EFFECTS OF ANTIMITOTIC AGENTS ON TUBULIN–NUCLEOTIDE INTERACTIONS

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Abstract—The interaction of antimitotic drugs with guanine nucleotides in the tubulin-microtubule system is reviewed. Antimitotic agent–tubulin interactions can be covalent, entropic, allosteric or coupled to other equilibria (such as divalent cation binding, alternate polymer formation, or the stabilization of native tubulin structure). Antimitotics bind to tubulin at a few common sites and alter the ability of tubulin to form microtubules. Colchicine and podophyllotoxin compete for a common overlapping binding site but only colchicine induces GTPase activity and large conformational changes in the tubulin heterodimer. The vinca alkaloids, vinblastine and vincristine, the macrocyclic ansa macrolides, maytansine and ansamitocin P-3, and the fungal antimitotic, rhizoxin, share and compete for a different binding site near the exchangeable nucleotide binding site. The macrocyclic heptapeptide, phomopsin A, and the depsipeptide, dolastatin 10, bind to a site adjacent to the vinca alkaloid and nucleotide sites. Colchicine, vinca alkaloids, dolastatin 10 and phomopsin A induce alternate polymer formation (sheets for colchicine, spirals for vinblastine and vincristine and rings for dolastatin 10 and phomopsin A). Maytansine, ansamitocin P-3 and rhizoxin inhibit vinblastine-induced spiral formation. Taxol stoichiometrically induces microtubule formation and, in the presence of GTP, assembly-associated GTP hydrolysis. Analogs of guanine nucleotides also alter polymer morphology. Thus, sites on tubulin for drugs and nucleotides communicate allosterically with the interfaces that form longitudinal and lateral contacts within a microtubule. Microtubule associated proteins (MAPs), divalent cations, and buffer components can alter the surface interactions of tubulin and thus modulate the interactions between antimitotic drugs and guanine nucleotides.

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1. MODES OF INTERACTION

The interaction of antimitotic drugs with tubulin and microtubules has been extensively reviewed (Lacey, 1988; Hamel, 1990) and is a very active area of research. These drugs are useful as biochemical and cytological probes, and have proven or potential utility as antineoplastic agents. This review will highlight a particular aspect of their interaction with tubulin: the interplay between antimitotics and nucleotides. This interaction will be discussed first in terms of a number of general molecular aspects. Specific drugs and topics will be discussed later in this chapter.

1.1. MECHANISM OF MICROTUBULE ASSEMBLY

The mechanism of microtubule assembly/disassembly has been reviewed elsewhere (Correia and Williams, 1983; Purich and Kristofferson, 1984; Dustin, 1984; Kirschnner and Mitchison, 1986;
Microtubules are composed of tubulin (an \(\alpha\beta\) heterodimer of 40% sequence-similar polypeptides, each of about 50 kDa) and numerous microtubule associated proteins. MAPs (Wiche et al., 1991), that decorate the exterior walls of the hollow microtubule structure. Typical purification schemes generate microtubule protein (MTP), tubulin plus MAPs, or purified tubulin (Williams and Lee, 1982). Multiple tubulin genes, gene products, and post-translational modifications occur in a species specific manner (Cleveland and Sullivan, 1985; Sullivan, 1988). There are two guanine nucleotide binding sites in a tubulin heterodimer. One, the exchangeable (or E-site) on the \(\beta\) chain, will rapidly exchange GTP for GDP in a Mg\(^{2+}\) dependent manner (Correia et al., 1987), and will hydrolyze GTP during microtubule formation. The other site (N-site) on the \(\alpha\) chain is noncatalytic, is always occupied by GTP, and is nonexchangeable for nucleotides, although it will slowly exchange free Mn\(^{2+}\) for bound Mg\(^{2+}\) (Correia et al., 1988).

In vivo, tubulin forms a helical rod composed of 13 protofilaments that run the length of the polymer (Scheele et al., 1982). In vitro conditions often support structures composed of 12–16 protofilaments (Scheele et al., 1982). It is not known how this variation affects ligand binding sites. Microtubules are 300 Å in diameter, by electron density maps from fiber diffraction studies (Besse et al., 1987a,b). The molecular mechanism of microtubule assembly is believed to proceed from a nucleation event, a highly unlikely event that exhibits a critical tubulin concentration, \(C_o\), below which polymerization does not occur. Nucleation probably involves protofilament formation and lateral association of protofilaments into a sheet structure that curves into a helical rod (Thompson et al., 1981; Detrich et al., 1985). Sheets are often observed by electron microscopy and the occurrence of a short sheet region with a microtubule is consistent with a helical lattice with a seam, referred to as the B-type lattice (Mandelkow et al., 1986; Linck, 1989; Mandelkow and Mandelkow, 1989). There is evidence that microtubules are cylindrical sheets, a two dimensional polymer constrained to a cylindrical surface, and that sheets and ribbons are overshoot products (Mandelkow and Mandelkow, 1989). Upon closure of the sheet, microtubule growth, called elongation, occurs in an endwise manner with heterodimers adding to both ends in vitro, although at different rates (Fig. 1). The fast growing end is the plus end (Fig. 1). The minus end is typically anchored to microtubule organizing centers (MTOC) like basal bodies or centriosomes (Euteneuer and McIntosh, 1981a,b), and thus growth in vivo typically occurs in the plus direction. A different critical concentration and rate of growth at the two ends is allowed thermodynamically because of the irreversible step of GTP hydrolysis that is coupled to subunit addition to the polymer (Kirschner and Mitchison, 1986). This hydrolysis step occurs concurrent with or soon after subunit addition and limits the size of the GTP cap, a region of tubulin heterodimers at both ends of the microtubule that contain GTP bound to their E-site. Subunit disassembly occurs by endwise loss of heterodimers, now containing GDP at the E-site (Melki et al., 1989), although at high Mg\(^{2+}\) concentration protofilament or ring structures may disassemble, thus accelerating the rates (Mandelkow and Mandelkow, 1985; O'Brien et al., 1990; Fig. 1). The hydrolysis of GTP and the conformation switch to the GDP form of tubulin within the microtubule is believed to include the release of MgPO\(_4\). This is similar to the mechanism of ATP hydrolysis in actin and actomyosin filaments (Korn et al., 1987; Carlier, 1975a,b; Howard and Timasheff, 1986).

![Fig. 1. An idealized mechanism of microtubule assembly/disassembly. GTP—tubulin heterodimers with GTP at the E-site are represented by dark ovals; GDP—tubulin heterodimers are represented by light ovals. Microtubule growth occurs at both ends in vitro. In vivo the slow growing end, the minus end, may be anchored at MTOC's. GTP hydrolysis occurs upon assembly and, after the release of HPO\(_4\)Mg, generates a microtubule that is stabilized by a GTP cap, a layer of GTP—tubulin subunits that prevent catastrophic disassembly. Disassembly may occur by the loss of oligomers. Subunits may also form nonmicrotubule polymers, typically double walled rings. Only the GDP—tubulin reaction is shown (Frigon and Timasheff, 1975a,b; Howard and Timasheff, 1986).](image-url)
The addition of GTP–tubulin subunits to the ends of a growing microtubule, and the hydrolysis of GTP after addition to the microtubule, leads to a gradient of GTP within the microtubule and thus to two forms of polymers, the GDP- and the GTP-form. This has been described as a phase transition (Hill, 1983). It is now widely believed (however see discussion in Carlier, 1991b) that only a few GTP-subunits (maybe as few as one GTP–tubulin layer) exist at the end of any given microtubule, consistent with a lateral cap model (Bayley et al., 1990) or a vectorial model (Carlier et al., 1984). This conclusion is strongly dependent upon the ability to rapidly fix and trap a population of microtubules during growth such that they retain \( \gamma^3\)P labeled GTP (Stewart et al., 1990). The temporal sequence of this event boils down to whether the delay after GTP–tubulin binding and before GTP hydrolysis, with the corresponding conformational change(s), is \( \mu\)sec or sec (see Carlier, 1991b for a discussion). Alternative theories endorse co-operative effects at the end of a cylindrical surface (Mandelkow and Mandelkow, 1989). Under steady state conditions, the removal of this cap allows for a conversion from an assembling or growing microtubule with GTP–tubulin at the ends to a disassembling or shrinking microtubule with GDP–tubulin at the ends. This process, referred to as dynamic instability, was first observed with microtubules nucleated from isolated centrosomes (Mitchison and Kirschner, 1984a,b) and is known to occur in vitro and in vivo. The effect is due to the fact that the off rate for GDP–tubulin heterodimers is ca. 100 fold faster than GTP–tubulin heterodimers (Gal et al., 1988; O'Brien et al., 1990). This may in part be due to the increased ability of GDP–tubulin heterodimers to form oligomers in a Mg\(^{2+}\) dependent manner (Frigon and Timasheff, 1975a,b; Howard and Timasheff, 1986; see Fig. 1), and is consistent with different conformations of GDP– and GTP–tubulin. The consequences of this are that (1) microtubules are simultaneously growing and shrinking in the same solution although maintaining a constant polymer mass, (2) GTP is hydrolyzed in an initial burst with microtubule polymerization and at a steady state with dynamic instability, and (3) the mean length of microtubules increases with time as a few microtubules completely depolymerize (catastrophic disassembly) and the heterodimers released exchange bound GDP for free GTP and add onto existing microtubules. Recovery or rescue from disassembly (tempered disassembly) is typically observed (Horio and Hotani, 1986; Bayley et al., 1990; O'Brien et al., 1990). The extent of microtubule dynamics is strongly dependent upon the buffer conditions. Glycerol (Kristofferson et al., 1986) and the presence of accessory proteins, MAPs (Kristofferson and Purich, 1981; Horio and Hotani, 1986; Keates, 1990), reduce dynamics. There is some support for treadmilling (Wegner, 1976) under these conditions, a vectorial process where growth occurs at one end and disassembly occurs at the other end, leading to a flux of subunits through the microtubule (Farrell et al., 1987; Hotani and Horio, 1988; see discussion in Correia and Williams, 1983).

Most of the above discussion pertains to in vitro experiments although the same concepts are often invoked in explaining in vivo results. For example, microtubules in nonneuronal cells are known to be dynamic (Schulze and Kirschner, 1986) and microtubule dynamics are believed to be involved in the formation of the mitotic spindle and chromosome movement (Gorbsky and Borisy, 1989; Mitchison, 1988). Thus, cellular requirements of a cytoskeleton are consistent with the dynamic instability model of microtubule assembly/disassembly (see Kirschner and Mitchison, 1986).

### 1.2. General Features of Antimitotic Drugs–Tubulin Interactions

As explained above, guanine nucleotides are involved in microtubule assembly and dynamics. Antimitotic agents that inhibit microtubule formation can be viewed as repressor molecules in this interaction. The activators are GTP and MAPs. The activity is dynamic assembly. In this context, then, our goal is to outline the various molecular means by which these effectors interact. Drugs that influence the interactions of GTP might reduce the affinity of its binding, alter the activity of the GTPase, or shift the conformation of GTP–tubulin to GDP–tubulin or to a nonmicrotubular form. The general principles of protein–protein interactions, enzyme kinetics and allosteric regulation pertain to this discussion (Nichol and Winzor, 1972; Oosawa and Asakura, 1975; Frieden and Nichol, 1981; Perutz, 1990; Wyman and Gill, 1990).

Most of the interactions to be discussed here are reversible interactions. Most drug binding to tubulin is rapidly reversible.\* Colchicine is the primary exception, due to a large activation barrier of binding, and thus an extremely slow off rate (see below). Taxol binding to tubulin is often referred to in the literature as being strong and irreversible, but its affinity for microtubules is only \( 10^9\)M\(^{-1}\) (Parness and Horwitz, 1981), and methods have been described for its recovery from microtubule solutions (Collins and Vale, 1987; Collins, 1991). Antimitotic drugs prevent microtubule assembly (colchicine, podophytoxin, vinca alkaloids, etc.) or prevent microtubule disassembly (taxol). Cell toxicity is thus believed to be

\*Sulfhydryl modifications are chemically reversible by reducing agents, however tubulin is often irreversibly changed by the modification of 1–2 SH groups/dimer, preventing complete recovery of polymerization activity. Sulfhydryl modification of tubulin has been reviewed in this series (Luduena and Roach, 1991).
due to preventing formation of the mitotic spindle, or, in the case of taxol, preventing disassembly of the cytoplasmic microtubules at metaphase, or preventing disassembly of the mitotic spindle and thus cell division during mitosis. Any disruption of microtubule dynamics may induce metaphase arrest (see Jordan and Wilson, 1990). Upon polymerization into a microtubule, tubulin becomes a GTPase and cleaves the terminal phosphate bond of GTP bound at the E-site. Thus, if a drug prevents microtubule formation or alters steady state dynamics, then it indirectly prevents or diminishes GTP hydrolysis. This is not in general believed to be a direct allosteric modification of the GTP binding site, but an indirect effect due to the inhibition of polymerization.

There are many potential mechanisms of drug inhibition of microtubule assembly. There are two kinds of stoichiometric effects. A direct steric mechanism involves drug binding at a site on the heterodimer that forms an interface within the microtubule and thus blocks longitudinal or lateral contact and bond formation. Alternatively, an indirect effect occurs when binding induces a conformational change in tubulin that interferes with microtubule bond formation. Both of these mechanisms involve sequestering of a drug–tubulin complex in a nonpolymerizable form. At stoichiometric concentrations, polymorphism, the formation of alternate nonmicrotubular polymer forms, may be induced. As long as the free tubulin concentration is less than $C_0$, polymerization is inhibited. For example, vinblastine and vincristine induce spiral protofilaments that are nonmicrotubular and lack GTPase activity. Vinca alkaloids also prevent microtubule assembly in a substoichiometric manner by means of a mechanism that is believed to cause poisoning of the ends of growing microtubules. A drug–tubulin complex adds to a growing protofilament, alters the conformation of that end and prevents the addition of tubulin subunits. This mechanism may involve copolymerization of tubulin and drug–tubulin molecules that alter the overall efficiency or energetics of growth (Sternlicht and Ringel, 1979). To put this in quantitative terms, vinblastine will inhibit mitosis at $7.5 \times 10^{-8} \text{M}$, but in vitro it binds to tubulin with an affinity of ca. $4 \times 10^8 \text{M}^{-1}$. This reflects the difference between inferring an effect due to substoichiometric poisoning by measuring microtubule assembly and a direct equilibrium binding measurement (see below).

Since treadmilling may predominate under certain conditions, poisoning of endwise growth may be selective or preferential for one end of a microtubule. (It will probably not be exclusive. Treadmilling can be expressed quantitatively with $S = 0$ meaning no treadmilling at steady state, and $S = 1$ meaning only association at one end and only disassembly at the other end. The experimental data puts $S$ at ca. 0.1 (Correia and Williams, 1983). This means both ends grow, one just grows faster.) Alternatively, drugs may induce alternate polymer formation and yet retain the activation of GTPase activity. Colchicine forms sheets or ribbons that have GTPase activity (Andreu and Timasheff, 1982e). Colchicine also induces weak GTPase activity in the heterodimer. It is not clear if GTP hydrolysis is coupled to colchicine–tubulin polymer formation although the evidence is that the GTPase activity increases with polymerization. Possibly the weak GTPase activity observed with heterodimers is actually coupled to small oligomer formation, perhaps as small as dimers of heterodimers (Heusele and Carlier, 1981). There is no evidence that colchicine–tubulin polymers are dynamic structures that assemble and disassemble in a GTP-dependent manner.

The molecular details of these interactions are not understood. Many studies of antimitotic analogs are beginning to unravel the structure–function requirements of these drugs (Lin et al., 1988; Batra et al., 1988; Liu et al., 1989; Andreu et al., 1991; Medrano et al., 1991; see Muzaffar and Brossi, 1991). However, the molecular structure of tubulin and microtubules is not likely to be known for some time (see Mandelkow and Mandelkow, 1989 for a discussion), and thus the topology of the binding sites remains a mystery. Below, attempts will be made to correlate binding at a particular site with consequences. The effects are not often additive, consistent or conclusive. In the future, upon obtaining the crystal structure of a drug–tubulin complex and viewing the molecular interaction involved, it will then be our task to explain how these contacts allosterically affect contacts within the microtubule.

Many workers persist in using a molecular weight of the heterodimer (110 kD instead of 100 kD) derived from SDS PAGE or denaturing sedimentation equilibrium experiments. The use of this erroneous value will systematically affect tubulin concentrations by ca. 9%, and will thus overestimate stoichiometries and binding constants determined for drug or nucleotide interactions with tubulin. Much of the tubulin literature uses the terms oligomer and aggregate interchangeably. Thus, there is no distinction made between reversible oligomer formation and irreversible aggregate formation. Ignoring the irreversible GTPase step, microtubule formation is reversible in the sense that heterodimers can be cycled between functional monomers and polymers. This is more like recycling an enzyme. Most drug induced polymorphic forms are reversible polymers and are sensitive to concentration effects. Irreversible tubulin aggregates are often irregular, denatured forms and are not our concern here.

### 1.3. Thermodynamic Linkage

The theory of thermodynamic linkage as applied to macromolecular interactions was first developed by Wyman (1964) and has been applied extensively to biological macromolecules (Wyman and Gill, 1990). It is based upon the thermodynamic principle that a
change in the activity of any species affects the activity of all other species in solution. The Wyman linkage relationship is

$$\left( \frac{\partial \ln K}{\partial \ln a_i} \right)_{T, P, n_3} = \Delta \tilde{v}$$

where $K$ is the equilibrium constant for some reaction to product at constant temperature, pressure, and protein concentration, $a_i$ is the activity of the ligand, and $\Delta \tilde{v}$ is the change in the apparent additional binding of component 3 to the protein, component 2, during the reaction. Note that it reflects a change in binding and not total amount bound, and is thus referred to as preferential interaction. This equation can be applied to protein stability, self-association, ligand binding, or solubility. In the context of microtubule assembly the binding of a ligand like a drug influences the binding of a heterodimer to the growing end of a microtubule. $\Delta \tilde{v}$ can be positive or negative and does not imply a specific binding site. For example, nonspecific electrostatic binding as described by Record et al. (1978) is an application of preferential interactions. The ligand need not bind uniquely to the polymer form, just more tightly. If a ligand preferentially binds to microtubules over heterodimers, then the addition of that ligand will shift the reaction to microtubule formation. For example, microtubule formation is accompanied by the binding of one $H^+$ (0.86) and one $Mg^{2+}$ (0.78) per heterodimer addition (Andreu et al., 1986). Thus microtubule formation is favored by lower pH and increasing $Mg^{2+}$ concentration. The sites of binding may be newly formed at the subunit interfaces in the polymer, but their location is irrelevant to the consequence; the extent and free energy of microtubule formation is increased.

Thermodynamic linkage applies to microtubule assembly in numerous ways. (1) Colchicine and a number of colchicine analogs induce large conformational changes in tubulin. Some of these conformational states have GTPase activity not coupled to microtubule assembly. Using a bifunctional model and ligand-linked conformational equilibria, a thermodynamic model has been proposed that is consistent with and explains the kinetics of colchicine binding (Andreu et al., 1991).

(2) The $Mg^{2+}$ induced assembly of tubulin into rings involves the binding of one additional $Mg^{2+}$ per heterodimer and proceeds by isodesmic chain growth and a ring closure step (Frigon and Timasheff, 1975a,b). The vinblastine-induced assembly of tubulin into spirals (Na and Timasheff, 1986a,b) can be described by a ligand-mediated plus ligand-facilitated polymerization model. As described below, there is one assembly-linked vinblastine binding site. The concentration dependence of microtubule and nonmicrotubule polymer formation magnifies the allosteric interactions between multiple ligands. This in turn is coupled to large changes in the solvent activity due to large changes in surface area upon assembly (Wyman and Gill, 1990).

(3) The concept of preferential interactions has been applied to explain the influence of solvent components on the induction of microtubule assembly. Typical examples are glycerol or DMSO nonspecific stimulation of assembly at very high concentrations. The driving force is the preferential hydration of tubulin and the minimization of surface area to avoid interaction between the solute, e.g. glycerol, and the macromolecule (Lee and Timasheff, 1987).

(4) In addition to nonspecific preferential interactions, there are a number of specific chelation equilibria that are important to the tubulin system. The original discovery of in vitro microtubule assembly conditions involved the addition of EGTA as a $Ca^{2+}$ chelator. $Ca^{2+}$ inhibits microtubule assembly, in the mM range for MTP and in the $\mu$m range for pure tubulin (Berkowitz and Wolff, 1981). Since $Mg^{2+}$ is required for strong GTP binding to the E-site, chelation of divalent cations in general will also inhibit assembly. Tropolone (Andreu and Timasheff, 1982a) and daunomycin (Na and Timasheff, 1977) are weak microtubule inhibitors due to binding to tubulin. However, they also bind $Mg^{2+}$ and thus could influence assembly by altering the free divalent cation concentration (Andreu and Timasheff, 1982d; Dabrowiak, 1980). $Mn^{2+}$ will substitute for $Mg^{2+}$ and in the early reports it was noted that $Mn^{2+}$ was less effective than $Mg^{2+}$ in promoting assembly (Buttlaire et al., 1980). However, those authors used 1mM EGTA in their buffers, a weak chelator of $Mg^{2+}$ but a strong chelator of $Mn^{2+}$ (Correia et al., 1988). In addition drugs may interact with buffer components. Hinman and Cann (1976) reported that chlorpromazine complexes with succrose and complicated the interpretations of their study of chlorpromazine interaction with mouse tubulin. Chlorpromazine competes with colchicine and in tissue culture resembles colcemide in arresting mitosis and disrupting organized microtubule structure (Appu Rao et al., 1978).

(5) It has been reported that vincristine stabilizes the colchicine binding activity of tubulin (Wilson, 1970) and that colchicine itself prolongs native conformation of the protein (Garland, 1978). This is a thermodynamic linkage between drug binding and tubulin stabilization. The native tubulin conformation is stabilized against denaturation by the additional energy of drug binding or nucleotide binding (cf. Brandts and Lin, 1990). The induction of polymer formation may provide additional stabilization. This probably contributes to the influence of vinca alkaloids or taxol on tubulin stability. Monod et al. (1963) predicted that "no direct interaction need occur between the substrate of the protein and the regulatory metabolite which controls its activity". In the examples listed above the driving forces are free energy changes mediated by coupled equilibria and allosteric interactions. The thermodynamic linkage principle is sufficient to understand
2. THE COLCHICINE SITE

2.1. COLCHICINE

Colchicine (Fig. 2) is the classic antimitotic; in fact, tubulin was once known as the colchicine binding protein (Wilson, 1970). Colchicine has four major effects on tubulin: (1) It induces a change in secondary structure as measured by circular dichroism, (2) it induces a weak GTPase activity in the heterodimer, (3) it inhibits microtubule formation substoichiometrically and thus inhibits the microtubule-linked GTPase activity, and (4) at elevated temperatures it induces sheet-like polymer formation at stoichiometric concentrations of drug. The kinetics (Lambeir and Engelborghs, 1981; Bane et al., 1984; Engelborghs and Fitzgerald, 1987; Hastie, 1989) and thermodynamics (Andreu et al., 1991; Medrano et al., 1989, 1991) of colchicine binding to tubulin have been extensively characterized. Roles for the A, B and C rings have been proposed. Colchicine is known to undergo a slow conformational change upon binding to tubulin and to induce a conformation change in tubulin. The slow kinetics of effectively irreversible colchicine binding are due to a large activation barrier ($E_a = 24$ kcal/mol), in part involving distortion of the central or B ring (Garland, 1978; Lambeir and Engelborghs, 1981; Banerjee et al., 1987). Removing the B ring increases the overall rate and decreases the activation barrier with a slight reduction in the overall affinity (Bane et al., 1984; Engelborghs and Fitzgerald, 1987). The specificity of binding is due to H-bonding and hydrophobic interactions at the trimethoxybenzene A ring and to stacking interactions at the 7 membered tropolone C ring. The link between colchicine and tubulin conformational changes has been experimentally inferred from kinetic studies (Garland, 1978; Lambeir and Engelborghs, 1981), measured by CD (Detrich et al., 1981; Andreu and Timasheff, 1982a) and quenched protein fluorescence (Andreu and Timasheff, 1982a), demonstrated by immunological methods (Morgan and Spooner, 1983), and inferred by the induction of GTPase activity in the heterodimer (David-Pfeuty et al., 1979; Andreu and Timasheff, 1981). The small CD changes are also induced by analogs of colchicine. MTC (2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-one) (Andreu et al., 1984), allocolchicine (Medrano et al., 1989) and tropolone methyl ether (Andreu and Timasheff, 1982b).

Podophyllotoxin (Fig. 2) is known to compete with colchicine for binding to tubulin and is believed to share a site with colchicine through the trimethoxybenzene moiety; yet podophyllotoxin does not induce a conformational change in tubulin or induce the GTPase activity. In fact, podophyllotoxin and tropolone, the C ring alone, can bind to tubulin simultaneously suggesting that only the A ring is shared in these binding sites (Cortese et al., 1977). Neither tropolone methyl ether nor vinblastine induces the GTPase activity (Andreu and Timasheff, 1981). An early report suggested that griseofulvin and daunomycin induce GTPase activity in heterodimers and do not compete with colchicine binding (David-Pfeuty et al., 1979), but this has not been followed up. A molecular understanding of this selectivity must await determination of a three dimensional structure of tubulin and a knowledge of the drug binding sites. Recent work suggests that the colchicine related site that induces GTPase activity requires binding to both the A ring and C ring subunits. Thus, GTPase activity is trimethoxybenzene-specific while the conformational change can be due to interactions, stacking or π stacking of the C ring alone (Lin and Hamel, 1981; Andreu et al., 1991; Medrano et al., 1991). This is also the mechanism of colchicine and colcemid dimerization (Engelborghs, 1981). The identity of the aromatic amino acid participating in this stacking is not known, but tryptophan has been suggested (Andreu and Timasheff, 1982a; Hastie, 1989). There is evidence that the B ring subdomain may also be involved in induction of the GTPase activity (Banerjee et al., 1987).

An alternative model of the colchicine binding site has been proposed based upon the rapid and reversible binding of combretastatin A-4 and A-2, natural products that have replaced the tropolone ring with a phenyl ring at the C-ring position (Lin et al., 1989). This model suggests a symmetry to the colchicine site that appears to be inconsistent with the head-to-tail asymmetry of the microtubule (see a complete discussion in Andreu et al., 1991). It is not clear from this report if these compounds induce large conformational changes in tubulin or alternate polymer formation. GTPase activity was assessed in 1.0 M glutamate, 4% DMSO under conditions where the drugs inhibit turbidity development but stimulated GTPase activity above the microtubule associated levels under otherwise identical (drug-free) conditions. The half-lives of colchicine displacement of these compounds from tubulin are much shorter.
than for colchicine (3.6 and 2.4 vs 405 min) but also longer than that for 2-methoxy-5-(2',3',4'-trimethoxy)tropolone, MTPT, a tropolone analog lacking the B ring (12–17 sec). Direct measurements of binding constants and rates of binding will greatly assist in evaluating the implications of these unusual colchicine analogs.

Since colchicine turns on the GTPase activity of the heterodimer, possibly mediated by small oligomer interactions (Heusele and Carlier, 1981), this will necessarily the interactions between divalent cations, guanine nucleotides, and tubulin. The affinity of the GTP Mg complex and of GDP may be altered. The conformation of the complex is clearly different, at least during hydrolysis where the triphosphate takes on the activated complex conformation. The transition state probably involves a pentavalent structure about the γ-phosphate with the fifth coordinate position occupied by an attacking water molecule (Osei et al., 1988; Angelastro and Purich, 1990). In the presence of stoichiometric GTP, hydrolysis will occur and the site will be occupied with GDP. It will be a low affinity Mgγδ site (Correia et al., 1987). The colchicine–tubulin sheets must also then consist of GDP occupied E-sites. A recent study of the colchicine–tubulin complex with nucleotides utilized GTP(γF), a nonhydrolyzable analog (Monasterio, 1985). Using NMR techniques, a distance of 6 to 8 Å was measured from Mn2+, a substitute for Mg2+, to the γF. GTP(γF) does have a reduced affinity for Mn2+ and Mg2+, and GTP(γF) inhibits microtubule polymerization (Monasterio, 1985; Monasterio and Timasheff, 1985a) although some incorporation may occur. Tubulin GTPase apparently requires two negative charges at the γ-phosphate, since GTP(γF) only has one (Monasterio and Timasheff, 1985b). Nonetheless, the distance measured by Monasterio (1985) probably reflects the conformation of the colchicine–tubulin complex trapped in a nearly active GTPase conformation.

The location and number of the colchicine binding sites is still under investigation. Cross-linking studies with potent alkylating agents, photoaffinity labeling, or direct UV cross-linking suggest the site is on the x chain or the γ and β chains (Schmitt and Atlas, 1976; Floyd et al., 1989; Wolff et al., 1991). Bromococlibine labeled the x and β chains but only x labeling is specifically inhibited by colchicine (Schmitt and Atlas, 1976). In the UV cross-linking studies the β chain was preferentially labeled, but both the β and x chains were labeled (β/x > 1), with the labeling at x correlated with structural changes, possibly loss of native structure (Wolff et al., 1991). The data are interpreted in terms of the colchicine site being at the interface between the subunits. An interface site is consistent with the genetic data that mutations in the β chain and the x chain can induce resistance to colchicine (Cabral et al., 1980), colcemid (Keates et al., 1981) or competitors of colchicine (Morris et al., 1979). Alternatively, a change in either chain can alter the conformation of the other and thus affect drug binding. Nonspecific weak sites may be induced by protein denaturation, possibly by the alkylating agents or the UV wavelength. A second colchicine binding site has an affinity 25-fold weaker than the high affinity specific site (Floyd et al., 1989). Proteolysis studies suggest a specific fragment, residues 339–390 of the x chain, is involved in colchicine binding (Serrano et al., 1984a). Colchicine modifies the trypsin cleavage pattern of tubulin, adding a cleavage site at Arg 390 (Serrano et al., 1984a). Subtilisin treatment, removal of the carboxy terminal regions of both chains, also affects colchicine binding (Avila et al., 1987). In addition, colchicine inhibits the crosslinking of Cys 239 and 354 on the β chain, suggesting that colchicine binding sterically or allosterically affects this region of the β chain (Luduena and Roach, 1991). EPR studies suggest that this is due to conformational changes and that these residues are not near the colchicine binding site. Deinum and coworkers (Deinum et al., 1981, Deinum and Lincoln, 1986) have measured the distance from the E-site to the essential cysteines to be greater than 10 Å, the distance between the essential cysteines to be at least 17 Å, and the high affinity colchicine site to be far removed from them all. Ward and Timasheff (1988) report that the colchicine site is > 24 Å from the E-site (but see below). Only minimum distances could be determined due to an absence of spectral change. These results are consistent with the observation that alkylation of essential cysteines inhibits polymerization but not colchicine binding (Kuriyama and Sakai, 1974; Ikeda and Steiner, 1978; Bai et al., 1989) or GTP binding (Nishida and Kobayashi, 1977).

Since binding of colchicine and colchicine analogs induces GTPase activity, this site on the surface of tubulin may be involved in lateral contacts within a microtubule that also promote the assembly-linked GTPase activity. It has been suggested that MAP binding favors proteolysis contacts that lead to curvature and rod closure (Correia and Williams, 1983). The structure of the colchicine–tubulin polymers, sheets instead of rods, is consistent with steric or allosteric effects that alter lateral contacts, and thus lead to a different curvature in the polymer. The sheets or ribbons induced by colchicine are similar to microtubules in that they share the same temperature, pH, divalent cation and nucleotide binding dependences. Both processes are entropically driven reactions with similar changes in heat capacity, ΔCp (Andreu and Timasheff, 1986). In conclusion, we have a large body of information but very little molecular understanding of where colchicine binds or how colchicine alters structure, GTPase activity or polymerizability.

2.2. PODOPHYLLOTOXIN

Podophyllotoxin (Fig. 2) binds slowly to tubulin (1000 m-1 sec-1 at 37°C with an activation energy of 14.7 kcal/mol) with reasonably high affinity,
1.8 × 10⁶ M⁻¹ at 37°C (Cortese et al., 1977; Engelborghs and Fitzgerald, 1987). The partial overlap of the binding site with the colchicine site is suggested by the observation that podophyllotoxin competes with colchicine and trimethoxybenzene or A ring analogs but not with tropolone or C ring analogs. Consistent with this selective competition, podophyllotoxin does not induce GTPase activity in the heterodimer or major conformation changes in tubulin. The slow rate of binding is consistent with a slow tubulin conformation change associated with binding at this site and previously described for colchicine analogs that lack the B ring. Morgan and Spooner (1983) have reported antibody studies that suggest a conformational change with binding of colchicine, podophyllotoxin or vinblastine binding to tubulin, but only colchicine induces significant changes detectable by CD measurements.

Batra et al. (1988) describe a number of podophyllotoxin analogs that weakly compete for the colchicine/podophyllotoxin binding site, inhibit microtubule polymerization stoichiometrically, and like colchicine, stimulate rather than inhibit tubulin-dependent GTPase hydrolysis. Microtubule polymerization is suppressed and thus these analogs are stimulating the heterodimer GTPase activity. These analogs all required the trimethoxybenzene ring for activity. The substitutions were at the 6 and 7 position of the central ring. If these analogs mimic colchicine then they most likely bind partially to the tropolone, C ring, site and are actually colchicine analogs. Competitive binding studies with tropolone methyl ether would be instructive. Modifications of podophyllotoxin with groups in the β configuration at the C-4 position of the C ring (the OH group of the central ring) result in reduced inhibition of tubulin polymerization but greater inhibition of topoisomerase (Liu et al., 1989).

Bhattacharyya et al. (1985) report that subtilisin treatment of the β subunit generates a polymer that is insensitive to cold, podophyllotoxin or GTP or GDP. Further cleavage of the α subunit restores the sensitivity to cold and podophyllotoxin and the requirement for GTP. The polymer forms are reported to be different, rings vs bundles and sheets, consistent with different energetics of formation and thus sensitivity to destabilizing agents. These results do not therefore suggest that the α and β carboxyl terminal regions interact with podophyllotoxin selectively.

Since podophyllotoxin does not induce heterodimer GTPase activity or alternate polymer formation, it constitutes a relatively simple system for studying the interaction of an antimitotic with steady state microtubules. Interactions with MAP-free tubulin should probe the dynamic instability characteristics of those microtubules. The mechanism of dynamic instability is intimately connected to the assembly-linked GTPase activity of tubulin and the differences between GTP–tubulin and GDP–tubulin at the ends of the microtubule. The mechanism of endwise poisoning provides a means of investigating the details of these reactions. Podophyllotoxin induces microtubule disassembly with a rate of 160 sec⁻¹, a rate very similar to the intrinsic rate of microtubule disassembly (Schilstra et al., 1989). The amount of disassembly is proportional to the amount of drug added, and thus the GTP–tubulin–podophyllotoxin ternary complex is elongation-inactive. The free tubulin concentration remains at the Cc value. These experiments are all performed with a GTP regeneration system to avoid complications of GDP–tubulin suppression of elongation or dynamics. Schilstra et al. (1989) also found a marked effect of DMSO on microtubule dynamics, and thus because methanol did not alter dynamics, they used 2% methanol in all their experiments. (These solvents are used to solubilize drugs at stock solution concentrations.) With stoichiometric or excess drug, complete polymer disassembly occurs. However, with substoichiometric drug amounts, under conditions where free tubulin heterodimers and microtubules still coexist, dynamics are dramatically reduced, with 50% suppression of dynamics occurring at 2–3 μM podophyllotoxin (9.5–14.3% drug/subunits in microtubules). These results are consistent with the ternary complex binding to the ends of microtubules but then coming off without elongating or allowing elongation. Kinetically, the ternary complex binds like GTP–tubulin but dissociates like GDP–tubulin. In the context of a lateral cap model, a single layer of ternary complex, GTP–tubulin–podophyllotoxin, prevents elongation and exchange, but allows for rapid disassembly to the Cc level. This is in contrast to the mode of action of colchicine. The affinity of colchicine–tubulin complex for microtubule ends has been estimated to be 0.3 μM, and the complex is an inhibitor of microtubule assembly and disassembly (Lambeir and Engelborghs, 1981).

### 2.3. Estrogenic Drugs

Estrogenic drugs inhibit microtubule assembly and induce the formation of twisted ribbons (Hartley-Asp et al., 1985; Sato et al., 1987; Chaudoreille et al., 1987, 1991; Sakakibara et al., 1990). There is no interaction with tryptophan residues or modification of free sulfhydryls. These drugs do not compete with GTP or vinblastine but they do weakly compete with colchicine. They induce twisted ribbons that are insensitive to Ca²⁺ and cold and, like vinblastine-induced polymers, are favored by Mg²⁺. Note that the same polymers are generated by estrogenic drugs, vinblastine, GMPCPP (Sandoval and Weber, 1979, 1980a,b), low pH and Ca²⁺ (Matsumura and Hayashi, 1976; Serrano et al., 1986b) and GMPPCP (Seckler et al., 1990), and that each of these effectors has a distinct binding site. Colchicine and estrogenic drugs compete for the same site but do not induce the same alternate polymer structures. Furthermore, the polymorphism is dependent upon the solvent;
it is Mg\(^{2+}\) dependent and more common in 1% methanol than in 1% DMSO (Donoso et al., 1979; Chaudoreille et al., 1991). A molecular explanation of these observations is not currently possible. Energetically each of these effectors favors twisted ribbon formation and thus twisted ribbons may expose binding sites for these effectors, or the corresponding effector sites on tubulin may be capable of allosterically or directly modifying lateral contacts within the polymer in a similar manner.

3. THE VINBLASTINE/MAYTANSINE SITE

3.1. VINCA ALKALOIDS

The vinca alkaloids, vinblastine and vincristine (Fig. 3), inhibit microtubule assembly stoichiometrically by endwise poisoning or stoichiometrically by causing the formation of spiral polymers that lack GTPase activity. There is no significant change in the CD spectra of tubulin upon binding vinblastine or vincristine. Most UV difference changes are in the drug and not in tubulin, except at low wavelengths. These changes in the spectra of the drugs are due to the lower dielectric constant of the binding pocket and reflect changes in the environment of the indole of the catharanthine moiety (Prakash and Timasheff, 1991). Both drugs induce the formation of rings, coils, and tubular aggregates. At sufficiently high drug and protein concentrations fragile paracrystals form. These are optically birefringent, parallel arrays of spiraled protofilaments or fibrils that require Mg\(^{2+}\) or Ca\(^{2+}\) in Pipes buffer. Paracrystal formation in vivo is stimulated by the presence of colchicine or colcemid, consistent with the requirement of a pool of unpolymerized tubulin (Takanari et al., 1990). Palanivelu and Luduena (1982) studied the interaction of colchicine, podophyllotoxin and sulfhydryl-oxidizing agents with vinblastine-induced tubulin spirals in the presence of tau, a low molecular weight MAP. These agents inhibit microtubule assembly but not vinblastine spirals, consistent with disruption of lateral and not longitudinal interactions. Thus, a colchicine–tubulin complex, derived from disassembled microtubules, can still be induced to form vinblastine spirals.

The formation of vinca alkaloid-induced polymers or paracrystals has been extensively characterized by Timasheff and coworkers (Lee et al., 1975; Na and Timasheff, 1980a,b, 1982, 1986a,b; Prakash and Timasheff, 1985, 1991). According to Na and Timasheff (1986a,b), the binding of the first vinblastine molecule is linked to spiral assembly. This is presumably a specific binding site (\(K = 4 \times 10^4 \text{ M}^{-1}\)) that is protein concentration dependent. There are at least two additional sites that are not protein concentration dependent and must be considered weak, nonspecific sites (\(K = 5 \times 10^3 \text{ M}^{-1}\)). The mechanism involves ligand-mediated plus ligand-facilitated spiral growth which means vinblastine can bind to both heterodimers or spirals. In addition, Mg\(^{2+}\) enhances vinblastine-induced tubulin self-association and thus must also enhance vinblastine binding to the association-linked binding site. Mg\(^{2+}\) alone induces alternate polymer forms in a GDP and GTP dependent manner (Frigon and Timasheff, 1975a,b; Howard and Timasheff, 1986). Thus Mg\(^{2+}\)-induced double walled rings may coexist with vinblastine spirals at high Mg\(^{2+}\) concentrations. A comparison of spiral formation in GDP vs GTP buffers has not been reported and thus we do not know if nucleotide content at the E-site contributes to this complex mechanism. The vinca alkaloid analog vinzolidine binds with a two-fold higher affinity to GDP–tubulin than to GTP–tubulin, but this could be due to a difference in extents of spiral assembly and not an intrinsic difference in the binding affinity (Codaccioni et al., 1988).

As recently verified by Singer et al. (1988), these results explain the wide variation in the number of sites and affinities reported in the literature (partially summarized in Luduena et al., 1986). Depending upon the method of measurement, the protein concentration, the drug concentration, the ionic strength, and the Mg\(^{2+}\) concentration, the number of sites for vinca alkaloids can vary dramatically. Studies of vinblastine binding to taxol and DMSO stabilized microtubules suggest a single binding site with an affinity of \(3-4 \times 10^4 \text{ M}^{-1}\) (Singer et al., 1989), very similar to the equilibrium constant of the two low affinity sites identified by Na and Timasheff (1986a,b). Vincristine-induced tubulin aggregation has been analyzed as ligand-mediated or ligand-facilitated (Prakash and Timasheff, 1985), but we must presume that the subsequent results of Na and Timasheff (1986a,b) reinterpreting the vinblastine data in terms of both mechanisms apply to vincristine as well. A thermodynamic interpretation of vinca alkaloid binding to tubulin using the bifunctional model previously applied to colchicine suggests that the cratic energy gained by covalently immobilizing the two parts of the molecule together is lost, unlike for colchicine (Andreu and Timasheff, 1981), possibly due to strain energy upon binding (Prakash and Timasheff, 1991). Thus the free energy of binding is due to the sum of the two constituents and is
not increased by the entropy lost due to covalent immobilization.

Photoaffinity studies demonstrate labeling of both α and β subunits by vinblastine in a 3:2 ratio (Safa et al., 1987). Irradiation was at 304 nm, potentially modifying and denaturing tubulin, and the high drug concentrations used precluded any control of labeling specific vs nonspecific sites. In a more recent study, a photosensitive derivative of vinblastine, photoactivated by light at 455 nm, was also crosslinked to both the α and β chains (Nasioulas et al., 1990). This analog reacts with tubulin in a similar manner to vinblastine, but the active photoproduc object product has a half-life of 400 min and thus may also react in a nonspecific manner.

Bowman et al. (1986, 1990) have suggested a requirement of GTP or GDP for vincristine binding to tubulin in cytosol from two human rhabdomyosarcoma xenographs. The effect is reported to be due to a factor, 20–50 kDa, possibly a GTP binding protein, that does not copurify with tubulin or MAPs.

The interaction of substoichiometric amounts of vinblastine with microtubules containing MAPs has been investigated by a double labeling procedure using [14C]GTP and [3H]GTP (Jordan and Wilson, 1990). The bovine brain MTP preparations are described as MAP rich and contain 30–40% MAPs. It is reported that these microtubules exhibit no dynamic instability, only steady state treadmilling. The presence of substoichiometric concentrations of vinblastine decreases the dissociation rate constant of tubulin at the A or plus end without changing the dissociation rate constant at the D or minus end. This is described as producing a kinetic cap at the plus or growing end of the microtubule, despite the fact that disassembly predominantly occurs at the plus end. These data are interpreted as being consistent with free drug rapidly and reversibly binding to high-affinity sites at one or both microtubule ends. Thus, vinblastine reduces the critical concentration but does not induce microtubule disassembly; rather it kinetically stabilizes the plus end.

Using their reported rate constants, both ends have identical affinities for tubulin, with association constants of 4.0–4.3 × 10^5 M<sup>-1</sup> at the plus end and 3.8–4.3 × 10^5 M<sup>-1</sup> at the minus end. The addition of vinblastine, 0.15 and 0.3 μM, reduces the association constants equally for both ends, 3.2 and 2.1 × 10^5 M<sup>-1</sup> respectively for the plus end, and 3.3 and 2.3 × 10^5 M<sup>-1</sup> respectively for the minus end. At steady state these microtubules are indeed treadmilling very slowly. Only during a transition induced by dilution, addition of drug, or new assembly will the ends appear to be different. In fact, the minus ends of these microtubules disassemble more slowly than the plus ends, possibly due to the MAP content. Given the identity of the association constants and the similarity of the rate constants, it appears that both ends are involved, with different ends being more active at various times.

The idea of a high affinity binding site at the end(s) of the microtubules comes from earlier vinblastine-MTP experiments (Wilson et al., 1982). In those experiments the estimates of affinities come from the [3H]-vinblastine concentration and the total tubulin concentration in a pellet. The data are consistent with a vinblastine–tubulin complex binding to the microtubule end(s), and thus a reasonable mechanism is vinblastine binding to free tubulin and the resulting complex adding to microtubules but then subsequent binding being inhibited by the presence of this complex. If the affinity of vinblastine for tubulin is taken from Na and Timasheff (1986a), 4 × 10^4 M<sup>-1</sup>, and the complex binds to a microtubule with the affinity of free tubulin (1 × 10^4 M<sup>-1</sup> in the experiments of Wilson et al. (1982), ca. 4 × 10^4 M<sup>-1</sup> in the experiments of Jordan and Wilson (1990)), then by thermodynamic coupling, the affinity of free vinblastine for microtubule ends is the product or 4–16 × 10^9 M<sup>-1</sup>. If vinblastine binding to the heterodimer alters this affinity for the ends, these values would be too large. In addition, affinities at different ends of the microtubules may be different since the geometries or topologies of the two ends are different. Previous estimates of these apparent affinities were 5.3 × 10^8 M<sup>-1</sup>, derived from inhibition of polymerization (Wilson et al., 1982). These values are not derived from the free concentration of tubulin in equilibrium with the ends, and do not take into account the loss of drug during the centrifugal separation that must occur due to rapid re-equilibration, although the 50% sucrose cushion may alter, most likely increase, the affinity and reduce this loss. Na and Timasheff (1986a,b) indicate the problems with measuring binding constants in this system, suggesting careful maintenance of free drug activity and consideration of all the competing equilibria in the calculations. This procedure appears to be a reasonable framework for interpreting the substoichiometric, endwise effects of drugs.*

### 3.2. MAYTANSINOIDS

Vinblastine and maytansine (Fig. 4) share and compete for a binding site on tubulin. Ansamitocin P-3 (Fig. 4) (an analog of maytansine), rhizoxin (Fig. 5) (a fungal antimiotic that only slightly resembles maytansine), and maytansine all inhibit vinblastine-induced tubulin aggregation. All of these...
drugs competitively inhibit vinblastine and vincristine binding (Bai et al., 1990b), and substoichiometrically inhibit microtubule assembly and assembly-linked GTP hydrolysis. For example, maytansine inhibits vinblastine and vincristine binding to tubulin with a $K_i$ of 0.5 and 0.4 $\mu M$ respectively (Luduena et al., 1986). Rhizoxin is a strong competitive inhibitor of maytansine and ansamitocin P-3 with a $K_i = 0.17$ $\mu M$. It inhibits vinblastine effects but not colchicine (Takahashi et al., 1987). Because maytansine does not induce alternate polymer formation and does inhibit induction of spirals by vinca alkaloids, maytansine has been referred to as a pure inhibitor of all tubulin polymerization. Luduena and coworkers (Roach and Luduena, 1984; Sullivan et al., 1990) use a bifunctional crosslinker EBI to alkylate cysteines in a class of $\alpha$ chains of tubulin and produce three new $\beta_1$ bands, two singly crosslinked and one doubly crosslinked (for a review see Luduena and Roach, 1991). GTP, maytansine, rhizoxin and vinblastine have common effects, enhancing the $\beta_s$ and inhibiting the $\beta_f$ crosslink, while colchicine, podophyllotoxin and nocodazole inhibit the $\beta_f^*$ and enhance the $\beta_s^*$ crosslinks. These results are consistent with the lack of competitive binding between these two classes of drugs and the conclusion that they target different regions of the heterodimer.

Maytansine and vinblastine appear to have an overlapping binding site near the E-site. In support of this, Huang et al. (1985) have shown that maytansine inhibits nucleotide exchange at the exchangeable site of tubulin. This could be a steric effect or due to a conformational change. Another indication that maytansine binds near the E-site is that maytansine and GTP have similar effects on alkylation (Roach and Luduena, 1984). Vinblastine does not affect nucleotide exchange, although it is not clear if these experiments have been done under conditions where spirals form. As mentioned above, vinca alkaloids stabilize colchicine binding activity and thus prevent tubulin denaturation. Maytansine, ansamitocin P-3 and rhizoxin do not stabilize tubulin. Taken together these data are evidence for competitive but overlapping binding sites and are consistent with a $\beta$ chain binding site for these agents.

Mutation of the $\alpha$ chain produces resistance to maytansine (Schibler and Cabral, 1985). Surprisingly, the mutation was also cross resistant to griseofulvin, vinblastine and colcemid and supersensitive to taxol. These results are consistent with a mutation that stabilizes microtubules, modifying a domain that affects subunit-subunit interactions (Cabral, 1983). In a more recent example (Schibler and Huang, 1991), a $\beta_2$-tubulin missense mutation in Chlamydomonas reinhardtii originally isolated as a colchicine resistant mutant is actually cross resistant to vinblastine and antimitotic herbicides and has increased sensitivity to taxol. Thus these mutations produce enhanced microtubule stability. Antimitotic resistant mutations of tubulin are often not at the drug binding sites. Rather they are mutations at the subunit-subunit interfaces that strengthen or diminish microtubule stability and are thus cross resistant with drugs specific for diverse sites (Rudolph et al., 1987).

3.3. PHOMOPSIN A AND DOLASTATIN 10

Phomopsin A (Fig. 6) and dolastatin 10 (Fig. 7) both prevent nucleotide exchange, and inhibit microtubule polymerization and assembly-linked GTP hydrolysis (Tonsing et al., 1984; Lacey et al., 1987; Luduena et al., 1989; Bai et al., 1990a,b). They both stabilize tubulin against decay, thus stimulating colchicine binding and diminishing binding by bis(8-anilino-naphthalene 1-sulfonate), Bis ANS, a probe sensitive to newly exposed hydrophobic regions. They both stabilize tubulin better than vinblastine, and were described in one case as providing indefinite stabilization (Luduena et al., 1990). Thus they may be

**$\beta_f^*$ refers to the crosslinking of cysteine 239 and 354 on the $\beta$ chain. $\beta_s^*$ refers to the crosslinking of cysteine 12 and 201 or 211 on the $\beta$ chain. Refer to Luduena and Roach (1991) for a current review of this research.
useful in crystallization. Phomopsin A inhibits EBI crosslinking in a manner very similar to maytansine and vincristine (Luduena et al., 1990). Phomopsin A induces 40 nm rings with MTP and purified tubulin, although drug induced depolymerization also causes coils and short helices, possibly during an endwise depolymerization mechanism (Tonsing et al., 1984). Dolastatin 10, like vinblastine, induces cold stable polymers (Bai et al., 1990a) although recent EM studies demonstrate they are rings similar to those reported for phomopsin A (P. Skehan and E. Hamel, personal communication). These agents are both noncompetitive inhibitors of vinblastine binding, suggesting a site distinct from vinblastine but overlapping with maytansine (Bai et al., 1990b). A topological model of the vinca alkaloid, maytansine, phomopsin A and dolastatin 10 sites has been proposed that places them in adjacent and overlapping regions on the β chain near the guanine nucleotide E-site (Bai et al., 1990b). Both steric and allosteric effects may be involved in these interactions.

4. TAXOL

The diterpenoid taxol (Fig. 8) is the sole antimitotic that stoichiometrically induces microtubule assembly and assembly-associated GTPase activity (Manfredi and Horwitz, 1984; Hamel, 1990). Nucleation is enhanced, thus microtubules are shorter, and growth proceeds through ribbon-like structures (Schiff et al., 1979) and extensive length redistribution (Caplow and Zeeberg, 1982; Carlier and Pantaloni, 1983). This may explain the observation of biphasic kinetics with taxol and MAP stimulated assembly (Kumar, 1981; Choudhury et al., 1987). Similar observations at low pH (Barton and Riazi, 1980; Bayley et al., 1983) may also be due to changes in morphology (Heusele et al., 1987). Microtubule annealing contributes to this process as well (Williams and Rone, 1988). The affinity of subunits for the end of a growing microtubule is increased by $-2 \text{ kcal/mol}$ (Schiff et al., 1979; see below) and dynamics are dramatically reduced (Wilson et al., 1985) although not eliminated (Caplow and Zeeberg, 1982). Other effectors also stimulate assembly and reduce dynamics in a specific (MAPs) or nonspecific (polylysine, glycerol, DMSO, sucrose, glutamate) manner. Taxol alone stimulates GDP–tubulin and nucleotide-depleted tubulin to assemble (Schiff and Horwitz, 1981; Carlier and Pantaloni, 1983). This is evidence that GTP hydrolysis is not absolutely required for microtubule polymerization, and that the free energy associated with the growth of GTP–tubulin can be derived from taxol binding to a stabilized microtubule. Taxol does not inhibit nucleotide binding or MAP binding to microtubules (Schiff and Horwitz, 1981; Vallee, 1982; Collins, 1991), although the E-site nucleotide within the assembled microtubule is nonexchangeable except through subunit exchange at the ends (Carlier and Pantaloni, 1983). Taxol binds to microtubules with a $K_d$ near 1 μM (Parness and Horowitz, 1981) and is reported to not bind to subunits (Collins and Vallee, 1987; Takoudju et al., 1988), although a gel filtration assay used to measure this is not sensitive to affinities weaker than ca. $1 \times 10^5 \text{ M}^{-1}$. * In fact, Carlier and Pantaloni (1983) estimated the affinity of taxol for the colchicine–tubulin complex to be 13 μM, or $7.7 \times 10^4 \text{ M}^{-1}$. This

*The insolubility of taxol in water may cause it to precipitate and pellet in excess of its affinity with microtubules and thus taxol may appear to bind irreversibly to microtubules. This is especially true at low temperature.
estimation involved the stimulation of colchicine—tubulin complex GTPase activity. The experiments were performed under conditions where the complex alone did not form colchicine—tubulin sheets, and no polymer formation was observed upon addition of taxol. Thus taxol appears to stimulate the GTPase activity of colchicine—tubulin heterodimers and probably binds to tubulin heterodimers in the absence of colchicine with a similar affinity. Because taxol binds to microtubules with high affinity, the location of this taxol binding site is suspected of being at the interface between subunits in the microtubule, but this is not known. The affinity of taxol for the colchicine—tubulin complex suggests a single subunit can bind taxol, and additional conformational changes within the microtubule could account for the affinity increase, corresponding to ca. 1.6 kcal/mol. Previous suggestions that nonhydrolyzable analogs of GTP bind at the taxol site are probably not correct (Manfredi and Horwitz, 1984).

Taxol induces microtubule bundle formation (Schiff and Horwitz, 1980; Turner and Margolis, 1984). Bundles are also induced in vivo by GMPCPP (Wehland and Sandoval, 1983). This may be mediated by a number of protein factors that are known to induce microtubule bundle formation in vivo and in vitro (Aamodt and Culotti, 1986; Amos, 1989; Campbell et al., 1989; Huitorel and Pantaloni, 1985).

Other drugs inhibit taxol-stimulated assembly. Studies in vivo and in vitro suggest inhibition of taxol-stimulated assembly by colchicine, colcemid, podophyllotoxin, and vinblastine (see Hamel, 1990 for a list). Estrogens inhibit taxol-induced assembly but this is reversed with the addition of GTP (Hartley-Asp et al., 1985). There are also many reports of resistance to antimitotic depolymerization of preformed taxol-microtubules (see Hamel, 1990). Taxol does not compete directly with colchicine, vinblastine or MAP binding sites, although experiments with drugs have not always distinguished competition for subunits or polymers (Schiff and Horwitz, 1981). A reasonable conclusion is that taxol competes with other drugs that shift the equilibrium to unpolymerized tubulin and alternate polymer forms. Thus, to interpret results, the relative affinity of these drugs, their relative concentrations, the total protein concentration, and the stability of alternate polymer forms are all involved in the competing equilibria and must be considered.

Taxol-stabilized microtubules are relatively insensitive to cold temperatures or Ca²⁺ in the presence of MAPs. However, Collins and Vallee (1987) found that a combination of Ca²⁺ or elevated ionic strength and low temperatures will depolymerize taxol-stabilized microtubules. In the absence of MAPs, cold temperatures alone will slowly depolymerize taxol-stabilized microtubules (Hamel et al., 1981). These results are tubulin concentration dependent. Thus, the free energy of microtubule stabilization by taxol and MAPs can be overcome by the free energy lost by the destabilizing effects of Ca²⁺ and cold temperatures. Howard and Timasheff (1988) have quantified the effects of taxol in terms of linkage free energy. In a phosphate, Mg²⁺-dependent buffer system, taxol enhanced subunit addition to a growing microtubule end by at least –3.0 kcal/mol. Taxol also enhances the addition of colchicine—tubulin complex to the alternate, sheet-like polymers it forms by –0.5 kcal/mol. This is much less enhancement than for microtubule formation, and consistent with this, taxol-stabilized colchicine—tubulin polymers depolymerize in the cold. Analysis by Wyman linkage theory (Wyman, 1964) gives 0.55 mol of taxol bound per mol of tubulin assembled, consistent with stoichiometric binding when corrected for unpolymerized tubulin and bound taxol, but possibly consistent with a bridging or cross-linking mechanism of taxol binding (ie. one mole of drug linking two dimers of tubulin). Previous experiments with [³H]taxol suggested a stoichiometry of 0.6 with MTP and 0.78 with PC-tubulin (Parness and Horwitz, 1981). In addition, taxol-induced assembly of tubulin does not require GTP, even in the cold, while taxol-induced colchicine—tubulin assembly requires GTP and elevated temperatures. This is buffer dependent. Thus, the free energy gained from GTP binding is required for the taxol enhanced colchicine—tubulin complex to assemble, while taxol alone can enhance tubulin assembly.

To suppress subunit and nucleotide exchange at the ends of taxol-stabilized microtubules, inferred by steady state GTP hydrolysis, much higher taxol concentrations were required than the amount needed to completely polymerize the tubulin (Carlier and Pantaloni, 1983). The data is consistent with a class of low affinity sites, perhaps at the ends of microtubules, or additional low affinity sites along the microtubule lattice. A model was suggested that makes the outer layer of GTP—tubulin subunits different in their affinity for taxol, possibly because they lack the extra subunit contacts that completely form the high affinity taxol binding site. As with the previous cases involving drug affects on the ends of microtubules, the interpretation of the data rests...
upon the framework of the model chosen, and thus strongly reinforces the idea that without a clear picture of the molecular events at the ends of microtubules, the effects of drugs on microtubule dynamics will continue to be problematic.

5. THE THIRD NUCLEOTIDE (ATP) BINDING SITE

There has been some suggestion of a third nucleotide binding site on tubulin that prefers ATP and modulates microtubule assembly synergistically with GTP, including the formation of double walled rings (Zabrecky and Cole, 1980, 1982a,b; O’Brien and Erickson, 1989). These effects are reminiscent of drug effects on tubulin and one could speculate that this third nucleotide binding site and antimitotic binding sites share common surfaces or regions of interaction. However, other workers suggest that ATP is interacting weakly with the E-site (Duanmu et al., 1986; Mejillano et al., 1990; Seckler et al., 1990) and that the problem with the previous work was not vacating the E-site of endogenous GDP or GTP nucleotides, by treatment with charcoal or alkaline phosphatase, or by hydrophobic chromatography (Hanssens et al., 1990), prior to measuring ATP binding. For example, Duanmu et al. (1986) have shown for ATP-stimulated microtubule formation a similar competitive inhibition by antimitotics and by GTP (GDP/ATP < 1), consistent with weak affinity for the E-site. ATP is hydrolyzed in their experiments, also consistent with interaction at the catalytic E-site. The ATP affinities measured by all of these workers, when corrected for contaminating nucleotides, are of the same magnitude, 3-7 × 10^4 M⁻¹, only the presumed site of binding differs. Buffer condition do vary in these studies. Seckler et al. (1990) report that, in the absence of Mg²⁺ and glycerol, GMPPCP binding to the E-site is too weak to accurately measure (Kd ≥ 400 μM). GDP and GTPMg bind at least 1000 fold tighter to the E-site than does ATP. It is the conclusion of this reviewer that tubulin has only two nucleotide binding sites and the E-site and their mutual modulation of the form of polymer and the extent of polymerization just as antimitotic drug binding to distal sites can. This has been observed with the nonhydrolyzable guanine nucleotides, GMPPCP, GMPPNP, and GMPCPP (Sandoval et al., 1977; Sandoval and Weber, 1979, 1980a,b; Hamel et al., 1983; Seckler et al., 1990). The same experimental difficulties which are due to relatively weak binding, have applied to studies using ATP and these GTP analogs. These observations are all consistent with allosteric interactions between drug binding sites and the E-site and their mutual modulation of the extent of polymer formation and polymer form.

6. N-SITE GTP

One of the two nucleotide binding sites on tubulin, the N-site, is nonexchangable, noncatalytic and believed to only turn over upon protein degradation in vitro (Spiegelman et al., 1977) or protein denaturation in vitro (Osei et al., 1990). Tubulin possesses two high affinity divalent cation binding sites (Kd = 1.0 - 0.2 μM) and it has been suggested that one site is at the N-site GTP and the other site is at the E-site GTP (Correia et al., 1988; Osei et al., 1990). GDP at the E-site is a low affinity metal site. Kd is 0.5 mM. Mn²⁺ will slowly exchange for Mg²⁺ at the N site (Correia et al., 1988), and it is possible that other divalent and trivalent cations may also exchange at this site. Recent Q-band EPR and electron spin echo envelope modulation spectroscopy experiments are consistent with Mn²⁺ at both sites being directly coordinated to the triphosphate of GTP (Correia et al., 1990). These results prove that the divalent cation at both sites directly interacts with GTP (Monasterio, 1985). More recent EPR studies on the colchicine-GDP-tubulin complex and on taxol-GDP-microtubules with Mn²⁺ bound to the N-site show a dramatic change in the Mn²⁺ EPR spectra (J. J. Correia and A. H. Beth, in preparation). These results are consistent with a tightening of the tubulin structure, at least in the proximity of the N-site divalent cation, by colchicine binding or by taxol induced microtubule formation. These results suggest a global, allosteric effect on tubulin structure upon drug binding. The E-site with GDP bound appears to be a low affinity metal site in the microtubule, just as it is in GDP-tubulin heterodimers (Correia et al., 1987). Thus, the N-site divalent cation is a spectroscopic probe of the influence of drug binding on tubulin-microtubule structure-function.

These results suggest caution in measuring distances from divalent cations by NMR (Monasterio, 1985) or fluorescence (Ward and Timasheff, 1988) techniques without verification of the location of the metal, the N-site and/or the E-site. For example, Monasterio (1985) used GTP(CF₃), Mn²⁺ and ¹⁹F and ³¹P NMR to study the localization of a high affinity divalent cation within the colchicine-microtubulin complex. His results suggested that at the E-site the divalent cation is within 6 to 8 Å of ³¹P at the triphosphate. The use of a guanine nucleotide analog insured that only E-site interactions are studied. Ward and Timasheff (1988) measured the distance from colchicine and ANS to Co²⁺ at the E-site by using energy transfer methods. They report that the binding of Co²⁺ is reduced when tubulin is complexed to colchicine, possibly consistent with the absence of Co²⁺ at the GDP filled E-site, and Co²⁺ now only bound to the N-site. They report greater than 28 Å between Co²⁺ and the ANS site and greater than 24 Å between Co²⁺ and the colchicine binding site. If the colchicine binding site is at the sβ interface this may represent the distance to the average of the two sites or the distance to the N-site.
and not to the E-site. Because the N-site is non-exchangeable for other nucleotide analogs, it should not affect drug binding or alter polymer morphology, but it is not known if different divalent cations at the E- or N-site can modulate polymer forms. Mn$^{2+}$ will stimulate microtubule assembly with a similar critical concentration, when corrections are made for a tighter affinity than Mg$^{2+}$ for GTP and the E-site (Correia et al., 1988). Divalent cations are known to bind to other sites and to alter polymer morphology (Serrano et al., 1986b, 1988). The Ca$^{2+}$ sites in the carboxyl terminal region may influence and regulate polymer morphology (Serrano et al., 1986b). The Zn$^{2+}$ site(s) in the N-terminus region may be at the interface(s) within the polymers (Serrano et al., 1988).

7. MICROTUBULE ASSOCIATED PROTEINS (MAPs)

Research in the tubulin-microtubule field was hindered for many years by a plethora of buffer conditions, widely differing assumptions and a tendency to treat all data, whether collected on pure tubulin or microtubule protein (MTP), as equivalent. MTP contains 70–80% tubulin and 20–30% associated proteins with numerous enzymatic functions that can alter the experimental conditions in subtle ways. These include GTPase, ATPase, nucleoside transferase, protein kinases, and molecular motors that transport microtubules or vesicles along microtubules in an energy dependent manner (Vallee, 1986, 1991). Clearly MAPs are present in vivo and the effects of drugs must be interpreted in the context of their effects on MAPs and tubulin–MAP interactions. The major brain MAPs, MAP-1, MAP-2 and tau, are known to bind to the carboxyl-terminal region of the α and β subunits (Serrano et al., 1984b, 1986a; Avila et al., 1987; Maccioni et al., 1989; Joly et al., 1989; Joly and Purich, 1990). As this region is known to influence and regulate polymer morphology, MAP binding must influence and modulate drug and nucleotide effects. Below a few examples of the interaction of MAPs with antimitotic drugs and nucleotides in the tubulin system are presented.

MAPs from pig brain do not affect the rate constant for the conformational change after colchicine initially binds to tubulin, but they do lower the apparent affinity of the drug for tubulin (Garland, 1978; Nunez et al., 1979; Lambeir and Engelborghs, 1981). Colchicine binding is slow, and thus the rate of ring disassembly is fast enough to provide tubulin heterodimers for interaction with colchicine. Colchicine can bind to MAP-induced rings. Biliger et al. (1991) have demonstrated that other MAPs may impart variable effects on drug binding either through shared binding sites or unique allosteric mechanisms. Colchicine sensitivity is MAP dependent for tubulin isolated from the Atlantic cod. In the presence of cod MAPs, cod tubulin is resistant to depolymerization by colchicine. In the presence of bovine MAPs, cod tubulin is partially resistant to depolymerization by colchicine. This is not due to an absence of colchicine binding ability, because in the absence of MAPs, cod tubulin is extremely sensitive to colchicine inhibition of polymerization. This MAP-dependent effect is reported to be a property of acetylated cod tubulin. Donoso et al. (1979) demonstrated that MAPs stimulated the formation of vincristine-induced spirals. In their absence microtubules were disassembled and at 0°C amorphous aggregates were formed. As mentioned previously, 10% DMSO stabilized microtubules and hindered the formation of vincristine-induced spirals. Luduena et al. (1981) observed a similar vinblastine-induced aggregation enhancement with tau and not MAP-2. In the absence of tau, with just tubulin and vinblastine, they saw no spiral formation. These experiments were done at 10 μM tubulin, 10 μM vinblastine. As shown by Na and Timasheff (1986a,b), higher tubulin and drug concentrations will promote spiral formation. The active MAP fraction in the work of Donoso et al. (1979) may be due to tau and not MAP-2, but concentration effects are clearly important. In subsequent work Luduena et al. (1984) investigated MAP concentration effects and found that MAP-2 enhanced vinblastine-induced aggregation, but with less effectiveness than tau. They continued to limit the tubulin concentration to 10 μM. MAP-2 was very effective at inhibiting the effect of tau. Both tau and MAP-2 readily induce microtubule formation. Under nonpolymerizing conditions both tau and MAP-2 stimulate the formation of tubulin rings, a 20S species for tau and a 30–39S species for MAP-2. In the presence of GMPCPP, tau and MAP2 induce different kinds of polymers, microtubules or ribbons (Sandoval and Weber, 1980b). These results suggest a difference between the activity of tau and MAP-2 in stimulating vinblastine-induced spirals, although it may only be due to a difference in affinities (Sandoval and Vandekerckhove, 1981). It is now well known that these MAPs share a tandem repeat sequence that binds to a region of the carboxyl terminus of β tubulin (Aizawa et al., 1988), although tau also interacts with a region near the N-terminus of the α chain (Littauer et al., 1986). Removal of the carboxyl terminal region by subtilisin precludes binding of MAP-2 and tau but enhances vinblastine-induced aggregation into spirals (Serrano et al., 1986a). This subtilisin digested tubulin assembles into microtubule-like structure more readily than undigested tubulin, and in the presence of vinca alkaloids, assembles more readily into spirals. Chemical modification of the carboxyl groups in the highly acidic region also enhances polymerization and decreases interactions with MAPs and other positively charged species (Mejillano and Himes, 1991). Thus, the carboxyl
terminal region regulates both lateral and longitudinal interactions between subunits in these polymers (longitudinal bonds in spiral formation and lateral and longitudinal bonds in microtubule formation).

Vallee (1982) developed a taxol, salt procedure for isolating MAPs from microtubules composed of pure tubulin. This procedure was used to demonstrate that different MAPs are enriched in different tissues of the brain. Thus, drug interaction with different tissues may, for the same reasons, be modulated by different MAP populations.

Hamel et al. (1983) suggested that MAPs may stimulate assembly by, in part, enhancing the affinity of GTP analogs for tubulin. This would constitute an additional linked free energy boost. The enhanced affinity was inferred indirectly by an effect on polymerization. Previous work by this group had demonstrated that MAPs stimulate taxol induced assembly such that it does not require GTP or warm temperatures (Hamel et al., 1981). In the absence of MAPs, GTP and warm temperatures are required. Alternatively, raising the glutamate concentration will promote taxol-microtubule formation. The effect of high concentrations of glutamate has been thermodynamically investigated by Arakawa and Timasheff (1984). As do other stimulators of assembly and stabilizers of protein structure, glutamate preferentially hydrates tubulin due to its unfavorable free energy of interaction with the surface of tubulin. Stimulation of polymerization is thus nonspecific, entropic and proportional to the surface area of tubulin. Assembly into microtubules decreases the surface area per g and thus further reduces unfavorable interactions. There is an important electrostatic component to this interaction, the negative charge on glutamate contributing to its exclusion from the surface of tubulin. In the absence of MAPs the predominant structures formed with taxol are sheets. This is in contrast to the results of Schiff and Horwitz (Schiff and Horwitz, 1981; Schiff et al., 1979), but the difference may be due to different buffers. Hamel et al. (1981) use 0.1 M to 1 M glutamate buffers, while Schiff and Horwitz (Schiff and Horwitz, 1981; Schiff et al., 1979) used 0.1 M MES buffer.

This final section is intended to stress the contribution MAPs can make in regulating or modifying drug-nucleotide–tubulin interactions. Similar free energy arguments involving surface free energy, conformational changes, competitive or noncompetitive binding, concentration effects, or alternate polymer reactions apply to the role of these effectors in vitro and in vivo. Thus a critical evaluation of microtubule protein interaction with antimitic drugs must include the role or influence of MAPs and other tubulin independent effects.

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REFERENCES


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