Amino acid substitutions at proline 220 of β-tubulin confer resistance to paclitaxel and colcemid

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Abstract

Chinese hamster ovary cells selected for resistance to paclitaxel have a high incidence of mutations affecting L215, L217, and L228 in the H6/H7 loop region of β1-tubulin. To determine whether other mutations in this loop are also capable of conferring resistance to drugs that affect microtubule assembly, saturation mutagenesis of the highly conserved P220 codon in β1-tubulin cDNA was carried out. Transfection of a mixed pool of plasmids encoding all possible amino acid substitutions at P220 followed by selection in paclitaxel produced cell lines containing P220L and P220V substitutions. Similar selections in colcemid, on the other hand, yielded cell lines with P220C, P220S, and P220T substitutions. Site-directed mutagenesis and retransfection confirmed that these mutations were responsible for drug resistance. Expression of tubulin containing the P220L and P220V mutations reduced microtubule assembly, conferred resistance to paclitaxel and epothilone A, but increased sensitivity to colcemid and vinblastine. In contrast, tubulin with the P220C, P220S, and P220T mutations increased microtubule assembly, conferred resistance to colcemid and vinblastine, but increased sensitivity to paclitaxel and epothilone A. The results are consistent with molecular modeling studies and support a drug resistance mechanism based on changes in microtubule assembly that counteract the effects of drug treatment. These studies show for the first time that different substitutions at the same amino acid residue in β1-tubulin can confer cellular resistance to either microtubule-stabilizing or microtubule-destabilizing drugs. [Mol Cancer Ther 2007; 6(10):2798–806]

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Introduction

Antimitotic agents target the α-tubulin and β-tubulin building blocks of the microtubule cytoskeleton. Drug toxicity is primarily associated with inhibition of mitotic spindle function that causes cells to block in mitosis. Some cells that experience a prolonged mitotic block trigger their own death through an apoptotic pathway, but other cells are able to bypass the mitotic checkpoint and exit mitosis without completing cell division (1–3). Cells able to progress to G_1 continue through the cell cycle several times and become large polyploid cells that eventually die through other mechanisms (2, 4, 5).

Drugs that target microtubules fall into two main groups: one promotes tubulin assembly and stabilizes microtubules, whereas a second larger group inhibits tubulin assembly and destabilizes microtubules. Tubulin has three well-defined binding sites for these agents. Paclitaxel (Taxol), the prototype for compounds that stabilize microtubules (6), binds to the β subunit in the microtubule polymer (7), inhibits microtubule dynamics at substoichiometric concentrations (8), and blocks cells in mitosis (9). Other microtubule-stabilizing drugs also bind to the same or overlapping sites. Examples include Taxotere, an analogue of paclitaxel, epothilones A and B, and discodermolide (6). It has been proposed that this class of drugs stabilizes microtubules by strengthening the lateral interactions between protofilaments (10). Clinically, paclitaxel is a very effective agent for treating many malignancies, including breast and ovarian carcinomas, lung cancer, head and neck tumors, melanomas, and gastric carcinomas (11).

Colchicine, colcemid, and the majority of known antimitotic drugs bind to free tubulin and inhibit microtubule assembly (12). Crystallographic studies indicate that the drug binds to β-tubulin near the intradimer interface where it stabilizes a ''curved'' tubulin conformation that inhibits microtubule elongation and destabilizes the polymer (13). Drugs that bind to this site are effective in treating a variety of diseases; for example, colchicine is used to reduce the inflammation associated with gout, griseofulvin is effective against some fungal infections, mebendazole is used to treat helminthiasis, and several drugs are in clinical trials for treating cancer (14, 15).

Vinca alkaloids, such as vinblastine, vincristine, and vinorelbine, occupy a third binding site that is predominantly in the α-tubulin subunit and sits at the interface between adjacent heterodimers in the protofilament (16). Occupation of this site destabilizes microtubules by inducing a tubulin conformation that favors spiral curvature in the protofilaments (16, 17). These drugs are important in the treatment of childhood leukemia, adult lymphomas, testicular cancer, and other neoplastic diseases (15).

Although many of these antimitotic agents have found important uses in treating cancer, the problem of drug

resistance has limited their effectiveness. Characterization of cultured cells able to survive toxic concentrations of these and other antimitotic drugs has identified several potential mechanisms for drug resistance. These include P-glycoprotein–mediated multidrug resistance (MDR; ref. 18), changes in microtubule assembly that counteract the effects of the drugs (19), alterations in drug binding affinity (20, 21), and changes in the expression levels of particular β-tubulin isotypes (22).

Single-step selections for drug resistance in Chinese hamster ovary (CHO) cells showed that the frequency with which a particular mechanism is seen depends on the drug that is used. For colchicine and vinblastine, 80% of resistant cell lines had the MDR phenotype, whereas the remaining cells had alterations in tubulin (23). In contrast, paclitaxel-resistant cell lines predominantly had tubulin mutations (92%) and exhibited a low frequency of MDR (24). Analysis of hundreds of resistant cell lines failed to reveal any additional mechanisms, suggesting that MDR and alterations in tubulin assembly represent the most common mechanisms of resistance to antimitotic drugs in this cell culture model.

Among resistant cell lines with mutations in tubulin, alterations in L215, L217, and L228 of β-tubulin occur at very high frequency (25). These altered residues are located in a loop that connects helix 6 with helix 7 (the H6/H7 loop) as well as in helix 7 itself. Consistent with a resistance mechanism that involves changes in microtubule assembly, this region has been implicated in forming lateral interactions with adjacent protofilaments in the microtubule wall as well as longitudinal interactions between heterodimers along each protofilament (26). In addition, this region is close to the paclitaxel binding site (7) and has been shown to undergo a sizable shift in position when tubulin adopts its assembled conformation (13). Given the importance of this region in microtubule assembly and in drug action and resistance, we initiated a mutagenesis study to determine whether alteration of other amino acids in the H6/H7 loop could influence sensitivity to both paclitaxel and colcemid.

Materials and Methods

Cell Lines, Plasmids, and Mutagenesis

The CHO tTApuro 6.6a cells used for transfection express a tetracycline-regulated transactivator and were maintained as described previously (25). CHO *Cβ1* cDNA (Genbank accession no. U08342) was modified to encode a COOH-terminal hemagglutinin antigen (HA) epitope tag and cloned into a pTOPneo vector to create *pTOP/HAβ1* that allows tetracycline-regulated transcription of the *HAβ1* cDNA (25).

Saturation mutagenesis of residue P220 was carried out by designing a pair of complementary primers that contained all possible nucleotides at the proline codon. These were used along with the QuikChange Site-Directed Mutagenesis kit (Stratagene) to generate plasmids containing mutant HAβ1-tubulin cDNAs. After transformation and growth of the bacteria in liquid culture, the mixed plasmids were purified using a QIAprep 8 Miniprep kit (Qiagen). A small aliquot of the culture was also streaked onto plates to isolate single bacterial clones. Plasmid DNA from 10 of these clones was sequenced to ensure that random nucleotides were incorporated into codon 220.

Transfection and Selection of Drug-Resistant Cell Lines

Cells were transfected with the pool of mutant plasmids using LipofectAMINE (Invitrogen). Approximately 16 h later, the cells were divided into 2- to 100-mm dishes in medium with 200 nmol/L paclitaxel, 50 nmol/L colcemid, or 2.5 μg/mL verapamil plus 25 nmol/L colcemid. The last selection was based on the observation that colcemid resistance selections frequently result in the isolation of cells with the MDR phenotype and that those mutants can be eliminated by inhibiting the transporter responsible for MDR with verapamil (23). After 10 to 14 days, visible colonies were isolated and maintained under selective conditions.

Immunofluorescence

Cells on sterile glass coverslips were rinsed in PBS, preextracted for 2 min at 4°C with microtubule buffer [20 mmol/L Tris-HCl (pH 6.8), 1 mmol/L $MgCl_2$, 2 mmol/L EGTA, 0.5% NP40] containing 4 μg/mL paclitaxel, and fixed in methanol at −20°C for 20 min. They were then washed and incubated with mouse α-tubulin antibody DM1A (1:100; Sigma-Aldrich) and rabbit polyclonal HA antibody (1:100; Bethyl Laboratories) for 30 to 60 min at 37°C. After three rinses in PBS, the cells were stained with a 1:50 dilution of Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG that included 1 μg/mL 4′,6-diamidino-2-phenylindole (all from Invitrogen). Cells were viewed using an Optiphot microscope (Nikon) equipped with a MagnaFire digital camera (Optronics).

Sequencing Mutant β1-Tubulin

To isolate genomic DNA, cells were rinsed with PBS, lysed with K buffer [20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.5% Tween 20, 0.1 mg/mL proteinase K], and incubated at 55°C for 45 min. Lysates were then heated to 100°C for 10 min to inactivate the proteinase and stored at −20°C. This DNA was used to PCR amplify the integrated HAβ1-tubulin cDNA using a 5′-untranslated region forward primer and a HA tag reverse primer, and the full amplicon was sequenced.

Site-Directed Mutagenesis and Selection of Paclitaxel- and Colcemid-Resistant Cells

Mutations found in drug-resistant cell lines were recreated in *pTOP/HAβ1* and transfected into CHO tTApuro 6.6a cells as already described. Stably transfected cell populations selected in 2 mg/mL G418 plus 1 μg/mL tetracycline were then plated into six-well dishes containing medium with 200 nmol/L paclitaxel or 50 nmol/L colcemid in the presence or absence of 1 μg/mL tetracycline to test for the ability of mutant HAβ1-tubulin to confer drug resistance. Cells were grown until visible colonies formed (10–14 days) and then stained with 0.5% aqueous methylene blue (27). The plates were rinsed with water to

remove excess stain and photographed with a D50 digital camera (Nikon).

Electrophoresis and Western Blots

Cells were lysed in SDS sample buffer, fractionated on 7.5% polyacrylamide minigels, and transferred to nitrocellulose membranes. The blot was stained using antibody Tub 2.1 for β-tubulin or DM1A for α-tubulin (both at 1:2,000 dilution; Sigma-Aldrich). Actin antibody C4 (1:5,000 dilution; Chemicon International, Inc.) was also included as a loading control. Immunoreactive bands were detected with goat anti-mouse IgG (1:2,000; Sigma-Aldrich) followed by SuperSignal West Pico chemiluminescent substrate (Pierce).

Measurement of Polymerized and Free Tubulin

To measure the fraction of tubulin assembled into microtubules, cells were grown for 2 days without tetracycline and lysed in 100 μL microtubule buffer containing 0.14 mol/L NaCl and 4 μg/mL paclitaxel to keep the polymerized microtubules intact (28). The lysates were centrifuged at 12,000 × *g* for 12 min at 4°C, and an equal amount of bacterial cell lysate containing glutathione *S*-transferase–tagged α-tubulin was added to each supernatant and pellet to control for possible losses of material during subsequent steps. Proteins were precipitated with acetone and resuspended in 100 μL SDS sample buffer, and equal volumes were analyzed by Western blotting with DM1A antibody and a Cy5-conjugated goat anti-mouse IgG (1:2,000 dilution; Chemicon International). Fluorescence detection was carried out with a Storm Imager (Molecular Dynamics, Inc.). The percent of total tubulin polymerized into microtubules was calculated by normalizing tubulin in the supernatant and pellet fractions to the amount of glutathione *S*-transferase-α-tubulin, dividing the normalized value from the pellet by the sum of the values from supernatant and pellet, and multiplying the quotient by 100.

Measurement of Drug Sensitivity

Approximately 100 to 300 cells were seeded into each of six duplicate wells of a 24-well dish containing medium with increasing concentrations of drug and incubated for 7 to 10 days until colonies were visible. The surviving cells were then stained with 0.5% methylene blue in water, dried, and photographed. To quantify the data, the dye was eluted into 200 μL of 1% SDS in 20 mmol/L Tris-HCl (pH 6.8), 100 μL were transferred to individual wells of a 96-well dish, and the absorbance was read at 630 nm using an Emax microplate reader (Molecular Dynamics). The highest absorbance in a series was set at 100%, and the relative values were plotted using pro Fit software (QuantumSoft).

Structural Predictions

Energy minimization and simulated molecular dynamics calculations were done using the Discover module within Insight software (Accelrys) and used class II consistent force field (CFF91; ref. 29). Structural coordinates of tubulin (1SA0; ref. 13) were taken from the protein data bank. Crystallographic waters, nucleotides, and paclitaxel were deleted. Builder module within Insight II was used to add protons, missing side chain atoms, and uncharged caps to the β-tubulin subunit. Biopolymer module within Insight II was used to replace P220 with leucine or cysteine. The mutant β subunit was first energy minimized with the steepest decent protocol using 100 steps and harmonic potential for bond energies to eliminate steric clashes. Then, each structure was subjected to molecular dynamics at 300 K using 100 initial time steps (1 fs/step) for equilibration followed by 1,000 steps of calculation using a dielectric constant of 1.000. The overall structure of β-tubulin was unchanged after the simulation. The Cα root-mean-square deviations for superimposition of the calculated mutant β subunit on the crystallographically determined structure were 1.83 Å for C220 and 1.72 Å for L220.

Results

Selection of Cells Resistant to Paclitaxel and Colcemid

The high frequency of mutations affecting leucine residues in the H6/H7 loop of β-tubulin in paclitaxel-resistant CHO cells (25) suggested that these leucines might play a unique role in the acquisition of resistance to paclitaxel, a microtubule-stabilizing drug. Moreover, no mutations in this region have yet been shown to confer resistance to drugs such as colcemid that destabilize microtubules. To explore whether targeted mutation of nonleucine residues in the H6/H7 loop might be capable of modulating sensitivity to both stabilizing and destabilizing drugs, we altered the highly conserved residue P220 with the expectation that changes at this locus should produce significant effects on structure and, presumably, drug sensitivity. Transfecting wild-type (WT) CHO cells with a pool of plasmids encoding all possible amino acids at the 220 locus allowed us to isolate a series of cell lines with resistance to paclitaxel or to colcemid. The selections were carried out under conditions in which cells transfected with mutant plasmids gave abundant resistant colonies (30–60 per dish), but control plates transfected with WT plasmid gave only 0 to 2 colonies. Sequencing of random clones revealed P220L and P220V substitutions in paclitaxel-resistant cell lines but P220C, P220S, and P220T substitutions in colcemid-resistant cells (Table 1). Multiple cell lines with these mutations were isolated, suggesting that the amino acid substitutions that were recovered are the ones most frequently associated with resistance. The possibility that redundant colonies bearing the same amino acid substitution are sister clones is refuted by the observation that multiple codons were found. Similarly, it is unlikely that the amino acid substitutions isolated in the drug-resistant cells are grossly overrepresented in the pool of plasmids because tubulin sequenced from individual clones of bacteria transformed with the mixed plasmids exhibited randomly encoded amino acids (data not shown).

Mutant HAβ1-Tubulin Is Responsible for Drug Resistance

To prove that the amino acid substitutions we identified were both necessary and sufficient to produce drug resistance, they were recreated by site-directed mutagenesis of *pTOP/HAβ1* and transfected into WT CHO cells.

G418-resistant cell populations were selected to enrich for cells that stably incorporated the transfected DNA, and these were then plated into medium containing a toxic concentration of antimitotic drug with or without tetracycline. Cells transfected with mutant cDNAs encoding P220L and P220V substitutions were able to grow in paclitaxel but cells transfected with the WT cDNA could not (Fig. 1A). Importantly, the ability of the transfected cells to grow in paclitaxel was lost when tetracycline was added to the medium to inhibit transcription of the mutant HAβ1-tubulin cDNA (Fig. 1A, *well 1*). Similar results were obtained for colcemid resistance using HAβ1-tubulin with P220C, P220S, or P220T substitutions (Fig. 1B).

Paclitaxel and Colcemid Select for Cells with Increased Production of Mutant HAβ1-Tubulin

If the mutations are responsible for drug resistance, one might predict that paclitaxel and colcemid would be more efficient agents than G418 for selecting cells with mutant HAβ1-tubulin production. In agreement with this prediction, cells transfected with mutant *pTOP/HAβ1* plasmids and selected in G418 produced barely detectable levels of HAβ1-tubulin (Fig. 2). The results were consistent with the observation that only a small percentage of G418-resistant cells had the ability to confer drug resistance (see Fig. 1). When the G418-resistant cells were reselected in paclitaxel or colcemid, however, the surviving cells produced much higher levels of mutant HAβ1-tubulin, indicating that the antimitotic drugs are more efficient than G418 at selecting cells with mutant tubulin. In addition, the cells selected with antimitotic drugs were uniformly positive for mutant HAβ1-tubulin production, whereas the G418-resistant cells were only ~50% positive as judged by immunofluorescence with antibodies to the HA tag (data not shown). The results strongly argue that the mutant tubulins are responsible for paclitaxel and colcemid resistance, and they help to explain why only a portion of the stably transfected cell population was able to form colonies in those drugs. An additional reason why all the transfected cells do not survive in drug is that some cells produce too little mutant tubulin to produce resistance, whereas other cells may produce so much mutant tubulin that it inhibits their growth.

Mutations at P220 Affect Microtubule Organization

To examine whether the P220 mutant tubulins could integrate into microtubules and affect microtubule organization, stably transfected cells were viewed by immunofluorescence 3 days after removing tetracycline to allow exogenous tubulin production. Cells transfected with WT HAβ1-tubulin cDNA (Fig. 3A) had normal cytoplasmic microtubule networks and mitotic spindles, consistent with previous studies (25, 30, 31). In the case of P220L and P220V (Fig. 3B and C), many of the transfected cells had extensively reduced microtubules, flat and large cell bodies, and multiple or enlarged nuclei. They also had multipolar spindles containing relatively few microtubules. This phenotype is identical to what has been reported for paclitaxel-dependent CHO cells (4, 24) and results from defective mitotic spindle formation, inhibited chromosome segregation, and reentry into G_1 without prior cytokinesis (32).

In contrast to the mutations that produced paclitaxel resistance, the P220C, P220S, and P220T mutations associated with colcemid resistance left the cells with abundant microtubules and normal-appearing bipolar spindles (Fig. 3D–F). In most cases, the cells actually seemed to have more microtubules than normal and they frequently exhibited evidence of microtubule bundling (e.g., Fig. 3E, *arrow*), changes that are commonly seen in normal cells that have been treated with microtubule-stabilizing drugs, such as paclitaxel. It thus seems that paclitaxel resistance and colcemid resistance mutations have opposing effects on microtubule assembly and organization.

Table 1. HAβI mutations in paclitaxel- and colcemid-resistant cell lines

Phenotype	Codon 220	Amino acid	nM IC_{50} paclitaxel	nM IC_{50} epothilone A	nM IC_{50} colcemid	nM IC_{50} vinblastine
WT	CCC	P	53.6 ± 2.7 (1)*	1.54 ± 0.02 (1)	34.5 ± 0.2 (1)	8.1 ± 0.3 (1)
Paclitaxel^R	CTC (6)†	L				
	CTT (2)	L	242.1 ± 9.1 (4.5)‡	4.8 ± 0.05 (3.1)‡	10.3 ± 0.3 (−3.3)‡	1.6 ± 0.2 (−5.1)‡
	CTA (1)	L				
	GTA (2)	V	286.6 ± 6.9 (5.3)	9.6 ± 0.2 (6.2)	ND§	ND§
Colcemid^R	ACC (4)	T				
	ACT (2)	T	18.4 ± 1.2 (−2.9)	0.56 ± 0.04 (−2.8)	56.9 ± 0.4 (1.6)	12.6 ± 0.3 (1.6)
	ACG (1)	T				
	ACA/ACC‖ (1)	T				
	TCT (1)	S	14.8 ± 0.8 (−3.6)	0.62 ± 0.03 (−2.5)	59.1 ± 0.6 (1.7)	12.4 ± 0.4 (1.5)
	AGT (1)	S				
	TGC (2)	C	9.6 ± 0.1 (−5.6)	0.34 ± 0.03 (−4.5)	69.5 ± 0.4 (2.0)	15.1 ± 0.3 (1.9)

NOTE: A single drug resistant isolate from each group (L, V, T, S, or C) was used to measure IC_{50}.

*Parenthesis indicate fold-resistance relative to wild-type cells. Negative numbers indicate fold-sensitivity.

† Brackets indicate number of isolate with a particular codon.

‡ All original paclitaxel resistant isolates of P220L were paclitaxel dependent. Therefore a subsequent clone with lower expression of mutant tubulin was isolated for this measurement.

§Not determined. The measurement could not be made because growth of the isolate was paclitaxel dependent.

‖This cell line contains 2 mutant HAβl-tubulin sequences, both of which encode the same amino acid. The isolate could be a mixture of 2 separate cell lines or a cell line that incorporated 2 separate plasmids. It was not further characterized.

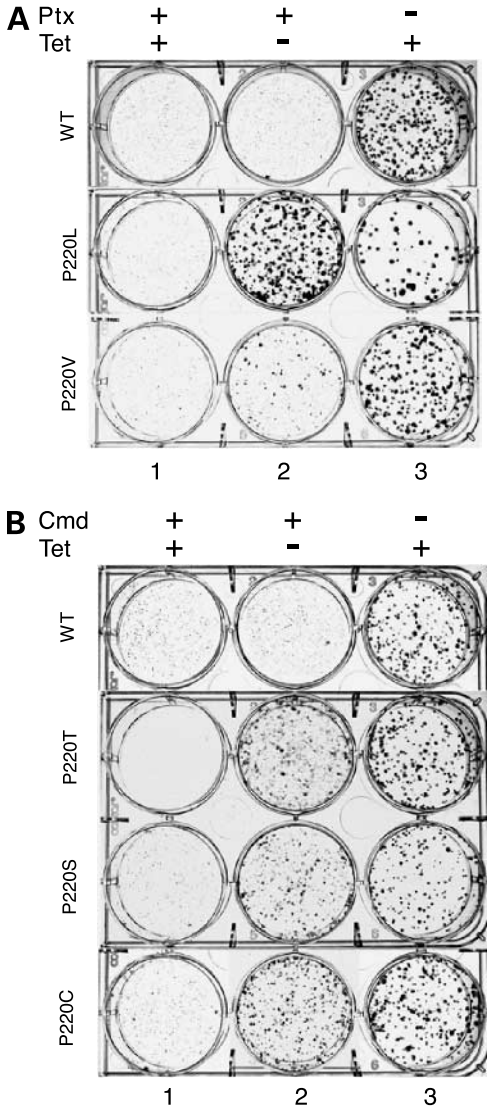

Figure 1. Drug resistance of cells transfected with mutant tubulin cDNA. CHO tTApuro 6.6a cells were transfected with WT HAβ1-tubulin cDNA, or with the same cDNA mutated to encode the indicated amino acid substitutions, and selected to obtain stable G418-resistant cell populations. These populations were plated into medium with tetracycline (*Tet*) to determine the number of viable cells (*well 3*), into medium with 200 nmol/L paclitaxel (*Ptx*; **A**) or 50 nmol/L colcemid (*Cmd*; **B**) to determine whether drug-resistant cells were present (*well 2*), and into medium with paclitaxel or colcemid plus 1 μg/mL tetracycline to turn off transgene expression and determine whether drug resistance was retained or lost (*well 1*). Cells were allowed to grow into colonies (10–14 d) and then stained with methylene blue. Note that 100 times as many cells were plated in wells 1 and 2 compared with well 3 for **A** and that 15 times as many cells were plated into wells 1 and 2 compared with well 3 for **B**.

Paclitaxel- and Colcemid-Resistant Cells Have Altered Microtubule Assembly

Estimates of microtubule density obtained from immunofluorescence microscopy are subjective and can vary greatly from cell to cell. To obtain an objective and quantitative measure of microtubule assembly in transfected cells, we lysed the cells in a microtubule-stabilizing buffer, centrifuged the lysate, and quantified tubulin in the pellet (microtubule) and supernatant (heterodimer) fractions (28). As judged by this assay, CHO cells transfected with WT *pTOP/HAβ1* had ~40% of cellular tubulin assembled into microtubules, a value that is essentially identical to previous reports for both WT and HAβ1-transfected cells (25, 28, 33–37). Cells transfected with *pTOP/HAβ1P220L* or *pTOP/HAβ1P220V* and selected for resistance to paclitaxel, on the other hand, had a much lower fraction of cellular tubulin assembly (Fig. 4). In contrast, cells transfected with *pTOP/HAβ1P220C*, *pTOP/HAβ1P220S*, or *pTOP/HAβ1P220T* and selected for colcemid resistance had a higher fraction of assembled tubulin. Thus, direct biochemical measurements on drug-resistant cell populations confirmed our visual impression from cell-to-cell observation that paclitaxel resistance mutations reduce microtubule assembly, whereas colcemid resistance mutations increase assembly.

Cells Resistant to One Drug Show Collateral Sensitivity to the Opposing Drug

Previous studies have shown that cells selected for resistance to paclitaxel frequently exhibit increased sensitivity to agents that inhibit microtubule assembly. Similarly, cells selected for resistance to inhibitors of microtubule assembly frequently exhibit increased sensitivity to paclitaxel and other drugs that stabilize microtubules (19, 38). To determine whether mutants with alterations in P220 follow a similar pattern, a clonogenic assay was used to determine

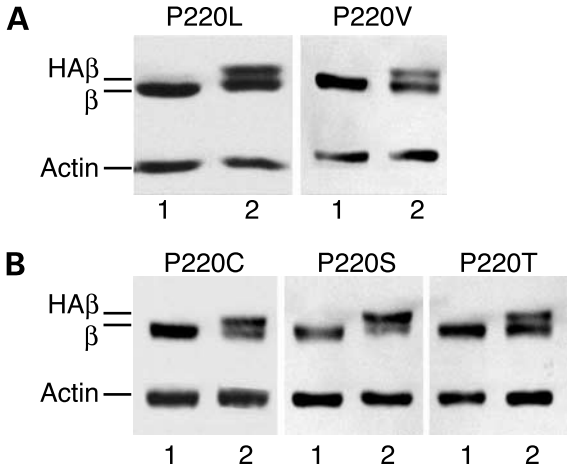

Figure 2. Elevated expression of mutant HAβ1-tubulin in drug-selected cells. *Lane 1,* CHO tTApuro 6.6a cells were transfected with pTOP/HAβ1 encoding each of the indicated amino acid substitutions and selected in the presence of 2 mg/mL G418 and 1 μg/mL tetracycline to obtain stably transfected cells. *Lane 2,* a portion of the total G418-resistant population was then reselected in αMEM without tetracycline but containing 200 nmol/L paclitaxel (**A**) or 50 nmol/L colcemid (**B**) to obtain stable drug-resistant populations. Cells surviving the drug selection were compared with the G418-resistant cells by growing both cell populations 24 h in αMEM, separating the proteins on SDS gels, and staining Western blots with an antibody that recognizes both transfected (*HAβ*) and endogenous (*β*) tubulin. Actin was also detected with a specific antibody and acted as a loading control.

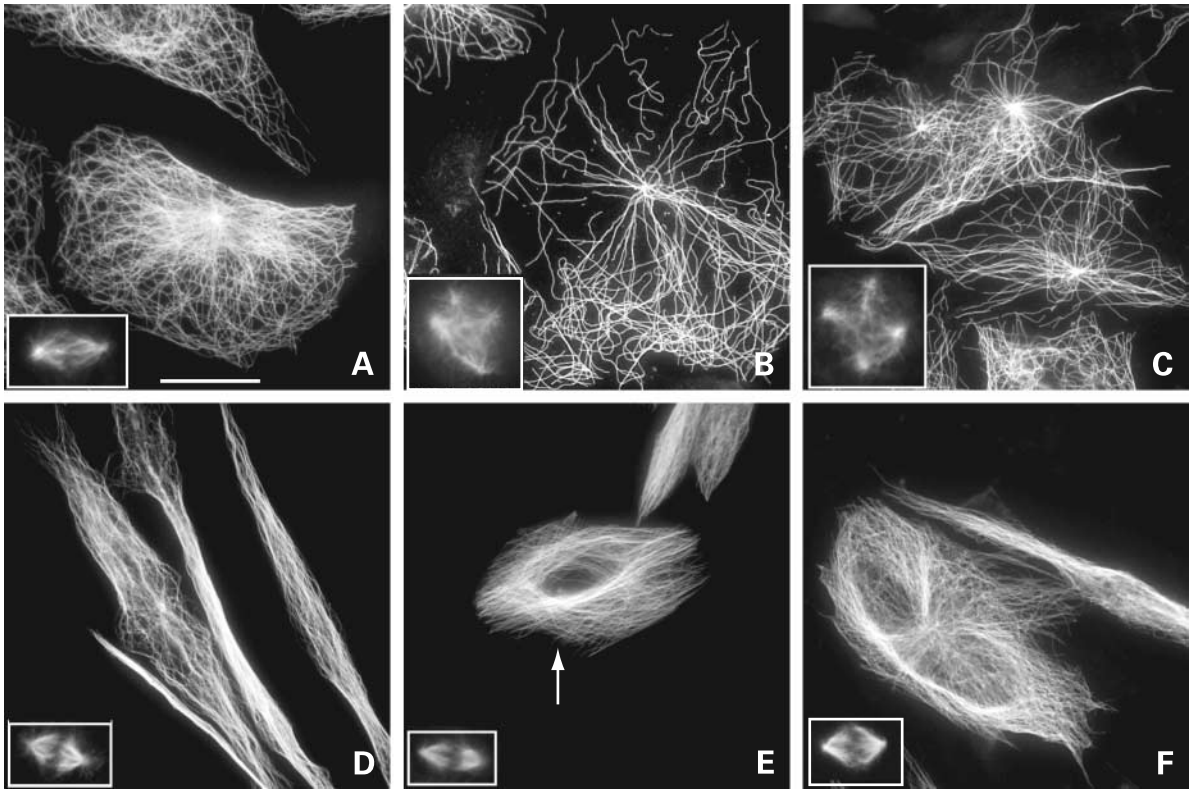

Figure 3. Immunofluorescence of transfected cells. CHO tTApuro 6.6a cells were transfected with HAβ1-tubulin cDNA (**A**) or with the same cDNA mutated to encode P220L (**B**), P220V (**C**), P220C (**D**), P220S (**E**), and P220T (**F**) amino acid substitutions. Stable G418-resistant populations were allowed to grow in αMEM without tetracycline for 3 d and then processed for immunofluorescence using an antibody specific for the HA tag. *Arrow,* a cell with bundled microtubules near the nucleus. *Insets,* mitotic cells. Bar, 20 μm (**A**).

the response of cells transfected with mutant β-tubulin to colcemid and vinblastine, two drugs that inhibit microtubule assembly but bind to distinct sites on tubulin (13, 16), and to paclitaxel and epothilone A, two microtubule-stabilizing agents that bind to a third site (7, 39). Examples of the dose-response curves are shown in Supplementary Fig. S1[3] and the full results are summarized in Table 1. Because the paclitaxel-selected P220L and P220V cell populations grew poorly in the absence of paclitaxel [i.e., they had a paclitaxel-dependent phenotype (see P220V in Supplementary Fig. S1A for an example)], a clone with lower expression of HAβ1P220L tubulin was isolated to be able to measure the response to colcemid and vinblastine. This cell line was not paclitaxel dependent but remained paclitaxel resistant as expected (Supplementary Fig. S1A, *squares* and *dashed line*). In addition, it was more sensitive than WT cells to the effects of colcemid (Supplementary Fig. S1B). As summarized in Table 1, cells with the P220L and P220V mutations were resistant not only to paclitaxel but also to epothilone A. Moreover, the P220L mutant chosen because it could grow in the absence of paclitaxel was more sensitive to both colcemid and vinblastine. Conversely, cells with P220C, P220S, and P220T mutations were resistant to colcemid and vinblastine but exhibited increased sensitivity to paclitaxel and epothilone A. Thus, the results are consistent with the resistance mechanism we proposed in previous publications and indicate that P220 mutant tubulins alter microtubule assembly in a manner that opposes the action of the selecting drug (19).

Drugs Counteract the Effects of the Mutations

Cells that produce P220L and P220V mutant tubulins exhibit reduced microtubule assembly and growth defects, whereas cells that produce P220C, P220S, and P220T mutant tubulins exhibit increased microtubule assembly and frequently have microtubule bundles (Figs. 3 and 4). If the mutations cause resistance by counteracting the effects of the selecting drug, then one would expect that the phenotypes of cells with mutant tubulin should be reversed by the selecting drug. To test this prediction, cells transfected with *pTOP/HAβ1P220L*, associated with paclitaxel resistance, and *pTOP/HAβ1P220S*, associated with colcemid resistance, were grown in the presence and absence of the selecting drug. Microtubules were viewed by immunofluorescence with antibodies to the HA tag for mutant tubulin and antibodies to α-tubulin for total tubulin (see Supplementary Fig. S2). In the absence of drug, cells that

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

produced high amounts of HAβ1P220L tubulin were large and multinucleated and had sparse microtubules compared with nonexpressing cells. In the presence of 100 nmol/L paclitaxel, however, the pattern reversed; that is, nonexpressing cells were large and multinucleated, whereas *pTOP/HAβ1P220L*-expressing cells appeared normal. In the case of *pTOP/HAβ1P220S*, cells that expressed the mutant tubulin cDNA exhibited microtubule bundles in the absence of any drug but appeared normal in the presence of 25 nmol/L colcemid. Nontransfected cells, on the other hand, were normal without drug but formed large multinucleated cells in the presence of colcemid. These observations support the notion that the phenotypic effects caused by mutant tubulin production result from alterations in microtubule assembly that can be compensated by adding a microtubule-stabilizing or microtubule-destabilizing agent.

Discussion

The H6/H7 loop of β-tubulin seems to play an important role in microtubule biology. In addition to its topological role in mediating lateral and longitudinal interactions in microtubule assembly (26), it forms part of the binding site for paclitaxel and other drugs that stabilize microtubules (7, 39), is close to the vinblastine binding site (16), and undergoes a sizable conformational shift when tubulin assembles into microtubules (13). It is also located near fenestrations in the microtubule wall, prompting the suggestion that this loop may control the entry of paclitaxel to its binding site in the microtubule lumen (40). Genetic evidence for the importance of this region comes from the observation that CHO cells selected for resistance to paclitaxel have a very high incidence of mutations affecting L215 and L217 in the H6/H7 loop as well as L228 in helix 7. These mutations seem to act by altering the assembly of microtubules (25). More recent studies have further shown that an L215I mutation in β-tubulin increases sensitivity to paclitaxel, most likely by increasing the binding affinity for the drug (37). Thus, a variety of structural, biochemical, and genetic studies have identified this region as having important significance in microtubule assembly, drug action, and drug resistance.

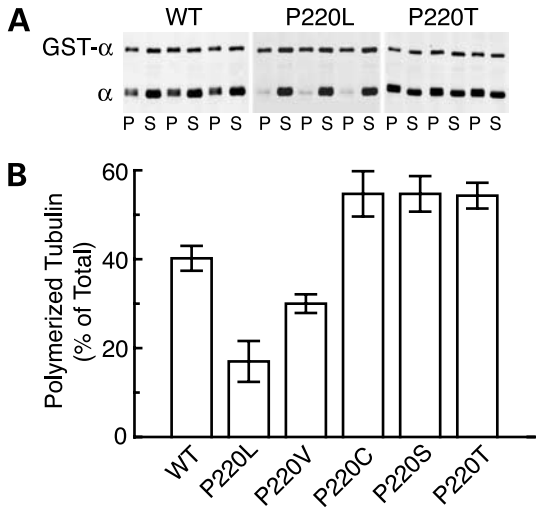

Figure 4. Microtubule polymerization. Cells selected for resistance to paclitaxel or colcemid and producing HAβ1-tubulin with the indicated amino acid substitutions were grown 24 h in the absence of the selecting drug and tetracycline, lysed in microtubule-stabilizing buffer, and centrifuged to separate microtubules in the pellet (*P*) from free tubulin in the supernatant (*S*). A constant volume of a bacterial cell lysate containing glutathione *S*-transferase (*GST*)-α-tubulin was added to each fraction to act as an internal control, and the proteins were separated on SDS polyacrylamide gels, transferred to nitrocellulose, and probed with an antibody to α-tubulin. Fluorescence emission from a Cy5-tagged secondary antibody was measured and used to calculate the percentage of total tubulin appearing in the microtubule pellet. An example of the fluorescence emission pattern from three cell lines run in triplicate is shown in **A**, and quantification for all of the cell lines is summarized in **B**. SDs were based on at least three independent experiments, each of which contained triplicate samples. $P < 0.05$, Student's *t* test.

To further address the role of this region in microtubule assembly and drug resistance, we targeted residue P220 because of its high degree of conservation in α-tubulin, β-tubulin, and γ-tubulin. Using saturation mutagenesis, we identified P220 substitutions that confer resistance to different antimitotic drugs with opposing effects on microtubule assembly. The properties of the resistant cells are consistent with our previously proposed model for drug resistance; that is, mutations that confer resistance to stabilizing drugs reduce microtubule stability (i.e., decrease the assembly of the polymer), whereas mutations that confer resistance to destabilizing drugs increase microtubule stability (i.e., increase the assembly of the polymer; refs. 19, 38). Although our studies measured microtubule polymer in interphase cells, the changes in microtubule assembly also affect spindle function, the main target of antimitotic drugs. In fact, many of the more extreme mutants we have isolated, including the paclitaxel-dependent cells expressing the P220L and P220V mutations described here, have obvious morphologic changes in their mitotic spindles and are unable to segregate chromosomes or complete cytokinesis (24, 32). Other drug-resistant cell lines have more subtle mitotic defects that only affect cell proliferation at elevated temperatures (1). The idea that mutations interfere with spindle function by altering microtubule stability is supported by the observation that paclitaxel-dependent cells, which have less stable microtubules, regain normal mitotic progression and proliferation, as well as normal morphology, when they are treated with paclitaxel, a microtubule-stabilizing drug (e.g., see Supplementary Fig. S2). How this change in stability relates to microtubule dynamics has not yet been adequately addressed, but one study of a paclitaxel-dependent mutant with multiple genetic alterations showed that the cytoplasmic microtubules in those cells were more dynamic than normal and that the increased dynamic behavior could be suppressed by adding paclitaxel (41).

Other potential mechanisms to explain the drug resistance of the mutants described here and in previous publications are much less likely (see refs. 19, 42 for a fuller discussion of this topic). For example, altered drug

binding is a common mechanism of resistance in lower eukaryotes that can grow as haploids and express a single β-tubulin gene (43). In higher organisms that are diploid and express multiple tubulin genes, however, this mechanism is unlikely to confer resistance because loss of drug binding produces a recessive phenotype. Moreover, it is difficult to envision how a mutation of a drug binding site can lead to the consistent pattern of resistance and increased sensitivity to multiple drugs that bind to three distinct sites on tubulin, or explain the paclitaxel dependence of cells with mutations such as P220L and P220V. In the few cases in which drug binding mutations have been reported in mammalian cells, they have been accompanied by functional haploidization; that is, they have lost expression from the WT allele, a condition we do not encounter in our single-step selections (20, 21).

The results presented here show for the first time that alterations at the same position of β1-tubulin can change microtubule structure in such a way that they lead to opposing effects on microtubule stability and drug resistance. Leucine and valine substitutions at P220 reduce microtubule assembly and confer resistance to paclitaxel and epothilone A, but cysteine, serine, and threonine substitutions increase microtubule assembly and confer resistance to colcemid and vinblastine. Moreover, paclitaxel counteracts the microtubule-destabilizing effects of P220L and P220V, whereas colcemid counteracts the microtubule-stabilizing effects of P220C, P220S, and P220T mutations—results that are consistent with the proposition that changes in microtubule assembly and stability underlie the mechanism of resistance. In recent years, other laboratories have reported drug resistance mutations in human cell lines that produce similar phenotypes and likely act through a similar mechanism (44–48).

It is intriguing that the substitutions able to confer paclitaxel resistance are both hydrophobic, whereas all three substitutions that conferred colcemid resistance have electronegative side chains. To examine the structural basis for the effects of these substitutions on microtubule assembly and drug sensitivity, we compared the colchicine- and paclitaxel-bound structures of β-tubulin as approximations for the nonassembled (''curved'') and assembled (''straight'') conformations, respectively (see ref. 49 for review). The analysis (Fig. 5A) showed that, relative to the colchicine-bound structure (1SA0, *maroon*), helix H7 of the paclitaxel-bound structure (1JFF, *light blue*) is shifted upwards and bends to the right, whereas H6 and the H6/H7 loop tilt downward toward the microtubule lumen. The position of the loop between H6 and H7, as well as the H6 helix itself, is likely to affect contacts made with the successive tubulin dimers in the protofilament. Downward movement of H6 positions the loop toward the straight conformation and enhances favorable contacts between tubulin dimers in adjacent protofilaments, resulting in an energetically more favorable microtubule structure. Upward movement, on the other hand, positions the loop toward the curved conformation and weakens tubulin interactions important for assembly.

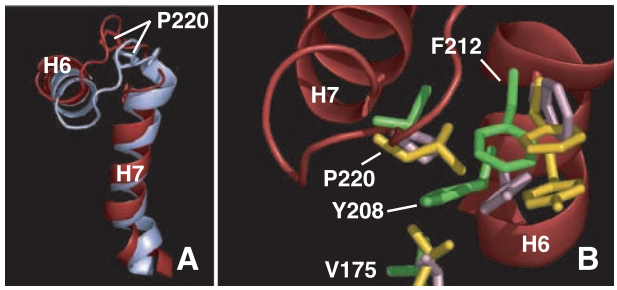

Figure 5. Model of the assembled and unassembled conformations of β-tubulin. **A,** the assembled or straight conformation (*light blue*) was drawn from the atomic coordinates 1JFF (50) and the unassembled or curved conformation (*maroon*) from 1SA0 (13). A luminal view of the loop containing P220 and flanking helices H6 and H7 is shown oriented with the plus end of the protofilament facing the top. Only the β subunits of the two structural models were superimposed (root-mean-square deviation = 1.808 Å) to minimize curvature introduced by stathmin in the 1SA0 model and to emphasize the structural changes produced by assembly. **B,** close-up of hydrophobic residues proximal to P220 (*pink sticks*) and their corresponding positions in models containing L220 (*yellow*) or C220 (*green*). To better show the positions of all the side chains, the molecule was reoriented from **A** by rotating 120° around the *X* axis and 180° around the *Y* axis. Structures were drawn using MacPyMOL (DeLano Scientific).

P220 is located in the H6/H7 loop among several hydrophobic amino acids. A leucine or valine substitution is likely to improve these hydrophobic interactions and stabilize the structure of the loop. To test this, we replaced P220 with leucine and did molecular dynamics calculations. The results show that L220 interacts with Y208, F212, and V175 (Fig. 5B). We propose that the resulting decreased flexibility of the loop stabilizes the assembly incompetent form of tubulin, diminishes the ability of tubulin to acquire the straight conformation, and thereby produces less stable microtubules and paclitaxel resistance. In contrast, S/T/C substitutions of P220 cannot maintain these hydrophobic interactions. Moreover, electrostatic repulsion between their alcohol or thiol groups and vicinal hydroxyl (Y208, T218, T219, and T221) and carboxyl (D224) groups is likely to move the loop toward the straight conformation. Consistent with this prediction, molecular dynamics calculations indicate that the C220 side chain points away from Y208 and F212. In short, the increased loop flexibility of S/T/C220 relative to WT P220 or mutant L/V220 forms of β-tubulin would favor the straight conformation, thereby stabilizing microtubules and conferring resistance to colchicine.

In summary, we have described a rapid method for identifying possible amino acid substitutions at a given locus that are associated with drug resistance. The approach has allowed us to report for the first time that different amino acid substitutions at the same residue in mammalian β-tubulin can confer resistance to drugs with opposing effects on microtubule assembly. It has also allowed us to show that substitution of hydrophobic side chains for P220 of β1-tubulin destabilizes microtubules but that substitution of amino acids with hydroxyl or sulfhydryl side chains stabilizes microtubules and leads to microtubule bundling. These studies lend further evidence for the importance of the H6/H7 loop in microtubule assembly and drug action.

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