

Paclitaxel-resistant cells have a mutation in the paclitaxel-binding region of β -tubulin (Asp²⁶Glu) and less stable microtubules

Malathi Hari,¹ Frank Loganzo,¹ Tami Annable,¹ Xingzhi Tan,¹ Sylvia Musto,¹ Daniel B. Morilla,¹ James H. Nettles,² James P. Snyder,² and Lee M. Greenberger¹

¹Oncology Research, Wyeth, Pearl River, New York and
²Department of Chemistry, Emory University, Atlanta, Georgia

Abstract

Resistance to paclitaxel-based therapy is frequently encountered in the clinic. The mechanisms of intrinsic or acquired paclitaxel resistance are not well understood. We sought to characterize the resistance mechanisms that develop upon chronic exposure of a cancer cell line to paclitaxel in the presence of the P-glycoprotein reversal agent, CL-347099. The epidermoid tumor line KB-3-1 was exposed to increasing concentrations of paclitaxel and 5 μ mol/L CL-347099 for up to 1 year. Cells grown in 15 nmol/L paclitaxel plus CL-347099 (KB-15-PTX/099) developed 18-fold resistance to paclitaxel and were dependent upon paclitaxel for maximal growth. They grew well and retained resistance to paclitaxel when grown in athymic mice. Cross-resistance (3- to 5-fold) was observed in tissue culture to docetaxel, the novel taxane MAC-321, and epothilone B. Collateral sensitivity (\sim 3-fold) was observed to the depolymerizing agents vinblastine, dolastatin-10, and HTI-286. KB-15-PTX/099-resistant cells did not overexpress P-glycoprotein nor did they have an alteration of [¹⁴C]paclitaxel accumulation compared with parental cells. However, a novel point mutation (T to A) resulting in Asp²⁶ to glutamate substitution in class I (M40) β -tubulin was found. Based

on an electron crystallography structure of Zn-stabilized tubulin sheets, the phenyl ring of C-3' NHCO-C₆H₅ of paclitaxel makes contact with Asp²⁶ of β -tubulin, suggesting a ligand-induced mutation. Optimized model complexes of paclitaxel, docetaxel, and MAC-321 in β -tubulin show a novel hydrogen bonding pattern for the glutamate mutant and rationalize the observed resistance profiles. However, a mutation in the paclitaxel binding pocket does not explain the phenotype completely. KB-15-PTX/099 cells have impaired microtubule stability as determined by a reduced percentage of tubulin in microtubules and reflected by less acetylated tubulin. These results suggest that a mutation in tubulin might affect microtubule stability as well as drug binding and contribute to the observed resistance profile. [Mol Cancer Ther 2006;5(2):270–8]

Introduction

The taxane paclitaxel binds to microtubules and inhibits their disassembly (1). Cells treated with paclitaxel are arrested in mitosis and eventually undergo death by apoptosis (2). The ability of paclitaxel and another taxane, docetaxel (Fig. 1), to kill tumor cells has made them useful chemotherapeutic agents against several types of cancers, including those derived from ovary, breast, head and neck, and lung as well as malignant melanoma (2). Nevertheless, resistance to paclitaxel has been an impediment to the wider use of paclitaxel in the clinic. Therefore, there has been an impetus to develop the next generation of taxanes as well as other tubulin-targeting agents that can overcome resistance to paclitaxel. One example is the novel taxane known as MAC-321 (Fig. 1; ref. 3).

Resistance to paclitaxel in laboratory cell line models has been extensively explored, and various mechanisms of resistance have been identified (4–6). One reported mechanism is the overexpression of ABC transporters, including P-glycoprotein/MDR1/ABCB1, ABCB4 (MDR2), ABCB11 (BSEP and SPGP; ref. 7–9), and ABCC1 (MRP7; ref. 10). These transporters actively pump out a wide variety of drugs, including microtubule agents such as taxanes and *Vinca* alkaloids, thus rendering the cells resistant to these drugs. Blocking P-glycoprotein is only partially sufficient to reestablish sensitivity to these agents, and this method has not been remarkably effective in the clinic (11). Another potential mechanism of drug resistance is the modulation of the cellular target of paclitaxel, tubulin. Expression of mutant tubulin or differential expression of tubulin isoforms has been shown to correlate with the resistance profile of drug-resistant cells (12–17). In some cases, the induced expression of mutant tubulins (18) and β -tubulin isoforms class III and V (19, 20) were shown to confer resistance to paclitaxel. Purified mutant tubulins also have altered

Received 6/10/05; revised 10/20/05; accepted 12/16/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: A preliminary version of this work was presented at the Annual Meeting of the AACR, July 2003, Washington, District of Columbia, abstract no. 5770.

The current address for J.H. Nettles is Novartis Institutes for Biomedical Research, Inc., 250 Massachusetts Avenue, Cambridge, MA 02139. The current address for L.M. Greenberger is Johnson & Johnson, Pharmaceutical Research & Development, 1000 Route 202, P.O. Box 300, B354B, Raritan, NJ 08869.

Requests for reprints: Frank Loganzo, Discovery Oncology, Wyeth, 401 North Middletown Road, Room 4709, Building 200, Pearl River, NY 10965. Phone: 845-602-4237; Fax: 845-602-5557. E-mail: loganzf@wyeth.com

Copyright © 2006 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0190

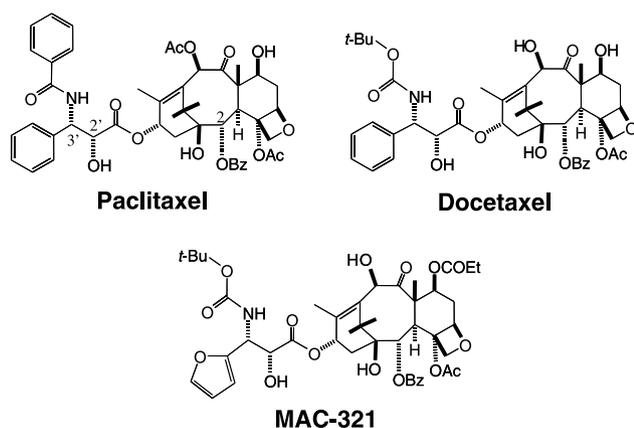


Figure 1. Chemical structure of paclitaxel, docetaxel, and MAC-321.

polymerization characteristics (15). One report noted increased expression of β -tubulin class I, III, and IVa isoforms in paclitaxel-resistant ovarian tumors (12). However, induced expression of β -tubulin class I in Chinese hamster ovary cells failed to confer resistance to paclitaxel (21). Other poorly understood paclitaxel resistance mechanisms include altered expression and phosphorylation status of microtubule-associated proteins (14) and anti-apoptotic proteins (22). Therefore, several mechanisms of resistance have been observed in cell culture systems; yet, it is not clear which are the predominate mechanisms that operate at the whole organism level in patients.

Cell lines selected for resistance to paclitaxel and to other microtubule agents can be generated by a single, high-dose exposure of paclitaxel (4, 23) or a dose-escalating maintenance in paclitaxel. The former has frequently given rise to selection of mutant cells expressing point mutations in tubulin and the latter to multifactorial resistance mechanisms, such as mutations in tubulin genes; differential tubulin isotype expression; overexpression of transporters, such as P-glycoprotein; or changes in expression of microtubule-associated proteins or proteins in the apoptotic pathway. During the course of development of novel taxanes that have much weaker interaction with P-glycoprotein (4), we investigated whether such taxanes would also overcome non-P-glycoprotein-mediated mechanisms of taxane resistance. To this end, we took the approach of blocking P-glycoprotein-mediated resistance by growing cells in the presence of CL-347099 (24), a so-called P-glycoprotein reversal agent that antagonizes P-glycoprotein-mediated active transport of drugs, along with paclitaxel. CL-347099 is more potent than verapamil in blocking P-glycoprotein but does not block Ca^{2+} channels. Coselection of P-glycoprotein-blocking agents combined with paclitaxel has been previously used to eliminate permeability mutants in selecting drug-resistant cells (15, 25, 26). In this report, the KB-15-PTX/099 cells (selected in paclitaxel and CL-347099) did not express P-glycoprotein but did have a novel point mutation in the major β -tubulin isotype HM40 (class I β -tubulin), which, based on crystal-

lographic data, makes contact with paclitaxel. Expression of this mutant β -tubulin is associated with both less stable microtubules and a dependence on the presence of paclitaxel for normal growth. These data suggest deficits in both drug binding and microtubule stability in paclitaxel resistance. The example described herein also helps to account for the differential resistance of closely related paclitaxel analogues.

Materials and Methods

Compounds

Paclitaxel, vinblastine, and Adriamycin were obtained from Sigma (St. Louis, MO). MAC-321 was obtained from Taxolog, Inc. (Fairfield, NJ). Docetaxel was obtained from MedWorld Pharmacy (Valley Cottage, NY). HTI-286, mitoxantrone, and bisantrene were synthesized at Wyeth (Pearl River, NY). Epothilone B was obtained from Calbiochem (San Diego, CA). The National Cancer Institute generously provided dolastatin-10. [^{14}C]paclitaxel (74 mCi/mmol) was obtained from Moravak Biochemicals, Inc. (Brea, CA). Nonradioactive compounds were solubilized as 1 or 10 mmol/L stocks in DMSO for *in vitro* studies.

Selection of Paclitaxel-Resistant Cell Lines and Cell Proliferation Assays

The KB-3-1 epidermoid carcinoma cell line was generously provided by Dr. M. Gottesman (National Cancer Institute) and was maintained as described (27). KB-3-1 cells were selected for resistance to paclitaxel by continuous exposure to increasing concentration of drug in the presence of 5 $\mu\text{mol/L}$ CL-347099 (KB-15-PTX/099) or in its absence (KB-15-PTX). The first selection concentration of paclitaxel (for both KB-15-PTX and KB-15-PTX/099) was 2 nmol/L, which was approximately the IC_{50} for KB-3-1 cells after 3 days of growth. The subsequent drug concentrations for the multistep selection were 2.5, 3.0, 3.5, 4.0, 5.0, 7.5, 10, and 15 nmol/L paclitaxel, in the presence of 5 $\mu\text{mol/L}$ CL-347099 for KB-15-PTX/099 over a period of 9 months, or 4, 8, and 15 nmol/L paclitaxel for KB-15-PTX cells over a period of 3 months. Cell proliferation assays were done as described previously (27).

Drug Accumulation Studies

Accumulation of radiolabeled paclitaxel in cells was conducted using 500 nmol/L [^{14}C]paclitaxel ($\sim 0.01 \mu\text{Ci}$) in serum-free media, similar to the method described previously (27).

Analysis of P-glycoprotein Expression

Drug transporter protein levels were determined by isolating cell membranes followed by SDS/PAGE and immunoblot analyses (27). P-glycoprotein was detected with PC03 antibody (Oncogene Science, Uniondale, NY). KB-8.5 cells (generous gift of Dr. M. Gottesman) were used as a positive control for the expression of P-glycoprotein. Levels of mRNA for drug transporter proteins were determined in cell lines by quantitative reverse transcription-PCR (3).

Tubulin Sequencing

Tubulin cDNA preparation and DNA sequencing were done as described (28). To obtain amino acid sequence,

tubulin protein was prepared from KB-3-1 and KB-15-PTX/099 cells (29). Purified microtubules were resuspended in $1 \times$ Laemmli sample buffer and run on a 7.5% SDS-PAGE gel with running buffer containing low-grade SDS (30). The proteins were transferred to Sequiblot polyvinylidene difluoride (Bio-Rad, Hercules CA) and stained with Coomassie blue R250. The separated β -tubulin was excised and sequenced to 30 cycles beginning at the NH_2 terminus on an Applied Biosystems Model 492 NH_2 -terminal protein sequencer.

In vivo Efficacy Studies

Athymic *nu/nu* female mice (Charles River Laboratories, Wilmington, MA) were implanted s.c. with 2.5×10^6 KB-3-1 or KB-15-PTX/099-resistant cells. When tumors attained a mass of 100 to 200 mg, animals were randomized into treatment groups of 10 mice each. Each group was treated i.v. with 1.25 mg/kg HTI-286 prepared in saline, 60 mg/kg/dose paclitaxel prepared in 6% ethanol/6% Cremophor EL/88% saline, or a saline vehicle control according to published methods (27). Tumor size was calculated: $(\text{length} \times \text{width}^2) / 2$.

Measurement of Tubulin Polymerization

Cells were grown in duplicate wells of 24-well dishes to 80% confluence and remained untreated or treated overnight with 15 nmol/L paclitaxel for 16 to 18 hours. The tubulin in microtubules was measured as described previously (31). Briefly, the cells were lysed in a microtubule-stabilizing buffer containing 20 mmol/L Tris-HCl (pH 6.8), 0.14 mol/L NaCl, 0.5% NP40, 1 mmol/L MgCl_2 , 2 mmol/L EGTA, and 4 $\mu\text{g}/\text{mL}$ paclitaxel as previously described (32). The lysates were centrifuged at $10,000 \times g$ for 20 minutes to obtain pellet fraction containing microtubules (*P*) and supernatant fraction containing soluble tubulin (*S*). The proteins in each fraction were precipitated with 5 volumes of acetone, precipitates were centrifuged, and pellets were air-dried. The pellets were resuspended in SDS-PAGE sample buffer, and equal volumes of sample were run on 10% Tris-HCl polyacrylamide SDS minigels (Bio-Rad). Proteins in the gel were transferred to nitrocellulose, and the blots were incubated with a mouse monoclonal anti- α -tubulin antibody (DM1A, 1:2,000, Sigma-Aldrich, Inc., St. Louis, MO). Immunoreactive protein bands were imaged using Cy5-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) and detected in the Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE). The percentage of assembled tubulin was calculated: $[P / (P + S)] \times 100$.

Measurement of Acetylated Tubulin

To determine relative amounts of acetylated tubulin, wild-type and mutant cell lines were grown to 80% confluence in 35-mm dishes and lysed with 1% SDS. Proteins were precipitated by adding 5 volumes of acetone, and the precipitate was collected by centrifugation for 5 minutes at $10,000 \times g$. The pellet was dissolved in SDS sample buffer and resolved by SDS-PAGE. After transfer of the proteins to nitrocellulose, the blots were incubated with monoclonal antibodies specific for acetylated tubulin (611b1, 1:1,000 dilution, Sigma) and actin (C4, 1:3,000

dilution; Chemicon, Temecula, CA). Detection and quantification of the immunoreactive bands was carried out using Cy-5-conjugated goat anti-mouse secondary antibody as described above for measuring tubulin assembly. The acetylated tubulin in each cell line was normalized to actin.

Computational Methods

Crystallographic coordinates for paclitaxel complexed with α,β -tubulin (1JFF) were obtained from the PDB (33). Refer to Supplementary Data for complete description of computational methods.³

Results

Isolation of Resistant Lines and *In vitro* and *In vivo* Resistance Profile

Paclitaxel-resistant cells were selected by a dose-escalating exposure to paclitaxel either in the absence or presence of CL-347099, a P-glycoprotein-blocking agent (24). The cell lines were designated as KB-15-PTX or KB-15-PTX/099, respectively. Drug sensitivity of the resistant cells was determined and is summarized in Table 1. The KB-15-PTX/099 cells showed 18-fold relative resistance to paclitaxel, which was ~ 4 -fold higher than resistance to three other tubulin-polymerizing agents (docetaxel, MAC-321, and epothilone B). In contrast, KB-15-PTX cells showed high relative resistance to paclitaxel (29-fold) and moderate resistance to docetaxel (10-fold) but little or no resistance to two polymerizing agents that are poor substrates for P-glycoprotein (MAC-321 and epothilone B). KB-15-PTX/099 cells showed 3-fold higher sensitivity to microtubule-depolymerizing agents vinblastine, dolastatin-10, and HTI-286 compared with parental cells. In contrast, KB-15-PTX cells were resistant to vinblastine and bisantrene, known substrates of P-glycoprotein, but sensitive to HTI-286 and mitoxantrone, which are poor substrates of P-glycoprotein. As expected, little resistance to MAC-321 was observed in the KB-15-PTX cells. Hence, the drug resistance profile of KB-15-PTX cells is consistent with a P-glycoprotein-associated phenotype, in contrast to the KB-15-PTX/099 cells.

The resistance of KB-15-PTX/099 cells was also tested in an *in vivo* xenograft model in athymic mice. Tumors derived from KB-15-PTX/099 cells were completely refractory to treatment with paclitaxel, whereas HTI-286 effectively inhibited tumor growth (Fig. 2). Tumors derived from the parental cells, KB-3-1, are responsive to both paclitaxel and HTI-286.

Analysis of P-glycoprotein Expression and Accumulation of [¹⁴C]Paclitaxel in Resistant Cells

The level of P-glycoprotein expression in the two paclitaxel-resistant cell lines was determined by Western blotting with an anti-P-glycoprotein antibody and quantitative reverse transcription-PCR (Fig. 3A and B, respectively). KB-15-PTX cells express high levels of

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

Table 1. Profile of KB-3-1 cells selected for resistance to paclitaxel

Compound	IC ₅₀ (nmol/L)*, KB-3-1	IC ₅₀ (nmol/L), KB-15-PTX/099	Relative resistance †	IC ₅₀ (nmol/L), KB-15-PTX	Relative resistance
Microtubule-polymerizing agents					
Paclitaxel	2.4 ± 0.4	44.2 ± 16.9	18.2	71.2 ± 17.6	29.3
Docetaxel	0.84 ± 0.46	4.1 ± 1.8	4.9	8.1 ± 1.9	9.7
MAC-321	0.57 ± 0.06	2.3 ± 0.2	3.9	1.1 ± 0.6	2.0
Epothilone B	0.61 ± 0.03	2.1 ± 0.1	3.4	0.84 ± 0.01	1.4
Microtubule-depolymerizing agents					
Vinblastine	0.82 ± 0.27	0.21 ± 0.04	0.3	3.0 ± 1.6	3.7
HTI-286	0.61 ± 0.11	0.20 ± 0.04	0.3	0.52 ± 0.08	0.8
Dolastatin-10	0.041 ± 0.010	0.012 ± 0.004	0.3	ND	ND
DNA-damaging agents					
Mitoxantrone	7.7 ± 2.0	23.6 ± 4.1	3.1	13.5 ± 3.1	1.8
Adriamycin	56.5 ± 41.3	76.0 ± 20.0	1.4	ND	ND
Bisantrene	68.9 ± 45.2	57.8 ± 46.5	0.8	764 ± 107	11.1

Abbreviation: ND, not determined.

*Mean IC₅₀ (nmol/L) ± SD from two to six independent determinations.

†Relative resistance = ratio of IC₅₀ of the resistant cell line to IC₅₀ of the parental line.

P-glycoprotein, whereas P-glycoprotein is undetectable in KB-15-PTX/099 cells. KB-8.5 cells, known to overexpress P-glycoprotein and which are 19-fold resistant to paclitaxel (3), were used as a positive control.

Although KB-15-PTX/099 cells lack P-glycoprotein expression, they are resistant to paclitaxel. To determine if this resistance is due to the ability of the KB-15-PTX/099 cells to selectively transport paclitaxel out of the cells, the accumulation of radioactive [¹⁴C]-labeled paclitaxel was determined. The data shown in Fig. 3C suggest that there is no significant difference in accumulation of [¹⁴C]paclitaxel in KB-3-1 and KB-15-PTX/099 cells, whereas KB-15-PTX

and KB-8.5 cells had significantly lower [¹⁴C]paclitaxel accumulation than parental cells, consistent with the observed expression of P-glycoprotein.

Dependence of KB-15-PTX/099 Cells on Paclitaxel for Growth

KB-15-PTX/099 cells were not only resistant to paclitaxel and other tubulin polymerizing agents but also partially dependent on these drugs. Low concentrations of paclitaxel, docetaxel, MAC-321, and epothilone B stimulated the growth of the cells (Fig. 4). On the other hand, growth of KB-15-PTX/099 cells was not stimulated by the microtubule-depolymerizing drugs vinblastine and HTI-286. This suggests that there is a common mechanism of resistance to paclitaxel and other tubulin-polymerizing agents in KB-15-PTX/099 cells. The common target for the polymerizing drugs is tubulin, and the broad resistance to these drugs suggests that KB-15-PTX/099 might have alterations in tubulin, tubulin isotype expression, and/or associated proteins that effect tubulin dynamics or the drug-binding site.

Sequencing of β -Tubulin Class I Isotype

To determine if the KB-15-PTX/099 cells have any mutations in tubulin that could mediate resistance to paclitaxel as described by other studies (14, 15, 18), the major tubulin isotypes, β -tubulin class I (HM40) and α -tubulin k- α -1, were sequenced. Sequencing revealed that class I β -tubulin of KB-15-PTX/099 cells encodes for a point mutation at residue Asp²⁶Glu (GAT to GAA) compared with the wild-type sequence (see Supplementary Fig. S1).³ This mutation is in the NH₂ terminus of β -tubulin and is part of the binding pocket of paclitaxel (ref. 34; see Discussion). No mutation was found in k- α -1-tubulin.

Amino acid sequencing confirmed that the Asp²⁶Glu mutation was expressed in the β -tubulin protein isolated from KB-15-PTX/099 cells. This mutant tubulin is functionally active because it was purified from paclitaxel-induced microtubules.

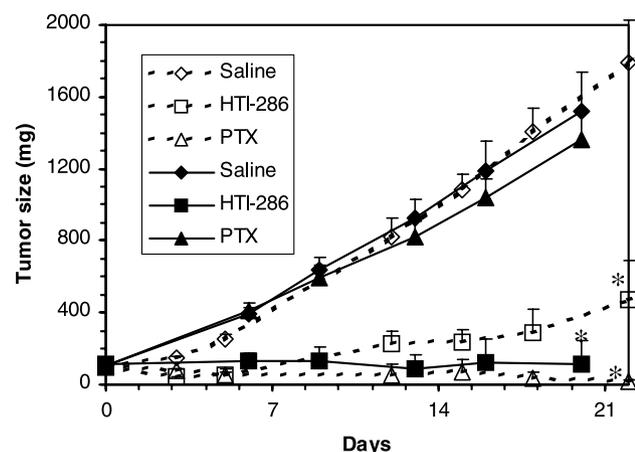


Figure 2. KB-15-PTX/099 cells are resistant to paclitaxel but retain sensitivity to HTI-286 *in vivo*. KB-3-1 (open symbols/dotted lines) or KB-15-PTX/099 (closed symbols/solid lines) tumor cells were implanted s.c. into athymic mice. Animals bearing established tumors were treated i.v. with saline (diamonds), 1.25 mg/kg HTI-286 (squares), or 60 mg/kg paclitaxel (triangles). Vehicle and drugs were administered weekly for three cycles. Points, mean of tumor size (mg) at each time point; bars, SD. *, $P < 0.01$, significant difference between treatments versus vehicle is indicated at the end of the treatment periods.

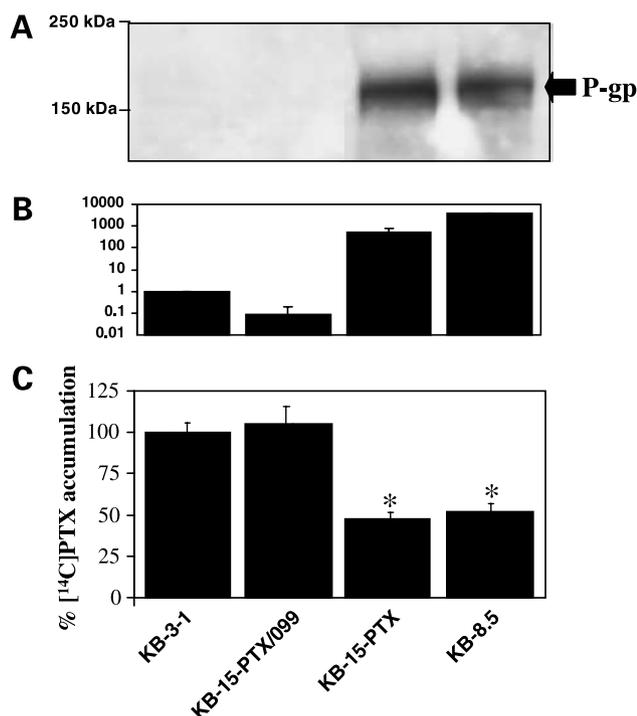


Figure 3. Analysis of P-glycoprotein levels and accumulation of paclitaxel in KB-3-1 parental and resistant cell lines. **A**, the indicated cell lines were analyzed for levels of P-glycoprotein (*P-gp*). Protein levels were determined by SDS-PAGE/immunoblot analyses. Positive control cell lines are included for P-glycoprotein expression (KB-15-PTX and KB-8.5). **B**, the cell lines were analyzed for mRNA expression by quantitative reverse transcription-PCR, with relative ratio of P-glycoprotein/actin set to 1 for KB-3-1 cells. **C**, the indicated cell lines were incubated for 2 h with [¹⁴C]paclitaxel ([¹⁴C]PTX) and levels of radioactive drug remaining in cells quantified by liquid scintillation counting. Representative experiment. Columns, percentage of drug remaining in resistant cells compared with parental cells (KB-3-1) set at 100%; bars, SD. *, $P < 0.01$, significant difference from KB-3-1.

Microtubule Stability in KB-15-PTX/099 Cells

Because KB-15-PTX/099 cells were resistant to several microtubule-stabilizing agents and concomitantly sensitive to destabilizing agents, we wanted to test whether the tubulin dynamics in the resistant cells had changed. The tubulin in microtubules (pellet fraction, *P*) was measured after separating it from free tubulin in the cytosol (supernatant fraction, *S*). KB-15-PTX/099 cells have lower levels of tubulin in microtubules compared with parental KB-3-1 cells when cells are grown in the absence of paclitaxel (Fig. 5A). This suggests that KB-15-PTX/099 cells have less stable microtubules. When the cells were grown in 15 nmol/L paclitaxel, it increased the tubulin in microtubules to 91% and 22% in KB-3-1 and KB-15-PTX/099 cells, respectively.

Tubulin that is post-translationally modified by acetylation in microtubules can be extremely long-lived (35). Thus, acetylated microtubules represent stable microtubules (36). Because KB-15-PTX/099 cells showed less tubulin in microtubules, they should also have less acetylated tubulin. We measured the acetylated microtubules in lysates of

KB-15-PTX/099 and KB-3-1 cells using an acetylated tubulin-specific antibody (Fig. 5B). The results show that KB-15-PTX/099 cells have lower levels of acetylated tubulin than parental KB-3-1 cells. Combined with the reduced amount of microtubules found in resistant cells, these data suggest that KB-15-PTX/099 cells have less stable microtubules. Refer also to the Supporting Information for a structural interpretation of this phenomenon.

Discussion

Selection of paclitaxel-resistant cells in the presence of a P-glycoprotein antagonist was undertaken to identify mechanisms of resistance other than P-glycoprotein overexpression. Paclitaxel resistance in KB-15-PTX/099 cells is not likely due to any ABC transporters (7, 8, 10) because no reduced accumulation of paclitaxel was observed in these cells. Moreover, because the cells are partially dependent on paclitaxel for growth, having a pump to transport the drug out of the cell would be deleterious for their growth. Additionally, transcriptional analysis of KB-15-PTX/099 cells did not show any ABC transporter mRNA overexpressed (data not shown), although this analysis might not have identified all known members of the ABC family of transporters and their mutational variants.

Induced Mutation and Resistance

Multiple data suggest that an alteration in tubulin is associated with paclitaxel resistance in KB-15-PTX/099 cells. These cells (*a*) are resistant to paclitaxel and cross-resistant to other tubulin polymerizing agents, yet are collaterally sensitive to microtubule depolymerizing agents; (*b*) are partially growth-dependent on paclitaxel or other tubulin polymerizing drugs; (*c*) contain a mutation in β -tubulin protein that substitutes glutamate for aspartate at position 26; and (*d*) have less stable microtubules compared with parental cells. Other point mutations in tubulin have been reported in cells selected for resistance to paclitaxel (in the presence of a P-glycoprotein inhibitor) or epothilones (6). Some of these mutations reside in contact sites for taxanes and epothilones (16), although in these cases, changes in microtubule stability are not observed nor

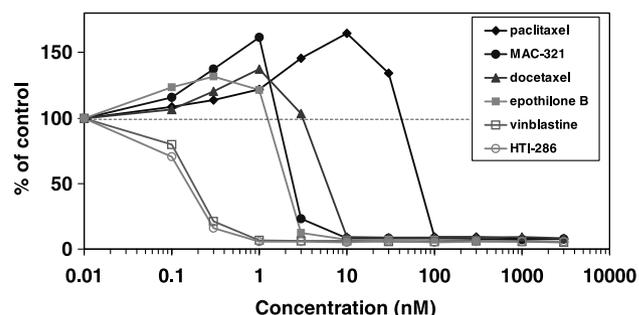
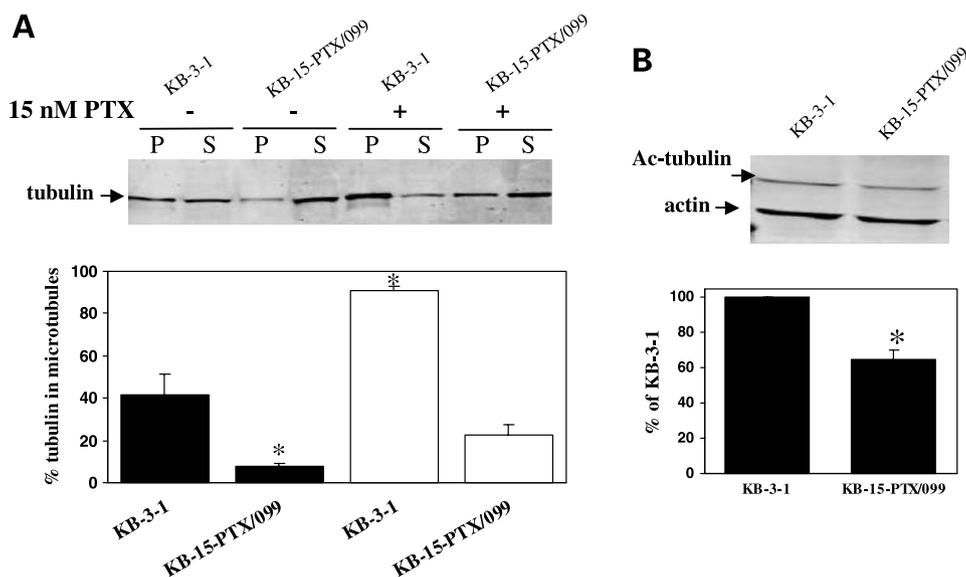


Figure 4. Dependence of KB-15-PTX/099 cells on microtubule-stabilizing agents. KB-15-PTX/099 cells were grown in the indicated tubulin agents, and growth was measured after 3 d. One hundred percent growth of untreated cells (dotted line). Microtubule-stabilizing agents (closed symbols). Microtubule-destabilizing agents (open symbols).

Figure 5. Comparison of microtubule stability in parental and paclitaxel resistant cells. **A**, cells were grown in the presence (*white columns*) or absence (*black columns*) of 15 nmol/L paclitaxel, and the amount of tubulin in microtubules was measured as described in Materials and Methods. The intensity of the tubulin band in the immunoblot was quantified. *Columns*, average of three measurements; *bars*, SD. *, $P < 0.01$, significant difference between indicated cell line and treatments versus KB-3-1 in the absence of paclitaxel. **B**, cells were grown in the absence of paclitaxel, and the amount of acetylated tubulin was measured by immunoblotting with an acetylated tubulin-specific antibody. *Columns*, average percentage of acetylated-tubulin from three experiments normalized to actin and KB-3-1 set to 100%; *bars*, SE. *, $P < 0.05$.



is there sensitivity to depolymerizing agents. In contrast, paclitaxel-resistant cells that have mutations outside the contact site typically have changes in microtubule stability and are collaterally sensitive to depolymerizing agents (17). The mutation in β -tubulin Asp²⁶Glu is unique because it seems to be in the contact site for paclitaxel but is also associated with a change in microtubule stability.

In support of the important role of Asp²⁶ in paclitaxel binding and tubulin function, a β -tubulin mutation converting Asp²⁶ to glutamate was also recently reported in paclitaxel-resistant Chinese hamster ovary cells (37). The exogenous expression of Asp²⁶Glu β -tubulin in a wild-type background conferred resistance to paclitaxel and sensitivity to colcemid and vinblastine. Expression of this mutant also caused decreased microtubule assembly as measured by tubulin in microtubules⁴ as well as acetylated tubulin. These data argue for a previously described model (38), in which a paclitaxel resistance mutation shifts the equilibrium from microtubules to soluble tubulin, thereby requiring paclitaxel for normal functioning of the microtubule cytoskeleton. According to this model, the destabilizing mutation will confer relative resistance to tubulin-polymerizing agents and sensitivity to depolymerizing agents. The phenotype of KB-15-PTX/099 cells is consistent with this model.

Importantly, the only tubulin that is expressed in KB-15-PTX/099 cells is mutant tubulin because no wild-type sequence is detectable in the mRNA sequence analysis and Edman degradation of purified β -tubulin. When cells are removed from drug for several passages, wild-type mRNA is also expressed (data not shown), suggesting that suppression of wild-type protein under selection pressure could also contribute to high levels of resistance. Reappearance of wild-type tubulin could also be explained by

the selection of revertants because cells bearing the Asp²⁶Glu mutation depend on paclitaxel for growth and may not survive extended passage without the drug.

Asp²⁶ Mutation

The mutation of Asp²⁶ to glutamate in β -tubulin detected in KB-15-PTX/099 cells is likely to reside in a contact site for paclitaxel based on biochemical and structural studies. First, based on an electron crystallography model using zinc induced sheets of tubulin (see Fig. 6), the phenyl ring of C-3' NHCO-C₆H₅ of paclitaxel makes contact with Asp²⁶ and near neighbor Val²³ on helix H1 of β -tubulin (33, 39). In both published models (33, 39), the Asp²⁶ (CCO₂) to NHCO-C₆H₅ heavy atom distances range from 3.6 to 4.7 Å, eliminating the possibility of an intervening water molecule. The Val²³ to C-3' phenyl distances are similar (3.5–4.3 Å). Second, a photoaffinity analogue of paclitaxel that contains the photoactive azido group on the C-3' NHCO-C₆H₅ ring of paclitaxel binds within residues 1 to 31 of β -tubulin (40). Third, when the C-3' NHCO-C₆H₅ of paclitaxel is replaced by a *tert*-butyl group in both docetaxel and MAC-321, these taxane analogues show 4-fold less resistance in KB-15-PTX/099 cells compared with paclitaxel. Fourth, in the recently published model for the binding of epothilone A to tubulin (41), the closest heavy atom contact between Asp²⁶ and the ligand is 6.7 Å, supporting the reduced fold resistance shown by epothilone B in KB-15-PTX/099 cells. Fifth, in yeast β -tubulin, Asp²⁶ is substituted with glycine. Epothilone A and B, but not paclitaxel, promote the *in vitro* polymerization of yeast tubulins (42). Because KB-15-PTX/099 cells preferentially express mutant β -tubulin, altered affinity to the drug could significantly contribute to the resistance profile. This has been observed in 1A9 cells made resistant to paclitaxel that harbor the mutation Phe²⁷⁰Val or Ala³⁶⁴Thr, where mutant tubulin mRNA is preferentially expressed. The paclitaxel-induced *in vitro* polymerization of tubulin is impaired (15) probably due to lack of paclitaxel binding.

⁴ F. Cabral, personal communication.

Binding Site Models for Paclitaxel, Docetaxel, and MAC-321

Figure 6 shows paclitaxel in wild-type tubulin from the computationally refined electron crystallographic structure (39), a pose we use as a standard for comparison. To the left, the C-3' phenyl is directed behind the plane of the paper towards the beta sheet strands B9-B10, whereas the benzamido phenyl is in front of it near the end of helix H1. The C-3' benzamido phenyl and the C-2 benzoyl phenyl straddle His²²⁷ on H7 at the bottom of the frame. To the left, the CH₂ and CO₂ moieties of Asp²⁶ fall near the benzamido group, whereas Val²³ on H1 is in hydrophobic contact with both the benzamido and C-3' phenyls (41). The terminal carboxylate of the Asp²⁶ side chain makes no hydrogen bonding interactions with the paclitaxel molecule, but the electron crystallographic complex suggests that there are favorable electrostatics with the polar groups at C-2' and C-3' on the paclitaxel C-13 side chain. For the paclitaxel-tubulin system, the long range electrostatics from Asp²⁶ to C-2' and C-3' are retained upon optimization. However, the docetaxel-tubulin model displays a hydrogen bond from one of the aspartate carboxy oxygens to the C-3' benzamido N-H as illustrated by Fig. 7A in accord with the 3-fold potency advantage for docetaxel. The figure likewise provides a reason for the difference. Although one of the *Ortho* methyl groups of docetaxel encounters the Asp²⁶ CH₂ center at the van der Waals radii sum limit (3.5 Å) and the carbamate oxygen avoids it altogether, both *ortho* and *meta* protons of the phenyl ring in paclitaxel reside at the 3.5 Å distance. The latter contacts prevent the benzamido N-H in paclitaxel from forming a hydrogen bond with the aspartate carboxylate (2.8 versus 2.0 Å for docetaxel, Fig. 7A), leaving electrostatics alone to anchor the N-H of paclitaxel in the protein.

To examine the effect of the Asp²⁶Glu mutation, we adopted a strategy that initially examined the different rotamers of the glutamate residue in the mutant in the energy-optimized models. There were eight possible conformers; however, the R2 rotamer provided the most informative model (refer to Supplementary Methods for discussion of other conformers).³ An overlay of the optimized complexes of docetaxel in the aspartate and glutamate binding sites (Fig. 8A) illustrates that the *Ortho* Bu group can achieve an

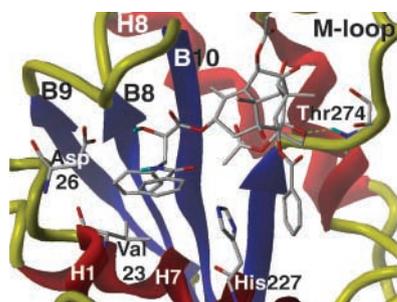


Figure 6. Paclitaxel ("T-Taxol") docked and optimized in β -tubulin. Model (from ref. 39) shows several protein side chains interacting with the bound ligand.

interaction geometry similar to that seen in the wild type, including hydrogen bonding between the Glu²⁶ carboxylate and the C-3' amide N-H. By contrast, Fig. 8B shows that the steric bulk of paclitaxel's C-3' benzamido phenyl, which prevented hydrogen bonding to Asp²⁶ in the wild type binding site, is responsible for a significant and deleterious ligand translation upon mutation to glutamate. For example, not only is the paclitaxel benzamido group shifted right in the figure, but the important C-2 side chain experiences a 1.8 Å root mean square displacement from its initial position.⁵ These movements cause the entire paclitaxel molecule (Fig. 8B, *yellow*) to be displaced upward and somewhat out of the binding pocket. This is consistent with the observed resistance to paclitaxel in KB-15-PTX/099 cells.

The situation for MAC-321 is very similar to that for docetaxel as depicted in Fig. 8A. However, an important difference for MAC-321 is the furan substituent at C-3'. Although there is no additional H-bonding, the furan ring possesses two edges, a polar and a hydrophobic one. As placed in the binding site, MAC-321's furan oxygen is directed toward the polar side of the pocket, whereas the ring CH=CH carbons are oriented toward the hydrophobic side. Because the C-3' phenyl ring of docetaxel has no similar orientational options, it would seem that the furan analogue possesses a unique feature that most likely adds an electrostatic component to its tubulin binding.

Analysis of nonbonded interactions in the separate β -tubulin models of paclitaxel, docetaxel, and MAC-321 implies that relative binding affinities to the Asp²⁶Glu mutant in the KB-15-PTX/099 cell line are paclitaxel < docetaxel \leq MAC-321. Careful thermodynamic studies show coupling between ligand binding and microtubule elongation for both paclitaxel and docetaxel (43, 44). If this is the case, then why do the compounds uniformly exhibit an increase in IC₅₀ in the mutant cell lines relative to wild type (Table 1)? In general, tighter binding drugs should increase rather than decrease cell kill, assuming that the only change in assay and cell conditions is the Asp²⁶Glu mutation. The puzzle is resolved in the observation that the mutant bearing cell line also has a reduced ratio of microtubules to free tubulin dimer relative to wild-type cells (refer to Fig. 5A). This means that a considerably higher concentration of drug is necessary to bring the microtubule level to that observed in wild type and then promote sufficient polymer stabilization to interfere with mitotic function. In this context, the tighter binders should be more effective in spite of the overall reduced efficiency from wild type to mutant. The relative resistance values of Table 1 are in complete accord with this interpretation.

Clinical Consequences

The basis for resistance to paclitaxel in the clinic is not well understood. Correlations with P-glycoprotein expression or resensitization of tumors that may overexpress P-glycoprotein with reversal agents remain poor (11). On

⁵ The RMSD of atomic position was calculated with a custom script provided by Dr. Michael Dolan of Tripos, Inc.

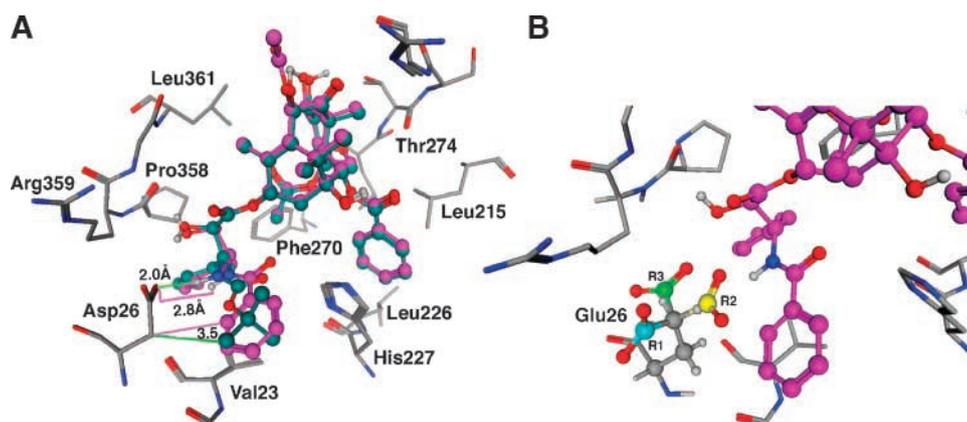


Figure 7. Model of paclitaxel and docetaxel in wild-type and mutant tubulin. Amber94/MOE energy optimized models of paclitaxel (*magenta*) and docetaxel (*teal*) in the binding site of wild-type and mutant β -tubulin; protein carbons (*white*). **A**, in the Asp²⁶ wild type, the protein atoms of each model are almost completely superimposable, with only a small displacement of His²²⁷ ($<0.1\text{\AA}$). The hydrophobic C-3' NHR end group of each ligand packs against the Van der Waal surfaces of both the Asp²⁶ CH₂ (heavy atom distances, 3.5 \AA) and the Val²³ side chains. The OtBu of docetaxel allows positioning of its C-3' N-H within hydrogen bonding distance (2.0 \AA) of the Asp²⁶ carboxylate, whereas the additional bulk of the paclitaxel phenyl keeps its N-H $\geq 2.8\text{\AA}$ away from the carboxylate. The corresponding reduction in noncovalent attraction may account for the lower activity of paclitaxel in wild-type systems. **B**, the Glu²⁶ mutants were derived from the wild-type models and potential rotamer positions analyzed (only paclitaxel shown). Three possible rotamer states (R1–R3) were identified for each ligand complex [cf. text and Table S1 (available at <http://mct.aacrjournals.org>)] to provide starting structures for further force field optimization.

the other hand, although initial reports of mutations in β -tubulin were shown to be associated with poor response to paclitaxel treatment in non-small cell lung cancer, they have been refuted by several investigators (45). Therefore, the clinical relevance of these data remains in doubt. The mutant line KB-15-PTX/099 when implanted as xenografts in athymic mice is completely unresponsive to paclitaxel given at the maximum tolerated dose on an optimal schedule, whereas the parental cells are completely inhibited by the same therapy. We have yet to examine the *in vivo* response of lower-level resistant cells that may contain tubulin mutations. Perhaps in the clinic, a more subtle condition exists, where low-level expression of a mutant tubulin and/or small changes in microtubule stability occur, and is enough to mediate resistance but has yet to be distinguished from

expression of predominantly wild-type tubulin with normal stability properties.

In summary, the KB-15-PTX/099 cell line, selected in increasing concentrations of paclitaxel in the presence of a P-glycoprotein reversal agent, has a point mutation in β -tubulin. The Asp²⁶Glu point mutation is at the drug binding site and is associated with a change in the stability of microtubules. The decreased stability of the microtubules requires drugs with increased affinity to maintain efficacy. The paclitaxel, docetaxel, and MAC-321 tubulin models described herein help to support a molecular mechanism underpinning the patterns of relative resistance in the KB-15-PTX/099 cell line. This is the first reported mutation that implicates both the effect of drug binding and microtubule stability changes in paclitaxel resistance.

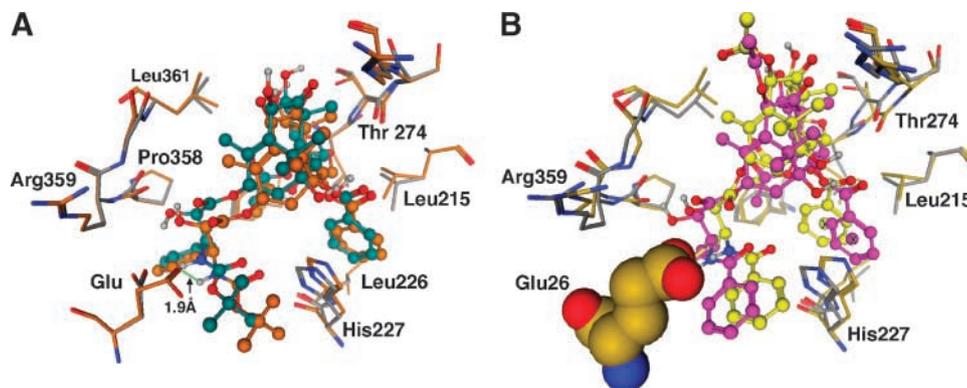


Figure 8. Overlay comparisons of wild-type versus mutant complexes of paclitaxel and docetaxel (Glu²⁶/rotamer R2). Protein carbons (*white*). **A**, docetaxel: wild type (*teal*), Glu²⁶ (*orange*). Optimization of the glutamate/R2 complex results in packing of the OtBu group between the tubulin residues Val²³ (data not shown; cf. Fig. 7) and His²²⁷. The orange ligand shows an AspCO₂-HN hydrogen bond while maintaining the locations of the baccatin core and the attached C-2 side chain. **B**, paclitaxel: wild type (*magenta*), Glu²⁶ (*yellow*). Glu26 (shown as Van der Waal surface) causes extensive rearrangement of the paclitaxel complex. The drug experiences a 1.8 \AA root mean square displacement of the C-2 heavy atom positions relative to the wild-type complex and moves partway out of the binding pocket.

Acknowledgments

We thank Dr. Thomas S. Rush III for critical review of the article.

References

- Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. *Nature* 1979;277:665–7.
- Rowinsky EK, Tolcher AW. Antimicrotubule agents. In: Devita Jr. VT, Hellman S, Rosenberg SA, editors. *Cancer principles and practice*, 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2001. p. 431–52.
- Sampath D, Discafani CM, Loganzo F, et al. MAC-321, A novel taxane with greater efficacy than paclitaxel and docetaxel *in vitro* and *in vivo*. *Mol Cancer Thera* 2003;2:873–994.
- Cabral F. Factors determining cellular mechanisms of resistance to antimitotic drugs. *Drug Resist Updat* 2000;3:1–4.
- Dumontet C. Mechanisms of action and resistance to tubulin-binding agents. *Expert Opin Investig Drugs* 2000;9:779–88.
- Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of taxol resistance related to microtubules. *Oncogene* 2003;22:7280–95.
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58.
- Childs S, Yeh RL, Hui D, Ling V. Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. *Cancer Res* 1998;58:4160–7.
- Lecureur V, Sun D, Hargrove P, et al. Cloning and expression of murine sister of P-glycoprotein reveals a more discriminating transporter than MDR1/P-glycoprotein. *Mol Pharmacol* 2000;57:24–35.
- Hopper-Borge E, Chen ZS, Shchavaleva I, Belinsky MG, Kruh GD. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. *Cancer Res* 2004;64:4927–30.
- Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med* 2002;53:615–27.
- Kavallaris M, Kuo DY, Burkhart CA, et al. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. *J Clin Invest* 1997;100:1282–93.
- Ranganathan S, Benetatos CA, Colarusso PJ, Dexter DW, Hudes GR. Altered beta-tubulin isotype expression in paclitaxel-resistant human prostate carcinoma cells. *Br J Cancer* 1998;77:562–6.
- Martello LA, Verdier-Pinard P, Shen HJ, et al. Elevated levels of microtubule destabilizing factors in a Taxol-resistant/dependent A549 cell line with an alpha-tubulin mutation. *Cancer Res* 2003;63:1207–13.
- Giannakakou P, Sackett DL, Kang YK, et al. Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. *J Biol Chem* 1997;272:17118–25.
- Giannakakou P, Gussio R, Nogales E, et al. 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* 2000;97:2904–9.
- He L, Yang C-PH, Horwitz SB. Mutations in beta-tubulin map to domains involved in regulation of microtubule stability in epothilone-resistant cell lines. *Mol Cancer Ther* 2001;1:3–10.
- Gonzalez-Garay ML, Chang L, Blade K, Menick DR, Cabral F. A beta-tubulin leucine cluster involved in microtubule assembly and paclitaxel resistance. *J Biol Chem* 1999;274:23875–82.
- Bhattacharya R, Cabral F. A ubiquitous beta-tubulin disrupts microtubule assembly and inhibits cell proliferation. *Mol Biol Cell* 2004;15:3123–31.
- Hari M, Yang H, Zeng C, Canizales M, Cabral F. Expression of class III beta-tubulin reduces microtubule assembly and confers resistance to paclitaxel. *Cell Motil Cytoskeleton* 2003;56:45–56.
- Blade K, Menick DR, Cabral F. Overexpression of class I, II or IVb beta-tubulin isotypes in CHO cells is insufficient to confer resistance to paclitaxel. *J Cell Sci* 1999;112:2213–21.
- Poruchynsky MS, Giannakakou P, Ward Y, et al. Accompanying protein alterations in malignant cells with a microtubule-polymerizing drug-resistance phenotype and a primary resistance mechanism. *Biochem Pharmacol* 2001;62:1469–80.
- Dumontet C, Duran GE, Steger KA, Beketic-Oreskovic L, Sikic BI. Resistance mechanisms in human sarcoma mutants derived by single-step exposure to paclitaxel (Taxol). *Cancer Res* 1996;56:1091–7.
- Greenberger LM, Collins KI, Annable T, et al. alpha-(3,4-dimethoxyphenyl)-3,4-dihydro-6,7-dimethoxy-alpha-[(4-methylphenyl)thio]-2(1H)-isoquinolineheptanenitrile (CL 329,753): a novel chemosensitizing agent for P-glycoprotein-mediated resistance with improved biological properties compared with verapamil and cyclosporine A. *Oncol Res* 1996;8:207–18.
- Schibler MJ, Barlow SB, Cabral F. Elimination of permeability mutants from selections for drug resistance in mammalian cells. *FASEB J* 1989;3:163–8.
- Jaffrezou JP, Dumontet C, Derry WB, et al. Novel mechanism of resistance to paclitaxel (Taxol) in human K562 leukemia cells by combined selection with PSC 833. *Oncol Res* 1995;7:517–27.
- Loganzo F, Discafani CM, Annable T, et al. HTI-286, a synthetic analogue of the tripeptide hemiasterlin, is a potent antimicrotubule agent that circumvents P-glycoprotein-mediated resistance *in vitro* and *in vivo*. *Cancer Res* 2003;63:1838–45.
- Loganzo F, Hari M, Annable T, et al. Cells resistant to HTI-286 do not overexpress P-glycoprotein but have reduced drug accumulation and a point mutation in alpha-tubulin. *Mol Cancer Ther* 2004;3:1319–27.
- Verdier-Pinard P, Wang F, Martello L, Burd B, Orr GA, Horwitz SB. Analysis of tubulin isotypes and mutations from taxol-resistant cells by combined isoelectrofocusing and mass spectrometry. *Biochemistry* 2003;42:5349–57.
- Best D, Warr PJ, Gull K. Influence of the composition of commercial sodium dodecyl sulfate preparations on the separation of alpha- and beta-tubulin during polyacrylamide gel electrophoresis. *Anal Biochem* 1981;114:281–4.
- Hari M, Wang Y, Veeraraghavan S, Cabral F. Mutations in alpha- and beta-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine. *Mol Cancer Ther* 2003;2:597–605.
- Minotti AM, Barlow SB, Cabral F. Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. *J Biol Chem* 1991;266:3987–94.
- Lowe J, Li H, Downing KH, Nogales E. Refined structure of alpha beta-tubulin at 3.5 Å resolution. *J Mol Biol* 2001;313:1045–57.
- Nogales E, Wolf SG, Downing KH. Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* 1998;391:199–203.
- Webster DR, Borisy GG. Microtubules are acetylated in domains that turn over slowly. *J Cell Sci* 1989;92:57–65.
- Palazzo A, Ackerman B, Gundersen GG. Cell biology: tubulin acetylation and cell motility. *Nature* 2003;421:230.
- Wang Y, Veeraraghavan S, Cabral F. Intra-allelic suppression of a mutation that stabilizes microtubules and confers resistance to colcemid. *Biochemistry* 2004;43:8965–73.
- Cabral FR, Brady RC, Schibler MJ. A mechanism of cellular resistance to drugs that interfere with microtubule assembly. *Ann N Y Acad Sci* 1986;466:745–56.
- Snyder JP, Nettles JH, Cornett B, Downing KH, Nogales E. The binding conformation of Taxol in beta-tubulin: a model based on electron crystallographic density. *Proc Natl Acad Sci U S A* 2001;98:5312–6.
- Rao S, Krauss NE, Heering JM, et al. 3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin. *J Biol Chem* 1994;269:3132–4.
- Nettles JH, Li H, Cornett B, Krahn JM, Snyder JP, Downing KH. The binding mode of epothilone A on alpha,beta-tubulin by electron crystallography. *Science* 2004;305:866–9.
- Bode CJ, Gupta ML, Jr., Reiff EA, Suprenant KA, Georg GI, Himes RH. Epothilone and paclitaxel: unexpected differences in promoting the assembly and stabilization of yeast microtubules. *Biochemistry* 2002;41:3870–4.
- Diaz JF, Menendez M, Andreu JM. Thermodynamics of ligand-induced assembly of tubulin. *Biochemistry* 1993;32:10067–77.
- Diaz JF, Andreu JM. Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: reversibility, ligand stoichiometry, and competition. *Biochemistry* 1993;32:2747–55.
- Kelley MJ, Li S, Harpole DH. Genetic analysis of the beta-tubulin gene, TUBB, in non-small-cell lung cancer. *J Natl Cancer Inst* 2001;93:1886–8.

Molecular Cancer Therapeutics

Paclitaxel-resistant cells have a mutation in the paclitaxel-binding region of β -tubulin (Asp²⁶Glu) and less stable microtubules

Malathi Hari, Frank Loganzo, Tami Annable, et al.

Mol Cancer Ther 2006;5:270-278.

Updated version	Access the most recent version of this article at: http://mct.aacrjournals.org/content/5/2/270
Supplementary Material	Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2006/03/01/5.2.270.DC1

Cited articles	This article cites 43 articles, 18 of which you can access for free at: http://mct.aacrjournals.org/content/5/2/270.full#ref-list-1
Citing articles	This article has been cited by 18 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/5/2/270.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/5/2/270 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.