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# 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.

## CONTENTS



## 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; Kirschner and Mitchison, 1986;

Mandelkow and Mandelkow, 1989; Engelborghs, 1990), but, to facilitate the discussion of drug and nucleotide effects, a brief overview is presented here. 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, $C_c$, 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 centrosomes (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,

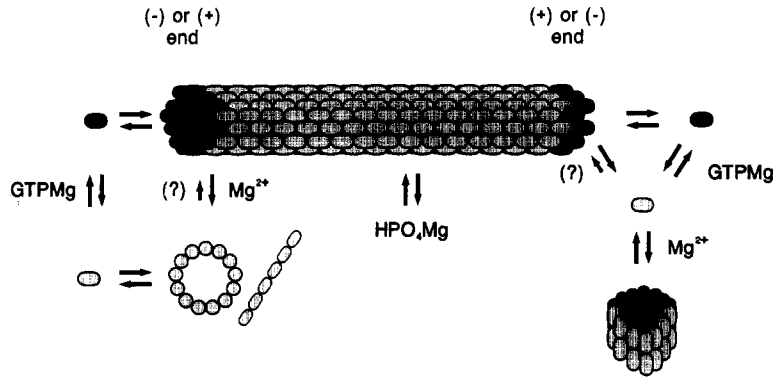


FIG. 1. An idealized mechanism of microtubule assembly/disassembly. GTP–tubulin heterodimers with GTP at the E-site are represented by dark ovals; GDP–tubulin heterodimers are represented by light ovals. Microtubule growth occurs at both ends *in vitro*. *In vivo* the slow growing end, the minus end, may be anchored at MTOC's. GTP hydrolysis occurs upon assembly and, after the release of $HPO_4Mg$, 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).

1991a), although the rate of $PO_4$ release appears to not be rate limiting for microtubules (Carlier *et al.*, 1989; Combeau and Carlier, 1989; Melki *et al.*, 1990).

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 $\gamma^{32}$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 $10^6\,M^{-1}$ (Parness and Horwitz, 1981), and methods have been described for its recovery from microtubule solutions (Collins and Vallee, 1987; Collins, 1991). Antimitotic drugs prevent microtubule assembly (colchicine, podophyllotoxin, vinca alkaloids, etc.) or prevent microtubule disassembly (taxol). Cell toxicity is thus believed to be

*Sulfhydryl modifications are chemically reversable 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 $C_c$, 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 $7.5 \times 10^{-8}$ M, but *in vitro* it binds to tubulin with an affinity of ca. $4 \times 10^4$ $M^{-1}$. 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, 1982c). 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

$$(\delta \ln K/\delta \ln a_3)_{T,P,m_2} = \Delta\bar{\nu}$$

where $K$ is the equilibrium constant for some reaction to product at constant temperature, pressure, and protein concentration, $a_3$ is the activity of the ligand, and $\Delta\bar{\nu}$ 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. $\Delta\bar{\nu}$ 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 and Timasheff, 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, 1977).

(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 sucrose 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

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