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Mechanisms of Taxol resistance related to microtubules

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Since its approval by the FDA in 1992 for the treatment of ovarian cancer, the use of Taxol has dramatically increased. Although treatment with Taxol has led to improvement in the duration and quality of life for some cancer patients, the majority eventually develop progressive disease after initially responding to Taxol treatment. Drug resistance represents a major obstacle to improving the overall response and survival of cancer patients. This review focuses on mechanisms of Taxol resistance that occur directly at the microtubule, such as mutations, tubulin isotype selection and post-translational modifications, and also at the level of regulatory proteins. A review of tubulin structure, microtubule dynamics, the mechanism of action of Taxol and its binding site on the microtubule are included, so that the reader can evaluate Taxol resistance in context.
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Introduction

The microtubule cytoskeleton is an effective and validated target for cancer chemotherapeutic drugs. A diverse range of structurally dissimilar compounds can interact with the tubulin/microtubule system and function as antimitotic agents. These antimitotic agents can be divided into two major classes: those that bind preferentially to α/β-tubulin heterodimers and inhibit polymer assembly, and those with a binding site on the polymer that stabilizes microtubules. The first class is exemplified by the vinca alkaloids. The prototypic microtubule-stabilizing drug is Taxol (Figure 1). More recently, other mechanistically similar but structurally unrelated natural products, including the epothilones, eleutherobin and discodermolide, have been developed, and are in various stages of preclinical/clinical development.

As with many cancer therapeutic agents, resistance remains a significant problem when using Taxol to treat malignancies. Chemotherapeutic failure may be related either to the tumor being inherently resistant to the drug and/or to the acquisition of resistance during treatment. Although Taxol has demonstrated antitumor activity against several cancers, the emergence of clinical drug resistance is a major limitation to its success. Resistance is often a multifactorial process that may originate through a series of modifications. In the case of Taxol, several potential mechanisms can be proposed to account for the resistance observed in human tumors and tumor cell lines. These include overexpression of the multidrug transporter P-glycoprotein (Gottesman, 2002), altered metabolism of the drug, decreased sensitivity to death-inducing stimuli (Blagosklonny and Fojo, 1999), alterations in microtubule dynamics and altered binding of Taxol to its cellular target, the microtubule (Dumontet and Sikic, 1999; Drukman and Kavallaris, 2002). This review will focus exclusively on potential mechanisms of resistance at the level of the microtubule.

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Structure and function of the microtubule cytoskeleton

Microtubule dynamics and function

In eucaryotes, microtubules are involved in a diverse range of cellular functions including mitosis and meiosis, motility, maintenance of cell shape and intracellular trafficking of macromolecules and organelles (Desai and Mitchison, 1997; Oakley, 2000; Sharp *et al*., 2000). Microtubules are hollow cylindrical tubes formed primarily by the self-association of α,β-tubulin heterodimers into polymers (Downing and Nogales, 1998; Nogales, 2000). The tubulin heterodimers (Figure 2) are associated in a head-to-tail fashion to form protofilaments, which associate in a lateral manner to form hollow microtubules. There is considerable flexibility in the number of protofilaments within a microtubule. *In vivo*, the cylinder is usually composed of 13 protofilaments with an overall diameter of 25 nm. After *in vitro* assembly of bovine brain tubulin, the number of protofilaments is usually 14, but can vary from 10 to 15. Since the lateral associations between protofilaments involve interactions between subunits of the same type, that is, the so-called B-type lattice, the protofilaments are arranged in a parallel array, thereby imparting polarity to the structure. Consequently, the β-chains of the tubulin dimer are exposed at one end (plus) of the polymer, and the α-chains at the other end (minus). In cells, microtubules are usually organized with their minus ends associated with the microtubule-organizing center (MTOC) near the nucleus, and radiate outward so that the plus ends are near the periphery of the cell. γ-Tubulin, a protein highly homologous to the α/β-tubulins, is

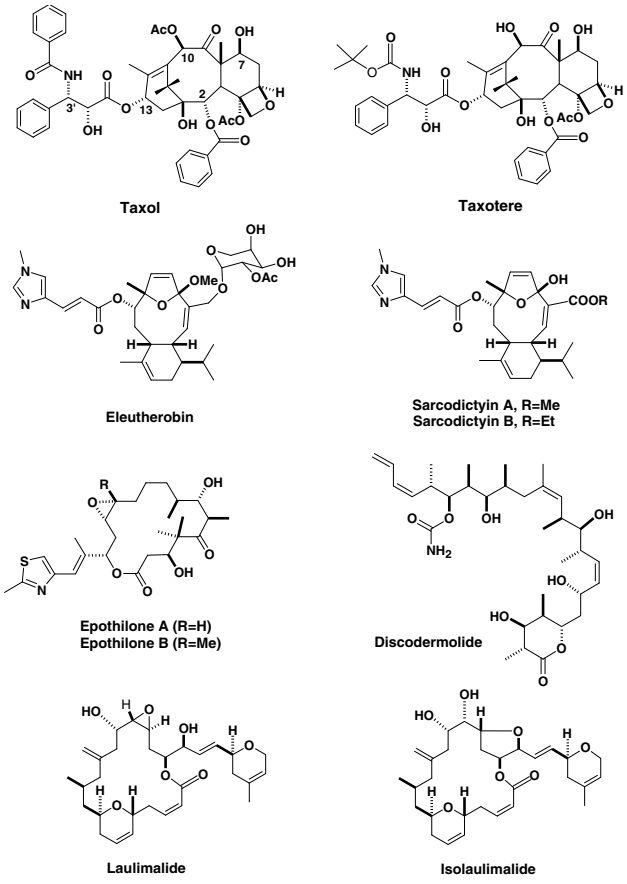

Figure 1 Structures of microtubule-stabilizing agents

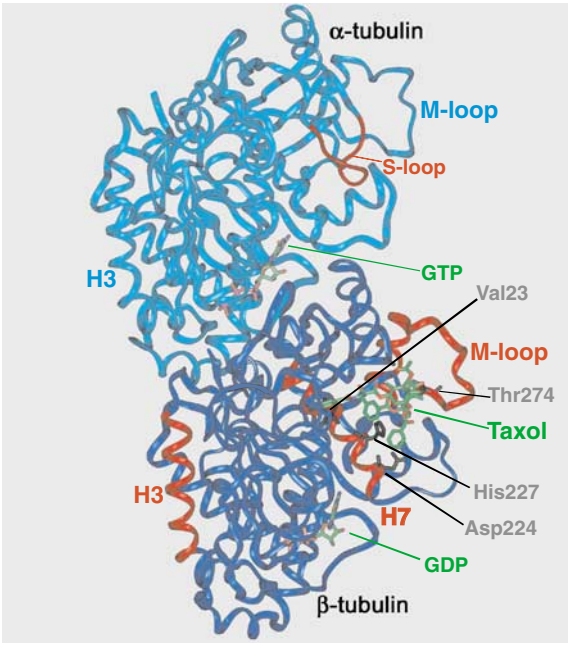

Figure 2 Three-dimensional model of α/β-tubulin heterodimer. Domains of β-tubulin that are discussed in the text, as well as the stabilizing loop (S-loop) unique to α-tubulin, are colored in red. Helix 3 (H3) and microtubule loop (M-loop) in α-tubulin are labeled in blue. Amino-acid residues close to Taxol are indicated in gray

localized at the MTOC, and plays an important role in microtubule nucleation by interacting with α-tubulin (Oakley, 2000).

Microtubules are highly dynamic, and exhibit a nonequilibrium behavior termed dynamic instability (Desai and Mitchison, 1997). In this process, microtubules undergo rapid stochastic transitions between growth and shrinkage, due to the association and dissociation, respectively, of tubulin dimers from the microtubule ends. The transition from growing to shrinking is termed a catastrophe, whereas the reverse behavior is referred to as a rescue. The orchestration of this dynamic instability is related to GTP binding and hydrolysis at the exchangeable or E-site of β-tubulin. GTP binds to both α- and β-tubulin, but in the case of α-tubulin, GTP is found at the nonexchangeable or N-site (Figure 2). Microtubule assembly requires β-tubulin to be charged with GTP, which is hydrolysed upon addition of the tubulin dimer to the elongating microtubule. After hydrolysis, the guanine nucleotide becomes nonexchangeable, and so microtubules are mostly composed of (GTP:α-tubulin/GDP:β-tubulin)$_n$, with the growing end capped with GTP (or GDP$\cdot$P$_i$):β-tubulin. In the GTP-cap model, microtubules, which are inherently unstable, are stabilized by GTP (or GDP$\cdot$P$_i$)-tubulin at the growing ends. When the GTP cap is lost, the microtubules rapidly depolymerize, with the protofilaments peeling outward. After depolymerization, the released dimers can exchange GTP for GDP at the E-site, and are thus primed for another cycle of polymerization. In contrast, microtubules containing nonhydrolysable GTP analogs are significantly more stable.

Numerous proteins that interact with microtubules and/or free tubulin dimers also have the potential to regulate both catastrophe and rescue rates (Nogales, 2000). The best characterized of these regulatory proteins are the microtubule-associated proteins (MAPs), which stabilize microtubules by decreasing catastrophes and/or increasing rescues. However, other proteins, such as stathmin, may regulate microtubule dynamics by increasing the catastrophe rate. Stathmin appears to bind exclusively to tubulin dimers and not to microtubules. The activities of many of these microtubule-stabilizing/-destabilizing proteins are themselves regulated by phosphorylation/dephosphorylation in a cell cycle-dependent manner.

The tubulin sequence/structure contains the necessary information for self-assembly of tubulin dimers into protofilaments and microtubules. The α- and β-tubulins (each $\sim$450 amino acids), although highly conserved, display extensive molecular heterogeneity at their C-termini. This structural diversity is a consequence of both the expression of several α- and β-tubulin isotypes (Sullivan and Cleveland, 1986; Stanchi *et al*., 2000), the products of distinct genes, and of numerous post-translational modifications occurring to both subunits (MacRae, 1997; Luduena, 1998). These modifications include polyglutamylation and polyglycylation of both subunits, acetylation, reversible tyrosination and excision of the C-terminal glutamate in nontyrosinable α-tubulin and phosphorylation of the class III β-tubulin.

Significantly, the majority of primary sequence divergence in the various tubulin isotypes and all of the post-translational modifications, except acetylation of lys_{40} of α-tubulin, occur within the C-terminal 20 amino acids of α- and β-tubulin subunits. While these C-terminal regions are highly variable among the isotypes within a species, the same regions are highly conserved within a single isotype, among species as diverse as human, mouse and chicken. The highly divergent C-termini may provide a mechanism for isotype-specific MAP binding. Moreover, each β-tubulin isotype has a unique pattern of expression ranging from highly specific expression for classes III, IVa and VI to constitutive expression for classes I and IVb. While the class II β-tubulin is predominately expressed in the brain, this isotype is also expressed at low levels in a variety of other tissues. The tissue distribution of the α-tubulin isotypes is less well established, primarily due to the lack of isotype-specific antisera. We have recently shown by mass spectrometry that k-α1 and α6 are the predominant α-tubulin isotypes expressed in the human breast and lung carcinoma cell lines. The issue of functional specificity of the multiple tubulin isotypes remains unresolved and somewhat controversial. However, the C-terminal isotype sequence conservation and their differential tissue expression strongly imply functional significance.

Molecular structure of tubulin

Nogales *et al*. (1998, 1999) have obtained, by electron crystallography, a model of the α/β-tubulin dimer fitted to a 3.7 Å density map using zinc-induced tubulin sheets stabilized by Taxol. This model is supported by a 2.8 Å X-ray diffraction map of FtsZ, a bacterial GTP-binding protein with some homology (~10%) to tubulin (Lowe and Amos, 1998). FtsZ also has the propensity to form protofilaments and sheets. Although α- and β-tubulin monomers share only 40% sequence homology, their overall folding patterns are very similar (Figure 2). Recently, models with improved resolution have been published (Lowe *et al*., 2001; Meurer-Grob *et al*., 2001). Each monomer structure can be divided into three major structural domains (Figure 3). The N-terminal domain (residues 1–206) is involved in nucleotide binding, and has a Rossman fold with alternating parallel β-strands (S1–S6) and helices (H1–H6). The central domain (residues 207–384) is involved in both longitudinal/lateral contacts between α- and β-tubulin monomers present in protofilaments, and is formed by an arrangement of mixed β sheets (S7–S10) and three helices (H8–H10). Taxol binds to a hydrophobic pocket within this central domain (see below). The C-terminal domain is formed by two antiparallel helices (H11 and H12)

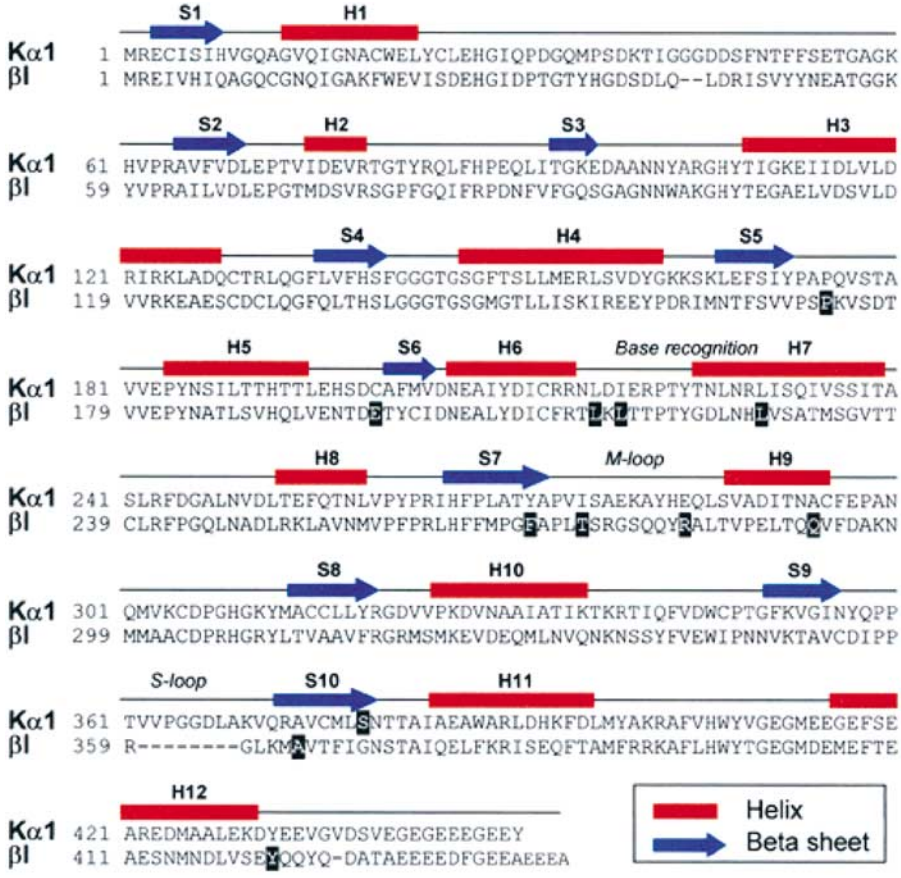

Figure 3 Primary sequences and secondary structure of the major human α- and β-tubulin isotypes. Helices (H1–H12) are represented as red rectangles and β-sheets (S1–S10) are represented as blue arrows. Mutations detected in Taxol-resistant cell lines are highlighted in black

that fold across the other two domains. However, the C-terminal 10 residues in α-tubulin and 18 residues in β-tubulin, which are highly charged, are not visible in this model. This C-terminal domain has been implicated in the binding of several regulatory and motor proteins including tau, MAP-2 and kinesin.

The inter- and intradimer contacts along the protofilaments were readily deduced, since the longitudinal dimer packing in zinc sheets and microtubules is the same. These longitudinal interactions are extensive, and are similar in both inter- and intradimer contacts. The model also provides a rationale for explaining the nonexchangeability/exchangeability of the guanine nucleotide-binding sites on each monomer. In the case of the N-site in α-tubulin, it is buried at the intradimer interface, thus accounting for the lack of exchange at this site. The guanine nucleotide at the E-site of β-tubulin, in contrast, is at the surface of the dimer allowing for exchange (Figure 2). After polymerization, the E-site becomes nonexchangeable, since it is buried at the interdimer interface.

A major difference between the two polymer types, that is, zinc sheets and microtubules, is the orientation of the protofilaments. In zinc sheets they are aligned antiparallel, whereas in microtubules they are parallel. This implies that the lateral contacts between protofilaments are different in the two polymer types. To visualize the lateral contacts in microtubules, the zinc sheet protofilament structure was docked into a 20 Å resolution map of microtubules obtained by cryoelectron microscopy. In the resulting model, the E-site of β-tubulin was exposed at the plus end of the microtubule and the N-site of α-tubulin at the minus end, in agreement with previous studies on the orientation of α/β-tubulin heterodimers in microtubules. The model of Nogales *et al*. suggests that the major lateral contacts between α–α and β–β monomers between protofilaments involve interactions between the microtubule loop (M-loop; residues 271–286) and the helix H3 and loop H1-S2 (Figure 2). The M-loop comprises part of the Taxol-binding site in β-tubulin (see below). Although the B-lattice is the predominant protofilament arrangement, many microtubules also contain a seam, in which the lateral contacts involve interactions between α- and β-tubulin monomers. Whether this seam plays a dynamic role in microtubule function is unclear.

In vitro *and* in vivo *mechanisms of taxol action*

Our research group was the first to examine the mechanism of action of Taxol, and although it became obvious that the drug was an antimitotic agent, it was also clear that Taxol was not a typical antimitotic drug, such as colchicine or the *vinca* alkaloids (Schiff *et al*., 1979; Schiff and Horwitz, 1980; Horwitz *et al*., 1986; Horwitz, 1992). These latter drugs bind primarily to tubulin dimers and prevent microtubule assembly. There is no evidence that Taxol can bind to the tubulin dimer. *In vitro*, Taxol binds to the microtubule polymer, enhancing the polymerization of tubulin (Parness and Horwitz, 1981; Manfredi *et al*., 1982). Microtubules formed in the presence of the drug possess unusual stability, and resist depolymerization by Ca^{2+}, cold temperature and dilution (Schiff *et al*., 1979). Taxol has the ability to polymerize tubulin in the absence of GTP, which under normal conditions is an absolute requirement for microtubule polymerization. The drug binds to the β-tubulin subunit in microtubules specifically and reversibly, with a stoichiometry, relative to the tubulin heterodimer, approaching one (Parness and Horwitz, 1981; Diaz and Andreu, 1993). Binding is reversible, since unlabeled Taxol can displace [^{3}H]Taxol from polymerized microtubules. *In vitro*, Taxol alters the kinetics of microtubule assembly. The overall effect of Taxol is to decrease the critical concentration of microtubule protein necessary for microtubule assembly. At a Taxol concentration of 5 μM, the critical concentration of tubulin required for assembly decreases by a factor of 20 from 0.2, to less than 0.01 mg/ml. Taxol also affects the structure of the microtubule polymer by reducing the number of protofilaments from a normal average of 13 to 12 (Diaz *et al*., 1998).

In cells, high concentrations of Taxol increase polymer mass, and also induce microtubule bundle formation in interphase cells, a phenomenon that has become a hallmark of Taxol binding (Schiff and Horwitz, 1980). However, microtubule bundle formation is a phenotypic consequence of Taxol binding that has a threshold effect. Therefore, at lower concentrations of Taxol, where only a fraction of the total Taxol-binding sites are occupied, the principal effect of the drug is suppression of microtubule dynamics without altering the polymer mass (Jordan *et al*., 1993; Derry *et al*., 1995). Interestingly, low concentrations of vinblastine, a microtubule-destabilizing drug, have similar effects on polymer dynamics as Taxol, suggesting that both drugs block mitosis by stabilizing spindle microtubule dynamics. However, we have shown recently that the two major classes of microtubule-based antimitotic agents, that is, the stabilizing and destabilizing drugs, exhibit different mitotic effects at low concentrations (Chen and Horwitz, 2002). Microtubule-stabilizing drugs, including Taxol, the epothilones and discodermolide, produced aneuploid populations of cells in the absence of a sustained mitotic block. In contrast, colchicine, vinblastine and nocodazole, all destabilizing drugs, did not induce aneuploidy at comparable concentrations. Exit from an aberrant mitosis appeared to be responsible for the aneuploidy, since multipolar spindles were induced by stabilizing, but not destabilizing, drugs. These studies imply that Taxol exerts its mitotic effects by alternate mechanisms, depending on the concentration of the drug utilized (Torres and Horwitz, 1998).

Taxol-binding site on the microtubule

In the absence of a high-resolution structure of tubulin, we used photoaffinity labeling to address the nature of the interaction between Taxol and its target protein (Rao *et al*., 1992, 1994, 1995; Orr *et al*., 1998; Rao *et al*., 1999, 2001). Initially, direct photoaffinity-labeling studies using [^{3}H]Taxol demonstrated that Taxol binds

specifically to the β-subunit of tubulin (Rao *et al*., 1992). However, the low extent of photoincorporation precluded a detailed analysis of the Taxol-binding site. The availability of a series of Taxol analogs, bearing photoreactive groups at defined positions around the taxane nucleus, afforded the opportunity to define the contact sites between the drug and β-tubulin. Photoaffinity labeling of microtubules using analogs with photoreactive groups at the C-2, C-3′ or C-7 positions also showed exclusive and specific photoincorporation into the β-tubulin monomer. By chemical/enzymatic digestion, and subsequent N-terminal amino-acid sequencing, it was possible to assign the residues in close proximity to the Taxol-binding site. Studies with [^{3}H]3′-(*p*-azidobenzamido)Taxol, where the arylazide was incorporated into the C-13 side chain, resulted in the isolation of a photolabeled peptide containing amino-acid residues 1–31 in β-tubulin (Rao *et al*., 1994). Studies with [^{3}H]2-(*m*-azidobenzoyl)Taxol, where the photoreactive group was attached to the B ring of the taxoid nucleus, demonstrated that a peptide containing amino-acid residues 217–233 of β-tubulin was involved in interacting with the 2-benzoyl group (Rao *et al*., 1995). Finally, when a benzophenone (BzDC) substituent was attached to the C-7 hydroxyl group of the C ring, specific photocrosslinking to Arg_{282} was observed (Rao *et al*., 1999).

In the electron crystallographic model obtained by Nogales and collaborators, the Taxotere-binding site is located at one side of the β-tubulin monomer that is believed to reside within the microtubule lumen. Although the tubulin model is derived from an unnatural polymer, there was excellent agreement between the binding site, as determined by photoaffinity labeling and electron crystallography. Using the three contact sites obtained through our photoaffinity-labeling studies, we proposed a model for the binding of Taxol to β-tubulin, based on the electron crystallographic model of α/β-tubulin (Rao *et al*., 1999). The composite model was developed using Taxol containing nitrenes at the *para* position of the C-3′ benzamido (labeling residues β 1–31) and the *meta* position of the C-2 benzoyl moieties (labeling residues β 217–233), and the BzDC group at the C-7 hydroxyl position (labeling β-Arg_{282}), and is based on the energy-minimized conformation of 7-BzDC Taxol, derived from the X-ray structure of Taxol. In this model, the nitrene on the C-3′ benzamido group is close to Val_{23}, in good agreement with the photoaffinity-labeling result, while the nitrene at the *meta* position of the C-2-benzoyl group fits into a pocket formed by the imidazole ring of His_{227} and the side chain of Asp_{224} (Figure 2). Finally, the photoreactive oxygen atom of the 7-BzDC group can be located at ~ 3 Å distance from the α-carbon of Arg_{282} in β-tubulin.

More recently, two additional models for the Taxol : microtubule interaction have been proposed. Based on data derived from a combination of fluorescence energy transfer (FRET) spectroscopy and solid-state rotational echo double-resonance (REDOR) NMR, Li *et al*. (2000) proposed an orientation for the microtubule-bound Taxol, which differs from that suggested by electron crystallography and photoaffinity-labeling studies. In this alternate model, Taxol is rotated 180° in its binding site compared to our model. An additional model proposed by Snyder *et al*. (2001) is based on docking individual conformers of Taxol, derived from X-ray crystal structures and NMR studies, into the experimental density map of the tubulin–Taxotere complex. In this model, the C-3′ benzamido and the 2-benzoyl groups are positioned as depicted in the earlier model of Rao *et al*. (1999). The C-7 hydroxyl group of Taxol is in close proximity to Thr_{274} (Figure 2).

The Taxol-binding site is close to the M-loop, which participates in lateral interactions with the H3 helix of the adjacent β-tubulin monomer in the microtubule (Figure 2). It has been proposed that Taxol-induced stabilization of microtubules is mediated via strengthening of lateral contacts between protofilaments, via a conformational change in the M-loop (Nogales, 2000). Our finding that 7-BzDC Taxol photoincorporates into Arg_{282} of the M loop may account for the unusual microtubule-binding properties of this Taxol analog. We observed that 7-BzDC Taxol did not promote tubulin polymerization; yet the analog can stabilize GTP-induced microtubules against cold-induced depolymerization (Rao *et al*., 1999). Based on our model of the 7-BzDC Taxol : tubulin interaction, it is likely that the analog can bind to small tubulin oligomers, but the presence of the bulky BzDC group in the vicinity of the M-loop prevents free tubulin dimers from associating with these stabilized nucleation centers. The M-loop has also been implicated in the unusual cold stability of Antarctic fish tubulin (Detrich *et al*., 2000). It has been suggested that two amino-acid substitutions within the M-loop of each α- and β-monomer (A278T/S287T in α and S280G/A285S in β) strengthens the lateral interactions between adjacent protofilaments by increasing the M-loop flexibility. It should be noted that the region of α-tubulin corresponding to the hydrophobic Taxol-binding pocket of β-tubulin is occupied by an eight-amino-acid loop, the S loop (residues 362–369, Figure 2) (Nogales *et al*., 1998, 1999; Downing, 2000; Nogales, 2000). It has been suggested that this segment of α-tubulin acts as an endogenous microtubule-stabilizing factor by promoting the lateral association between protofilaments. We had proposed, a number of years ago, that Taxol was a mimetic of a naturally occurring microtubule-stabilizing factor (Horwitz *et al*., 1986).

Binding site for non-taxane-based microtubule-stabilizing drugs

Several natural products, all with unique structures unrelated to that of Taxol, have been reported to have similar mechanisms of action as Taxol (Figure 1; He *et al*., 2001). Epothilones A and B, isolated from a Myxobacterium fermentation broth, were found to induce tubulin polymerization, arrest cells in mitosis and cause the formation of microtubule bundles (Bollag *et al*., 1995). Epothilone B was reported to be more potent than Taxol and epothilone A in promoting microtubule assembly *in vitro*. Discodermolide was

isolated from a marine sponge and reported to induce the assembly of microtubules *in vitro* more rapidly than Taxol, and to cause mitotic arrest and microtubule bundling (Hung *et al*., 1996; ter Haar *et al*., 1996; Kowalski *et al*., 1997). Interestingly, the combination of Taxol and discodermolide exhibited a synergistic cytotoxic interaction in human carcinoma cell lines (Martello *et al*., 2000). A fourth microtubule-stabilizing agent, eleutherobin, was isolated from a marine soft coral and shown to have activity comparable to that of Taxol (Long *et al*., 1998; Hamel *et al*., 1999). The epothilones, discodermolide and eleutherobin, are all competitive inhibitors of the binding of [^{3}H]-Taxol to microtubules, suggesting that these drugs interact at the same or an overlapping binding domain on β-tubulin (He *et al*., 2001). The laulimalides are another group of natural products that display microtubule-stabilizing activity (He *et al*., 2001). However, it appears that laulimalide binds at a site on the tubulin polymer that is distinct from the taxane-binding site (Pryor *et al*., 2002).

We discovered that 2-*m*-azido baccatin III, a Taxol analog lacking the C-13 side chain but with a *meta* azido benzoyl group at the C-2 position, possesses all of the activities that are characteristic of Taxol (He *et al*., 2000). Although not as active as Taxol, it does promote microtubule assembly in the absence of GTP, stabilizes microtubules and competitively inhibits the binding of [^{3}H]-Taxol to the microtubule protein. The observation that the C-13 side chain is not an absolute requirement for biological activity in a taxane molecule allowed us to propose a new common pharmacophore model between Taxol and epothilone (He *et al*., 2000). In this model, the thiazole side chain of epothilone corresponds to the C-2 side chain of 2-m-azido baccatin III, and binds in the pocket formed by His227 and Asp224. The macrolide ring system of the epothilones overlaps with the taxane ring system. This model of the epothilone : tubulin interaction is essentially equivalent to one of the two models proposed by Fojo's group, based on β-tubulin mutations identified in epothilone-resistant cells (Giannakakou *et al*., 2000).

Although Taxol does not promote the *in vitro* assembly of yeast tubulin, it has been recently demonstrated that the epothilones do (Bode *et al*., 2002). Comparison of the primary sequences of mammalian and yeast tubulins show sequence variations at several positions known to be important for Taxol binding. These include K19A, V23T and D26G substitutions in the N-terminal domain of β-tubulin, residues that make contact with the 3′-benzamidophenyl group of Taxol. In our proposed model (He *et al*., 2000), the epothilones do not make contact with the N-terminal domain of β-tubulin, potentially explaining their ability to interact with yeast tubulin.

Resistance to taxol in cell lines

Alterations in microtubule dynamics

Since the Taxol-binding site is present only on polymerized tubulin, and not on tubulin dimers, selection of a less stable polymer, that is, a polymer with increased microtubule dynamics, could potentially offer a survival advantage for a tumor challenged with a microtubule-stabilizing drug such as Taxol. Two potential models describing the relationship between resistance to microtubule-active drugs and cellular microtubule dynamics have been proposed. According to Cabral and coworkers, Taxol-resistant cell lines contain 'hypostable' microtubules in which the equilibrium between the dimer and polymer is shifted towards the former (Cabral *et al*., 1986; Cabral and Barlow, 1989; Minotti *et al*., 1991). As such, these cells will display increased resistance to polymer-binding drugs like Taxol, and increased sensitivity towards tubulin dimer-specific agents, such as vinblastine and colchicine. In addition, this model offers a potential explanation for the intriguing observation that some Taxol-resistant cell lines have an absolute requirement for low concentrations of Taxol for normal cell growth. In these drug-dependent cells, the stability of the polymer is apparently perturbed to such an extent that normal cell function is compromised, and the cells require low concentrations of Taxol for survival. Based on the observation that low concentrations of microtubule-stabilizing and -destabilizing drugs inhibit microtubule dynamics without altering polymer mass, Wilson and Jordan have suggested that in Taxol-resistant cell lines, the equilibrium between weakly and highly dynamic microtubules has been shifted towards the latter (Derry *et al*., 1995; Wilson and Jordan, 1995; Jordan and Wilson, 1998; Goncalves *et al*., 2001).

The dynamics of individual rhodamine-labeled microtubules in Taxol-sensitive and -resistant A549 cell lines, derived from a human lung carcinoma, have been quantified by digital time-lapse microscopy (Goncalves *et al*., 2001). The A549-T12 and -T24 cell lines, nine- and 17-fold resistant, respectively, to Taxol were selected by continual exposure of the parental drug-sensitive, cell line to increasing concentrations of drug. Significantly, both resistant cell lines are also dependent on low concentrations of Taxol (2 nM) for growth, and become blocked in the G_2/M phase of the cell cycle if the drug is removed. Both resistant cell lines exhibited increased dynamic instability compared with the parental, drug-sensitive, cell line. Several potential mechanisms can be envisaged by which microtubule dynamics could be modulated in a Taxol-resistant cell line, and include altered tubulin isotype expression, mutations to tubulin that affect either longitudinal/lateral interactions or binding of regulatory proteins, alterations to tubulin through post-translational modifications that modify regulatory protein binding, and altered expression or post-translational modifications to tubulin-/microtubule-regulatory proteins.

Altered expression of β*-tubulin isotypes*

Inherent differences in the assembly properties, microtubule dynamics and drug interactions among some of the β-tubulin isotypes have been revealed by *in vitro* analysis of immunoaffinity-purified isotypes prepared

from bovine brain tubulin (3% class I, 58% class II, 25% class III and 13% class IV β-tubulin) (Banerjee *et al*., 1990, 1992; Lu and Luduena, 1993, 1994; Panda *et al*., 1994; Derry *et al*., 1997). It was reported that microtubules assembled from βIII-tubulin had distinct assembly properties compared to βII-, βIV- or unfractionated tubulin (Banerjee *et al*., 1990; Lu and Luduena, 1993, 1994). βIII-tubulin required the highest critical concentration of tubulin for assembly, exhibited a distinct delay in nucleation and proceeded at a slower rate compared to other isotypes. Since the differences in assembly occurred in the absence of MAPs, this would suggest that the various tubulin isotypes, by themselves, can modulate microtubule dynamics (Panda *et al*., 1994). In fact, microtubules containing only βIII-tubulin exhibited a dynamicity more than double that of βII- and βIV-derived microtubules (Panda *et al*., 1994). As a result, microtubules composed exclusively of βIII-tubulin are less stable than microtubules composed of either βII- or βI-tubulin. Nevertheless, when βII-microtubules were spiked with βIII-tubulin, the resulting microtubules exhibited decreased, not increased dynamicity. Derry *et al*. (1997) demonstrated that microtubules composed of either βIII- or βIV-tubulin were considerably less sensitive to the suppressive effects of Taxol on microtubule dynamics, than microtubules assembled from βII or unfractionated tubulin. Collectively, these *in vitro* studies suggest that microtubule dynamics, and the effects of Taxol on this process, can be modulated by the β-tubulin isotype composition. Such studies have formed the basis for the idea that altered cellular expression of β-tubulin isotypes, especially βIII and βIV, could be an important determinant in cellular resistance towards Taxol. However, there are two major caveats to these *in vitro* studies. First, the α-tubulin isotype composition of the immunoaffinity-purified β-tubulin isotypes has not been determined. Bovine brain tubulin has three major α-tubulin isotypes, α1, α2, and α4, all of which are extensively post-translationally modified. Preferential association between specific α- and β-isotypes could complicate the analysis of any *in vitro* studies. Second, βI, not βII, is the major β-tubulin isotype in non-neuronal cells, and the influence of βIII- and βIV-tubulins on βI-microtubule dynamicity has not been determined. So, although mammalian brain tubulin is a rich and readily available source of tubulin for *in vitro* studies, its tubulin composition is probably not representative of many human cancer cell lines and tumors.

There have been numerous reports of altered expression of individual β-tubulin isotypes in cells that have been selected for resistance to antimitotic agents (see Table 1). Analysis of β-tubulin isotypes in Taxol-resistant cells has been performed by utilizing isotype-specific primers for RT–PCR analysis, as well as isotype-specific antibodies for Western blot analysis and/or immunofluorescence. In the Taxol-resistant non-small lung carcinoma cell lines A549-T12 and A549-T24, described above, RT–PCR analysis demonstrated that the class III and IVa isotypes, which were barely detectable in the parental cell line, increased ~2–3-fold, in the A549-T12, and ~fourfold in the A549-T24 cell lines (Kavallaris *et al*., 1997). The increase in βIII tubulin in A549-T24 cells was confirmed by immunofluorescence. Likewise, a twofold increase in class IVa β-tubulin mRNA and protein level was noted in a K562 erythroleukemia cell line that was ninefold resistant to Taxol (Jaffrezou *et al*., 1995). Selection of a human prostate carcinoma cell line, DU-145, with Taxol produced alterations in the expression levels of both class III and Iva (Ranganathan *et al*., 1998a). The DU-145 cell line, which was fivefold resistant to Taxol, had an ~threefold increase in total α- and β-tubulin, and a fourfold increase in class III protein with a ninefold increase at the RNA level. Nicoletti *et al*. (2001), using RT–PCR, analysed β-tubulin isotype composition in a subset of 17 cancer cell lines from the National Cancer Institute-Anticancer Drug Screen. In these cell lines, βI was the major tubulin isotype accounting for 85–99% of all the β-tubulin mRNA. Significantly, when the sensitivities of these cell lines towards antimitotic drugs, including Taxol, vinblastine, vincristine and rhizoxin, were correlated with the absolute levels of mRNA expression for the various β-tubulin isotypes, it was found that sensitivity towards Taxol, but not the three other antimicrotubule drugs, correlated with βIII-tubulin levels. After βI, the βIII mRNA was the next predominant message expressed in these cell lines, with levels ranging from 0.5 to 14%.

All these studies imply that altered expression of β-tubulin isotypes, especially class III and IVa, may be correlated with Taxol sensitivity. This hypothesis is supported by analysis of tubulin isotypes in cells not selected for drug resistance. A study of brain cell lines with different intrinsic levels of class III β-tubulin showed that all were able to accumulate Taxol to a similar extent (Ranganathan *et al*., 1998b). However, the two cell lines with elevated levels of class III protein were ~5.5-fold less sensitive to Taxol, compared to the cell line that had no detectable levels of class III. Studies in HT29-D4, a human colon adenocarcinoma cell line, that is used as a model for epithelial cell differentiation, also support a role for βIII in determining cellular sensitivity towards Taxol (Carles *et al*., 1999). Undifferentiated HT29-D4 cells are malignant and proliferate rapidly. After galactose-induced differentiation, the cells take on the appearance of polarized epithelial cells. Interestingly, the cytotoxicity of Taxol towards HT29-D4 cells depends upon their differentiation status. Although bundling of microtubules occurred in undifferentiated cells in the presence of Taxol, the microtubules of the differentiated cells failed to bundle even though they accumulated twofold more drug than the undifferentiated cells. RT–PCR and immunoblot analyses have demonstrated that the class I, II, III, IVa and IVb β-tubulin isotypes were expressed in HT29-D4 cells. However, a selective increase in class III β-tubulin mRNA and protein occurred upon differentiation. In other studies, cell lines overexpressing EGFRvIII and HER2 oncogenic growth factors had decreased sensitivity to Taxol (Montgomery *et al*., 2000). Significantly, Taxol-induced polymerization was suppressed in these

Table 1 Alterations in tubulin composition associated with resistance to Taxol

Cell line[a]	*Alteration in tubulin content*[b]		*Fold resistance to Taxol*[c,d]	*Reference*
	Tubulin (fold increase)[d]	*Detection method*		
Taxol-selected				
NCIH460/T800 (lung)	α-Tubulin (↑)	WB	1000[e]	Kyu-Ho *et al.* (2000)
A549-T12 (lung)	βIII (2)	RT–PCR	9	Kavallaris *et al.* (1997)
	βIVa (3)	RT–PCR		
A549-T24 (lung)	βIII (4)	RT–PCR	17f	Kavallaris *et al.* (1997)
	βIII (↑)	NB IF		
	βIVa (4)	RT–PCR		
H69/Tx1 (lung)	Acet α-tubulin (↑)	WB	4.7	Ohta *et al.* (1994)
MCF-7-PTX30 (breast)	Tyr α-tubulin (2)	WB	ND	Banerjee (2002)
	βIII (2)	WB		
	βIV (1.5)	WB		
S2/TXT (pancreas)[g]	βII (2.4)	RT–PCR	9.5[e]	Liu *et al.* (2001)
	βIII (2.3)	RT–PCR		
DU-145-Pac10 (prostate)	α- and β-tubulin (3)	IF IF	5	Ranganathan *et al.* (1998a)
	βIII (4)	WB		
	βIII (9)	RT–PCR		
	βIVa (5)	RT–PCR		
1A9PTX22 (ovary)	βI (1.8)	RT–PCR	24	Giannakakou *et al.* (1997)
	βIVa (0.03)	RT–PCR		
K562-KPTA5 (erythroleukemia)	βIVa (2)	RT–PCR	9	Jaffrezou *et al.* (1995)
	βIV (↑)	WB		
J7-T1 (murine macrophage-like)	βI (1.9)	RT–PCR	2200[e]	Haber *et al.* (1995)
	βII (21)	RT–PCR		
Taxol-resistant ovarian tumor samples	βI (3.6)	RT–PCR	NA	Kavallaris *et al.* (1997)
	βIII (4.4)	RT–PCR		
	βIVa (7.6)	RT–PCR		
Not drug-selected				
NIH3T3-HC2 (murine fibroblast)	βIVa (2.5)	RT–PCR	3.3	Montgomery *et al.* (2000)
	βIVb (3.1)	RT–PCR		
HT29-D4 (colon)	βIII (1.7)	RT–PCR	1000	Carles *et al.* (1999)
	βIII (↑)	WB		
	Tyr α-tubulin (↓)	WB		
SF 295 vs SF 539 (glioblastoma)	βIII (↑)	WB	5.5	Ranganathan *et al.* (1998b)
SNB75 vs SF539 (glioblastoma)	βIII (↑)	WB	5.5	Ranganathan *et al.* (1998b)
17 Human cancer cell lines	βIII (↑)	RT–PCR	↑	Nicoletti *et al.* (2001)

[a]Human cell lines except where noted. [b]WB, Western blotting; RT–PCR, reverse transcriptase–polymerase chain reaction; IF, immunofluorescence; NB, Northern blotting; acet, acetylated; tyr, tyrosinated. [c]IC_{50}-resistant cell line/IC_{50} parental cell line. ND, not determined; NA, not available. [d]Vertical arrows indicate relative increase or decrease. [e]Expresses high levels of Pgp. [f]Expresses very low levels of Pgp. [g]Selected with Taxotere

cells compared to cells expressing wild-type EGFR. Increases in class IVa β-tubulin were observed in both oncogene-transfected cell lines. Introduction of a mutation into the kinase domain of the receptor, thereby inhibiting EGFRvIII kinase activity, partially reversed resistance to Taxol and decreased expression of the class IVa β-tubulin by 50%. These studies are highly significant since they suggest that certain oncogenes can alter drug sensitivity by modulating β-tubulin isotype levels.

Alterations in β*-tubulin isotype levels by transfection studies*

To validate definitively that tubulin isotype composition can modulate Taxol sensitivity, specific isotype levels must be modulated in drug-naive cells using either protein overexpression or antisense oligonucleotide approaches. In three reported transfection experiments, stable overexpression of class I, II and IVb β-tubulin genes in Chinese hamster ovary (CHO) cells (Blade *et al.*, 1999), the class II β-tubulin gene in NIH 3T3 cells (Burkhart *et al.*, 2001) and βIII in a human prostate carcinoma cell line (Ranganathan *et al.*, 2001) failed to confer resistance to Taxol. However, downregulation of class III β-tubulin by antisense oligonucleotides in Taxol-resistant A549-T24 cells resulted in a 40–50% decrease in both class III mRNA and protein levels, and was associated with a 39% increase in sensitivity to Taxol (Kavallaris *et al.*, 1999).

It is important to consider why overexpression of tubulin isotypes in drug-naive cells failed to confer a resistant phenotype, while downregulation of βIII tubulin in a drug-resistant cell line was modestly effective in altering drug sensitivity. Attempts to modulate specific β-tubulin isotype levels in cells are complicated by compensatory changes in the expression levels of other β-tubulin isotypes. The mechanisms of transcriptional regulation of α- and β-tubulin synthesis are distinct. Cellular β-tubulin levels are autoregulated by cotranslational degradation of mRNAs. This negative feedback control utilizes a tetrapeptide, MREI, in the N-terminus of β-tubulins, to induce a signal for message degradation.

βI-Tubulin is the major isotype in all of the transfected cells and, as discussed previously, is the least

studied of the isotypes in terms of *in vitro* microtubule assembly and dynamics. It has yet to be established whether βIII or βIV isotypes can alter the dynamics of microtubules composed predominately of βI tubulin. It is also possible that the levels of overexpression achieved in the above transfection experiments were not sufficient to alter microtubule dynamics and thus produce a resistance phenotype. RT–PCR analysis and isotype-specific antibodies were used in these studies to quantify isotypic changes. Unfortunately, quantitation of tubulin mRNA levels may not accurately reflect the protein profile in transfected cells. Likewise, antibody-based methods can only give relative, not absolute, levels of a specific tubulin isotype in cells. Our research group has recently described a mass spectrometry-based method for analysing human tubulin isotype composition (Rao *et al*., 2001). By incorporating stable isotope quantitation into this method, we anticipate that we will be able to determine absolute levels of each isotype in cells and tissues.

Finally, altered expression of β-tubulin isotypes may not be directly related to the resistant phenotype, but represents a secondary effect that may require the participation of additional isotype-specific regulatory proteins. Since it is known that some MAPs bind to the highly divergent, but isotope-specific C-terminal regions of tubulin, it would not be unexpected if such regulatory proteins exist and are coordinately expressed along with their respective isotype upon drug selection. This scenario would explain why simple overexpression of tubulin isotypes in drug-sensitive cells cannot produce a resistance phenotype; yet alterations in drug sensitivities of resistant cell lines can be observed using an antisense approach.

Alterations in α-tubulin isotype composition

The α-tubulin isotype composition also has the potential to affect the drug sensitivity of cells (Table 1). Under *in vitro* conditions, tubulin enriched by immunoaffinity purification in the tyrosinated α1, α2 isotypes was shown to assemble three times faster than the nontyrosinated forms (Banerjee and Kasmala, 1998). At the cellular level, the lung carcinoma cell line, NCI-H460/T800, an MDR-expressing cell which is 1000-fold resistant to Taxol compared to the parental cell line, overexpresses its α-tubulin protein, but not at the mRNA level (Kyu-Ho Han *et al*., 2000). Downregulation of k-α1-tubulin in this resistant cell line using an anti-sense DNA construct caused a 45–51% increased sensitivity towards Taxol in three independent clones. Furthermore, overexpression of k-α1-tubulin in the parental H460 cells caused a 2.5-fold increase in resistance towards Taxol. Interestingly, both the antisense and sense clones also displayed altered sensitivities towards vinblastine and colchicine, but not to nocodazole.

Point mutations in tubulin leading to alterations in microtubule dynamics

Tumor cell lines selected for resistance to Taxol often demonstrated altered migration of α- and β-tubulin by two-dimensional gel electrophoresis. Several lines of evidence suggest that many of the Taxol-resistant cells contained a less stable microtubule polymer. Some of the selected cell lines were Taxol-dependent, and exhibited lower levels of microtubule assembly than the parental drug-sensitive or the Taxol-independent, but resistant, cell lines (Table 2). Moreover, many of the Taxol-resistant lines were hypersensitive to

Table 2 Tubulin mutations associated with resistance to Taxol

Cell line[a]	*Mutation*[b]	*Fold resistance to drug*[c]			*Taxol dependence*[d]	*Impairment of drug-induced polymerization*	*Reference*
		Taxol	*Vinblastine*	*Colchicine*			
Taxol							
A549-T12	αSer379Ser/Arg	9	1.5	1.0	+ + + +	Taxol no	Martello *et al*. (2003)
A549-T24[e]	αSer379Ser/Arg	17	1.4	1.3	+ + + +	Taxol no	Martello *et al*. (2003)
1A9PTX10	βPhe270Val	24	0.5	ND[f]	–	Taxol yes; EpoB no	Giannakakou *et al*. (1997)
1A9PTX22	βAla364Thr	24	0.4	ND	–	Taxol yes; EpoB no	Giannakakou *et al*. (1997)
CHO-Tax mutants	βLeu215His	2–3	ND	ND	–	Taxol no	Gonzalez-Garay *et al*. (1999)
	βLeu215Arg				–		
	βLeu215Phe				+ + + +		
	βLeu217Arg				–		
	βLeu228Phe				+ + + +		
	βLeu228His				+ + + +		
MDA-MB-231/K20T	βGlu198Gly	19	1.0	ND	–	Taxol no	Wiesen *et al*. (2002)[g]
Epothilone A or B							
A549.EpoB40	βGlu292Glu	22	0.5	0.6	+	EpoB yes	He *et al*. (2001)
HeLa.EpoA9	βPro173Pro/Ala	6.4	0.9	0.6	+	ND	He *et al*. (2001)
HeLa.EpoB1.8	βTyr422Tyr/Cys	2.8	1.6	0.4	+ +	ND	He *et al*. (2001)
1A9/A8	βThr274Ile	10	ND	ND	–	Taxol yes; EpoA yes	Giannakakou *et al*. (2000)
1A9/B10	βArg282Gln	6.5	ND	ND	–	Taxol yes; EpoA yes	Giannakakou *et al*. (2000)

[a]The drug used for selection is indicated. [b]Location of mutation is described in text and Figure 3. [c]IC_{50}-resistant cell line/IC_{50} parental cell line. [d]+ + + +, total dependence; + +, medium dependence; +, low dependence; –, no dependence. [e]Expresses very low levels of Pgp. [f]Not determined. [g]Proceedings of the AACR 93rd Annual meeting, 2002, Vol. 43, p. 788, #3906

microtubule-destabilizing drugs, such as vinblastine or colchicine, that bind to free tubulin dimers. A detailed analysis of class I β-tubulin mutations in Taxol-resistant CHO cell lines, isolated by single-step selection, revealed a cluster of mutations at leucines 215, 217 and 228 (Gonzalez-Garay *et al*., 1999). It was concluded that resistance in these cells was due to the mutations that altered microtubule dynamics by affecting the lateral/longitudinal interactions important for microtubule assembly. By destabilizing microtubules, these mutations apparently counteract the stabilizing effects of Taxol. Importantly, using a tetracycline-regulated expression system, it was shown that the low-level expression of β-tubulin containing any one of these mutations conferred Taxol resistance in CHO cells.

Three new epothilone-resistant cell lines have been selected in our laboratory in A549 and HeLa cells. These resistant cell lines are crossresistant to the taxanes and do not express the MDR1 gene. Sequence analysis of the class I β-tubulin from these resistant cell lines revealed that there were single point mutations at β292 (Gln to Glu), β173 (Pro to Ala) and β422 (Tyr to Tyr/Cys), respectively. These mutations are near the M-loop, the nucleotide-binding site and the C-terminus, regions that are involved in stabilizing the lateral contacts between adjacent protofilaments, the hydrolysis of GTP and the binding of MAPs, respectively (Figure 3). It is likely that these mutations decrease the endogenous stability of the microtubule to compensate for the activities of microtubule-stabilizing drugs. Consistent with this hypothesis, it was found that these resistant cell lines became more sensitive to microtubule-destabilizing drugs such as vinblastine and colchicine.

Sequencing of the class I β-tubulin gene in the A549-T12 cells did not reveal any mutations. However, a heterozygous point mutation in K-α1 tubulin was found at residue 379 (Ser to Ser/Arg) (Martello *et al*., 2003). The expression of both the wild-type and mutated α-tubulins in the A549-T12 cell line was confirmed by mass spectrometry (Verdier-Pinard *et al*., 2003). This region of α-tubulin is near the C-terminus, and is close to the proposed sites of interaction for both MAP4 and stathmin.

Post-translational modifications to tubulin

As mentioned previously, the structural diversity of the tubulin protein family is further increased by extensive post-translational modifications. All of the post-translational modifications, except acetylation, occur within the C-terminal 20 amino acids of α- and β-tubulin chains. Since several MAPs have been shown to interact with the C-terminal region of tubulin, it is possible that reversible post-translational modifications to this region of tubulin could regulate its interaction with MAPs, thus modulating microtubule dynamics. It is known that the ability of several structural and motor MAPs, including tau, MAP-2 and kinesin, to interact under *in vitro* conditions with the microtubule cytoskeleton is regulated by the level of polyglutamylation of the α-and β-tubulins (Boucher *et al*., 1994; Larcher *et al*., 1996; Bonnet *et al*., 2001). The levels of tubulin glutamylation and tubulin polyglutamylase activity were shown to be cell cycle dependent (Bobinnec *et al*., 1998; Regnard *et al*., 1999). Although enzymatic activity peaked in G_2 phase, the level of glutamylated tubulins was maximally elevated in mitosis, suggesting a complex regulation involving both polyglutamylase and deglutamylase activities. Removal of phosphate from βIII tubulin by protein phosphatase 2A inhibited MAP-2-stimulated *in vitro* microtubule assembly (Khan and Luduena, 1996).

Utilizing 2-D gel electrophoresis, P19 embryonal carcinoma cells demonstrated increased expression of the more acidic isoforms of βIII tubulin after Taxol treatment (Laferriere and Brown, 1996). The lack of ^{32}Pi incorporation into the more acidic βIII isoform suggests that glutamylation was responsible for the shift in isoelectric point. In the case of a Taxol-resistant human small lung cell carcinoma, increased acetylation of α-tubulin was observed (Ohta *et al*., 1994) (Table 1). However, it is likely that these modifications reflect substrate preference, namely polymer over dimer, of the modifying enzymes, and are not directly associated with Taxol resistance. To date, however, there is little evidence that altered post-translational modifications are a major determinant of cellular sensitivity towards Taxol or any tubulin-directed antimitotic agent.

Altered expression/post-translational modifications of tubulin-/microtubule-regulatory proteins

Proteins that regulate microtubule dynamics by interacting with tubulin dimers or polymerized microtubules clearly have the potential to modulate the sensitivity of a cell towards Taxol. Stathmin, a microtubule destabilizer, and MAP4, a microtubule stabilizer, represent such proteins that regulate the dynamics of cellular microtubules. Stathmin is a soluble cytoplasmic protein that can bind to tubulin dimers and stimulate microtubule catastrophes (Belmont and Mitchison, 1996; Cassimeris, 2002). This destabilizing activity is regulated by phosphorylation, and is lost when stathmin is fully phosphorylated (Marklund *et al*., 1996; Horwitz *et al*., 1997). MAP4 is the predominant human non-neuronal MAP, and the microtubule-stabilizing function of MAP4 is also regulated by phosphorylation (Chapin *et al*., 1995; Chang *et al*., 2001). MAP4 alters microtubule dynamics by increasing the rescue frequency, without affecting the catastrophe frequency. Phosphorylation of MAP4 results in a loss of this microtubule-stabilizing activity. The overexpression/activation of stathmin and/or the downregulation/inactivation of MAP4 should increase the dynamicity and decrease the stability of microtubules. Such changes in cancer cells could reduce the microtubule-stabilizing potency of Taxol, and confer a mechanism of resistance to the drug. Inversely, the potency of microtubule-depolymerizing drugs like vinca alkaloids could be enhanced.

Downregulation of stathmin, by a stathmin antisense construct stably transfected into K562 erythroleukemia cells, produced a synergistic inhibition of their growth and clonogenicity when treated with low concentrations of Taxol, and were more resistant to vinblastine

compared to control mock-transfected cells (Iancu *et al*., 2000, 2001). In contrast, overexpression of stathmin in human lung carcinoma cells sensitized the cells to vindesine and vincristine, but did not significantly decrease their sensitivity to Taxol or Taxotere (Nishio *et al*., 2001). Moreover, stathmin inhibited *in vitro* Taxol-induced polymerization of microtubules (Larsson *et al*., 1999). Altogether, these data indicate that overexpression of active stathmin in cancer cells could decrease their sensitivity to Taxol by opposing the microtubule-stabilizing effect of Taxol.

Alterations in expression of various forms of MAP4 are also predicted to modulate cancer cell sensitivity to microtubule-interacting drugs. Inhibition of MAP4 expression by an antisense approach decreased microtubule polymer levels in HeLa cells, whereas overexpression of MAP4 increased microtubule stability. MAP4 expression has been shown to be transcriptionally repressed in the presence of wild-type p53 (Murphy *et al*., 1996), and Hait and co-workers demonstrated that inactivation of p53 in murine fibroblasts increased their sensitivity to Taxol, but decreased their sensitivity to vinblastine (Zhang *et al*., 1998). This group confirmed this trend when they induced p53 by treating the same cells with DNA-damaging agents, and observed a decrease in Taxol sensitivity and an increased sensitivity to vinblastine (Zhang *et al*., 1999). MAP4 phosphorylation and dissociation from microtubules correlated with a decrease in Taxol sensitivity in Taxol-resistant ovarian cell lines (Poruchynsky *et al*., 2001). In contrast, the expression of nonphosphorylated forms of MAP4 is increased in vinblastine-resistant cells (Kavallaris *et al*., 2001).

The protein levels of stathmin and MAP-4 have been quantified in the Taxol-sensitive and -resistant A549 cell lines. The stathmin protein levels in the A549-T12 and -T24 resistant cell lines were increased $\sim$twofold compared to the parental drug. Since stathmin activity is regulated by phosphorylation, its phosphorylation status was also evaluated. In the parental A549 cells, exposure to increasing concentrations of Taxol caused a shift from the nonphosphorylated and active form of stathmin to the fully phosphorylated and inactive protein. Significantly, no shift in phosphorylation was observed in the two Taxol-resistant cell lines. With regard to MAP4, the parental cell line expressed exclusively the nonphosphorylated and active form of the microtubule-stabilizing protein, whereas A549-T24 cells predominately expressed the phosphorylated and inactive form. A549-T12 cells that display lower resistance to Taxol than the A549-T24 cell line expressed both forms of MAP4. These changes in the tubulin-/microtubule-regulatory proteins observed in the Taxol-resistant A549 cell lines would be predicted to act in concert, to increase the dynamicity of their microtubules.

The regulation of microtubule dynamics by interacting proteins is complex, and is likely to involve a variety of proteins in addition to stathmin and MAP4. For example, the expression levels of MAP4 and E-MAP-115, another MAP expressed in cells of epithelial origin, were quantified during HT29-D4 cell differentiation. Levels of MAP-4 did not vary during differentiation. However, extremely low levels of E-MAP-115 were present in undifferentiated cells, and the levels were upregulated significantly during the differentiation process. Overexpression of E-MAP-115 in MCF-7 and HeLa cells increased their sensitivity towards Taxol (Gruber *et al*., 2001).

Altered binding of taxol to the microtubule

The acquisition of mutations that confer altered binding of a drug to its primary target is a recurring theme in drug resistance. Not unexpectedly, examples of altered drug binding have been identified in cell lines resistant to microtubule-stabilizing drugs. Two independent Taxol-resistant human ovarian carcinoma cell lines, 1A9PTX10 and 1A9PTX22, have been isolated, and are 24-fold resistant to Taxol, but are hypersensitive to vinblastine (Giannakakou *et al*., 1997) (Table 2). These cell lines were not Taxol dependent, and the resistant phenotype was sustained even after the cells were cultured for 3 years in the absence of drug. The total tubulin contents of both the resistant and the parental cells were similar and all the cells had the same fraction of tubulin in the polymerized state, suggesting that microtubule dynamics in these Taxol-resistant cells was not altered. However, the isolated tubulins from the resistant cells polymerized poorly in the presence of Taxol, suggesting that these mutations abrogated Taxol binding. Interestingly, these Taxol-resistant cells retained sensitivity to epothilone B and to 2-*m*-azidobenzoyl-Taxol, both of which are considerably more potent microtubule-stabilizing drugs than Taxol. Sequence analysis of the major β-tubulin isotype βI in these cell lines revealed that the 1A9PTX10 cells had a Phe_{270}-to-valine substitution, whereas the 1A9PTX22 cell line had an Ala_{364}-to-threonine substitution. From molecular modeling studies, Phe_{270} is close to the region of tubulin that makes important contacts with the taxane ring system of Taxol (see above). It is possible that replacing the phenyl ring at this position by the less bulky side chain of valine could disrupt Taxol binding to the mutant tubulin. Epothilone-resistant cell lines were isolated after exposure of the human ovarian carcinoma cell line to epothilone A or epothilone B (Giannakakou *et al*., 2000). These epothilone-resistant cell lines exhibited impaired epothilone- and Taxol-induced tubulin polymerization (Table 2). One cell line had a mutation leading to a threonine-to-isoleucine change at amino acid 274, and the other had a mutation leading to an arginine-to-glutamine change at amino acid 282. This arginine residue is the site of photoincorporation of 7-BzDC Taxol (see above; Rao *et al*., 1999). Based on molecular modeling studies, it was suggested that the Thr_{274}Ile substitution could disrupt the hydrogen bond between the side chain hydroxyl group of threonine and the C7-hydroxyl of the epothilones (Giannakakou *et al*., 2000).

Alterations in signaling pathways

Key proteins that mediate various signaling pathways are often localized to microtubules (Gundersen and

Cook, 1999; Hollenbeck, 2001; Cardone *et al*., 2002), and microtubule-targeting drugs, such as Taxol, have the potential to modulate these pathways. One well-documented example of a signaling pathway that interacts with microtubules is the extracellular signal-regulated kinase (ERK1 and 2), a component of the mitogen-activated protein kinase (MAPK) family. We and others have documented activation of the ERK-signaling cascade in response to microtubule disruption (Shinohara-Gotoh *et al*., 1991; Schmid-Alliana *et al*., 1998; McDaid and Horwitz, 2001). In fact, we have demonstrated additivity between Taxol and MEK inhibition, utilizing a commercially available MEK inhibitor, U0126 (McDaid and Horwitz, 2001). Other groups have demonstrated similar findings with respect to Taxol and other drugs that induce MEK/ERK activation (e.g. UCN-01) (Dai *et al*., 2001). Our study clearly demonstrated that it is the degree of activation of this signaling pathway that governs whether the interaction between Taxol and MEK inhibition is additive/synergistic, or antagonistic. The mechanism for the enhanced cytotoxic effects of Taxol in the presence of MEK inhibitors may be related to the repression of the survival-signaling function of the ERK/MEK pathway, and to enhanced microtubule polymerization, since it has been proposed that MAPK activation inhibits microtubule stabilization (Shinohara-Gotoh *et al*., 1991).

Although Taxol has been shown to activate MAPK *in vitro*, there are currently no data available from patients treated with Taxol. However, a recent report has suggested that active MAPK is expressed in approximately 48% of primary human breast cancer tumors, and is potentially a marker of breast cancer metastasis since its expression is elevated in lymph node metastases (Adeyinka *et al*., 2002). Since Taxol is FDA approved for the treatment of ovarian, breast and lung carcinomas, it may be possible to potentiate clinical responses in these disease types by combining Taxol-based chemotherapy with signal transduction inhibitors, including EGFR inhibitors and farnesyl transferase inhibitors that target oncogenic ras signaling. In this strategy, Taxol-based chemotherapy may have enhanced efficacy in patients who would otherwise respond poorly. This strategy is currently being investigated in current clinical trials (Tolcher, 2001; Esteva *et al*., 2002; Forero *et al*., 2002). Indeed, the FDA has approved the use of trastuzumab (the humanized anti-ErbB2 antibody) and Taxol as first-line treatment of ErbB2 (HER2)-overexpressing metastatic breast cancer, based on the results of a randomized phase III clinical trial, showing that this combination produced higher response rates and longer survival duration than treatment with chemotherapy alone (Slamon *et al*., 2001). It has been demonstrated that ErbB2 overexpression inhibits Cdc2 activation and Taxol-induced cell death in breast cancer cells, via deregulation of the G2/M cell cycle checkpoint (Yu *et al*., 1998), and more recently that ErbB2-overexpressing breast cancer cells and primary tumors have elevated levels of inhibitory phosphorylation of Cdc2 on tyrosine (Y)15 (Tan *et al*., 2002), providing a mechanistic rationale for the association between ErbB2 overexpression and Taxol resistance. As discussed previously, there may exist a novel relationship between oncogenic growth factor signaling, and the modulation of tubulin isotypes (Montgomery *et al*., 2000), although this hypothesis will require validation in human tumors that express oncogenic forms of receptor tyrosine kinases. Interestingly, overexpression of EGFRvIII, a receptor variant of the EGFR gene that has the most common alteration of the EGFR gene, a deletion encompassing exons 2–7, is associated with constitutive activation of the pERK (Montgomery *et al*., 1995) and phosphatidylinositol 3-kinase pathways (PI3k/AKT) (Moscatello *et al*., 1998), consistent with increased cellular survival. It has recently been demonstrated that overexpression of a catalytically active subunit of PI3k in ovarian cancer cells confers Taxol resistance, which is reverted upon inhibition of the PI3k pathway utilizing a selective inhibitor (Hu *et al*., 2002). Thus, aberrant expression of key signaling molecules required for the control of cellular survival may confer Taxol resistance, and one current focus of future chemotherapy in the treatment of cancer is the circumvention of this type of resistance, utilizing selective inhibitors of these proteins to increase drug sensitivity.

Taxol resistance in patients

Taxol, in combination with the platinum agents, has been accepted as the standard chemotherapy in patients with advanced ovarian cancer. This combination has also shown activity in patients with breast and non-small-cell lung cancer. Despite the clinical success of Taxol in treating a number of solid malignancies, several disease types are intrinsically resistant to the drug, notably gastrointestinal tumors, thereby limiting its therapeutic applications. It is thought that the high expression of P-glycoprotein in the gastrointestinal tract mediates Taxol resistance. This supposition has been supported by the observation that patients with advanced colorectal tumors have demonstrated clinical responses to epothilone B, which is not a substrate for P-glycoprotein (Calvert *et al*., 2001).

The majority of patients with advanced cancer eventually develop progressive disease after initially responding to Taxol treatment. Drug resistance, whether intrinsic or acquired, represents a major obstacle in improving the response and survival of cancer patients, and these problems related to resistance have motivated a search for novel antimitotic agents that have the potential to improve the Taxol prototype. The ideal Taxol prototype would display activity in a broad range of malignancies, have manageable toxicities, a reduced propensity for acquired clinical resistance, and ideally produce an enhanced degree and duration of antitumor response. The epothilones and discodermolide, both of which are being evaluated in clinical trials, fulfill some of these criteria, although their toxicity profiles are

still being assessed and it remains to be seen if they will attain the same clinical success as Taxol.

Alterations in tubulin isotype composition in tumors

An analysis of β-tubulin isotype expression levels in Taxol-sensitive and- resistant human ovarian epithelial tumors by RT-PCR was undertaken (Kavallaris *et al*., 1997). Resistance to Taxol was defined as disease progression during treatment, or relapse within 6 months following treatment. Taxol-resistant ovarian tumor samples displayed significant increases in class I (3.6-fold), class III (4.4-fold), and class IVa (7.6-fold) β-tubulin compared to primary untreated ovarian tumors. In contrast, no correlation was observed between β-tubulin mRNA expression and Taxol sensitivity in mouse xenografts established from 12 human ovarian carcinomas taken before or after the initiation of Taxol treatment (Nicoletti *et al*., 2001).

Tubulin mutations in human tumors

A recent study identified β-tubulin mutations in serum DNA isolated from 33% of patients with non-small-cell lung cancer (Monzo *et al*., 1999). This finding was considered extremely significant, since it validated *in vitro* data from numerous laboratories documenting the acquisition of mutations in Taxol- and epothilone-resistant cell lines that correlated with increasing levels of resistance. Moreover, this report suggested a relationship between the location of the mutations on β-tubulin and response to Taxol-based chemotherapy, since patients with and without mutations had dramatic differences in median survival, a finding, which if validated, would have profound implications in determining treatment options for patients with NCSLC. Several groups sought to confirm this initial study; however, the results have not been corroborated in these prospective studies (Kelley *et al*., 2001; Kohonen-Corish *et al*., 2002; Sale *et al*., 2002; Tsurutani *et al*., 2002), although silent polymorphisms have been reported. A recent study analysing 62 human breast cancer tumors also concluded a lack of β-tubulin mutations in these tumors, and documented the presence of a silent polymorphism at codon 217 (Hasegawa *et al*., 2002). All of these studies addressed the issue of concomitant amplification of tubulin pseudogenes during the analyses, an artifact that appears to be circumvented by cDNA sequencing (Tsurutani *et al*., 2002). We have identified a unique polymorphism, utilizing cDNA sequencing, at the extreme C-terminus of β-tubulin in a patient with advanced breast cancer (McDaid *et al*., 2002). This patient had a partial response to BMS-247550, an epothilone B analog currently in clinical development, although the relevance of this polymorphism to her response is unknown. In addition, a Taxol-resistant cell line that harbors a mutation in α-tubulin has recently been identified (Martello *et al*., 2003), suggesting that nucleotide alterations may not be confined to β-tubulin, but may arise in multiple locations, resulting in perturbation of normal microtubule function. Therefore, the prevalence of sequence variants of tubulin in human tumors, and the relevance, if any, of these variants to response to Taxol-directed chemotherapy is still a subject of debate.

The study by Monzo *et al*. utilized genomic DNA that was extracted from circulating tumor DNA isolated from patient serum samples. The β-tubulin gene has many pseudogenes, seven of which have been reported to date (Wilde *et al*., 1982a, b; Lee *et al*., 1983). The existence of pseudogenes makes it difficult to analyse the precise nucleotide sequence of β-tubulin using genomic DNA. One report (Tsurutani *et al*., 2002) has documented the amplification of nonspecific nucleotide sequences in β-tubulin, depending on whether genomic DNA or cDNA is utilized. There is also the possibility that circulating tumor DNA may have additional nucleotide alterations compared to DNA from a primary tumor, due to the clonal expansion of tumor cells and the metastatic process. However, whether these micrometastases give rise to secondary tumors that may have tubulin alterations remains to be determined. Owing to the potential clinical relevance of sequence variants in tubulin to microtubule-directed chemotherapy, a sensible recommendation for future studies would be to carry out a systematic analysis of the genetic basis of these nucleotide alterations, utilizing rigorous strategies that eliminate the possibility of detecting pseudogenes.

Tubulin-/microtubule-regulatory proteins in human cancers

Stathmin mRNA levels are known to be upregulated in breast carcinoma cells from patients with more aggressive disease, and in acute leukemias, lymphomas and various carcinomas (Hanash *et al*., 1988; Nylander *et al*., 1995; Bieche *et al*., 1998; Curmi *et al*., 2000). In the case of MAP4, a recent phase 1 clinical study of sequential doxorubicin/vinorelbine indicated partial correlation with induction of p53 and decreased MAP4 expression in peripheral blood mononuclear cells and in tumors (Bash-Babula *et al*., 2002).

Summary

Acquired Taxol resistance may be mediated by a number of putative mechanisms, based on data accrued from the selection of cells with Taxol *in vitro*. These include, but are not limited to, overexpression of P-glycoprotein, alterations in tubulin and aberrant signal transduction pathways and/or cell death pathways. The real contribution of these potential mechanisms is ultimately dependent on the extent of dysregulation of normal cellular integrity in cancer cells. As our knowledge of drug resistance increases, based on *in vitro* models in resistant cells, it is apparent that there are multiple mechanisms responsible for the resistant phenotype in cells cultured *in vitro*. The contribution of the various drug-resistant phenotypes to acquired

Taxol resistance in actual human tumors has yet to be precisely defined.

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