Physiochemical Aspects of Tubulin-Interacting Antimitotic Drugs

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Abstract: (250 words): A diverse group of natural biological compounds bind to microtubules and suppress microtubule dynamics. Here we review the mechanism of microtubule assembly and dynamics as well as structural features that are important for nucleotide binding, GTP hydrolysis and stabilization of longitudinal and lateral protofilament contacts. Specific emphasis is placed upon the polar structure of the microtubule, the exposure of the nucleotide hydrolysis site at the + end and the conformational and configurational plasticity of the microtubule lattice. These features have important implications for the mechanism of dynamic instability and the disruptive action of antimitotic drugs. We then discuss the various classes of tubulin binding drugs emphasizing their site and mode of binding as well as the structural and energetic basis for their effects on microtubule assembly and dynamics. A common feature of tubulin-interacting compounds is a linkage to assembly, either the stabilization of a microtubule lattice by compounds like taxol or epothilone A, or the preferential formation of alternate lattice contacts and polymers at microtubule ends by compounds like colchicine, vinca alkaloids and cryptophycin-52. Finally, we explore the likely possibility that these drugs also disrupt the regulation of microtubule dynamics. Future generations of these compounds may be selectively developed to directly target the proteins that regulate mitotic spindle dynamics.

INTRODUCTION

Microtubules are highly dynamic macromolecular assemblies that are organized in a polar, spatial and temporal, cell cycle specific manner. The organization is regulated by numerous factors including the intrinsic ability of microtubule subunits, tubulin heterodimers, to form nonequilibrium, dynamic polymers. This process is referred to as dynamic instability and requires microtubule dependent GTP hydrolysis [1]. The extent of dynamic behavior depends upon the competing role of both stabilizing proteins, MAPs (tau, MAP1, MAP2, MAP4, XMAP215), and dynamics regulatory proteins that destabilize microtubules (stathmin, XKCM1, XKIF2, katanin) [2,3]. It is now well established that antimitotic drugs cause cell cycle arrest at G2/M and can do so in the absence of any significant depolymerization of the mitotic spindle [4,5]. Antimitotic and cytotoxic activity is believed to primarily arise from suppression of dynamics in the mitotic spindle, the inhibition of spindle assembly and/or the disruption of spindle-checkpoint functions [6-9]. Our goal here is to provide the physiochemical basis for the microtubule centric mode of action of antimitotic drugs. After elucidating the fundamental mechanism of microtubule dynamic instability and the role of dynamics in mitotic spindle function, we will emphasize the structural features of tubulin and microtubules that dictate the formation of polar structures and the hydrolysis of GTP. With this introduction as a basis, we will then describe the molecular features and energetic characteristics of antimitotic drugs. These drugs in general share the characteristic of binding to and stabilizing polymers, either microtubules or alternate polymer forms (eg. spirals or rings). A central assumption in this presentation is that these drugs preserve their \textit{in vitro} molecular features while interacting with the microtubule body and/or microtubule ends to disrupt dynamics \textit{in vivo} [10,11]. In addition, these drugs or future generations of these drugs may disrupt other processes (dynamics regulation, posttranslational modifications) leading to enhanced utility and selectivity.
MECHANISM OF MICROTUBULE ASSEMBLY AND DYNAMICS

The mechanism of microtubule assembly has been extensively investigated since the early seventies when \textit{in vitro} microtubule polymerization was first achieved [12,13]. The mechanism of assembly was initially described as nucleated condensation, based upon work by Oosawa and colleagues [14], and established a requirement for formation of an unstable nucleus (protofilaments and sheets) that could elongate to a steady state polymer mass. Nucleation, either spontaneously \textit{in vitro} or from centrosomes \textit{in vivo}, limits the number of microtubules formed, while elongation occurs exclusively at microtubule ends. This end-wise growth and disassembly has important implications for microtubule regulation and the mode of action of antimitotic drugs. For the structural reasons discussed in the next section, assembly is favored by the presence of GTP, Mg$^{2+}$ and high ionic strength. By analogy with the mechanism of actin filament assembly [15] Margolis and Wilson applied the concept of treadmilling to microtubule growth [16]. Double labeling, pulse chase experiments suggested the net addition of subunits to the fast growing or + end and the net loss of subunits from the slow growing or – end. This mechanism requires an irreversible step, GTP hydrolysis, to establish a flux of subunits through the polymer, adding tubulin-heterodimers to the + end and dissociating heterodimers from the - end. Subsequently it was discovered that in fact both ends of the microtubule are capable of undergoing independent excursions of growth and disassembly [17,17a], where the growth phase involves the addition of GTP containing subunits while the rapid disassembly phase involves the loss of GDP containing subunits. The establishment of this nonequilibrium process, occasionally referred to as a phase change, requires GTP hydrolysis. Treadmilling appears to occur \textit{in vivo} when microtubule ends are stabilized by other factors (centrosomes and kinetochores) and thus a steady state flux can occur through fixed length structures like mitotic spindles [18]. This process may also involve ATP hydrolysis and reflect the role of a molecular motor or motors. Dynamic microtubules are required for spindle assembly and progression into anaphase [6,8,9].

To investigate the properties of dynamic microtubules the field has taken advantage of the technique of video enhanced differential interference contrast (DIC) microscopy [19,20]. In these experiments microtubules are nucleated off axonemal seeds and the growth and disassembly excursions are captured on video for subsequent kinetic analysis. The phenomenon is described in terms of five rate or lifetime parameters, the rate of microtubule growth and disassembly, and the frequency of microtubule catastrophe, pause and recovery (see Table 1). In general, the + end of a microtubule grows faster than the – end, and the – end disassembles at the same rate [21] or faster [22] than the + end, but the range of values observed are broad and overlapping [21]. The frequency of catastrophe is either slightly greater at the + end [22] or the same at opposite ends [21], with the difference in part being due to the use of a time based (1/sec) versus a length based (1/µm) unit. Likewise, the rescue frequency is either slightly greater at the - end [22] or within error the same at opposite ends [21]. A definitive interpretation of these data in terms of uninuclear or bi-molecular events has been lacking due to a number of additional complexities in the system. First, microtubules are known to have a distribution of protofilaments per structure, varying from 9-18 \textit{in vitro} and occasionally \textit{in vivo} (23; see [24] for a recent discussion). Furthermore, the protofilament number can vary within the same polymer and during the growth or disassembly phase. Thus, the quaternary structure at the ends of a microtubule is heterogeneous and varies with time. Secondly, both assembly and disassembly are cooperative processes that partially reflect lateral interactions at microtubule ends, and the fact that tubulin can add to or dissociate from microtubules as curved protofilament polymers [22]. This phenomenon is especially dependent upon the stabilizing activity of Mg$^{2+}$ ion and the presence of regulatory protein factors [2,25]. Thirdly, it is still not entirely understood what structural or conformational changes must occur to convert a microtubule end from a growing to a disassembling phase. There has been insightful analysis that evaluates negative stain EM and cryoelectron microscopy data of growing and shortening microtubules in terms of major structural transitions from growing sheets to intermediate closed cylinders to depolymerizing ram’s horns or peeling protofilaments [1,22]. However, detailed analysis of the dynamic
Table 1  Effects of Drugs on Microtubule Dynamics

<table>
<thead>
<tr>
<th>Drug</th>
<th>duration (µm)</th>
<th>rate</th>
<th>frequency (µm)</th>
<th>Reference (comment)</th>
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<tbody>
<tr>
<td></td>
<td>g</td>
<td>s</td>
<td>pause</td>
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<tr>
<td>colchicine</td>
<td>-</td>
<td>-</td>
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<tr>
<td>naphthopyran</td>
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<td>estramustine</td>
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<tr>
<td>taxol</td>
<td>ne/-</td>
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<tr>
<td>taxol</td>
<td>ne</td>
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<td>taxol in cells</td>
<td>nd</td>
<td>nd</td>
<td>ne/+</td>
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<tr>
<td>vinblastine</td>
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<td>vinflunine</td>
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<td>vinorelbine</td>
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<td>vinblastine</td>
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<td>cryptophycin-52</td>
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<td>cemadotin</td>
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Unless indicated all parameters correspond to the + end of a microtubule. Abbreviations used: g = growth; s = shortening; ne= no effect; - means decreases; + means increases; ne/- means small decrease; ne/+ means small increase; nd, not determined or cannot infer from time based data. Duration of growth and shortening and frequency of catastrophe and rescue are listed in units of length to emphasize their occurrence per molecular event rather than per a rate dependent time scale. Many of the results are dose dependent and the summary reflects the overall trend of the data.

properties of microtubules reveals that each phase exhibits a distribution of rates or frequencies that can only be accounted for by stochastic models [22,26-28]. Consistent with this, Chretien and Fuller [24] have recently described 14 types of microtubule structures that define the range of energetic configurations possible between subunits and protofilaments, and may account for the stochastic nature of microtubule dynamics. To accommodate different protofilament numbers and helical repeats the protofilaments are skewed about their axis and longitudinally shifted to modify lateral interactions. It is therefore likely that each structural configuration has an intrinsically different rate of growth and disassembly, and random conversion between configurations alters those rates, thus giving rise to stochastic dynamic instability. Drug binding will disrupt these processes, but it is apparent that the mode of action of a drug will be complicated by (and must necessarily reflect) the numerous energetic and structural configurations at a microtubule end. To emphasize this point, it is worth noting that the depolymerizing ram’s horns observed during normal microtubule disassembly [22] are also observed in the presence of vinblastine [5,29] and in the presence of depolymerizing proteins like XKCM1 [30].

As discussed and emphasized in this review, the mode of action of these antimitotic drugs involves the suppression of microtubule dynamics. The effects of drugs on microtubule dynamics have been extensively investigated by video enhanced DIC microscopy, primarily in the labs of Wilson and Jordan (see Table 1). These studies primarily involve the + end of steady state microtubules. The data are typically presented as mean values +/- standard errors and not histograms of the broad distribution of values that typically occur [21]. In
the absence of drugs the rates of assembly/disassembly can vary widely and are dependent upon many factors including the microtubule end being studied, the presence of GTP hydrolysis, the concentration and ratio of GTP/GDP-tubulin in solution, the divalent cation concentration and presence of stabilizing MAPs [21,22,27,31-33]. Some of these factors, but not all, can be explained by a stabilizing influence on either a straight microtubule lattice or a curved peeling protofilament. An interpretation of most of the data however still awaits a detailed molecular understanding of dynamic instability (discussed below).

Upon the addition of antimitotic drugs the situation gets far simpler. With few exceptions these drugs decrease both the growing and shortening rate and duration, increase the rescue frequency, and increase the time spend in a pause state. Table 1 is organized to summarize these trends. For example, a decrease in the rate of growth or shortening is indicated by a minus sign. The duration of growth and shortening and the frequency of catastrophe and rescue are expressed in units of length, either µm or per µm. Many authors also express these data in terms of time of growth or catastrophes per unit time. However, time based values are a function of the rates and thus not truly independent. It seems more reasonable to ask how long a growth event occurred in units of length because it directly measures how many subunits were added [21,27]. Alternatively, catastrophes per unit length grown allows a correlation with number of subunits added and thus some measure of the probability of a conformation or configuration change occuring that leads to catastrophe [24]. The vast majority of the data indicate decreasing rates of growth and shortening (-) with a few no effects (ne), and one instance of an increase in rate (+). Likewise the pause data, expressed in units of the time spent, is in general increased with a few no effects or weak effects (taxol), and a few surprising decreases in the pause time. Many drugs increase the frequency of rescue, with taxol in general having no effect. There are also a number of disparate features in the data. Drugs effects can be gradual (taxol) or they can occur very abruptly and cooperatively (vinca alkaloids and cryptophycin). Drugs can behave differently at low doses, so-called substoichiometric, compared to high or stoichiometric levels (colchicine, vinca alkaloids and taxol). The effects can be at both microtubule ends, at one end, or some combination thereof in a drug concentration dependent manner. These features have mechanistic implications that will be discussed in detail below.

**IMPLICATIONS OF THE STRUCTURE OF TUBULIN FOR DRUG BINDING AND SUPPRESSION OF DYNAMICS**

The structure of the tubulin hetero-dimer was obtained by electron crystallography of zinc induced tubulin sheets [34]. The structure contains information at 3.7 Å on all but the final 10-18 carboxyl tail residues [35,36]. The disordered carboxyl tail is the site for subtilisin digestion [37], numerous post-translational modifications [38,39] and MAP binding [40,41]. Since zinc sheets are composed of protofilaments, the structure contains information on the polymerized longitudinal contacts both within and between dimers. Tubulin heterodimers have two nucleotide-binding sites, an N-site on the α-chain, always nonexchangeable, and an E-site on the β-chain, exchangeable when tubulin is in the heterodimeric form. The crystallographic structure reveals that both nucleotides become nonexchangeable when buried at a polymer interface. The N-site is buried at the intra-dimer interface, and the E-site is buried at the inter-dimer interface in microtubules, spirals and ring polymers. This structural feature further establishes a direct influence of nucleotides on stabilizing straight or curved quaternary structures in these polymers since the nucleotide is located at the interface itself [10,42]. Thus, the requirement for GTP in microtubule assembly reflects in part a direct structural and energetic influence on the microtubule lattice. Mg\(^{2+}\) is required for assembly in part because GTP binds as a Mg-GTP complex [43]. This structural feature is also differentially displayed at the ends of the microtubule. The nucleotide is exchangeable at the + end because the β-subunit and the E-site are exposed, and not exchangeable at the minus end because the α-subunit (but not a nucleotide site) is exposed [44-46]. As discussed below, this also has implications for how the nucleotide state of terminal tubulin subunits differentially cap and stabilize both ends of the microtubule.

Within the zinc-induced sheet GTP is hydrolyzed, but the sheet lattice keeps the
subunits in a straight quaternary structure. Thus, we have no direct structural information on the differences between the curved vs straight quaternary state other than the suggestion that an inter-dimer kink causes the curvature [35,36]. (As discussed below drugs like vinca alkaloids and dolastatin 10 may directly induce a similar kink at the inter-dimer interface.) It has been proposed that hydrolysis induces strain in the microtubule structure and this unfavorable energy accounts for the rapid dissociation rate of GDP-tubulin from microtubules [47]. The assembly of tubulin into microtubules and other polymeric forms like rings and spirals is favored by high Mg\(^{2+}\) concentration and high ionic strength. This in part overcomes the repulsive electrostatic influence of the highly charged carboxyl-tail region of each subunit. Limited subtilisin cleavage removes the carboxyl tail region at the end of H12 (see [34] for the assignment of this terminology), and dramatically enhances formation of rings or microtubules, depending upon the nucleotide conditions in solution [37]. In addition, increasing the ionic strength reduces the energetic difference between the GDP and GTP bound state. (These electrostatic effects are occurring against the background of the major entropic driving force for microtubule and spiral assembly, the burial of hydrophobic surfaces and the release of bound water [10,49-51].) Restated in energetic terms, GDP favors the curved quaternary structure in rings, spirals and dissociating protofilaments, while GTP favors the straight quaternary conformation of a microtubule or zinc-induced sheet lattice. The preference for GDP in rings and spirals is approximately 0.54 - 0.9 kcal/mol at each dimer interface [10,52]. In microtubules the preference for GTP is much larger (> 3-4 kcal/mol; [47,50]) since GDP-tubulin will not assemble in the absence of taxoids [53]. In this instance the unfavorable energy comes from both repulsive effects at the interface between heterodimers and disruption of lateral contacts between protofilaments. The disruption of lateral contacts need only reveal itself at microtubule ends in the form of curved protofilaments or ram’s horns, since this is the site of subunit addition and dissociation [22,35]. The inability of GDP-tubulin to form microtubules also partially reflects the energetically preferred formation of curved protofilaments and rings [53a].

The high-resolution structure of the tubulin dimer has also been docked into a 20 Å reconstruction of the microtubule lattice [51]. This model required no significant alteration of the structure and suggests a high degree of conservation between protofilaments in microtubules and two-dimensional zinc sheets. The binding site for taxol is located on the inside surface at a location labeled the M loop, between two adjacent protofilaments. The binding sites for MAPs (the carboxyl tail and H12; see [54]), molecular motors (H11 and H12), and the dynamics regulator stathmin [55], are located on the outside surface of the microtubule. Subtilisin digestion removes the binding site for most MAPs (not EMAP; [56]) and suggests structural MAPs bind primarily at the disordered and highly modified carboxyl tail regions [41]. Motor proteins bind to subtilisin digested tubulin, but recent work suggests that removal of the carboxyl tail regions disrupts processivity [57]. This is consistent with motor domains binding to helices H11 and H12 of the β-subunit on the outer surface of the microtubule, but then sliding to the next high affinity site while being electrostatically tethered to the carboxyl terminus of tubulin. Image reconstruction of microtubules decorated with motor domains suggests contacts on both the α- and β-subunit [58,59] possibly reflecting interactions from both the motor domains and the neck coiled-coil. It is currently not known what structural features are altered in the KinI family members XKCM1 and XKIF1 to target them to microtubule ends for disassembly, although an energetic preference for binding to and stabilizing curved protofilaments appears to be a sufficient explanation [30]. We emphasize these interactions and structural features because it is highly likely drugs may selectively disrupt or mimic these activities (see discussion below).

By homology with the bacterial protein FtsZ [60], it is now evident that tubulin is its own GTPase-activating protein. A catalytic residue, Glu-254, on the α-subunit from the next dimer in the protofilament is located in the inter-dimer interface and is directly involved in the cleavage of GTP bound to the β-subunit. This demonstrates the mechanism by which GTPase activity is tightly linked to self-association, and establishes a
structural requirement that GTP hydrolysis can occur only upon dimer-dimer contact. Note this necessarily applies to both microtubule assembly and colchicine-induced GTPase activity [10,35,36]. This further suggests that a straight quaternary structure, associated with microtubules and GTPase activity, positions Glu-254 correctly near the γ-phosphate on β-tubulin, while a curved quaternary structure, like rings and vinca-induced spirals, lack GTPase activity due to a shift in the interface that possibly displaces Glu-254 preventing hydrolysis. A role for α-Glu-254 in microtubule-induced hydrolysis is consistent with the high-resolution structure of zinc sheets [34,60], while the displacement of α-Glu-254 in spirals awaits structural verification. In this context it is worth noting that the colchicine binding site is located at or near the intra-dimer interface, 40 Å from the inter-dimer interface, while the vinca alkaloid binding site is located near helices H5 and H6 at the inter-dimer interface [36]. The implications of this hydrolysis mechanism for dynamic instability have only recently been considered [35,36,61]. It is now generally accepted that a single layer of subunits can laterally cap [62] and stabilize the ends of microtubules [1,63,64]. At the + end of a microtubule GTP-tubulin must be the predominant cap that stabilizes microtubules for growth since another layer of (GTP-containing) subunits must bind to initiate hydrolysis. For GDP-Pi-tubulin to be at the cap the terminal GTP-subunits must dissociate to expose the hydrolyzed nucleotide products they catalyzed. This state will probably be kinetically brief since Pi has a very weak affinity for GDP-tubulin [65]. At the – end of a microtubule the nucleotide site is buried and hydrolysis may occur at any time after addition. [The rate of this hydrolysis may depend upon the presence and nucleotide content of neighbor subunits (see [35]).] Thus, at the – end either GTP or GDP-Pi may act as the capping structure, depending upon the relative rate of assembly and hydrolysis. The assumption is these nucleotide caps will stabilize a (GTP favored) straight quaternary structure, growing sheets or intermediate closed microtubules, against a transition to a (GDP favored) curved quaternary structure, peeling protofilaments. The extent of stabilization as discussed above may vary with protofilament number and helical lattice structure as well as the configuration of nucleotide states at the microtubule ends (see [35] for more details). It is not yet clear if these structural arguments will quantitatively explain the differential stability and dynamics observed at microtubule ends.

Antimitotic drugs can be classified into various groups based upon their site of binding and their mode of interaction. Since we currently have only one structural verification of a drug-binding site, taxol [34,51], this categorization is undoubtedly in a state of uncertainty. Furthermore, since new microtubule associated binding proteins and activities are being discovered with enormous rapidity, the exact in vivo mode of drug activity is equally uncertain. Nonetheless, we will initially focus on a tubulin-centered vision of drug action. There are three general classes of drug binding sites on tubulin, the colchicine binding site, the taxol site and the vinca alkaloid site. Most other drugs appear to bind in competitive or noncompetitive fashion with at least one of these drugs, suggesting they share overlapping binding motifs [42,66] [There are exceptions like estramustine and naphthopyran (see Table 1) but not enough is known about those sites to discuss in detail.] There are also three general modes of interaction, tubulin-sequestering drugs like colchicine, drugs that induce alternate polymers like vinca alkaloids, and drugs that stabilize microtubules like taxol. To compound the complexity of this designation system, colchicine, taxol and vinca alkaloid-like drugs can also bind at or near the ends of microtubules and substoichiometrically (relative to the total tubulin concentration) poison microtubule assembly and dynamics. Given the end-wise mechanism of microtubule assembly and the importance of dynamics in regulating mitosis, it is generally assumed that the end-wise binding mode is most important in determining the cytotoxicity and antimitotic activity of these drugs [7]. The next sections present a detailed analysis of energetic linkages between subunit binding, microtubule binding and end-wise effects on microtubule assembly and dynamics.

ENERGETIC LINKAGES FOR NEW AND EMERGING CLASSES OF DRUGS

There have been numerous reviews of microtubule assembly and drug interactions from a physicochemical perspective in the past [1,42,67-71]. The major recent progress in the field has been achieved by the solving of the high-resolution
structure of tubulin and the docking of that structure into a microtubule lattice [34,51]. Thus, we are now better able to appreciate at a structural level many of the thermodynamic and allosteric linkages that occur in the system. A common theme that is structurally evident is that the mode of action of tubulin-interacting compounds is strongly linked to nucleotide effects (see [42] for detailed background), and nucleotide hydrolysis intimately regulates the assembly and dynamics of microtubules. The next three sections discuss this concept for three classes of tubulin-interacting drugs.

The traditional means of quantifying microtubule assembly is to measure the critical concentration, Cc, the tubulin concentration below which nucleation of microtubule assembly will not occur. Lee and Timasheff [49] demonstrated that this value corresponds to the propagation constant for polymer growth (Cc = 1/Kp) if one assumes helical polymerization where all the binding sites are equivalent. Thus at 1 μM Cc the Kp equals 1 x 10^6 M\(^{-1}\) and, at 37°C, corresponds to -8.5 kcal/mol of stabilization free energy. The free energy of stabilization is the (average) sum total of all interactions that occur in the system upon subunit addition. The measurement of Kp values has provided great insight into the thermodynamics of microtubule assembly and the role of solution conditions, MAPs and drugs [49,50,72,73]. However, as the actual structure and nucleotide dependence of microtubule ends suggests [24,62], it is well established that the energetics and kinetics of subunit addition are dauntingly complex (see discussion in [35,62,68]). In the discussion of individual drug sites and their effects on microtubule dynamics presented below, this complexity, while not understood, is a central focus and a challenging area for future experimental exploration.

**DRUGS THAT BIND AT THE COCHICINE SITE**

An excellent starting point for understanding the energetic linkages involved in antimitotics is the classic tubulin binding drug colchicine (see [74,75] for more background). Colchicine binds irreversibly to the tubulin heterodimer at a site near the intra-dimer [76]. (It is worth noting Downing and Nogales [76] docked colchicine into this site by molecular modeling, and more detailed QSAR analyses of many colchicine derivatives are currently in progress [75,77,78].) The irreversible character arises from a large activation barrier and significant conformational changes within tubulin but not the drug [79]. This tubulin-colchicine complex (TC) substoichiometrically inhibits microtubule assembly and activates a weak GTPase activity in the dimer. The inhibition activity is presumably due to the fact that colchicine binding alters contacts within the microtubule and disrupts normal microtubule growth. The colchicine site faces the microtubule interior and thus, in the absence of structural data, we must concur that the unfavorable energy comes from a conformational change in the tubulin component of the complex. A caveat to this observation is that at high concentrations the TC complex will assemble into sheet like polymers that resemble a microtubule protofilament lattice [72]. Thus, this altered lattice can favorably accommodate the conformational changes in the TC complex. These structural effects are likely to be subtle, however, because TC-induced sheets form in an entropically driven manner with a similar critical concentration and heat capacity change, ΔCp, to that of normal microtubules [72,80].

Colchicine-induced GTPase activity has been interpreted historically in terms of a conformational change within the dimer. Given the catalytic group is located on another dimer [60], GTP hydrolysis requires self-association, and thus any conformational change induced by colchicine binding is actually stabilizing the straight quaternary inter-dimer interface. This interpretation is supported by the fact that GTPase activity is TC concentration-dependent [81]. It is also supported by the fact that tubulin spontaneously undergoes weak Mg\(^{2+}\)-dependent self-association into curved protofilaments and rings. Recently Chaudhuri, et al. [82] probed the structure of the TC complex by a protein footprinting study and observed a conformational change far from the colchicine binding site. The perturbed region was identified by the protection of cys295, 305, 315 and 316 from labeling with N-ethylmaleimide (NEM) in the presence of colchicine. These residues, 295-316 on the α-subunit, correspond to H9 and B8 near the top of the α--subunit, a region that is very close to the interface with the β-subunit on another dimer.
within a protofilament, and directly involved in lateral contacts between protofilaments [51]. Thus, colchicine binding alters the conformation of residues near the longitudinal and lateral protofilament interfaces and potentially destabilizes a normal microtubule lattice. This could also account for the stabilization of small TC polymers and the activation of GTPase activity in those polymers. (Note Chaudhuri, et al. [82] restricted their interpretation to a conformational change and neglected the importance of changes in the inter-dimer interfaces and the disruption or stabilization of different polymer forms. We suggest the protection of cysteine residues they observed is in part due to the colchicine-induced formation of small oligomers in their solutions.)

Thus, the proper mechanistic interpretation of colchicine binding to tubulin requires an inclusion of effects on the destabilization of a microtubule lattice and the formation of alternate polymer forms. The drug itself binds at a site away from the interdimer interface but induces conformational changes that destabilize the microtubule lattice. It is not understood how on the one hand colchicine favors GTPase activity and presumably a straight quaternary structure, yet disfavors the straight microtubule lattice. Presumably effects on lateral or protofilament packing disrupt a typical closed microtubule structure and favor sheets. Dynamics data (Table 1; [83,84]) suggest the TC complex substoichiometrically suppresses microtubule growth, and favors both a pause state and rescue from disassembly. Thus colchicine opposes the typical effects of GTP hydrolysis and stabilizes the ends of a microtubule, possibly because it disfavors the formation of curved peeling protofilaments. This effect will be long lasting in cells due to the irreversible binding of colchicine to tubulin [85]. Colchicine binds to tubulin heterodimers containing the β3 isotype slower than to the other isotypes [86]. This suggests isotypes may regulate the action of drugs in vivo (see overview in [6]), although the overall colchicine affinity for these isotypes varies by only a factor of 2-3.

Drugs like podophyllotoxin reversibly compete for the colchicine site but do not induce GTPase activity or induce conformational changes. This appears to explain why many of these drugs have weak or stoichiometric effects on microtubule assembly [87]; they do not disrupt the lattice and act more as true sequestering drugs. RPR112378 and RPR115781 [88] and T138067 [89] are representatives of a new but related family of microtubule assembly inhibitors. They also bind at the colchicine site without increasing GTPase activity (GTPase not measured for T138067). However, they do induce the formation of cold stable aggregates, and their cytotoxicity is due to covalent interaction at the intra-dimer interface with cysteine 239. These covalent interactions should also be highly cytotoxic and, it has been suggested, effective against MDR expressing tumor cells [89]. (It is not yet clear if irreversible binding will in general make these compounds inappropriate for clinical use [85].) Thus, the colchicine class of compounds (including combretastatin- and staganacin-related compounds; [66]) will most effectively disrupt dynamics when the induction of a conformational change induces GTPase activity, disrupts lateral contacts, but stabilizes both + and - microtubule ends. This hypothesis is supported by the recent observation on a family of colchicine analogs that the ability to inhibit microtubule assembly is not a simple function of the tubulin binding affinity [77]. Hidden in this apparent contradiction (disrupts but stabilizes) may be information about both the lateral and longitudinal interactions at a microtubule end that trigger dynamic instability.

**DRUGS THAT BIND AT THE TAXOL SITE**

Taxol was present in the electron crystallography experiments that were used to solve the structure of tubulin [34]. These data, in conjunction with the microtubule docking analysis [51], have been interpreted to explain the ability of taxol to stabilize microtubules [35,90]. A number of other natural compounds, epothilones A and B, eleutherobin and discodermolide, appear to bind to the taxol site and stabilize microtubules in a similar manner [91]. Taxol binds to microtubules, but not heterodimers, at the M loop, located between B7 and H9 on the β-subunit, and stabilizes lateral contacts between protofilaments. The drug also interacts with H7, the core helix [51,90], and is proposed to structurally counteract the destabilizing effects of GTP hydrolysis [35]. These sites of interaction are consistent with photoaffinity labeling [92,93,93a] and missense mutations that confer drug resistance [91,94,94a]. There are a number of caveats worth mentioning.
First, the original analysis actually docked the extended crystal structure of taxotere, and not taxol, into the appropriate electron density from zinc sheets because the taxol coordinates were not available [76]. Taxol prefers to bind to 12 protofilament microtubules, and taxotere to 13 protofilament microtubules [95], but the image reconstruction analysis was done on a 15 protofilament structure. The kinetics of taxol binding appear to be insensitive to the protofilament number [96], and it is assumed that the lateral plasticity of microtubule protofilaments involves contacts stabilized by taxol. Nonetheless, there continues to be some controversy about the correct conformation of bound taxol, collapsed or extended [91,97], or perhaps an intermediate “T-shaped” conformation [98]. However, there are reports of multiple, nucleotide-dependent binding sites for taxol [99]. Furthermore, the kinetics of binding and the microtubule structural rearrangements induced by binding have been interpreted to suggest that the site is more solvent exposed than the current buried, lumen-binding model implies [95,96]. In fact the data suggest a remarkable ability of microtubules to rapidly change protofilament number upon taxol/taxotere binding/exchange. How entire protofilaments can be rapidly added or removed without microtubule disassembly is not clear. Finally, there are reports that the effect of taxol on microtubule dynamics is dependent upon the isotype composition of a microtubule in vitro (disassembly rates vary by a factor of two; Table 1; [100]) and thus potentially dependent upon the cell type in vivo [101].

As discussed above, our focus here will be on the effects of taxol on microtubule dynamics. Consistent with the mode of action of other antimitotic drugs, taxol suppresses dynamics and kills cells without altering the total mass of assembled microtubules (Table 1; [101,102]). Studies on dynamics in vitro reveal that at the lowest drug concentrations (< 100 nM) taxol selectively suppresses the rate and extent of shortening at the + end of microtubules (Table 1; [103]). At intermediate concentrations (100 nM – 1 µM) taxol inhibits growth and shortening at both microtubule ends. [The effects of taxol on microtubule dynamics are surprisingly gradual, possibly reflecting a modulating effect of the large number of binding sites along the microtubule length (see below).] These data are consistent with both structural and energetic observations. Li, et al. [99] observed heterogeneous taxol binding sites on microtubules assembled with GTP-tubulin, and suggest taxol binds to a high affinity site associated with GTP at the E-site. Upon hydrolysis the site is converted to a low affinity taxol binding site (3.2 kcal/mol weaker). It is not clear why taxol binding would not induce formation of the high affinity site, unless lattice cooperativity and heterogeneity prevent simple linkage. Diaz, et al. [95] do observe rapid taxol-induced changes in (MAP-free) microtubule structure and protofilament number. In a more recent fluorescent-taxol kinetic study Diaz, et al. [96] also observed a homogeneous class of taxol binding sites. These results raise the possibility that the probe used by Li, et al. [99] may influence the result. Nonetheless, the results by Li, et al. [99] imply the high-resolution taxol-binding site [34], being used to construct pharmacophores of taxol [98] and epothilone [91], may correspond to the low affinity site or a mixture of low and high affinity sites. Thus, if both high and low affinity sites occur, taxol should prefer to bind to the GTP containing cap region or rapidly polymerizing regions at microtubule ends. This is supported by recent studies in vivo with different fluorescent taxoids which demonstrate a predominant interaction with newly polymerized sites [104]. The structural restrictions of GTP-hydrolysis (see discussion above) suggest the + end, but not the – end, of a growing microtubule must have GTP-tubulin in the cap. The selective suppression of shortening at the + end is thus entirely consistent with a nucleotide, GTP-cap specific interpretation of the low taxol concentration dynamics data. Taxol does not induce the high affinity [103], but rather the sites preexist and are coincident with the GTP-cap region. Taxol will thus initially and selectively bind to the dynamic + ends of microtubules. It is not clear if this means binding occurs to growing sheets or closed intermediates or both, but growth was not suppressed in the low concentration regimen (< 100 nM). [This suggests that taxol may also selectively, but weakly (see [101]), inhibit the interaction of microtubules with dynamics regulators, which are, by their preference for peeling protofilaments, end binders.] This analysis of the effects of taxol on microtubule dynamics and structure clearly suggests that the controversy concerning the conformation of bound taxol and the location of the taxol-binding site will necessarily continue for some time. It may only be resolved when high-resolution structural data is
collected on the ends of microtubules, and on sheets in different nucleotide states, for example using GMPCPP as a GTP analog. The extrapolation of these results to cell culture or clinical settings is complicated by the ability of cells to concentrate taxol, by binding to microtubules, and thus saturate a significant fraction of the potential sites [4,5,7,102]. Taxol may actually cause mitotic arrest in the concentration regime described here and in Table 1 as > 100 nM.

DRUGS THAT BIND AT THE VINCA ALKALOID SITE

Vinca alkaloids, like vinblastine and vincristine, induce tubulin to form alternate spiral polymers. The thermodynamics of formation of spirals and large paracrystals have been intensively investigated by Timasheff and coworkers [105,106] and more recently by Lobert and Correia [10,11]). The reaction is best described by a ligand-mediated indefinite polymerization mechanism that is entropically- driven and favored by GDP, Mg$^{2+}$ and high ionic strength [10,48]. These factors are all consistent with the structure of the interface buried during spiral formation. Like microtubule protofilament assembly, the interface is primarily hydrophobic and contains a nucleotide-metal complex. Vinblastine crosslinks to a site near H5 and H6, at the interdimer interface, that interacts with the next dimer in a protofilament [35]. It has thus been proposed that this class of drugs (vinca alkaloids, dolastatin 10) sterically distorts or kinks the interface to a curved quaternary structure that is favored by GDP [10]. This enhancement by GDP implies vinca alkaloids will form spirals that will propagate into a microtubule GDP containing lattice and dissociate or unzipper microtubules by peeling spiral protofilaments. These spirals are stable, equilibrium polymers that slowly dissociate, consistent with the formation of mixed polymers that contain microtubule cores and blossoms-of-spirals protruding from both ends [29]. These data are also consistent with the interpretation that even a single drug molecule can bind at a microtubule end at an interdimer interface and thus form a kinked spiral protofilament, albeit short. Based upon the microtubule subunit structure and the dynamics data described below, the binding of drug or drug-tubulin complexes may be mechanistically different at opposite microtubule end [5,10,35,107]. The most recent work has focused on structure activity relationships in a family of vinca alkaloid congeners [11], investigating the relationship between the ability to induce spirals, inhibit microtubule assembly and cytotoxicity against the leukemia L1210 cell line. These vinca alkaloid derivatives differ in their ability to form spirals by up to 500-fold [11]. The results demonstrated a strong correlation between the ability to make spiral polymers and cytotoxicity. There was no correlation with inhibition of microtubule assembly in a turbidity assay, probably due to the formation of mixed polymers. The interpretation of these data is that spiral formation at microtubule ends suppresses dynamics and enhanced spiral formation correlates with the lifetime of suppression. A similar conclusion has been reached based upon paracrystal condensation, turbidity and pelleting assays [108]. It is likely that the ability to form paracrystals correlates with the average spiral size and thus the agreement is understandable. β-tubulin isotypes interact with clinically useful vinca alkaloids with nearly the same spiraling potential [109], suggesting the differential antitumor efficacy observed for these drugs is not due to differences in isotype compositions, but there is disagreement on this point [6].

At substoichiometric concentrations vinblastine differentially suppresses dynamics at opposite microtubule ends (Table 1; [107]). At the + end the effect exhibits a steep and cooperative concentration dependence [5,107]. Both growing and shortening rates are suppressed equally while the time spent in a pause state increases. At the – ends there is no effect on growth or shortening rates; there is only an effect on the catastrophe frequency. At higher drug concentrations spiral polymers grow off both ends and dynamics are suppressed extensively and equally. As discussed elsewhere in this review, these data offer insight into the differential structure and dynamics of microtubule ends. At the + end and at high drug concentrations, these data are consistent with vinca alkaloids being able to bind to microtubule ends where they induce spiral polymers that disrupt dynamics. Spiral polymers are equilibrium polymers that do not hydrolyze GTP and thus do not display dynamic instability. The steep concentration dependence observed at the + end is consistent with a highly cooperative, linked self-
association process that may reflect unique features of vinblastine binding at microtubule + ends. At higher concentrations the formation of mixed polymers with blossoms-of-spirals at both ends reflects longitudinal cooperativity in the formation of peeling spiral polymers, and possibly lateral cooperativity between adjacent peeling spiral polymers [10,108]. Other related drugs may simply alter the lattice to a more stable nucleotide insensitive configuration. For example, approximately 20 molecules of cryptophicin 52 appear to cooperatively bind to both ends of a microtubule, suggesting the formation of some kind of a unique capping structure [110,111].

The low concentration data requires a more detailed look at the differential structure of the microtubule ends. The + end has a terminal β-subunit with an exposed nucleotide binding site and thus a site for a stabilizing GTP-cap. The –end has a terminal α-subunit and a buried GTP-site that can undergo hydrolysis. In the case of taxol the argument has been made that taxol may prefer a GTP-lattice over a GDP lattice thus accounting for the + end preference. However, vinca alkaloids favor GDP in the interdimer interface and thus in this instance should not have an energetic preference for the + end. We favor a kinetic interpretation of the data and stress the differential end structure and concentration of solution reactants [10]. At low drug concentration the solution will contain mostly free drug and low concentrations of drug-tubulin complex and small drug-induced spirals. The + end of the microtubule can bind either free drug or liganded tubulin complexes. Drug or complex binding to the + end should induce a kink or spiral that will disfavor hydrolysis and microtubule growth and shortening. It is possible that the altered lattices or structures formed upon the initial binding of vinca alkaloids to a microtubule end resemble the paused state in normal assembly. However, at the – end only liganded tubulin complexes can bind, and not free drug. The only assumption here is vinca alkaloids bind to β-tubulin and not α-tubulin. Since the spiral complex concentrations are low relative to GTP-tubulin they will compete inefficiently and thus neither growth or shortening is affected. Raising the drug concentration raises the spiral complex concentration and thus both ends become sites for dynamic suppression. Why does the - end exhibit increased catastrophe at low drug concentrations? It is possible that liganded complexes bind with low efficiency but favor or induce a curved lattice conformation that resembles catastrophe at the – end. Thus, once again we are left with the requirement for understanding the structure and lattice conformation of different microtubule phases.

**OTHER PLAYERS AND THE REGULATION OF DYNAMICS**

If the effect of drugs is primarily on suppression of microtubule dynamics, then factors that regulate microtubule dynamics in a cell cycle- and phosphorylation-specific manner should be considered as essential elements of the process. Factors like XKCM1 and OP18 (or stathmin) bind specifically to microtubule ends and cause dissociation of tubulin dimers [25,61]. In the case of XKCM1, a kinesin related member of the KIN I protein family, disassembly is induced at both ends of the microtubule, the process is catalytic and involves ATP hydrolysis [30]. ATP hydrolysis is required to release the dissociated dimer from the microtubule, and the use of nonhydrolyzable ATP (AMPPNP) or GTP (GMPCPP) analogs also causes an accumulation of spiral and curved polymers at the microtubule ends. The blossom-of-spirals formed with GMPCPP undoubtedly reflects some stabilization of peeling protofilaments or sheets. It is not currently understood how the binding of XKCM1 or XKIF2 to microtubules is targeted to both microtubule ends and what features cause disruption of lateral contacts and dissociation of dimers. Stathmin binds to two tubulin dimers and holds them in a curved, GDP conformation [55], analogous to the curved, GDP conformation associated with disassembling protofilaments. Mg-induced ring formation and vinca-alkaloid spirals. There appears to be two related activities of stathmin that are pH dependent [112]. At low pH, 6.8, stathmin primarily acts as a sequesterer that binds to two tubulin dimers and holds them in a curved conformation [55], analogous to the curved, GDP conformation associated with disassembling protofilaments. Mg-induced ring formation and vinca-alkaloid spirals. There appears to be two related activities of stathmin that are pH dependent [112]. At low pH, 6.8, stathmin primarily acts as a sequesterer that binds to two tubulin dimers and holds them in a curved, helical conformation. Consistent with the energetics of other straight and curved quaternary structures, this interaction is favored by GDP binding [113]. At high pH, 7.5, stathmin binds to the microtubule + end and disrupts assembly and dynamics directly, possibly partially facilitated by the lower stability of microtubules at higher pH. Presumably a similar curvature induced at microtubule ends by stathmin (or XKCM1)
binding is the mechanism for destabilizing lateral contacts. It has been suggested stathmin destabilizes microtubule + ends by inducing GTP hydrolysis \[114\], but that interpretation seems to be inconsistent with binding studies \[113\], where stable stathmin-(GTP-tubulin)\(_2\) complexes form, and the formation of a curved interdimer interface \[55\], which should disfavor GTP-hydrolysis.

McNally \[61\] has proposed the + end specificity of stathmin tells us something about the structure or the stability of the + vs the – end and specifically suggested the – end lacks a GTP-cap. This interpretation is supported by the hydrolysis mechanism discussed above \[114\]. Nogales \[35\] has recently suggested the – end is stabilized by the terminal contact of GTP containing \(\alpha\)-subunits which imparts additional stability to the minus end, regardless of the nucleotide content of the \(\beta\)-subunit. The + end, in the absence of a GTP-cap, is stabilized by the terminal contact of GDP containing \(\beta\)-subunits which favors lateral peeling and dissociation. By this structural argument the + end is more prone to form peeling protofilaments and possibly act as a target for stathmin binding, while the minus end is more prone to remain in a closed quaternary conformation and resist stathmin induced conformational changes. (Note the distinction between sufficient interaction to bind vs. binding and inducing curvature and dissociation.) This interpretation is also favored by microtubule cutting experiments where severed microtubules undergo catastrophes at the new + end but pause or recover growth at the – end \[115\]. A more direct and steric explanation might be that the globular domain of stathmin binds to the exposed \(\beta\)-subunit, at or near the + end nucleotide site, but cannot bind to a buried \(\beta\)-subunit at the – end. Additional work is required to rectify these models with both the structure and the dynamics of microtubule ends. Specifically it might be helpful to compare the structure of + vs. – ends during growing and shortening phases to see if – ends peel less frequently.

There is no experimental evidence that drug binding to microtubule ends directly interacts with these factors. However, given that these factors induce disassembly and thus probably favor a curved conformation to break lateral bonds, we can anticipate that drugs may act in either a cooperative or an anti-cooperative manner with these factors depending upon their detailed interaction with microtubule ends. For example, vinca alkaloids favor spiral formation and since stathmin (and presumably XKCM1) induces a curvature similar to a vinca induced spiral, we expect they might cooperate in dissociating microtubules or creating blossoms-of-spirals at the ends. Alternatively, taxol binds between protofilaments and favors a straight topology. Thus, taxol would oppose the effect of XKCM1 and stathmin \[30\]. Furthermore, since vinca-alkaloids (and possibly taxol) specifically bind to microtubule ends their influence would occur at the earliest stages of binding and thus the lowest effective concentrations. The competition of taxol with dynamics regulation would be modulated by the fact that it binds at numerous sites along the microtubule, and thus disassembling microtubules would continuously reach a site of bound taxol that would resist disassembly. For example, in interphase cells only microtubule ends near the cell periphery exhibit dynamic behavior \[1\], and thus the rest of the microtubule network will temper the influence of taxol by binding and essentially sequestering drug. However, during M phase, the fact that mitotic spindle microtubules are in continuous flux would essentially guarantee that taxol will disrupt dynamics. This may explain taxols remarkable clinical utility \[7\].

The suggestion that antimitotic drugs might act at other sites or disrupt other factors is consistent with the idea that each protein interacts with numerous other regulatory factors, potentially in a cell type- or cell cycle-specific manner. New generations of drugs are being developed and tested for their selective ability to disrupt one of these factors but not all \[116\]. Depending upon the screening method it should be possible to select for drugs that disrupt dynamics, motility, or cytoskeletal organization in a cell cycle specific manner. Thus, testing of the current family of antimitotics discussed here might reveal unusual activities that future rounds of synthesis and QSAR can evolve into highly specific drugs.

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