Our studies confirm the importance of the H6−H7 loop of β-tubulin in microtubule assembly and resistance to antimitotic drugs. They also identify the first mammalian mutation shown to specifically increase sensitivity to paclitaxel.

Microtubules make up an essential component of the eukaryotic cytoskeleton and are required for chromosome segregation, vesicle transport, ciliary and flagellar motion, and placement of the endoplasmic reticulum and Golgi apparatus. They are formed from heterodimers of α- and β-tubulin which assemble end to end into linear protofilaments, 13 of which associate laterally to become the walls of most microtubules found in nature. Because microtubules are essential and exist in a dynamic steady state with free tubulin heterodimers, many plant and animal species have developed poisons that kill cells by interfering with microtubule assembly and thereby blocking mitosis. Paclitaxel (Taxol) is the prototype for a subclass of antimitotic drugs that bind microtubules and stabilize them to disassembly. These drugs are becoming increasingly important for treating breast cancer, ovarian carcinoma, non-small cell lung carcinoma, head and neck carcinomas, melanoma, and other malignant diseases (*1*). Despite the efficacy of these drugs, however, many patients who initially respond acquire resistance to further therapy. In an effort to understand this phenomenon, a number of laboratories have turned to cell culture systems to isolate and characterize cells resistant to paclitaxel and other drugs that affect microtubule assembly (reviewed in refs *2* and *3*). Although these studies have identified several potential mechanisms by which tumor cells may acquire resistance, none has yet been definitively shown to cause resistance in a clinical setting. Nevertheless, it is likely that one or more of these cellular-based mechanisms of resistance will be seen once optimal drug dosing and pharmacokinetic problems are worked out.

Tubulin mutations affecting microtubule stability constitute a major mechanism of cellular resistance to antimitotic drugs. These mutations act in a manner that opposes drug action; i.e., mutations that increase microtubule stability confer resistance to drugs that inhibit microtubule assembly (e.g., colcemid), whereas mutations that decrease microtubule stability confer resistance to drugs that promote assembly (e.g., paclitaxel) (*4*, *5*). In the case of paclitaxel, we previously reported that many of the resistance mutations affect a loop connecting helix 6 and helix 7 of β-tubulin (*6*). Among nine paclitaxel resistant Chinese hamster ovary (CHO)[1] cell lines, six had mutations affecting leucine 215 (L215), one had an L217 substitution, and two had amino acid substitutions at L228 which is located in helix 7. Furthermore, the amino acid substitutions were not random: of the six possible amino acid changes (H, F, R, V, I, and P) permitted by a

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single-base substitution of the CTC leucine codon in CHO β-tubulin, only the first three were recovered in the mutant cells.

In the studies described here, we used site-directed mutagenesis and transfection of a β1-tubulin cDNA to answer two fundamental questions. (1) Can amino acid substitutions other than H, F, or R at L215 produce paclitaxel resistance? (2) Do all substitutions produce paclitaxel resistance by a similar mechanism? The answers to these questions are important for understanding the structure−function relationship of the H6−H7 loop in microtubule assembly, for identifying constraints that determine which mutations are capable of conferring resistance to paclitaxel, and for gaining insights into the mechanism of drug action.

EXPERIMENTAL PROCEDURES

Isolation and Growth of Cell Lines. Strain CHO 10001 (*7*) is a subclone of strain CHO Pro^{-}5 (*8*) and served as the parental cells for all the cell lines used in this study. The cells were grown in an α modification of minimal essential medium (αMEM) containing 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 50 units/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C and 5% CO_2. For transfection experiments, CHO tTA 6.6a was isolated by transfecting CHO cells with a plasmid containing the cDNA for a tetracycline-regulated transactivator (*9*) and a puromycin resistance gene. A subclone of the transfected cells was identified for its ability to support tetracycline-regulated expression and maintained in αMEM containing 10 μg/mL puromycin and 1 μg/mL tetracycline (*6*).

Site-Directed Mutagenesis and Transfection. Wild-type CHO β1-tubulin cDNA (GenBank accession number U08342) (*10*), a class I isotype (*11*), was modified to express a nine-amino acid hemagglutinin (HA) tag at the C-terminus of the protein and was cloned into pTOP, a tetracycline-regulated mammalian expression vector containing a neomycin resistance gene (*6*). Site-directed mutagenesis of this HAβ1-tubulin cDNA was carried out using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing the full cDNA. Transfection into CHO tTA 6.6a cells was performed using Lipofectamine (Invitrogen, Carlsbad, CA) as described by the manufacturer, except that 1 μg/mL tetracycline was maintained throughout the procedure and subsequent growth of the cells to prevent expression of the transfected cDNA and thereby prevent any potential toxicity from the presence of mutant tubulin gene products. Geneticin (G418, Mediatech Inc., Herndon, VA) was used at 2 mg/mL to select stably transfected cell lines, and in some cases, these cells were further selected in 200 ng/mL paclitaxel with no tetracycline.

Electrophoretic Techniques. Samples were dissolved in SDS gel buffer (*12*), and proteins were resolved on 7.5% polyacrylamide gels using a minigel apparatus (Bio-Rad, Hercules, CA). Electrophoretic transfer of proteins onto nitrocellulose paper (Schleicher and Schuell, Keene, NH) was carried out as previously described (*13*), and the transferred proteins were immunodetected using mouse monoclonal antibodies to α-tubulin (DM1A, 1:2000 dilution, Sigma-Aldrich), β-tubulin (Tub 2.1, 1:2000 dilution, Sigma-Aldrich), or actin (C4, 1:5000 dilution, Chemicon International Inc., Temecula, CA), followed by a peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution, Sigma-Aldrich). Immunoreactive bands were detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposure to X-OMAT film (Eastman Kodak, Rochester, NY).

Immunofluorescence. Cells growing on glass coverslips were rinsed in PBS, lysed for 1 min at 4 °C in microtubule buffer (MTB) [20 mM Tris-HCl (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, and 0.5% Nonidet P-40] containing 4 μg/mL paclitaxel, and fixed in methanol for at least 10 min at −20 °C. The fixed cells were rehydrated in PBS and incubated in a 1:50 dilution of mouse monoclonal antibody 12CA5 (Roche Diagnostics Corp., Indianapolis, IN) specific for the HA tag, followed by a 1:20 dilution of Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). After being washed in PBS, the cells were inverted over a 5 μL drop of Gel/Mount (BioMeda Corp., Foster City, CA) and viewed by epifluorescence with an Optiphot microscope (Nikon, Inc., Melville, NY) equipped with a 60×, 1.4 n.a. oil objective.

Microtubule Assembly. The fraction of total tubulin assembled into microtubules was determined as previously described (*5*, *14*). Briefly, cells were grown to near confluence, lysed in MTB containing 0.14 M NaCl and 4 μg/mL paclitaxel (Sigma-Aldrich), and centrifuged at 12000*g* to separate polymerized from free, soluble tubulin. An equal volume of bacterial lysate containing glutathione *S*-transferase (GST)−α-tubulin fusion protein was added to each fraction to control for losses at all subsequent steps, and equivalent volumes of each fraction were resolved on SDS−polyacrylamide gels. Western blots of the gels were stained with antibody DM1A for α-tubulin followed by Cy5-conjugated goat anti-mouse IgG (Chemicon). Fluorescence emission was captured with a Storm imager (Molecular Dynamics Inc., Sunnyvale, CA) and quantified using NIH Image J (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The fraction of total tubulin appearing in microtubules was calculated by first normalizing the amount of tubulin in supernatant and pellet fractions to the amount of the GST−α-tubulin fusion protein in each fraction, and dividing the resultant value from the pellet by the sum of the values from the pellet and supernatant. The quotient was multiplied by 100 to convert it to a percentage.

Drug Resistance. Dose−response curves for each of the cell lines to various antimitotic drugs were determined by plating an equal number of cells (typically 100−200) into replicate wells of a 24-well dish containing increasing concentrations of a drug and allowing the cells to grow until they formed macroscopic colonies (7 days). The cells were then stained with 0.5% methylene blue in water as previously described (*7*), gently rinsed in deionized water to remove excess stain, dried, and photographed. To quantify the results, the stain from each well was eluted with 200 μL of 1% SDS in 20 mM Tris-HCl (pH 6.8), and 100 μL of the eluate was transferred to individual wells of a 96-well dish. The absorbance at 630 nm was read using an Emax microplate

[1] Abbreviations: CHO, Chinese hamster ovary; Cmd, colcemid; EpoA, epothilone A; HA, hemagglutinin; IC_{50}, concentration of drug that inhibits growth by 50%; MEM, minimal essential medium; MTB, microtubule buffer; PBS, phosphate-buffered saline; Ptx, paclitaxel; Tax, Taxol; tet, tetracycline.

Table 1: Summary of β-Tubulin Mutations in Paclitaxel Resistant Mutants

cell line	nucleotide change[a]	amino acid substitution
Tax 4−5[b]	CTC → **T**TC	L215F
Tax 10−5[b]	CTC → **T**TC	L215F
Tax 18	CTC → **T**T**T**	L215F
Tax 6−21	CTC → **T**TC	L215F
Tax 1−4	CTC → C**A**C	L215H
Tax 2−4	CTC → C**A**C	L215H
Tax 4−9	CTC → C**A**C	L215H
Tax 1−19	CTC → C**G**C	L215R
Tax 6−9	CTC → C**G**C	L217R
Tax 2−5	CTC → C**A**C	L228H
Tax 11−3	CTC → **T**TC	L228F

[a] Base changes are shown in bold. [b] Not previously reported.

reader (Molecular Dynamics), and values at each drug concentration were expressed as a percentage of the value for the same cell line without drug. These relative values were then plotted using pro Fit (QuantumSoft, Uetikon am See, Switzerland), and IC_{50} values were calculated as the concentration of drug that inhibited growth of the cells by 50%.

RESULTS

Most Amino Acid Substitutions at L215 Confer Paclitaxel Resistance. In our previous genetic selections for paclitaxel resistance, leucine 215 was the most frequently altered amino acid, and recently, we identified two further mutants with a lesion at this same site (see Table 1). Because only three unique amino acid substitutions were found among 11 resistant cell lines, we asked whether there are constraints on which amino acids at this position can confer resistance. To address this question, HAβ1-tubulin cDNA was altered by site-directed mutagenesis to encode eight different amino acids (A, E, F, H, I, M, P, and V) at position 215, and each mutagenized cDNA was transfected into a wild-type CHO strain using a tetracycline-regulated expression system (*6*). Stably transfected cell populations were first selected in medium containing 2 mg/mL G418 and 1 μg/mL tetracycline to repress transcription of the potentially toxic mutant tubulin cDNA. As judged by immunofluorescence, these cell populations were typically 40−60% positive for expression of the transgene when tetracycline was removed (e.g., see Figure 1). Next the G418 resistant populations were assayed for their ability to form colonies in αMEM (to check toxicity resulting from mutant tubulin expression), αMEM with 1 μg/mL tetracycline (control), or αMEM with 200 ng/mL paclitaxel (to check drug resistance). The concentration of paclitaxel used in this experiment reduces the cloning efficiency of wild-type CHO cells by 4−6 orders of magnitude and is the same that we have previously used in our genetic selections for paclitaxel resistance.

The results summarized in Table 2 demonstrate that all the mutations, with the exception of the conserved L215V and L215I substitutions, gave significant numbers of paclitaxel resistant colonies. Although L215M gave a much lower frequency of resistant colonies compared to the other amino acid substitutions, a repeat experiment using five times as many cells gave a clear indication of resistance. L215I and L215V, on the other hand, did not give resistant colonies after repeated transfections, indicating that these two amino

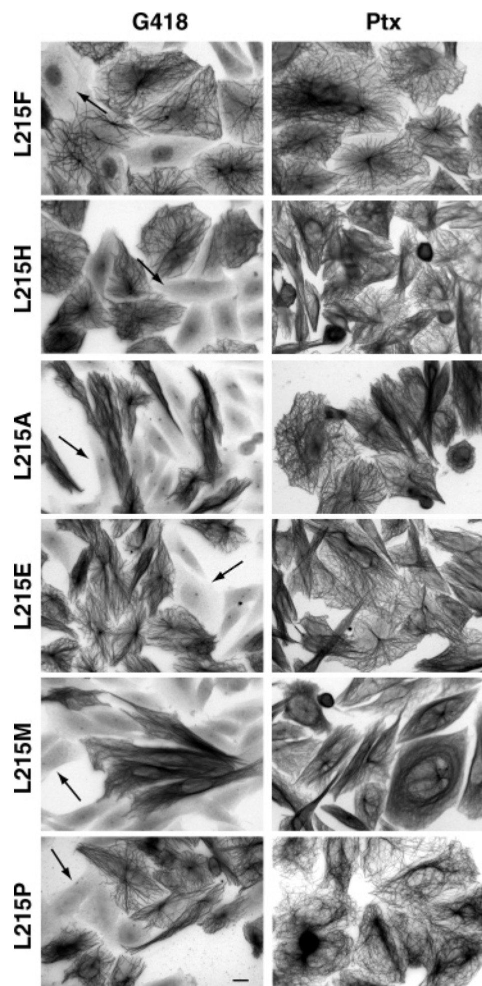

FIGURE 1: Immunofluorescence of cells transfected with mutant β-tubulin cDNAs. CHO tTA 6.6a cells were transfected with a CHO HAβ1-tubulin cDNA encoding each of the indicated L215 amino acid substitutions and a C-terminal HA epitope tag. The transfected cells were selected in G418 or paclitaxel (Ptx), and the resistant cells were stained with an antibody to the HA tag. Note that cells selected in G418 contain many negative cells (arrows) but that cells selected in paclitaxel are all positive for expression of the mutant tubulin. The images shown are all inverted from the original fluorescence image to provide better contrast. The bar is 10 μm.

Table 2: Site-Directed Mutagenesis of β-Tubulin L215

		colonies in α-MEM with		
mutation	IMF[a]	no addition	1 μg/mL tet	200 ng/mL Ptx
L215H	100%	97	204	20
L215F	100%	142	410	36
L215I	NA[b]	1100	1025	0
L215V	NA[b]	554	540	0
L215P	100%	257	403	49
L215A	100%	165	186	15
L215M	100%	387	393	1 (6)[c]
L215E	100%	183	258	14

[a] The percentage of cells surviving a paclitaxel resistance selection that stained positive for transfected tubulin by immunofluorescence (IMF). [b] Not applicable. No cells survived the paclitaxel selection. [c] Number of colonies obtained in a second selection using 5 times as many cells.

acid substitutions do not confer resistance to paclitaxel even in a forced expression system. One surprise from these experiments was the observation that the L215P substitution was able to confer resistance to paclitaxel even though it

was not recovered in our previous genetic selections. An explanation for this result came from further characterization of this mutation described in the sections that follow. Finally, it should be noted that in some instances (L215H, L215F, L215P, and L215E) significantly fewer colonies were obtained when tetracycline was omitted from the medium, suggesting that under nonselective conditions (no paclitaxel), expression of the mutant tubulin was toxic to a subset of the cells, presumably those with the highest levels of expression.

Paclitaxel Provides a Stringent Selection for Cells that Express Mutant Tubulin. To determine whether expression of the mutant tubulins has visible effects on the microtubule network and to further document whether the expression of the mutant tubulin is required for paclitaxel resistance, we examined G418 resistant and paclitaxel resistant cell populations by immunofluorescence microscopy using an antibody specific to the HA tag. Our prior experience indicated that G418 resistant cell populations are typically only ~50% positive for expression of the transgene on a cell-to-cell basis (*6*, *15*). In contrast, if expression of the mutant β-tubulin is required for paclitaxel resistance, we predicted that the paclitaxel resistant population would be essentially 100% positive for expression. The results of this experiment are shown in Figure 1. It is important to note that all the mutant β-tubulins were able to assemble into microtubules without causing gross distortions of microtubule organization. As expected, only ~50% of the cells in the G418 resistant cell population (Figure 1, left column) were positive for the transfected mutant tubulin. In the paclitaxel resistant population on the other hand, 100% of the cells expressed the mutant tubulin (Figure 1, right column). The observation that paclitaxel is a more stringent selection than G418 for cells that express the transfected tubulin cDNAs supports the results summarized in Table 2 and provides additional evidence that expression of the mutant tubulin genes is responsible for the paclitaxel resistance phenotype. The L215I and L215V mutations could not be analyzed in this way because they failed to give any paclitaxel resistant colonies. Although all the mutant tubulins were able to assemble into the microtubule cytoskeleton, immunofluorescence microscopy left us with the impression that some mutant tubulins (e.g., L215H, L215E, L215F, and L215P) produced a decrease in the density of the microtubule network. The mutant tubulins that produced this apparent decrease were the same that reduced the cloning efficiency of transfected cells when grown in the absence of tetracycline (Table 2).

Paclitaxel Resistant Cells Have Reduced Levels of Microtubule Assembly. We previously reported that paclitaxel resistant cells have an attenuated ability to form microtubules compared to wild-type cells or to cells transfected with wild-type β-tubulin cDNA (*5*, *6*, *16*, *17*). To determine whether cell lines expressing HAβ1-tubulin with various substitutions at L215 also have attenuated microtubule assembly, we separated cellular microtubules from free, soluble tubulin by centrifugation and measured the relative amount of tubulin in pellet and soluble fractions. The results summarized in Figure 2 demonstrate that cells transfected with wild-type HAβ1-tubulin cDNA have approximately 39% of their tubulin polymerized into microtubules, a value that is identical to what we have previously measured in numerous experiments with wild-type CHO or HAβ1-transfected cells

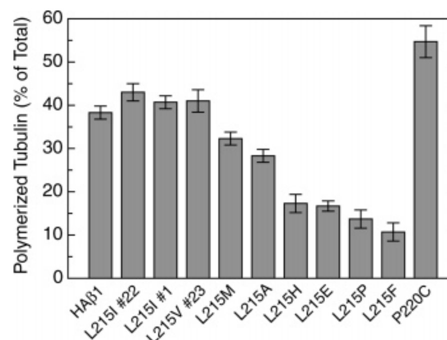

FIGURE 2: Tubulin assembly in cells transfected with mutant β-tubulins. Cells transfected with each of the indicated β-tubulin cDNAs were selected in paclitaxel or isolated as single G418 resistant clones (HAβ1, L215I#22, L215I#1, and L215V#23), grown overnight in the absence of any drugs, lysed in a microtubule stabilizing buffer, and centrifuged to separate soluble from polymerized tubulin. Each fraction was run on an SDS gel, transferred to nitrocellulose, probed with an antibody to α-tubulin, and quantified. The results show the percentage of the total tubulin that appeared in the polymerized (pellet) fraction. Standard deviations from the mean were calculated from at least three independent experiments.

(*5*, *6*, *16*, *17*). In contrast, cell lines expressing HAβ1 with a mutation at L215 frequently have a reduced fraction of polymerized tubulin, consistent with our previous measurements in paclitaxel resistant CHO cells. The only exceptions were the cell lines expressing HAβ1-tubulin with an L215I or L215V mutation, each of which failed to confer paclitaxel resistance. It thus appears that most substitutions at L215 impair microtubule assembly and confer paclitaxel resistance, but the two conserved L215I and L215V substitutions do not. As a control, we also examined cells transfected with a cDNA containing a P220C mutation that confers resistance to colcemid and increased sensitivity to paclitaxel (S. Yin and F. Cabral, unpublished studies). Unlike the mutations that cause paclitaxel resistance, the P220C mutation causes increased rather than decreased levels of microtubule assembly when expressed in CHO cells.

Mutant Tubulins Differ in Their Ability To Inhibit Microtubule Assembly. To determine the relative potencies of the mutations in disrupting microtubule assembly, we measured how much mutant HAβ1-tubulin was produced in each of the paclitaxel resistant cell populations. Because all the cell lines were selected in the same concentration of drug, one might predict that the amount of mutant tubulin should inversely correlate with the ability of the amino acid substitution to disrupt microtubule assembly; i.e., weakly disruptive tubulins would need to be expressed at higher levels than strongly disruptive tubulins to achieve the same resistance to paclitaxel. While this might be true to a first approximation, other factors such as the overall ability of the transfected gene to be expressed could also affect the results. Nevertheless, there did appear to be some notable differences in the ability of the mutant tubulins to disrupt microtubule assembly and produce paclitaxel resistance (Figure 3). For example, L215E, L215F, and L215H caused considerable microtubule disruption at moderate levels of expression, but L215M and L215A caused less disruption at similar or higher levels of expression (compare Figures 2 and 3). Of particular interest, L215P conferred paclitaxel resistance at very low levels of expression, and the gel had to be overexposed to detect the mutant HAβ1-tubulin. This

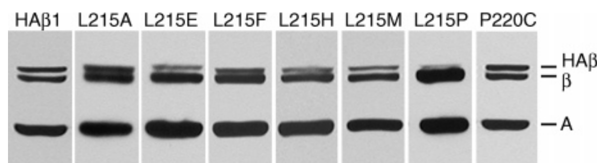

FIGURE 3: Production of HAβ-tubulin in transfected cells. Stable transfected cell lines were selected in 200 nM paclitaxel (for L215 mutants) or in G418 (for HAβ1 control), and proteins were resolved on SDS gels. Western blots probed with an antibody to β-tubulin and with an antibody to actin (A) are shown. Note that some of the samples were processed at different times; therefore, the ratio of tubulin to actin may vary between experiments because of differences in the dilution and activities of the two antibodies, but the ratio of transfected (HAβ) to endogenous (β) tubulin is the same from one experiment to the next. Also note that the blot for L215P had to be overexposed to detect the mutant HAβ-tubulin which was not visible at shorter exposures. P220C represents a colcemid resistant cell line used as a control in several experiments.

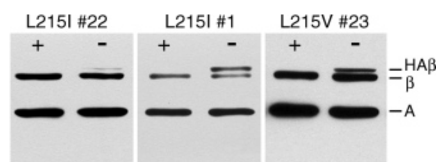

FIGURE 4: Production of transfected tubulin in G418 resistant clones. CHO tTA 6.6a cells were transfected with an HAβ1-tubulin cDNA encoding each of the indicated amino acid substitutions, and stable G418 resistant clones were isolated. The cells were grown in the presence (+) or absence (−) of tetracycline, and proteins were resolved on SDS gels. Western blots were probed with antibodies to tubulin (β and HAβ) and actin (A). Note that the transfected tubulin is only produced when tetracycline is absent from the growth medium.

outcome may explain why an L215P mutation was never recovered in our normal genetic selections for paclitaxel resistance; i.e., CHO cells have a fixed stoichiometry of β-tubulin subunits such that a mutant allele of β1-tubulin would produce ~35% of the total β-tubulin in the cell (*18*, *19*). It is likely that at that level of expression, an L215P mutation would so severely disrupt microtubule assembly that the cells would be unable to survive even in the presence of paclitaxel.

The L215I Mutation Confers Increased Sensitivity to Paclitaxel. Our inability to isolate paclitaxel resistant cell lines expressing L215V and L215I could be due to an inability of those amino acid substitutions to produce resistance, or to inadequate expression of the mutant tubulin. It was further possible that expression could cause resistance, but only to concentrations of paclitaxel below the one used in the initial selection. To distinguish among these possibilities, stably transfected cell lines were isolated by selecting colonies resistant to G418. Clones that were positive for expression of the mutant tubulin by immunofluorescence were then further examined for their level of mutant tubulin production by Western blot analysis, and three of those clones are shown in Figure 4. One clone, L215I#22, had a very low level of expression, while a second clone, L215I#1, had a high level of expression of the transfected β-tubulin. A third clone, L215V#23, had an intermediate level of expression. Production of the mutant tubulin in all three clones could be repressed by growing the cells in the presence of tetracycline.

To determine whether these cell lines have any resistance to paclitaxel, a growth assay in the presence of increasing concentrations of the drug was carried out. The results, summarized in Figure 5 and Table 3, showed that rather than conferring resistance to paclitaxel, production of mutant tubulin containing the L215I substitution (clone L215I#22) made cells more sensitive to the drug. Mutant tubulin containing an L215V substitution (clone L215V#23), on the other hand, produced little or no enhanced sensitivity or resistance to paclitaxel.

Previously, we demonstrated that most cell lines selected for resistance to microtubule disruptive drugs such as colcemid exhibit increased sensitivity to paclitaxel and other drugs that promote microtubule assembly, whereas cells selected for resistance to paclitaxel exhibited enhanced sensitivity to colcemid and other drugs that disrupt microtubule assembly (*3*, *4*). To highlight the differences between these previous mutants and the cells containing the L215I mutation, cells transfected with HAβ1-tubulin cDNA containing an L215H mutation, first identified in a mutant cell line selected for paclitaxel resistance (*6*), and cells transfected with HAβ1-tubulin containing the P220C mutation that confers colcemid resistance are included in Figure 5 for comparison. Cells transfected with HAβL215H are approximately 5-fold resistant to paclitaxel, 4-fold resistant to epothilone A, another drug that binds to the paclitaxel binding site and promotes microtubule assembly (*20*, *21*), and 2-fold more sensitive to colcemid than cells transfected with HAβ1-tubulin cDNA containing no mutations. Conversely, cells transfected with HAβP220C are 2.5-fold more resistant to colcemid, 2-fold more sensitive to paclitaxel, and 4-fold more sensitive to epothilone A. These results are consistent with a model for drug resistance based on changes in microtubule stability and are supported by the observation that cells expressing HAβL215H have an attenuated assembly of microtubules, but cells expressing HAβP220C have enhanced microtubule assembly (Figure 2). In contrast to these results, cells that express HAβL215V have normal sensitivity to all three drugs. Cells expressing HAβL215I, on the other hand, are 4-fold more sensitive to paclitaxel, but retain normal sensitivity to epothilone A and colcemid. Also in contrast to the other cell lines, cells that express HAβL215V or HAβL215I have near-normal levels of polymerized tubulin (Figure 2). Thus, L215V appears to be a phenotypically silent alteration in these assays, but L215I produces a phenotype that is distinct from any of the paclitaxel resistant mutants we have isolated to date.

HAβL215I-Tubulin Acts Substoichiometrically. To determine whether enhanced sensitivity to paclitaxel is influenced by the level of HAβL215I expression, two stable clones, L215I#1 and L215I#22, were compared for their sensitivity to paclitaxel (Figure 6). Both cell lines had near-wild-type sensitivity to paclitaxel when grown in the presence of tetracycline to inhibit transcription of the transfected cDNA, but had similar levels of enhanced sensitivity to paclitaxel when grown without tetracycline, a condition under which they produce vastly different amounts of mutant tubulin (see Figure 4). Thus, the ability of the L215I mutation to enhance sensitivity to paclitaxel is not strongly influenced by the amount of mutant β-tubulin produced in the cell.

This result contrasts sharply with mutant tubulin subunits that confer resistance to paclitaxel by altering the stability of microtubules. Figure 7 shows the result of an experiment in which a G418 resistant cell population from cells

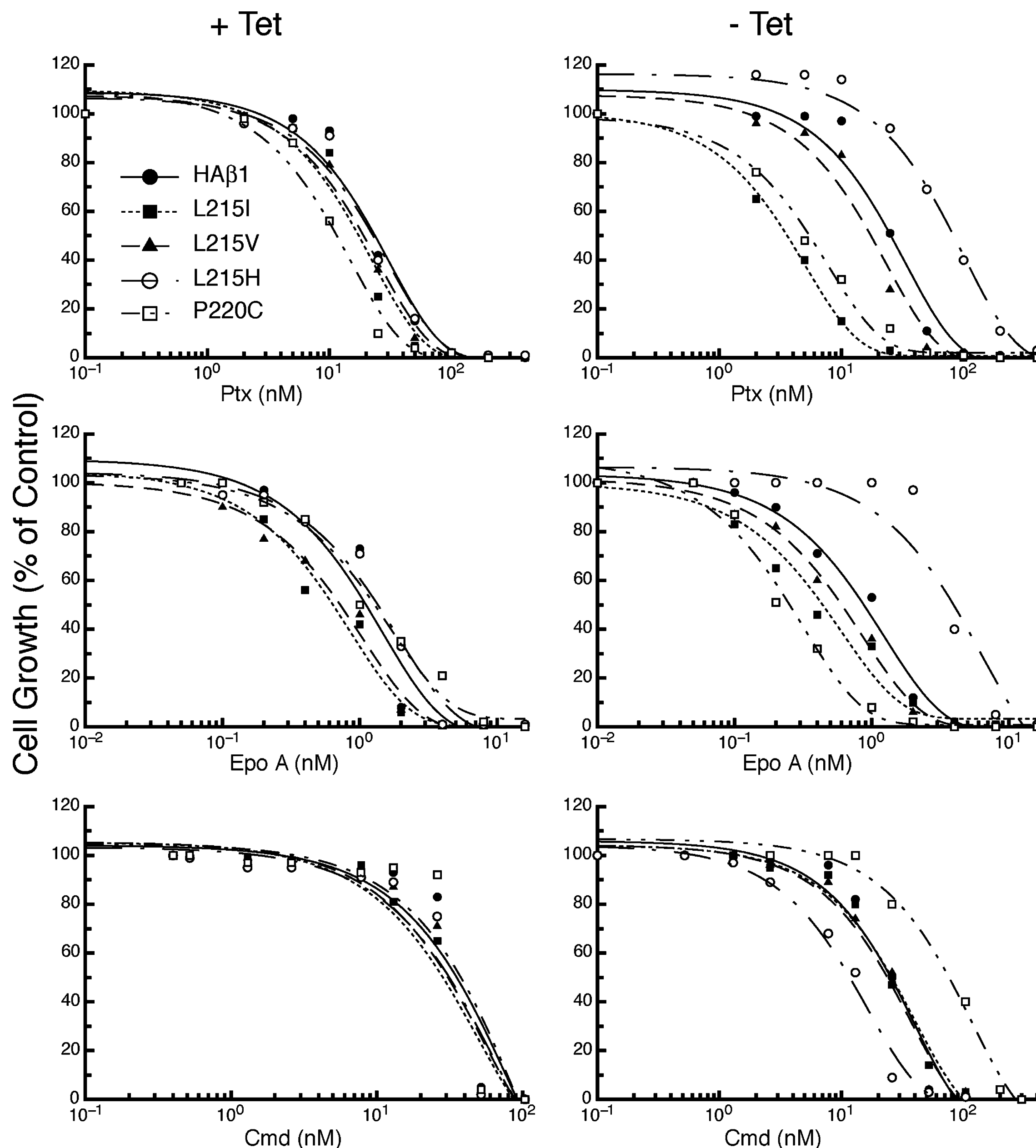

FIGURE 5: Sensitivity of transfected cells to antimitotic drugs. Stable cell populations transfected with wild-type (HAβ1) and mutant HAβ-tubulin cDNAs were replated in increasing concentrations of each of the indicated drugs in the presence or absence of tetracycline, and their growth was measured after 7 days. The data (average of two to four experiments) are plotted as the growth at a given concentration of drug relative to the growth of the same cells without drug set at 100%. The data points were fitted using a single exponential: (●) HAβ1, (■) L215I#22, (▲) L215V#23, (○) L215H, and (□) P220C. The relative resistance for each cell line compared to that of HAβ1 transfected cells is summarized in Table 3.

transfected with HAβ1-tubulin containing an L215F mutation was reselected in varying concentrations of paclitaxel. As the resistance level of the cells increased, there was a corresponding increase in the level of expression of the mutant tubulin, a result that is consistent with a model in which the incorporation of mutant tubulin weakens subunit–subunit interactions and thereby destabilizes the microtubule lattice.

DISCUSSION

The replacement of L215 of β-tubulin with amino acids varying in size, charge, and hydrophobicity produced two distinct phenotypes. The most prominent phenotype was similar to that of the mutants we and others have previously isolated (*22*–*29*); i.e., there was a decreased level of microtubule assembly, cross resistance to epothilone A, and increased sensitivity to colcemid. Also in common with previously isolated mutants, cells that had large decreases in the level of microtubule assembly experienced a mitotic delay that was associated with a dysfunctional mitotic spindle and missegregated chromosomes. These changes caused a failure of cytokinesis and led to the accumulation of large, multinucleated cells as we have reported for other CHO tubulin mutants (*24*, *27*, *29*). Smaller and larger amino acids, hydrophobic and polar amino acids, and basic and acidic amino acids all produced similar biochemical, pharmacological, and morphological changes and therefore appeared to act through a common mechanism. All the amino acid

Table 3: Effect of Mutant Tubulin Expression on Drug Sensitivity[a]

cell line	Ptx	EpoA	Cmd
HAβ1	1	1	1
L215I#22	−4.2	−1.1	−1.1
L215V#23	1	−1.1	−1.1
L215H	4.9	3.9	−2.2
P220C	−2.3	−4.2	2.5

[a] IC_{50} values for each cell line were calculated from two to four experiments in the presence and absence of tetracycline and then divided by the corresponding IC_{50} for HAβ1. These normalized values were then computed to give the fold resistance [IC_{50}(−tet)/IC_{50}(+tet)] or the fold sensitivity [IC_{50}(+tet)/IC_{50}(−tet)] for a cell line and compared to the same calculation for HAβ1 set to 1. Increased sensitivity is indicated by negative numbers. Fold increases or decreases deviating from 1 by less than 20% are considered insignificant on the basis of the standard deviations for the IC_{50} values (range of 7−23% of the average values).

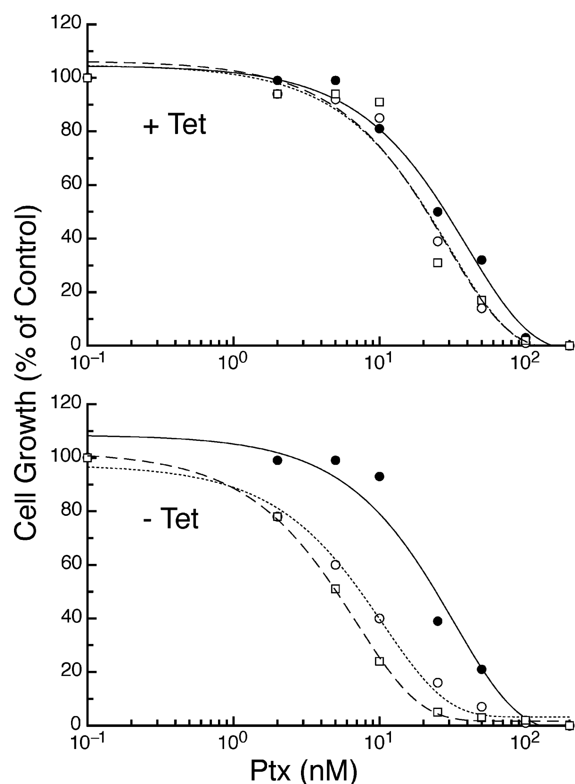

FIGURE 6: Effect of expression of the L215I mutation on paclitaxel sensitivity. Stable cell lines expressing small (L215I#22) or large (L215I#1) amounts of mutant HAβ-tubulin were compared for their sensitivity to paclitaxel as described in the legend of Figure 5: (●) HAβ1 control, (○) L215I#1, and (□) L215I#22. Note that both mutant cell lines have similar sensitivity to paclitaxel despite large differences in mutant gene expression (see Figure 4).

substitutions, however, were not equally effective in producing these effects. For example, the L215P substitution was able to significantly disrupt microtubule assembly and confer resistance to paclitaxel when produced at extremely low levels. The especially potent effects of the L215P mutation may explain why it was never isolated in genetic selections of paclitaxel resistant CHO cells where β-tubulin subunits are produced at fixed and relatively high stoichiometries (*19*). The qualitatively similar disruption of microtubule assembly by substitution of diverse amino acids at L215 suggests that this residue may be more involved in determining the conformation of the H6−H7 loop than in a specific amino acid contact required for microtubule assembly.

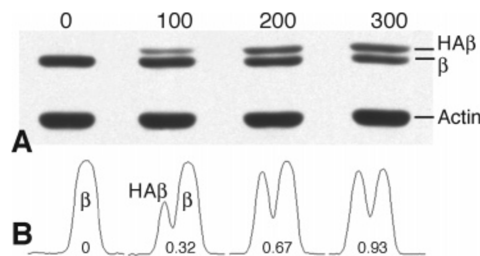

FIGURE 7: Production of mutant HAβ-tubulin in cells selected in varying concentrations of paclitaxel. Cells transfected with HAβ1-tubulin containing an L215F mutation were selected in the indicated nanomolar concentrations of paclitaxel. The resistant cells were then lysed in SDS and the proteins resolved on a polyacrylamide gel. A Western blot of the gel was probed with antibodies to tubulin and actin. HAβ represents transfected mutant β-tubulin and β endogenous β-tubulin. Panel B shows a scan of the tubulin bands, and the numbers indicate the ratio of HAβ to β. Note that an increase in the extent of mutant HAβ-tubulin production accompanies the increase in paclitaxel resistance.

A second distinct phenotype was produced by the conserved L215I substitution. In this case, there was little or no change in microtubule assembly, yet the cells exhibited a 4-fold increase in sensitivity to paclitaxel. Thus, L215I does not act by the same mechanism as the other amino acid substitutions. In addition to not causing a decrease in the level of microtubule assembly, L215I produced little, if any, enhanced sensitivity to epothilone A, a drug that binds to the same pocket and shares a similar mechanism of action with paclitaxel. Also in contrast to the other substitutions, it produced no change in sensitivity to colcemid, a drug that binds to a distant site (*30*). Finally, the L215I substitution was able to enhance sensitivity to paclitaxel at a very low stoichiometry to endogenous tubulin, and the sensitivity was not augmented at higher expression levels. On the other hand, all of the other substitutions (except L215V) produced an increase in paclitaxel resistance that was proportional to the level of expression of the mutant protein.

We can only speculate about the mechanism by which substitution of an isoleucine for L215 increases sensitivity to paclitaxel. One attractive possibility is that the change increases tubulin's affinity for the drug. Such a mechanism is consistent with the fact that L215 is within 4 Å of the C2 acetobenzoyl group of paclitaxel (Figure 8A). In comparison, the distance between L215 and the C3 hydroxyl in epothilone A is 3 Å (Figure 8B). Substitution with an isoleucine does not affect the epothilone sensitivity perhaps because it is not possible to improve on what is already a very close contact. In the case of paclitaxel, however, the side chain geometry of isoleucine 215 could potentially lead to a closer interaction with the C2 benzoate, producing a higher affinity and/or a more favorable docking of the drug onto β-tubulin. Alternatively, the mutation might mediate a more assembly competent tubulin conformation upon paclitaxel binding, thereby enhancing the effectiveness of the drug. Any such change in conformation, however, would have to specifically affect the action of paclitaxel.

If L215I tubulin binds paclitaxel with higher affinity, this subunit becomes a marker for the site of action of the drug. It is well established that paclitaxel binds preferentially to microtubule polymers in a 1:1 stoichiometry with the assembled tubulin heterodimers (*31*). The presence of a small number of subunits with increased affinity for the drug would

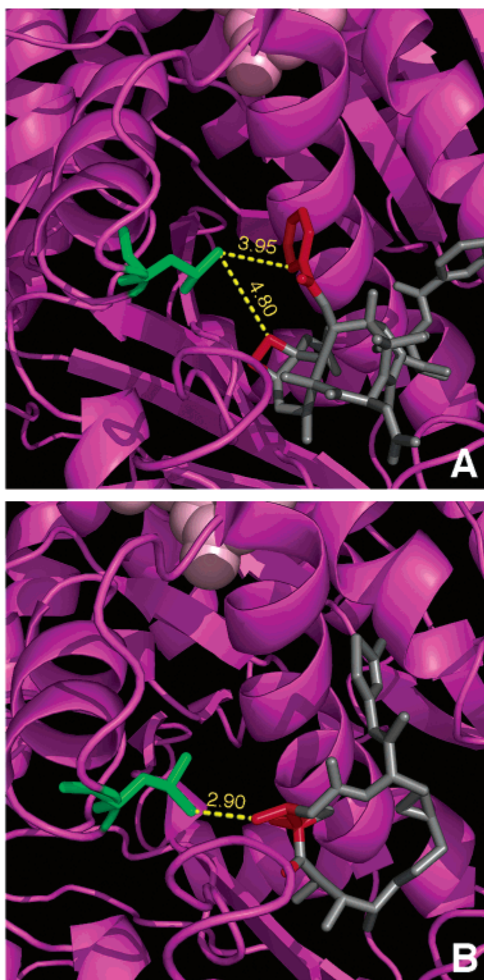

FIGURE 8: Paclitaxel binding pocket of β-tubulin. Structural models were drawn using the atomic coordinates for tubulin with paclitaxel (A) [PDB entry 1JFF (*37*)] and tubulin with epothilone A (B) [PDB entry 1TVK (*20*)]. MacPyMOL (www.pymol.org) was used to find drug atoms (colored red) within 5 Å of L215 (colored green) and to calculate the distances shown in the figure. Pink spheres represent a portion of the GDP bound to the β-tubulin subunit.

therefore mark the areas of the microtubule to which paclitaxel first binds. We estimate that mutant tubulin in cell line L215I#22 accounts for less than 5% of the total tubulin (see Figure 4), an amount that would provide less than one subunit per turn of the microtubule helix. Because this small amount of L215I tubulin produces a maximal increase in drug sensitivity, we infer that paclitaxel must act in a substoichiometric manner, a conclusion that is supported by in vitro studies of the effects of paclitaxel on microtubule dynamics (*32*). Our immunofluorescence data further indicate that L215I tubulin does not assemble into any specialized areas; rather, it appears to be evenly distributed along the microtubule (data not shown). Thus, paclitaxel must stabilize microtubules by affecting relatively few, dispersed lateral and/or longitudinal contacts. We speculate that one way this could potentially happen is that microtubules might depolymerize until at least one paclitaxel-bound mutant tubulin subunit becomes exposed and stabilizes the end. Because 13 protofilaments make up the wall of most mammalian microtubules, microtubule lengths would not significantly decrease before such a subunit was exposed, even if the level of mutant expression were lower than the 5% of total tubulin seen in L215I#22.

Given that L215I can strengthen the binding or enhance the effectiveness of paclitaxel, one might predict that other amino acid changes at that position should also affect the binding or effectiveness of the drug. In fact, even though most amino acid substitutions at L215 produce a decrease in the level of microtubule assembly, they could additionally be causing reduced paclitaxel binding affinity that produces no phenotype. As we have previously argued, weakened binding is a loss of function that would result in a recessive phenotype (*3*). Strengthened binding or increased drug sensitivity, on the other hand, is a gain of function that results in a dominant phenotype. The ability of L215I to enhance the sensitivity to paclitaxel at a low stoichiometry thus explains why a reduced level of drug binding would fail to produce a phenotype in diploid cells; i.e., there would still be abundant wild-type subunits present to bind the drug with normal affinity and produce a dominant phenotype (in this case normal sensitivity to paclitaxel).

The high frequency of L215 mutations found in paclitaxel resistant CHO cells suggests that the H6−H7 loop is important in microtubule assembly and in the mechanism of action of paclitaxel. Structural models place the loop facing the lumen of the microtubule where it is situated to form interdimer contacts along each protofilament as well as lateral interactions between protofilaments (*33*). Its location near fenestrations in the microtubule wall has also led to the suggestion that it could play a role in controlling the accessibility of paclitaxel to its binding site in the microtubule lumen (*34*). This idea, however, would not explain the changes in microtubule assembly or the changes in colcemid sensitivity that we find associated with most of the mutations in this area. A recent comparison of tubulin structures derived from assembled ("straight" conformation) tubulin versus nonassembled ("curved" conformation) tubulin found significant changes in the positions of the H6−H7 loop and helix 7 that appear to affect longitudinal contacts between heterodimers in a protofilament (*30*). Thus, mutations in the H6−H7 loop could be affecting specific subunit contacts involved in microtubule assembly; perhaps more likely, they could be altering the ability of tubulin heterodimers to assume an assembly competent conformation.

An alignment of the residues comprising the H6−H7 loop indicates that L215 is absolutely conserved in β-tubulin from all species in which it has been sequenced, and the conservation extends even to the other tubulin family members, α- and γ-tubulin. The very high degree of conservation at this position reinforces the data demonstrating its importance in the structure and assembly of tubulin, and is consistent with the observation that most substitutions at L215 perturb microtubule assembly and produce paclitaxel resistance. The absence of species with isoleucine and valine at this position suggests that even though these amino acid substitutions fail to perturb microtubule assembly sufficiently to produce paclitaxel resistance, they may cause more subtle changes that are deleterious to living organisms. In support of this notion, we have observed that high levels of expression of L215I and L215V can slow the growth of transfected cells (unpublished data).

Given that some laboratories have reported mutations affecting other regions of β-tubulin in paclitaxel or epothilone resistant cells (*25*, *28*, *29*, *35*, *36*), one might ask why our mutations are so heavily concentrated in the H6−H7 loop.

One possibility is that the characteristics of CHO cells coupled with the magnitude of the selecting drug concentration produce favorable growth of cells with mutations in this region. We previously reported that CHO β-tubulin is composed of 70% β1, 25% β4, and 5% β5 (*19*). All the mutations we have uncovered affect β1-tubulin; thus, a mutant allele would alter approximately 35% of the total cellular tubulin, a value that could differ in other cell types. To be recovered in the drug resistance selection, a mutation would have to disrupt microtubule assembly enough to allow the mutant cells to survive in the elevated concentration of paclitaxel but not be so severe that the growth of the cells is compromised. Presumably, those conditions are met for H6−H7 loop mutations in our cells using our method for selection, but they may not be met for other cell types undergoing different selections. Additional factors could include the codon used for leucine in different cell types and the level of resistance achieved by the mutant cells. Whereas our mutants are selected for low resistance (2−5-fold), most other laboratories use multiple steps to select for much higher resistance (∼20-fold), and this could well influence the kinds of mutations that are recovered. It is unlikely that the high frequency of H6−H7 loop mutations in our resistant cell lines is due to a mutational hot spot because selections for resistance to other drugs map to other regions of tubulin (*7*, *14*). We propose instead that this region may be critical for the action of paclitaxel in stabilizing microtubule structure.

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REFERENCES

1. Mekhail, T. M., and Markman, M. (2002) Paclitaxel in cancer therapy, *Expert Opin. Pharmacother. 3*, 755−766.
2. Orr, G. A., Verdier-Pinard, P., McDavid, H., and Horwitz, S. B. (2003) Mechanisms of Taxol resistance related to microtubules, *Oncogene 22*, 7280−7295.
3. Cabral, F. (2000) Factors determining cellular mechanisms of resistance to antimitotic drugs, *Drug Resistance Updates 3*, 1−6.
4. Cabral, F., Brady, R. C., and Schibler, M. J. (1986) A mechanism of cellular resistance to drugs that interfere with microtubule assembly, *Ann. N.Y. Acad. Sci. 466*, 745−756.
5. Minotti, A. M., Barlow, S. B., and Cabral, F. (1991) Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin, *J. Biol. Chem. 266*, 3987−3994.
6. Gonzalez-Garay, M. L., Chang, L., Blade, K., Menick, D. R., and Cabral, F. (1999) A β-tubulin leucine cluster involved in microtubule assembly and paclitaxel resistance, *J. Biol. Chem. 274*, 23875−23882.
7. Cabral, F., Sobel, M. E., and Gottesman, M. M. (1980) CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered β-tubulin, *Cell 20*, 29−36.
8. Stanley, P., Callibot, V., and Siminovitch, L. (1975) Selection and characterization of eight phenotypically distinct lines of lectin-resistant Chinese hamster ovary cells, *Cell 6*, 121−128.
9. Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters, *Proc. Natl. Acad. Sci. U.S.A. 89*, 5547−5551.
10. Boggs, B., and Cabral, F. (1987) Mutations affecting assembly and stability of tubulin: Evidence for a non-essential β-tubulin in CHO cells, *Mol. Cell. Biol. 7*, 2700−2707.
11. Lopata, M. A., and Cleveland, D. W. (1987) In vivo microtubules are copolymers of available β-tubulin isotypes: Localization of each of six vertebrate β-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens, *J. Cell Biol. 105*, 1707−1720.
12. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
13. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
14. Hari, M., Wang, Y., Veeraraghavan, S., and Cabral, F. (2003) Mutations in α- and β-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine, *Mol. Cancer Ther. 2*, 597−605.
15. Blade, K., Menick, D. R., and Cabral, F. (1999) Overexpression of class I, II, or IVb β-tubulin isotypes in CHO cells is insufficient to confer resistance to paclitaxel, *J. Cell Sci. 112*, 2213−2221.
16. Barlow, S. B., Gonzalez-Garay, M. L., and Cabral, F. (2002) Paclitaxel-dependent mutants have severely reduced microtubule assembly and reduced tubulin synthesis, *J. Cell Sci. 115*, 3469−3478.
17. Bhattacharya, R., and Cabral, F. (2004) A ubiquitous β-tubulin disrupts microtubule assembly and inhibits cell proliferation, *Mol. Biol. Cell 15*, 3123−3131.
18. Ahmad, S., Singh, B., and Gupta, R. S. (1991) Nucleotide sequences of three different isoforms of β-tubulin cDNA from Chinese hamster ovary cells, *Biochim. Biophys. Acta 1090*, 252−254.
19. Sawada, T., and Cabral, F. (1989) Expression and function of β-tubulin isotypes in Chinese hamster ovary cells, *J. Biol. Chem. 264*, 3013−3020.
20. Nettles, J. H., Li, H., Cornett, B., Krahn, J. M., Synder, J. P., and Downing, K. H. (2004) The binding mode of epothilone A on α,β-tubulin by electron crystallography, *Science 305*, 866−869.
21. Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E., and Woods, C. M. (1995) Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action, *Cancer Res. 55*, 2325−2333.
22. Cabral, F. (1983) Isolation of Chinese hamster ovary cell mutants requiring the continuous presence of taxol for cell division, *J. Cell Biol. 97*, 22−29.
23. Cabral, F., Abraham, I., and Gottesman, M. M. (1981) Isolation of a taxol-resistant Chinese hamster ovary cell mutant that has an alteration in α-tubulin, *Proc. Natl. Acad. Sci. U.S.A. 78*, 4388−4391.
24. Cabral, F., Wible, L., Brenner, S., and Brinkley, B. R. (1983) Taxol-requiring mutant of Chinese hamster ovary cells with impaired mitotic spindle assembly, *J. Cell Biol. 97*, 30−39.
25. He, L., Yang, C. H., and Horwitz, S. B. (2001) Mutations in β-tubulin map to domains involved in regulation of microtubule stability in epothilone-resistant cell lines, *Mol. Cancer Ther. 1*, 3−10.
26. Martello, L. A., Verdier-Pinard, P., Shen, H.-J., He, L., Torres, K., Orr, G. A., and Horwitz, S. B. (2003) Elevated levels of microtubule destabilizing factors in a taxol-resistant/dependent A549 cell line with an α-tubulin mutation, *Cancer Res. 63*, 1207−1213.
27. Schibler, M., and Cabral, F. (1986) Taxol-dependent mutants of Chinese hamster ovary cells with alterations in α- and β-tubulin, *J. Cell Biol. 102*, 1522−1531.
28. Verrills, N. M., Flemming, C. L., Liu, M., Ivery, M. T., Cobon, G. S., Norris, M. D., Haber, M., and Kavallaris, M. (2003) Microtubule alterations and mutations induced by desoxyepothilone B: Implications for drug-target interactions, *Chem. Biol. 10*, 597−607.
29. Wang, Y., Veeraraghavan, S., and Cabral, F. (2004) Intra-allelic suppression of a mutation that stabilizes microtubules and confers resistance to colcemid, *Biochemistry 43*, 8965−8973.
30. Ravelli, R. B. G., Gigant, B., Curmi, P. A., Jourdain, I., Lachkar, S., Sobel, A., and Knossow, M. (2004) Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain, *Nature 428*, 198−202.
31. Manfredi, J. J., Parness, J., and Horwitz, S. B. (1981) Taxol binds to cellular microtubules, *J. Cell Biol. 94*, 688−696.
32. Derry, W. B., Wilson, L., and Jordan, M. A. (1995) Substoichiometric binding of taxol suppresses microtubule dynamics, *Biochemistry 34*, 2203−2211.
33. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) High-resolution model of the microtubule, *Cell 96*, 79−88.
34. Diaz, J. F., Barasoain, I., and Andreu, J. M. (2003) Fast kinetics of taxol binding to microtubules, *J. Biol. Chem. 278*, 8407−8419.

35. Giannakakou, P., Sackett, D. L., Kang, Y.-K., Zhan, Z., Buters, J. T. M., Fojo, T., and Poruchynsky, M. S. (1997) Paclitaxel-resistant human ovarian cancer cells have mutant β-tubulins that exhibit impaired paclitaxel-driven polymerization, *J. Biol. Chem. 272*, 17118−17125.
36. Giannakakou, P., Gussio, R., Nogales, E., Downing, K. H., Zaharevitz, D., Bollbuck, B., Poy, G., Sackett, D., Nicolaou, K. C., and Fojo, T. (2000) A common pharmacophore for epothilone and taxanes: Molecular basis for drug resistance conferred by tubulin mutations in human cancer cells, *Proc. Natl. Acad. Sci. U.S.A. 97*, 2904−2909.
37. Lowe, J., Li, H., Downing, K. H., and Nogales, E. (2001) Refined structure of $\alpha\beta$-tubulin at 3.5 Å resolution, *J. Mol. Biol. 313*, 1045−1057.

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