MECHANISMS AND KINETICS OF MICROTUBULE PERTURBING AGENTS

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Microtubules are cellular cytoskeletal components that play an integral part in many cell functions. Compounds that bind to microtubules and alter their dynamics are highly sought as a result of the clinical success of paclitaxel and docetaxel. A series of analogues of the microtubule stabilizing dictyostatin were examined to probe biological and biochemical structure-activity relationships. The results were consistent with previous reports showing that 16-normethyldictyostatin and 15*Z*,16-normethyldictyostatin lose potency in pacitaxel-resistant cell lines that have a Phe270-to-Val mutation in the taxoid binding site of β-tubulin. 6-*epi*-Dictyostatin and 7-*epi*-dictyostatin were potent analogues of dictyostatin, and 6-*epi*-dictyostatin was chosen for milligram scale for pre-clinical studies.

The thalidomide analogue 5HPP-33 was identified as an easily synthesized small microtubule perturbing agent, and experiments with isolated tubulin were performed to determine its mechanism of action. Tubulin polymerization was used to determine the effect of 5HPP-33 on normal microtubule formation. In experiments utilizing microtubule associated proteins (MAPs) to induce polymer formation, 5HPP-33 inhibited tubulin polymerization, but under a different set of conditions appeared to form and stabilize microtubules. The polymer was imaged using electron microscopy, which showed that 5HPP-33 caused the formation of spirals and rings. Due to 5HPP-33 failing to compete with known radiolabeled microtubule perturbing agents for their respective binding sites, a tritiated version of 5HPP-33 was synthesized. The

binding experiments performed showed that [^{3}H]5HPP-33 had a slight affinity for isolated MAPs, and this was the reason for the discrepancy between the tubulin polymerization experiments. A binding site for 5HPP-33 could not be determined, making it a possible novel microtubule perturbing agent.

(–)-Pironetin is a microtubule inhibitor that appears to form a covalent linkage to the tubulin heterodimer. Although immunoflourescent images showed (–)-pironetin to work in the same manner as vinblastine, cellular and biochemical experiments proved that (–)-pironetin is mechanistically different from vinblastine.

The tubulysins are known microtubule destabilizers and bind to the vinca domain on β-tubulin. Three analogues of the tubulysins were synthesized and their effects on cell growth and microtubule perturbation experiments were determined. WZY-111-63C (N^{14}-desacetoxytubulysin H) was found to be 50 times more cytotoxic than paclitaxel and vincristine.

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ABBREVIATIONS

ATP	Adenosine 5'-Triphosphate
BSA	Bovine Serum Albumin
ddGTP	2',3'-Dideoxyguanosine-5'-Triphosphate
DEAE	Diethylaminoethyl
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycoltetraacectic Acid
FITC	Fluorescein Isothiocyanate
GFA	Genetic Function Approximation
GTP	Guanosine 5'-Triphosphate
HF	Heat of Formation
MAPs	Microtubule Associated Proteins
MDEC	Minimum Detectable Effective Concentration
MES	*N*-Morpholino Ethane Sulfonate
MR	Molar Refractivity
MSG	Monosodium Glutamate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
PBS	Phosphate Buffered Saline
QSAR	Quantitative Structure Activity Relationships
SAR	Structure Activity Relationships
Tris	Tris(hydroxymethyl)aminoethane

PREFACE

I have been incredibly lucky that there have been so many people in my life that have provided me with emotional and intellectual support. First, I would like to thank my advisor Dr. Billy Day for the support and guidance he has provided me with over the years. I have learned much about the fields of medicinal and biological chemistry through his mentorship. I would like to thank my committee members Dr. Steve Weber, Dr. Scott Nelson, and Dr. Sam Poloyac for serving on my committee and helping me to grow as a graduate student.

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My research projects have been a highly collaborative effort and many people have been involved in the projects on which I have worked. I would like to thank Prof. Dennis Curran and all of the chemists that have worked on the synthesis of dicytostatin and all of the analogues. I would like to thank Prof. Andreas Vogt for his important contributions without which my publication in *Molecular Pharmacology* would not have been possible. He has also provided his vast scientific knowledge to many helpful discussions. I would like to thank Dr. Pui-Kai Li at The Ohio State University for providing the 5HPP-33 project and giving me the chance to work on it. In addition, I would also like to thank the chemists for that worked on the pironetin and tubulysin projects, and Profs. Nelson and Wipf. I would be remiss if I did not thank Dr. Ernest Hamel at the NIH for not only allowing me to work in his lab, but the invaluable scientific information he has provided me with over the years. There is not a time in my graduate career where Dr. Hamel did not provide me with extremely helpful advice when I asked for it.

Finally, I would like to thank my home away from - the School of Pharmacy. If not for their late night moral support I would not be able to write this thesis today. I would like to thank the girls of the 8th floor, Dr. Marci Chew, Nisa Ghonem, Trish Butera and Megan Klamerus. My sincere appreciation is given to the people in the office of Salk 731 for letting me use the computers there, for providing me with delicious food (Shringi Sharma), and engaging in stimulating conversations. Last but certainly not least, I would like to thank my family and fiancé for their unwavering love and support.

1.0 INTRODUCTION

Microtubules play a role in many cellular processes. They provide a means of transportation from the cell periphery to the nucleus, give structural support to the cell as a part of the cytoskeleton, and are the major component of flagella and cilia. Microtubules also play a crucial role in mitosis, serving in the separation of sister chromatids during anaphase and in preparation for the division of the parent cell into two daughter cells. Microtubules are not static structures, rather they interconvert between rapid stages of depolymerization and polymerization. This process is known as dynamic instability. Regulation of dynamic instability is controlled by a number of factors *in vivo*. Agents that can interfere with the normal function of microtubules and arrest cell division are of great importance in cancer treatment.

1.1 MICROTUBULE STRUCTURE, FUNCTION AND REGULATION

1.1.1 Microtubule structure and function

Microtubules are noncovalent polymers formed from the heterdimers of the protein tubulin.[1] The heterodimeric protein is composed of α and β isoforms of similar amino acid sequence, and each isoform has a molecular mass of ~50 kDa.[2] The microtubule is a tube composed of longitudinal strands of heterodimers called protofilaments. These protofilaments, typically 14 of which, interact laterally to form the microtubule, in which the heterodimers are arranged in a left-handed, three-start helix. The interior of the microtubule is known as the lumen. Panel A in Figure 1 shows the tubulin heterodimer, and Panel B provides a diagram of microtubule formation. Microtubules are arranged in a B-type lattice, where lateral interactions occur between adjacent α monomers and adjacent β monomers.[3] These interactions are uniform throughout the helix, except at the seam where the path switches from α to β interactions or *vice versa*.

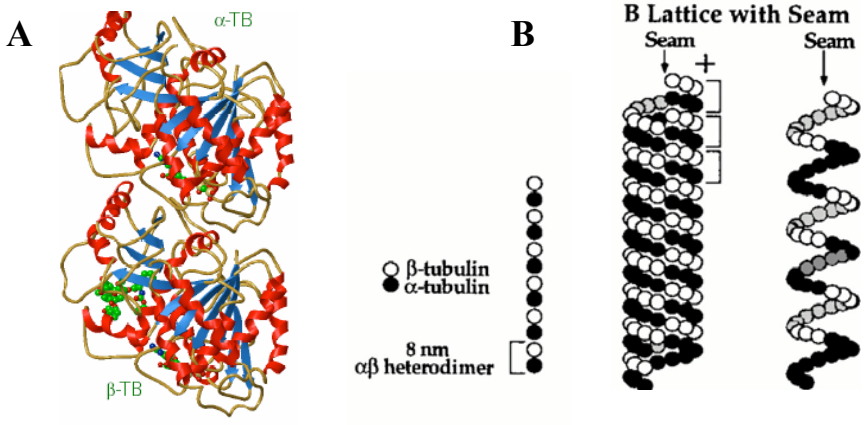

Figure 1. The tubulin heterodimer and diagram of microtubule formation
Panel A shows the quaternary strucuture of the tubulin heteromdimer containing α and β subunits. The monomers share a 50% homology at the amino acid level [adapted from Nogales, E *et al.*, **Nature**, 1998(391): 199[4]]. Panel B shows the formation of protofilaments from head-to-tail interactions of heterodimers. Heterodimers then associate to form the microtubule B lattice. The lateral interactions within the B lattice are between homologous monomers, but there is an exception at the seam of the microtubule [adapted from Desai A and Mitchison T, **Annu. Rev. Cell Dev. Biol.**, 1997(13): 83-117[1]].

Both α and β tubulin contain a binding site for the nucleotide guanosine triphosphate (GTP).[6] The GTP at this site is hydrolyzed after incorporation of the dimer into the microtubule, and remains bound to β tubulin as guanosine diphosphate (GDP). Microtubules have a distinct polarity because of the formation of a layer of newly added GTP containing heterodimers.[5] As the microtubule lengthens, the GTP is hydrolyzed in these heterodimers leaving heterodimers containing GDP in their exchangeable site. The end from which heterodimers contain GDP and readily dissociate from is known as the (–)-negative end, and the end where addition of heterodimers occurs containing a layer of GTP containing heterodimers is known as the (+)-positive end. The subunits containing GDP are released from the (–) end. The released heterodimeric subunits can then actively exchange the bound GDP for GTP, and these subunits can be used in another cycle of microtubule polymerization. The binding site for GTP on α tubulin differs in function from that of the β tubulin monomer. It is known as the non-exchangeable site or N-site due to the lack of exchange for GDP to GTP during microtubule depolymerization and the fact that GTP bound at this site is not hydrolyzed during polymerization.[7]

The process of microtubule formation occurs in three phases termed nucleation, elongation and steady-state.[8] Nucleation is the slow formation of a small microtubule nucleus from soluble tubulin. After formation of this nucleus, elongation of the microtubule rapidly occurs. Elongation is the rapid addition of heterodimers to the (+) end of the microtubule. After a period of elongation, the microtubule reaches a steady state. The steady state occurs when the lengthening and shortening of the microtubule occur at equal rates.

Microtubule polymerization and depolymerization does not usually occur in a continuous manner with equivalent addition and subtraction of subunits at each end.[9, 10] This phenomenon, called treadmilling, does occur, but more often the microtubule never reaches an equilibrium length. Experiments have shown that while a given population of microtubules may have an average length, a single MT rapidly interconverts between depolymerization and polymerization.[11] This rapid change is called dynamic instability and allows for microtubule function within the cell.

Dynamic instability is characterized *in vitro* by four parameters.[12] These parameters are the rate of microtubule growth, the rate of microtubule shortening, the frequency of catastrophes and the frequency of rescues. Catastrophe is the transition of the microtubules from a polymerization state to that of depolymerization and rescue is exactly the opposite process.

GTP hydrolysis powers dynamic instability with the free energy provided by the release of phosphate. The primary role of GTP hydrolysis is destabilization of the microtubule lattice by the formation of weaker lateral interactions within the GDP bound β-tubulin subunits.

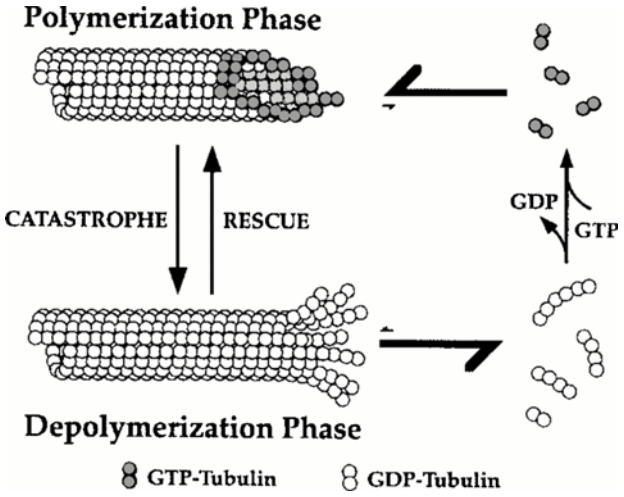

Figure 2. Diagram of microtubule dynamics and the role of GTP [adapted from Desai and Mitchison, **Annu. Rev. Cell Dev. Biol.**, 1997(13): 83-117[1]].

GTP-induced polymerization causes instability. The plus ends of the microtubule contain a small layer of GTP-containing subunits that help to stabilize the microtubule and drive polymerization.[11] The loss of this cap permits rapid depolymerization due to the instability of the GDP-bound tubulin.

1.1.2 Role of microtubules in mitosis

Mitosis is the process by which the nucleus of the cell divides and produces two genetically identical daughter cells from a parent cell.[13] Division of the cytoplasm follows mitosis in a process known as cytokinesis. Mitosis is divided into five stages that occur sequentially, prophase, prometaphase, metaphase and anaphase, with cytokinesis occurring after the last stage.

Microtubules are the main functional component of the mitotic spindle.[14] The bipolar mitotic spindle is made up of the centrosome, which consists of a pair of centrioles, the centrosome matrix and an aster. The attachment of the (–) ends of microtubules to the centrioles produces an array of radiating microtubules known as an aster.[15] The centrosome matrix consists of a number of proteins, which help in the nucleation of the microtubules.

Microtubule dynamics plays a very important role in all stages of mitosis. Figure 3 represents the three phases of mitosis, namely metaphase, anaphase and telophase, in which microtubules are known to play a prominent role.

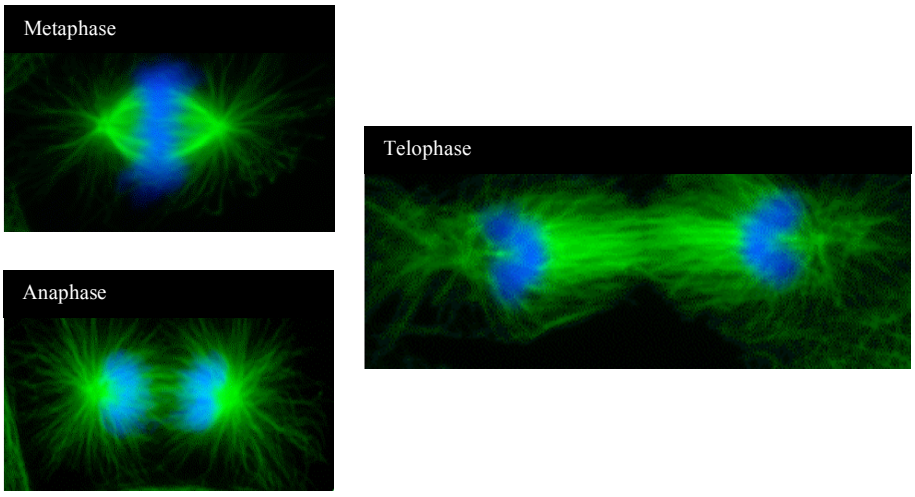

Figure 3. Immunoflourescence images displaying the function of microtubules in three phases of mitosis [adapted from *http:// mitichison.med.harvard.edu/research/microtubules/html*].
The stages of mitosis were visualized using Hoechst to stain chromatin (blue) and a fluorescently labeled secondary antibody to stain microtubules (green).

During metaphase, the microtubules attach to the kinetochores of the sister chromatid pairs, allowing for their correct orientation along the metaphase plate.[16] The cell then enters anaphase where the microtubules function in separating the sister chromatid pairs by, in concert with motor proteins, pulling them to opposite poles of the cell. A membrane forms around the separated chromatin in telophase and prepares for the division of cytoplasm during cytokinesis.[17]

1.1.3 Regulation of microtubule dynamics

The rates of polymerization and catastrophes *in vivo* are both greater than that observed *in vitro* using purified tubulin.[18] This is probably due to the *in vivo* regulation of microtubules through signal transduction pathways, post-translational modifications and binding of regulatory proteins to the microtubule lattice. Cells can alter the expression of proteins that destabilize or stabilize microtubules. Certain proteins that interact with the microtubules are inactive when phosphorylated, and the cell can alter the phosphorylation levels of these proteins.

The most well known microtubule-stabilizing factors *in vivo* are the microtubule-associated proteins (MAPs). MAPs bind to the microtubule and stabilize the lattice by cross-linkage of heterodimers.[19] The crosslinkage functions to stifle heterodimer dissociation, decrease catastrophes and increase rescue.[20] There are four types of well-characterized MAPs: MAP1, MAP2, tau in neurons, and MAP4 in non-neuronal cells. MAP binding in neurons to microtubules is in a ratio or 1:4-10, and binding is primarily through electrostatic interactions with the acidic C-terminal of the microtubule proteins. MAP binding is negatively regulated by phosphorylation, and over-phosphorylation of tau plays a role in the pathogenesis of Alzheimer's disease.[1]

Proteins that increase the rate of catastrophes and decrease rescues include kinesins, stathmin, and microtubule severing proteins.[21-23] Kinesins are motor proteins that move by walking from the (–) to (+) ends of microtubules. It has been shown in *Xenopus* eggs that immunodepletion of kinesin, XKCM1, causes an increase in microtubule length and a decrease in catastrophe frequency.[24]

Stathmin/Op18 is a small protein that binds in a ratio of 1:2 to tubulin heterodimers as determined by electron microscopy.[25] Stathmin not only increases the catastrophe frequency but also lowers the amount of soluble tubulin available. X-ray crystallography studies with the stathmin like domain (SLD) of RB3, a neuron-specific homologue of the stathmin family of proteins, show that binding of the protein to tubulin changes the conformation of the protofilament.[26] As presented in Figure 4, binding of SLD-RB3 to tubulin causes the heterodimer to curve.

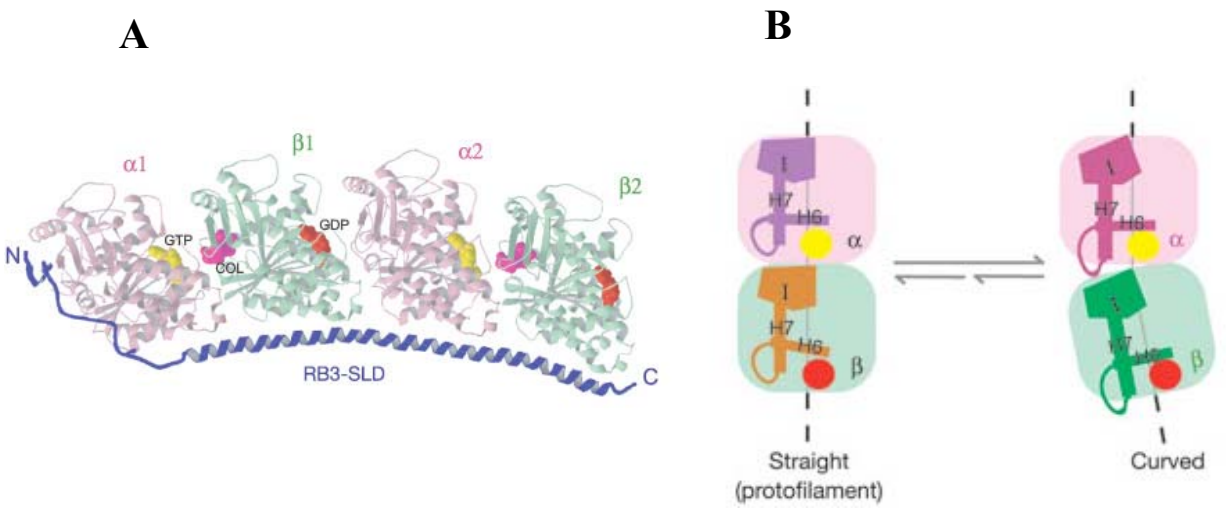

Figure 4. Conformational changes to the tubulin heterodimer associated with SLD-RB3 binding.
A.) The stathmin like binding domain of RB3 bound to two heterodimers. B.) The (🔴) represents the GTP exchangeable site on β tubulin and (🟡) represents the nonexchangeable site on α tubulin. The intermediate domain (I) is rotated toward the nucleotide binding domain in the straight protofilament to allow for lateral interactions to occur [adapted from Ravelli R, **Nature**, 2004(428): 198-202[26]].

The binding of SLD-RB3 is associated with movement in the H6-H7 loops and the intermediate domain in the both the α and β subunits. This movement causes a loss of favorable lateral and longitudinal interactions within and between the protofilaments.

The microtubule severing proteins seem to play multiple roles within the cell. Katanin, a microtubule severing protein in the ciliate protozoa *Tetrahymena,* promotes microtubule assembly in ciliary microtubules, but decreases polymer mass of internal microtubules.[27] Post-translational modifications to tubulin seem to be markers for the severing of the microtubules.

1.2 MICROTUBULE PERTURBING AGENTS

The importance of microtubules to mitosis and the potential to interfere with delicate dynamics of microtubules has lead to the search and development of many microtubule interacting compounds. Microtubule perturbing agents can be roughly classified into two broad groups based upon the mechanism by which they disrupt normal microtubule function. These include agents that stabilize microtubule formation and agents that destabilize or inhibit polymer formation.[28] These agents can further be classified by the location of their binding site to tubulin and/or the microtubule.

1.2.1 Paclitaxel and other known microtubule stabilizers

Paclitaxel was originally isolated from the bark of the yew tree *Taxus brevifolia*.[29] Its ability to stabilize microtubules and increase the polymer mass was recognized in 1979 by Schiff and Horwitz.[30] Paclitaxel was clinically approved in 1995 and has been shown effective for the treatment of breast, ovarian and non-small cell lung carcinomas.[28] The binding of paclitaxel to the microtubule stabilizes the lateral interactions between heterodimers. Paclitaxel binds to the β-tubulin subunit and preferentially binds to polymerized tubulin. Interestingly, high resolution cryoelectron microscopy studies of zinc cation- and paclitaxel-stabilized sheets of tubulin and computational models of microtubules from these sheets strongly suggest that the taxane binding site (*i.e.*, where paclitaxel binds) is in the lumen of the microtubule. Studies with cyclostreptin, a microtubule stabilizer that binds covalently to the taxoid site, have shown that taxane site binding may be a two-step mechanism.[31] Taxane site agents may first bind to an external site on the microtubule surface, and then transfer to an interior site within the lumen.

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