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Drugs That Target Dynamic Microtubules: A New Molecular Perspective

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Abstract

Microtubules have long been considered an ideal target for anticancer drugs because of the essential role they play in mitosis, forming the dynamic spindle apparatus. As such, there is a wide variety of compounds currently in clinical use and in development that act as antimitotic agents by altering microtubule dynamics. Although these diverse molecules are known to affect microtubule dynamics upon binding to one of the three established drug domains (taxane, vinca alkaloid, or colchicine site), the exact mechanism by which each drug works is still an area of intense speculation and research. In this study, we review the effects of microtubule-binding chemotherapeutic agents from a new perspective, considering how their mode of binding induces conformational changes and alters biological function relative to the molecular vectors of microtubule assembly or disassembly. These “biological vectors” can thus be used as a spatiotemporal context to describe molecular mechanisms by which microtubule-targeting drugs work.

Keywords

microtubules; anticancer drugs; tubulin-binding

1. INTRODUCTION

Microtubules are ubiquitous cellular polymers that play diverse roles within the cell, such as maintenance of cell structure, protein trafficking, chromosomal segregation, and mitosis. Composed of cylindrical tubes of the protein tubulin, microtubules serve as the cytoskeleton of the cell and function as highways for intracellular transport (including movement of organelles, vesicles, proteins, or signaling molecules by motor proteins throughout the cell).[1] Microtubules are able to perform a wide variety of tasks through several means, including interactions with microtubule-associated proteins (MAPs), expressing different tubulin isotypes, and posttranslational modifications of tubulin. Although they constitute the robust cellular cytoskeleton, they are not static polymers. Instead they are highly dynamic polymers that alternate relentlessly between periods of growth and shortening, which are intervened by phases of no detectable activity, a “paused” phase. This excessively agitated behavior of

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microtubules, also known as dynamic instability, is marked by ongoing hasty growth and shrinkage and is fundamental to the multifarious workings of microtubules in the cell. Although energetically expensive, the speedy reorganization of dynamic microtubule arrays is responsible for establishing a highly elegant bipolar mitotic apparatus called the mitotic spindle. The mitotic spindle functions to accurately and precisely segregate the replicated chromosomes into two daughter cells during cell division. Thus, owing to their crucial role in the orchestration of mitotic events, microtubules serve as pharmaceutically validated targets for anticancer chemotherapy.[2,3] No wonder microtubule-active compounds have met with exceptional clinical success and are frequently referred to as spindle poisons.[4–7] Currently available chemotherapeutic regimes include taxanes and a variety of vinca alkaloids for the treatment of a wide spectrum of human malignancies. Intriguingly, though, these two drug classes target different cancer types. Taxanes (paclitaxel and docetaxel) are employed primarily for the management of a range of solid cancers such as breast, ovarian, gastroesophageal, as well as cancers of the head and neck, and nonsmall cell lung cancer. On the other hand, vinca alkaloids, such as vincristine, vinblastine, and vinorelbine, are most often used for hematological malignancies, such as lymphomas and leukemias. Although it is an overly simplistic classification, drugs that interfered with microtubule structure and impeded their function were divided into stabilizers (taxanes) and destabilizers (vincas).[2] Yet another drug-binding site on tubulin, known as the colchicine-binding site, elicits the same “destabilizing” phenotype as seen with the vincas, even though colchicine-site-binding drugs do not share structural similarity with vincas at the molecular level.[8] Though colchicine, owing to its extreme toxicity, is not itself used as an anticancer agent, colchicine-domain-binding drugs, such as combretastatins, 2-methoxyestradiol (2-ME), and chalcones, are being actively investigated for their anticancer action. Recently, an emerging class of microtubule-modulating agents, noscapinoids, sets itself apart from the conventional stabilizers and destabilizers and is currently gaining momentum. Based on the parent molecule, noscapine, which is in Phase I/II clinical trials, noscapinoids perhaps traverse a “middle-path,” as they are neither a true stabilizer nor a destabilizer, and they offer a “kinder and gentler” chemotherapeutic approach.[9–11]

No matter the class, all microtubule-binding agents belongs alter microtubule dynamics at low concentrations; thus their most potent mechanism of action seems to be the specific inhibition of the dynamics of mitotic spindle microtubules. Attenuation of microtubule dynamics engages cell cycle surveillance mechanisms to arrest cell division in mitosis. This mitotic stall may then lead to various irremediable chemotherapeutic outcomes including mitotic death, mitotic exit, apoptotic death, or aneuploidy.[12] Several antimitotic drugs with diverse binding sites on tubulin are in various stages of clinical development and the armamentarium of microtubule-binding drugs continues to grow.

In this review, we describe the growing number of both experimentally and clinically validated antimitotic drugs in the context of their binding position within a microtubule. To accomplish this, we have constructed an *in silico* model of a microtubule in its biologically relevant state (a thirteen protofilament, three-start polymer) in order to illustrate the relationship between the binding position of relevant drugs within tubulin heterodimers and the microtubule superstructure itself. When viewed from this larger context, the actions of antimitotic drugs affecting microtubule dynamics can be well characterized, as their binding positions relative to vectors formed by the growth of straight or peeling protofilaments can be used to elucidate their activity as it relates to the directionality of these vectors. This novel concept of “biological vectors” thus may provide a new perspective to predict the efficacy of tubulin-binding drugs in four-dimensional space.

2. MICROTUBULE ARCHITECTURE: AT A GLANCE

The discovery of tubulin, the major component of microtubules, was intimately coupled with the identification of the colchicine-binding site. Colchicine, the first identified antimitotic agent, was extremely valuable in the purification of tubulin heterodimers, the subunit comprising the microtubules.[13] The biological unit in solution is composed of α-tubulin and β-tubulin heterodimers. Microtubules are long, filamentous, ubiquitous tubulin polymers arranged in the form of slender filamentous tubes that can be many micrometers long. They are intrinsically robust polymers that resist compression and bending. At the structural level, each hollow microtubule is assembled from 13 parallel protofilaments, each comprised of alternating α- and β-tubulin subunit molecules along the longitudinal axis of the microtubule (Fig. 1). The building block of each protofilament is a tubulin heterodimer, formed from a very tightly linked pair of α-tubulin and β-tubulin monomers.

Although microtubules can be assembled in vitro from high concentrations of purified tubulin subunits, the *in vivo* assembly is highly controlled by a microtubule organizing center (MTOC) called the centrosome.[14–17] In interphase cells, the centrosome, which is centrally located adjacent to the nucleus (Fig. 2), radially emanates microtubules that project toward the cellular periphery. The tandem arrangement of tubulin heterodimers results in an intrinsic heterogeneity between the two ends of the microtubule, resulting in different kinetics of addition and subtraction of heterodimers at the two ends. This explains the basis of the "polar" characteristics of the microtubule, i.e., the existence of the plus (+) and minus (−) end.[18] Although the plus end is capable of rapid growth, the minus end is sluggish. The direction of the αβ dimer in relation to the polarity of the microtubule lattice displays β-tubulin monomer at the plus end and the α-tubulin is exposed at the minus end. Furthermore, the plus ends are most often free in the cytoplasmic space or reach as far as the plasma membrane. The slugglishly growing or the "lazy" minus end, on the other hand, is frequently embedded in the centrosome.

A. Restless Microtubules, Intriguing Dynamicity

The assembly of a microtubule by the polymerization of αβ tubulin dimers occurs in two phases: nucleation and elongation. Formation of a short polymerization nucleus precedes elongation or polymer growth at each end by the reversible, noncovalent addition of tubulin subunits.[4] For net polymer elongation, the association of tubulin heterodimers into the growing microtubule is faster than microtubule depolymerization. However, at steady state, growth of microtubule polymer due to αβ-heterodimer addition is counterbalanced by shrinkage due to disassembly into αβ-tubulin subunits.[1,19] Thus, a polymerized microtubule switches between episodes of growth and shrinkage, a property called dynamic instability.[16,18,20–22] Dynamic instability can be described as a combined function of four factors: the rate of microtubule growth, rate of shortening, frequency of transition from a growing or paused (neither growing nor shortening) state to a shortening state (an event referred to as "catastrophe"), and conversely the frequency of transition from a shortening state to a growing or paused state (termed "rescue"). Microtubules thus are not simple equilibrium polymers,[21,23] and this frenzied behavior of microtubules is an intense none-quilibrium behavior, which is energy-expending. Since heterodimers of tubulin co-purify with two moles of guanine nucleotide per mole of αβ dimer,[13] it is assumed that hydrolysis of GTP, the energy carrier, fuels dynamic instability.

Structurally similar subunits of 55 kDa each, α- and β-monomers both possess a GTP-binding region that can accommodate one molecule of GTP. Although the GTP molecule is tightly bound to the α-tubulin monomer at the dimer interface and is never hydrolyzed or exchanged, β-tubulin can bind both GTP and GDP in an exchangeable fashion.[24] Thus, the hydrolyzable GTP site on the β-monomer is also called the Exchangeable (E)-site.

Heterodimers of tubulin can only be polymerized when GTP is present on the E-site. Hydrolysis of GTP powers the "aggressive" behavior of microtubules and has crucial implications for the plethora of functions performed by microtubules. GTP-bound β-tubulin subunit of the free heterodimeric tubulin molecule integrates into the microtubule structure. Shortly thereafter, this GTP molecule is hydrolyzed to GDP, which in turn remains bound to the tubulin in the GDP form. In the event of rapid microtubule growth (that is, if the rate of subunit addition is high), a new GTP-containing subunit is added on the microtubule polymer before the GTP of the previously added subunit is hydrolyzed. This results in accrual of GTP-containing subunits at the tip of the microtubule which is referred to as a "GTP cap." On the other hand, a slow rate of subunit addition to the polymer offers enough time for hydrolysis of GTP into GDP, thereby exposing GDP at the tip of the microtubule. Conformational changes induced upon GTP hydrolysis cause a reduction in the binding affinity of the subunits for neighboring subunits in the polymer; as a result, dissociation is favored and the filament is forced into a curved shape. This strains the straight protofilament and causes it to splay apart orthogonally from the microtubule much like the peeling of a banana, as shown in Figure 3.[25] The conformational changes caused by the switch from GTP- to GDP-tubulin are seen at both the intra and interdimer interfaces.[26] GDP-tubulin protofilaments show approximately 12 and 18° kinks at these interfaces, respectively, vs. straight, GTP-capped protofilaments, and the sum of these angles results in the characteristic protofilament peel.[27] It has been shown that the depolymerization rate of microtubules is about 100 times faster from an end containing GDP-tubulin compared with that from one containing GTP-tubulin.[5] Thus, GTP-tubulin polymerizes into straight protofilaments, which induces growth of the microtubule, while GDP-tubulin results in depolymerization and curved protofilaments. The presence of the protective "GTP cap" on the microtubule tip averts dissociation of the GDP protofilaments, maintaining the rigid cylindrical shape of the microtubule. Furthermore, in the presence of GTP cap, the plus ends of microtubules can rapidly oscillate between states of growth and shrinkage, which is greatly reduced at the minus end.[5]

Yet another dynamic instability behavior that microtubules display is "treadmilling." It is a well-known process by which tubulin dimers added on to the microtubules at their plus ends are released from the minus ends.[23,28] In doing so, the polymer length stays constant while there is a constant flux of tubulin dimers from the microtubule minus end to the plus end. The rapid treadmilling of microtubules has been shown to occur during metaphase and anaphase, where it may be essential in the relay of intracellular signals from the kinetochore to the poles.[29] Although quite different, treadmilling and dynamic instability are not mutually exclusive activities and often occur in concert. Some microtubules simultaneously display both behaviors while others primarily show one or the other. The extent to which individual microtubule populations display dynamic instability and treadmilling behaviors is largely contingent on intracellular conditions.[30] There is a complex set of mechanisms that determine the dynamics and activities of microtubules *in vivo*, including the level of expression of different tubulin isotypes, posttranslational modifications, and the activity of MAPs. In humans, there are six isotypes of α-tubulin and seven isotypes of β-tubulin, and the level of expression of each isotype varies in different tissues and cells.[31–36] Each isotype can further be divided into different subtypes according to posttranslational modification including phosphorylation, polyglutamylation, polyglycylation, acetylation, detyrosination/tyrosination, or even removal of the penultimate glutamate residue in α-tubulins.[5] Of course, tubulin-binding drugs have different affinities for different isotypes, which affects the overall efficacy in different cancers.

B. Microtubules: Performance of Multifarious Functions

The functional diversity of tubulin helps microtubules to serve many essential roles in cellular morphogenesis, cell polarity, migration, intracellular signaling, and division. Since microtubules fill up the cytoplasmic space and interact with many signaling proteins and organelles, they are perfectly equipped to cascade signaling molecules throughout the cell and thus maintain the network of signaling circuitries. Microtubules serve as “road-tracks” for molecular motors as well as “highways” on which cellular trafficking occurs, such as transport of vesicles and mitochondria.

C. Microtubules: A Spectacular Role in Propelling Mitosis

Beyond catering to a plethora of crucial physiological functions, microtubules and their inherent dynamicity are vital for the cell division process. In particular, exquisite spatiotemporal regulation of microtubule dynamics during mitosis is of special importance (Fig. 4). During mitosis, microtubule dynamicity increases 20- to 100-fold enabling spatial organization and quick remodeling of interphase arrays to assemble the elegant mitotic machinery within a matter of minutes.[37] The nucleation rate of microtubules at the centrosomes also increases by sevenfold during mitosis.[37–39] Assembly of GTP-tubulin and disassembly of GDP-tubulin are both thermodynamically favorable in the cytoplasm and can thus perform mechanical work.[21] The rapid dynamics of spindle microtubules is necessary for the capture of chromosomes during prometaphase, as the spindle fibers have to “explore” the cytoplasmic space to find and make productive attachments to the kinetochores.[38] This is accomplished through alternating phases of rapid growth to long distances (usually around 5–10 mm), followed by nearly complete shortening.[5] After all the chromosomes establish connections at their kinetochore region with the microtubules springing from both opposite poles, they are all aligned along the metaphase plate in a process known as congression. Only after this precise alignment, can mitosis proceed past the metaphase-anaphase checkpoint into anaphase. Thereafter, the sister chromosomes are synchronously separated and pulled to opposite ends of the dividing cell. The extremely rapid dynamics of microtubules plays an important role in the intricate movement of chromosomes. The push (by polymerizing) and pull (by depolymerizing) of chromosomes by mitotic spindle fibers generates ~50pN of force.[40] Even a single misaligned or absent chromosome from the metaphase plate can stall mitosis and prevent the cell from progressing beyond the checkpoint, resulting in an arrested prometaphase/metaphase state that eventually leads to induction of apoptosis. Most microtubule-targeting drugs exploit this mechanism to eliminate cancer cells, as their high rates of proliferation increase vulnerability to mitotic checkpoint-induced apoptosis.

Microtubule dynamics thus play a crucial role in the orchestration of the mitotic process, with the institution of a perfectly bipolar mitotic machinery that dictates the separation of duplicated chromosomes into two identical sets before cleavage of the cell into two daughter cells (Fig. 4).

D. Microtubules: Partner Proteins

Speed of microtubule growth and shrinkage and frequency of transitions (catastrophe and rescue rate) are the four variables that describe microtubule dynamics.[41] These parameters are amenable to regulation by different microtubule-modulating factors either positively, by increasing the frequency or speed, or negatively, by suppressing transitions or reducing the speed. An arsenal of microtubule-modulating factors is expressed by cells that either promote assembly or disassembly, or display more specific roles only on a subset of microtubules.[5] Essentially, microtubule dynamics-modulating factors can largely be divided into microtubule-stabilizing and -destabilizing factors. Microtubules can be stabilized by preventing catastrophe, rescuing a depolymerizing microtubule, and by decreasing shrinkage

speeds. Likewise, microtubules can be destabilized by inducing catastrophes, preventing rescues, or increasing shrinkage speeds. MAPs bind in a nucleotide-insensitive manner to the microtubule lattice.[21] Most MAPs identified to date are posttranslationally regulated by phosphorylation, with the more phosphorylated forms attenuated in their capacity to stabilize microtubules.[42,43] Since the binding interaction of MAPs to microtubules is largely electrostatic, employing the acidic C-terminal domains of both α- and β- tubulin,[44] phosphorylation perhaps inhibits MAP function by reducing the affinity of the MAP for the microtubule lattice.[45] MAP inactivation by phosphorylation has been shown to reduce the frequency of rescue and can increase microtubule turnover *in vivo*.[46]

1. Microtubule-Stabilizing Proteins—Microtubule-stabilizing proteins (MAPs) act through suppression of catastrophes, promotion of growth, and reduction of shrinkage speeds.[5,42,47–50] A large group of MAPs[47] that stabilize microtubules against disassembly include MAP1, MAP2, MAP4, tau, and DCX (doublecortin). The most abundant and ubiquitous MAP is MAP4, which is present in non-neuronal cells and stabilizes microtubules. One major MAP, the tau protein, has been shown to antagonize the microtubule-destabilizing activity of XKCM1 (Xenopus kinesin catastrophe modulator-1)[51] and can offer protection against katanin-dependent microtubule severing.[52]

One major class of MAPs, called the +TIPs (microtubule plus-end tracking proteins), are known to specifically bind to growing microtubule plus ends[53,54] and can intensely influence microtubule dynamics. Many +TIPs such as CLASPs (cytoplasmic linker protein-associated proteins) and ACF7 (ATP-dependent chromatin assembly and remodeling factor 7) act as microtubule rescue and stabilizing factors at the cell cortex,[55,56] whereas CLIPs (cytoplasmic linker proteins) act as cytosolic rescue factors.[56,57] One of the best studied +TIPs is EB1 (end-binding protein 1), which forms comet-like structures at the tips of growing microtubules. In addition, EB1 and EB3 allow relentless microtubule growth in cells by impeding catastrophes.[58] The binding of EB1 to microtubule plus ends aids in the promotion of microtubule polymerization.[59] This binding interaction is facilitated by its amino-terminal calponin homology domain. In addition, EB1 binds other +TIPs, for example, the tumor suppressor adenomatous polyposis coli, p150glued, and cytoplasmic linker protein CLIP-170.[59,60] The interaction of EB1 with its binding partners regulates multifarious microtubule-mediated cellular actions, including cell division, cell migration, and morphogenesis.[59,60] Beyond its role as a regulator of microtubule dynamicity and related cellular activities, EB1 overexpression has been recently observed in human cancers, such as gastric, esophageal squamous cell, and hepatocellular carcinoma.[61–63] Another recent report recognizes the oncogenic function of EB1 in breast cancer, wherein the authors showed that EB1 stimulated Aurora-B activity in breast cancer cells, and EB1 expression correlated with enhanced activity of Aurora-B in clinical breast cancer samples.[64] Another MAP, XMAP215 has been shown to potently increase microtubule polymerization rate by ~10-fold *in vitro* at the plus ends.[65–67] Interestingly, XMAP215 also augments the depolymerization rate and reduces rescue frequency (at the minus end), thereby enhancing microtubule turnover. The capacity of XMAP215 to affect the two ends of a microtubule in a different manner is intriguing and has been an area of investigation.

Accumulating evidence suggests that the sensitivity of cancer cells to microtubule-targeting agents is MAP-dependent thus indicating a role of MAPs in tumor cell resistance to such agents. Consequently understanding molecular mechanisms that underlie MAP expression in cancers will enhance therapeutic efficacy of microtubule-targeting drugs.[68]

2. Microtubule-Destabilizing Proteins—The best studied and most potent microtubule depolymerizers are the nonmotile kinesins from the kinesin-13 family, which includes three mammalian members: Kif2A, Kif2B, and Kif2C/MCAK (mitotic centromere-associated

kinesin).[69] Kinesin-13s serve dual roles: an ATP-dependent, catastrophe-promoting activity and an ATP-independent, tubulin-sequestration activity.[70] MCAK has been shown to bind both plus and minus ends *in vitro*[69,71] and displays the highest affinity for curved protofilaments that resemble shrinking microtubules.[72] Kif2A has been shown to promote catastrophes at the cell cortex.[73,74] Different kinesin-13 family members serve varying roles; while Kif2A and Kif2B are essentially associated with the centrosome, Kif2C/MCAK predominates at the kinetochores,[73,74] thus suggesting that these proteins are likely to affect diverse microtubule subpopulations differently.[75]

Other members of the microtubule-destabilizing class include members of the kinesin-8 and kinesin-14 family that promote microtubule depolymerization in cells. Kinesin-8s, namely Kip3 and Kif18A, disassemble MTs solely from the plus end in a length-reliant way, where long microtubules are depolymerized more efficiently than short ones.[76–78] This suggests that kinesin-8s participate in controlling mechanisms that dictate microtubule length and thus can be crucial for the alignment of chromosomes at metaphase mid-plate.[77,78] Some kinesin-14 homologs, such as HSET (human spleen, embryonic tissue, and testes) and XCTK2 (Xenopus C-terminal kinesin 2), are also known to control spindle length.[79]

Yet another negative regulator of microtubule stability is Oncoprotein 18 (Op18)/stathmin, a small protein (19 kDa) which is highly expressed in leukemic cells. Stathmin physically interacts with tubulin dimers and increases the catastrophe rate of microtubules.[80] Recent studies have shed light on the complex formed between stathmin and tubulin thus presenting a mechanistic model of stathmin's action on microtubules.[81] Stathmin possesses two equal affinity binding sites for tubulin heterodimers. Interaction of both binding sites results in a ternary tubulin–stathmin complex,[80,82] which has a kinked geometry that averts the incorporation of sequestered tubulin subunits into protofilaments (Fig. 5).[83] This characteristic ability of stathmin to induce a bent conformation of tubulin subunits at microtubule ends, which resembles the “peeling” of microtubule ends, partly explains the catastrophe-inducing activity of stathmin that has been well-reported.[84,85] Additionally, the stathmin-like protein RB3 has been used to stabilize tubulin in a bent conformation for crystallographic studies to determine the binding sites of several depolymerizing drugs.[86,87]

3. Motor Proteins—Motor proteins are another important class of MAPs that are indispensable for microtubule function. Understanding motor protein diversity, direction of motor protein movement, and microtubule organization is crucial to gain insight into cellular strategies of intracellular transport. Without aid from motor molecules, microtubules would not be competent to perform their spectrum of functions, including mitosis, secretory transport, and organelle movement.[88] Primarily, motor proteins bind to microtubules and utilize the energy derived from ATP hydrolysis to “walk” steadily along them. They can ferry the membrane-enclosed organelles, for example, mitochondria, golgi stacks or secretory vesicles, to various destined locations in the cell. Motor proteins also cause cytoskeletal filaments to slide past each other, a process vital in cell division. Two major classes of microtubule-dependent motor proteins are collectively grouped as kinesins and dyneins, which in turn comprise a huge number of proteins.

a. Kinesins: Kinesins are relatively simple in organization with most of them being 500 kDa or smaller in size. Kinesins contain between one and four copies of a principal polypeptide that grasps the motor domain, a key element for the generation of ATP-dependent force along microtubules. The kinesin superfamily, which are categorized by a range of distinct “tail” domains[89,90] confer distinctive cargo-binding capacity on each kinesin motor protein. Most kinesins are plus end-directed, although a few display minus end directed behavior. Importantly the kinesin superfamily proteins have specific roles in spindle assembly and

chromosome segregation during cell division, although some of them are involved in transport.

b. Dyneins: Dyneins, on the other hand, are a family of minus end-directed microtubule motor proteins that are less diverse but are much larger than kinesins. They play crucial roles in vesicle trafficking and are important for the proper localization of intracellular organelles.[88] Typically, dyneins are 1- to 2-MDa protein complexes comprised of two to three dynein heavy chains and many variable intermediate and light chains. Each heavy chain is about 500 kDa and holds the motor domain. Kinesins and dyneins generally move in opposite directions along microtubules, and thus together they manage bidirectional vesicle transport.

Although we have begun to dissect the activities of these individual players that interact with microtubules, our knowledge of how and to what extent these players cross-talk with each other to generate a certain effect on a microtubule array is far from complete. It is not even clear if most of the MAPs that control microtubule dynamics (both positively and negatively) are known; thus, our knowledge in this respect perhaps represents only the tip of the iceberg. Nonetheless, the ever-expanding list of MAPs, knowledge of their phosphorylation states, and information about their effects on microtubule dynamics continues to accumulate.

3. MICROTUBULES: UNSURPASSED ANTICANCER TARGETS THUS FAR

The workings of microtubules in concert with several microtubule stabilizing and destabilizing molecules are known to harmonize various physiological functions. In particular, owing to their indispensability in mitosis and cell division, microtubules epitomize the finest anticancer target identified so far. No wonder drugs belonging to this class continue to be among the most commonly prescribed agents in cancer chemotherapy.[4,91] Although most microtubule-interfering agents either stabilize or destabilize tubulin via binding on known tubulin-binding sites, there do exist some compounds that bind to tubulin on undefined sites or target microtubules indirectly by altering their posttranslational modification. However, since microtubules cater to several important functions in resting and differentiated cells, including normal cell division and mediating intracellular transport, antimicrotubule drugs display their downside by causing a variety of undesirable side effects such as severe peripheral neuropathies, immunosuppression, myelosuppression, and gastrointestinal toxicity. Therefore, novel drugs that are “kinder and gentler” to microtubules are being continually investigated and the search for a “magic-bullet” is still on.

A. Targeting Microtubules: Tubulin-Binding Drugs

Natural products present a wealth to medicine and have resulted in the development of drugs ranging from penicillin from the mold *Penicillium notatum* to the well-known chemotherapy drug taxol from the Pacific Yew tree. Indeed, half of all drugs that hold therapeutic value have been derived from naturally occurring toxic molecules. The microtubule-binding drugs are no exception, and most of them have been discovered in large-scale screens of natural products. Their discovery and early development dates back about 50 years when the vinca alkaloids were isolated from periwinkle leaves (*Catharanthus roseus*). Conventionally, microtubule-binding drugs are categorized into two groups: microtubule stabilizers, including taxanes and epothilones, and microtubule destabilizers, including a variety of vinca alkaloids and colchicine (Fig. 6). However, it is becoming appreciated that all microtubule-active drugs at low nanomolar concentrations attenuate microtubule dynamicity rather than altering net polymer mass.[4,92,93] Based on this notion, barriers between the two classes seem to be disintegrating and these drugs should be referred to instead as

"suppressors of dynamic instability." Nevertheless, they occupy different sites on their cellular target and historically are categorized based upon their binding sites on microtubules rather than their mode of action. For example, the taxane site exists on β-tubulin within the microtubular lumen, the vinca domain surrounding the GTP binding site on β-tubulin, and the colchicine-binding site at the interface between the α- and β-tubulin dimers.[94] Figure 6 shows immunomicrographs of breast cancer MCF-7 cells that were treated with paclitaxel (middle) and vinblastine (right). As expected, control cells (left) depict radial arrays of interphase microtubules (green) with the nucleus (red). In contrast, paclitaxel-treated cells showed overpolymerized microtubules in a bundled sheet-like pattern, whereas vinblastine treatment causes depolymerization of the microtubular network (Fig. 6).

B. Assembly Promoters: Stabilizing Drugs

1. Taxane Site Binders—"Stabilizers and Overpolymerizers"—Taxanes are microtubule-binding drugs that target specific sites within the lumen of polymerized microtubules (Table I). They act by binding to GDP-bound β-tubulin molecules and stabilizing them by changing their conformation to the more stable GTP-bound β-tubulin structure.[95] This change aligns the dimer's biological vector with the vector of microtubule growth, increasing incorporation into the microtubule and its subsequent stabilization. This interaction between neighboring dimers results in an equilibrium shift from the soluble to the polymerized form of tubulin, resulting in the bundled phenotype of interphase microtubules.[96] Until recently, the most significant microtubule stabilizers have been the taxanes and the drugs that bind to the taxane site, including paclitaxel (taxol) (Fig. 7), docetaxel (taxotere), taxol analogs, and other similar molecules. These have been widely used as cytotoxic agents targeting a wide range of tumors. Their cytotoxic effect has clearly been attributed to their ability to bind tubulin, stabilize protofilaments leading to microtubule over-polymerization, and ultimately death by apoptosis.[97]

a. Taxoids: Paclitaxel (taxol) has been the mainstay of therapy for several solid neoplasms including breast, ovarian, and prostate neoplasms. Originally discovered from the bark of the Pacific Yew tree (*Taxus brevifolia*) in the 1960s, the mechanism of action was not reported until 1980. Paclitaxel was FDA-approved in 1992 for the treatment of ovarian cancer. To improve on the pharmacology of paclitaxel, its semisynthetic analog, docetaxel, was introduced as a second-generation taxane derived from a precursor found in the European Yew tree (*Taxus baccata*). Indeed, docetaxel is more water-soluble than paclitaxel and turns out to be more active than paclitaxel against cancer cell proliferation; thus, it is currently being employed in chemotherapeutic regimens to treat breast and prostate malignancies.

Paclitaxel facilitates tubulin assembly under all reaction conditions including low protein concentrations, lower temperature, absence of MAPs, and absence of GTP, resulting in highly resistant tubulin polymers with shorter and highly polymerized microtubules.[98–100] Although the specific microtubule phenotype in paclitaxel-treated cells is highly variable, most cells show astoundingly abnormal microtubule arrays that appear sheeted and bundled. Significantly thick bundles of microtubules seemingly not originating from MTOC have also been repeatedly seen.[101] This emphasizes that paclitaxel elevates microtubule nucleation as well as elongation. The electron crystal structure of tubulin complexed with paclitaxel reveals that the binding site for paclitaxel is on the β-subunit (Fig. 8) and is located inside (lumen) the polymer surface (Fig. 9).[102] It is believed that paclitaxel reaches its binding site through small openings on the microtubule surface or due to dynamic fluctuations in microtubule structure.[1] Paclitaxel's attachment to its binding site on the inside of the microtubule tends to stabilize the microtubule lattice, thus increasing polymerization. This can be attributed to the conformational change in paxlitaxel-bound tubulin that maintains a

straight biological vector in the dimer, which aligns with the biological vector of the growing microtubule, and thus enhances its affinity to the surrounding tubulin molecules. The major factors limiting further clinical development of these overpolymerizing drugs include tumor resistance, dose-limiting side effects causing various toxicities, and possible hypersensitivity. One of the mechanisms explaining tumor resistance is the inherent expression of multidrug resistance proteins like P-glycoprotein (Pgp), which is an ABC (ATP-binding cassette) transporter. Upon expression, these transporters act as drug efflux pumps causing diffusion of substrate drugs out of tumor cells.[97] Clinical administration of taxane has been associated with acquired tumor resistance due to Pgp overexpression.[103] Resistance to taxanes can also be attributed to another mechanism causing hindrance in the interaction of the drug with β-tubulin. This is the result of overexpression of the β-III isoform of tubulin in tumor cells, which is usually a feature of neuronal cells.[104] Another severe issue affecting the development of taxanes is their limited solubility, making their administration difficult. Owing to this, it is formulated in various agents like cremophor or polysorbate which present a high risk of hypersensitivity to the receiving patients. This problem has been attenuated by implementing certain modifications in paclitaxel delivery (Abraxane, ANG1005) or using premedications. In addition, significantly high toxicity including immunosuppression and peripheral neuropathy associated with taxanes make them an inappropriate candidate for long-term clinical use. The compelling need to develop better agents has led to several advances aimed at developing newer agents with improved efficacy and specificity.

b. Epothilones: Epothilones (Fig. 7) are microtubule-stabilizers belonging to the macrolide drug family. Naturally produced by myxobacterium *Sorangium cellulosum*, epothilones A and B represent a novel class of antimicrotubule drugs. They were initially isolated for their unique antifungal activity and cytotoxic activity,[105] which was associated with mitotic arrest and polymerization of microtubules.[106] Both epothilone A and B appear to be competing with paclitaxel for its binding site, but their unique molecular framework binds at a site close to the taxane-binding site. Epothilones show microtubule-stabilizing activities similar to paclitaxel but display somewhat distinct mechanisms.[107] Owing to their bacterial origin, these drugs have the advantage of ease of production. Additionally, they are not susceptible to Pgp-mediated drug efflux, thus they are useful in the treatment of taxane-resistant tumors.[108] Various synthetic and semisynthetic analogs have been developed with reduced toxicity and enhanced stability. **Patupilone**, which is naturally occurring epothilone B, has been shown to be 20 times more effective than paclitaxel. It has successfully been shown to permeate the blood–brain barrier and has a toxicity profile limited to diarrhea with nominal neurotoxi-city.[109]**Ixabepilone**, which is a derivative of epothilone B, is yet another significant member. It has been shown to have 2.5-fold higher cytotoxicity compared with paclitaxel and is equally effective in taxane-resistant tumors.[110] Some of the side effects associated with this drug include sensory neuropathy and fatigue.[111] Since these drugs share similar structures and binding sites, their variable toxicity profiles are confounding. This may be attributed to differential tissue distribution and metabolism.[97,109] Several other semisynthetic epothilone B and epothilone D analogs are under development and in clinical trials.

c. Laulimalide and Peloruside A: Laulimalide, an antimitotic agent, with a complex structure is derived from marine sponges and binds to a unique site on α-tubulin, but it has microtubule-stabilizing effects similar to paclitaxel.[112] Though significantly effective against taxane-resistant tumors like epothilones laulimalide seems to have a narrow therapeutic index. On the other hand, Peloruside A, also isolated from marine sponges, shares structural similarity with epothilones but binds to the laulimalide-binding site on α-tubulin.[113] The diversity in structure but similarity in binding site and vice versa is indeed an intriguing aspect of these drugs. Nevertheless, a distinct binding site but similar

polymerization effects of these drugs presents an opportunity for synergism with taxanes, which might result in combination drugs with improved anti-proliferative effects.[114]

d. Discodermolide and dictyostatin: Both discodermolide and dictyostatin are naturally isolated from marine sponges, in which microtubule toxins act as a critical defense mechanism.[115] Similar to paclitaxel, they induce tubulin assembly, hypernucleation, increased microtubule stability, and decreased depolymerization. They form microtubule bundles and spindle aberrations. They are structurally similar and possess a therapeutic advantage for cells expressing β-tubulin III isoforms.[115] Their binding site seems to be distinct from that of paclitaxel, yet they exhibit synergism with paclitaxel.[116]

C. Assembly-Demoters: "Destabilizing" Drugs

The other major class of antimitotic drugs, the depolymerizers, includes those that bind to the vinca domain (Fig. 10) or colchicine domain of tubulin and act to destabilize the microtubule structure at high concentrations. However, as with the polymerizing drugs, they are generally dosed at much lower concentrations clinically, and they act by disrupting microtubule dynamics vs. merely reducing polymerization. This blocks dividing cells in mitosis, eventually leading to apoptosis.[4]

1. Vinca Domain-Binders–"Depolymerizers"—Essentially, the vinca domain is a "target" site for agents that interfere with the binding of vinblastine, a vinca alkaloid, on tubulin. These vinca domain-binders are classified into "vinca site" binders and "peptide site" binders in the vinca domain of tubulin.[117] Among the huge group of microtubule-destabilizing agents, which also include the colchicine-site-binding agents, vinca alkaloids have proven to be quite successful in the clinic. There have been many heterocyclic compounds of varied chemical nature (Fig. 10, Table II) obtained from mother nature, primarily isolated from plants, microbes, and marine organisms, that bind to the vinca domain of tubulin. As we already reviewed, dynamic instability of microtubules is a consequence of GTP hydrolysis on the β-subunit that follows tubulin polymerization and then exchange of GDP for GTP for regeneration of GTP-tubulin.[118] The agents binding to the vinca domain of tubulin differ in these mechanisms while interacting with tubulin. They also engage in the inhibition of cross-links formed between the β-subunits of tubulin.

a. "Vinca-site" binders: The vinca alkaloids, a class of antimitotic compounds derived from the periwinkle plant, *Catharanthus roseus*,[119] act by binding to the β-subunit near the GTP-binding site on tubulin (Fig. 11)[120] and arresting mitosis at prometaphase.[2,8,91] Vinblastine and vincristine are the first-generation vinca alkaloids,[118] which have undergone significant clinical development. Binding near the hydrolyzable GTP site, they alter the dimer conformation, inhibit tubulin-dependent GTP hydrolysis, and GDP–GTP exchange. The dimeric conformational change also changes the dimeric biological vector from a straight "growing" vector to a curved "peeling" vector. At low concentrations, vincas bind to the plus ends of microtubules (Fig. 12), reducing the dynamics and further leading to mitotic arrest.[121] For this reason, they are also referred to as "end poisons."[117,118,121] At higher concentrations, the vinca alkaloids have affinity for free tubulin heterodimers, again potentially forming an altered, curved geometry of the dimeric biological vector, thus favoring the formation of paracrystals, spirals, and tubules.[122–124] Vindesine, vinorelbine, and vinflunine are semisynthetic vinca alkaloids,[118,119] among which vinflunine has been found to have better efficacy when compared with the parent, vinblastine.[125,126] The cross-linking of cys239–cys354 is enhanced by vinblastine, but the cys12–cys201/211 cross-link formation is inhibited.[127]

b. Maytansinoids: Another class of plant-derived anticancer compounds is the maytansinoids, of which maytansine, an ansa macrolide, is derived from a higher plant, *Maytenus ovatus*.[128] Several other related compounds are obtained from *M. serrata*, *Colubrina texensis*, and *Putterlickia verrucosa*. Structurally similar ansamitocins, derived from a gram-positive species of *Nocardia*, also exhibit antiproliferative activity.[129]

Maytansine differs from vinca alkaloids in its method of inhibiting the assembly of microtubules. It does not result in the formation of spiral aggregates, as observed in the case of vincas, but instead inhibits the aggregates formed by vinblastine and results in their disintegration.[130] Studies show that the binding site of maytansine is inhibited by vinca alkaloids, especially vincristine (a competitive inhibitor).[131–133] The binding strength of this macrolide has been shown to be higher than that of vincas.[130]

c. Rhizoxin: Derived from the fungus, *Rhizopus chinensis*,[134] rhizoxin is a macrocyclic lactone with significant antitumor activity similar to vincristine. This macrolide has been found to inhibit the binding of vinblastine to tubulin as it shares a common binding site with maytansine, which does not match but might overlap with the vinblastine-binding site.[127,135] Despite this similarity, rhizoxin has a better efficacy against human and murine tumors than maytansine.[136] Unlike vinblastine, rhizoxin does not induce aggregate formation at high concentrations.[127,135,137]

d. Phomopsin: Phomopsin A from *Phomopsis leptostomiformis*[137] and ustiloxins from *Ustilaginoidea virens*[138] are structurally similar peptides of fungal origin causing mitotic arrest at micro-molar concentrations. Phomopsin A not only inhibits tubulin polymerization events but also prevents tubulin-dependent nucleotide hydrolysis and nucleotide exchange, very similar to maytansine and ustiloxin A.[129,131,139] The highly potent ustiloxins were shown to inhibit microtubule assembly, and ustiloxin A stabilizes the conformation of tubulin. Several studies show that phomopsin A results in the formation of spiral aggregates of tubulin.[129]

e. Halichondrins: Halichondrins and halistatins are microtubule-destabilizing anticancer agents of marine origin.[129,137] Halichondrin B, a lactone polyether, was derived from the marine sponge, *Halichondria okadai*, and the latter, halistatin, also is from an unrelated sponge, *Axinella* species.[129,140] Halistatin affects *in vitro* tubulin assembly and studies have shown disappearance of microtubules after mitotic arrest. Also, it inhibits the tubulin-dependent GTP hydrolysis and nucleotide exchange. Halichondrin B inhibits the binding of vinblastine to tubulin in a noncompetitive fashion.

f. "Peptide-site" binders: Microtubule polymerization is prevented by a number of linear and cyclic compounds of marine and microbial origin.[117] These compounds result in extraordinary amounts of polymer in aggregates with explicit morphological differences. Also, they cause microtubule destabilization by inhibiting GTP hydrolysis.[141,142] As the binding sites of these natural compounds on tubulin coincide partly with that of vinca alkaloids, and most of them are peptides, cyclic peptides, modified peptides, or depsipeptides, they are known to occupy the "peptide-site" of the vinca domain.[117] Dolastatins, spongistatins, and cryptophycins are the best-studied anticancer agents binding to the "peptide-site" of vinca domain.[117]

g. Dolastatins and spongistatins: Dolastatins are natural peptides derived from a species of sea hare, *Dolabella auricularia*,[127,129] and are derviatives of a pseudo-peptide that contains four unusual amino acids. Among the several dolastatins derived from this shell-less mollusc, Dolastatin 10 is the most potent antimitotic agent.[129] Similar to halichondrins, this peptide results in the arrest of cells in mitosis followed by dissolution of microtubules.

Dolastatin 10 inhibits the binding of vincristine to tubulin in a noncompetitive manner[143] and also inhibits tubulin-dependent GTP hydrolysis along with nucleotide exchange.[143–145] Dolastatin 10 inhibits the binding of phomopsin A and rhizoxin to tubulin.[129,146] A despipeptide, dolastatin 15, obtained from *Dolabella auricularia*, is equally as potent as Dolastatin 10 but acts in an exactly opposite fashion; it does not involve the inhibition of nucleotide exchange (despite inhibiting GTP hydrolysis) and induction of tubulin aggregation.[147]

Spongistatins (1–9), a series of lactone polyethers obtained from marine sponges, namely *Spongia* species and *Spirastrella spinispirulifera*, are cytotoxic agents that strongly inhibit the binding of vinblastine and dolastatin 10.[129,137,144] Similar to halichondrins and dolastatins, spongistatins result in mitotic arrest of cells and disintegration of intracellular microtubule assembly.[148] Spongistatins inhibit the formation of dolastatin 10-induced aggregates, but as such they do not induce aggregate formation.[129,149] Spongistatin 1[148] inhibits nucleotide exchange and Spongistatin 3 inhibits the cys12–cys201/211 cross-links formation.[129,148,149]

h. Cryptophycins: Cryptophycins, isolated from a terrestrial cyanobacterium, a *Nostoc* species,[129] exhibit strong destabilizing properties by blocking the hydrolysis of GTP by isolated tubulin.[150] They inhibit the binding of vinblastine to tubulin but not colchicine. Cryptophycins are not a substrate of Pgp, which favors their explicit antitumor activity against multiple cancers.[151] At lower concentrations, cryptophycins are known to interrupt microtubule dynamic instability. The synthesis of cryptophycins *in vitro* has been achieved,[152,153] and its analogs cryptophycin-1, cryptophycin-52, and cryptophycin-24 have been developed. However, cryptophycin-52 failed clinical trials because of very high toxicity.[154] Despite high toxicity, studies to develop better cryptophycin analogs with less toxicity are still being conducted, considering their high activity. Cryptophycins have two kinds of binding sites, a high affinity binding site and a group of low-affinity binding sites.[155,156] It interferes with the vinblastine-binding site on tubulin in a noncompetitive manner.[127,157]

In spite of differences in origin and structure, mostly all above-mentioned drugs bind at the same site or overlap in the “vinca domain.” However, they display distinct phenotypic patterns. For example, vinblastine[126] and vincristine result in nonmicrotubule polymers of tubulin, unlike rhizoxin and maytansine. Rhizoxin does not form tubulin aggregates[135] and maytansine[130] causes the disintegration of vinblastine tubular polymers.[117,127,129]

2. Colchicine-Domain Binders—The colchicine-binding site is located in the center of the tubulin dimer, right at the interface of α- and β-tubulin monomers. It lies in the lumen of the filament, instead of being on the interacting surface. Binding to the colchicine site is followed by a conformational change involving an intradimer bending, where the tubulin monomers undergo twisting around the interface. This change in the conformation allows the inclusion of colchicine-tubulin complex inside the microtubule filament.[158–160]

Colchicine, a tropolone derivative, continues to be involved in the treatment of acute gouty arthritis and is presently used in the treatment of familial mediterranean fever.[161] It was first isolated from the meadow saffron, Colchicum autumnale, as one of the earliest microtubule-targeting agents.[6,161] Colchicine played a primary role in determining properties of microtubules and its tubulin subunits because of its very strong binding to tubulin, which was originally referred to as high affinity colchicine-binding protein. Owing to its severe toxicity to normal tissues at doses required for antitumor effects, neither colchicine nor other related compounds have been successful as chemotherapeutic agents.[6,13,162,163]

a. Colchicine: The structure of colchicine comprises of three hexameric rings, A-B-C (Fig. 13). Colchicine binds to the intradimeric α-β interface of tubulin heterodimers (Fig. 14), contiguous to the GTP-binding domain of the α-tubulin subunit.[86,118,164,165] It is known to bind to the unpolymerized tubulin subunits in a two-step reaction process that begins with the formation of an initial pre-equilibrium complex, which is reversible and bound with low affinity. This is followed by slow conformational changes in tubulin, which finally leads to the formation of a poorly reversible final-state tubulin-colchicine (TC) complex having high activation energy. This conformational change in tubulin heterodimers, followed by the addition of the TC complex in small numbers along with large numbers of soluble tubulin molecules at the ends of microtubules (*purple cylinder in* Fig. 12), is responsible for the suppressed assembly dynamics of microtubule ends despite their competency to grow.[166–169] Colchicine and its analogs, which bind to the colchicine-binding site, suppress microtubule dynamics at lower concentrations and induce depolymerization of microtubules at higher concentrations.

About six α-tubulin and seven β-tubulin isotype classes that have been identified in mammals are expressed in a tissue-specific manner.[170,171] The $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ tubulin dimers have been purified from bovine brain.[172] The B-ring of colchicine is crucial for an inhibitory role in the tubulin binding; the colchicine analog lacking this ring [2-methoxy-5-2′,3′,4′-trimethoxyphenyl]tropone (MTPT) binds to tubulin almost immediately and the complex displays maximum reversibility among the colchicine analogs.[173–176] The tubulin isoforms display significant variability in their drug-binding properties; $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ interact much faster with colchicine and its B-ring-modified analog desaretamidocolchicine than does $\alpha\beta_{III}$.[177,178] Lately, microtubule depolymerizers, such as colchicine and other drugs that bind to the colchicines site, have gained intense interest as potential cancer chemotherapeutic agents (Table III). Among these are combretastatins, 2-ME, their several analogs, and others (Fig. 15).[6,179–181]

b. Combretastatins: Naturally occurring combretastatins are antimitotic agents isolated from the bark of the South African tree, *Combretum caffrum*.[129] Combretastatins A-4[182] (Fig. 15) and combretastatins A-2 are the simplest and most potent members of this class of compounds. Besides acting as an inhibitor of colchicine-binding and tubulin polymerization, they are also known to act as antineoplastic agents by binding to tubulin, inhibiting cell cycle progression at mitosis, and ultimately leading to death by apoptosis.[6,129,183,184] Combretastatins are known to inhibit angiogenesis, thus have been used to selectively target tumor vasculature as an alternative to the conventional chemotherapy.[185] These agents that target tumor vasculature work by disrupting and eliminating the tumor core but are unable to eliminate the outer shell of the tumor, unless they are used in combination with other drugs like paclitaxel.[118,186] Tubulin-dependent GTP hydrolysis has been observed as an effect of high concentrations of combretastatins, sufficient to cause complete inhibition of assembly. Combretastatin-4A exhibits greater assembly inhibition due to a substantial amount of GTP hydrolysis as compared with combretastatin-2A, where minimal hydrolysis has been observed along with simultaneous assembly.[129,187]

c. 2-methoxyestradiol: 2-ME is an endogenous, naturally occurring, mammalian derivative of the primary estrogenic hormone β-estradiol (Fig. 15). It shows antitumor activity and antiangiogenic properties in rapidly growing tumors through a direct apoptotic effect on endothelial cells.[181] However, it binds with low affinity to the estrogen receptors α and β, suggesting that its antiproliferative activity is independent of receptor binding.[181] Unique properties of 2-ME are inhibition of angiogenesis and bioavailability of orally available formulations. Studies indicate that 2-ME inhibits the rate but not the extent of tubulin assembly, as it is a weak competitive inhibitor of colchicine binding to tubulin.[188] It has been observed that micro-tubules show altered forms in the presence of 2-ME, and this

suggests that 2-ME binds to tubulin after it has assembled into a polymer rather than forming a tubulin-2ME complex and participating in the polymerization reaction.[188,189]

d. Podophyllotoxin: Podophyllotoxin and its analogs are extracted from dried roots of *Podophyllum peltatum* and have been employed for the treatment of constipation, gout, tuberculosis, syphilis, liver cirrhosis, rheumatism, as well as cancer.[190] Podophyllotoxin inhibits colchicine binding to the colchicine-binding domain by competitive inhibition and demonstrates a more rapid and reversible binding to tubulin compared with colchicine. However, recent studies suggest that podophyllotoxin and colchicine-binding sites show partial overlap.[129] Unlike any other colchicine-binding compounds, podophyllotoxin shows tubulin-dependent GTP hydrolysis without any interference in GDP/GTP exchange of tubulin. Two semisynthetic, less toxic derivatives of podophyllotoxin have been developed, namely etoposide and teniposide. Etoposide is a more potent topoisomerase II inhibitor and a weak inhibitor of tubulin, whereas teniposide shows less usage in clinical treatment, although both of these compounds are used in combination chemotherapeutics.[129,190]

It was determined from the ternary structure of tubulin that podophyllotoxin binds to the same binding site as that of colchicine, but it adopts a slightly different orientation. This was inferred from studies that showed similar binding of the trimethoxyphenyl ring of colchicine and podophyllotoxin in the same hydrophobic domain of β-tubulin surrounded by the same amino acids. Although colchicine and podophyllotoxin bind to the same pocket on β-tubulin, these molecules do not show complete overlap in binding site and thus show considerable differences in their binding features.

e. Chalcones: Chalcones are another class of colchicine domain-binding agents. Chalcones were first isolated from ferns (Pityrogramma calomelanos)[191] and multiple evergreen plants like *Calythropsis aurea*,[192] *Piper aduncum*,[193] *Fissistigma lanuginosum*[194] from the myrtle, matico, and magnolia families, respectively. Chalcones (Fig. 15), like benzacetophenone groups synthesized to contain a trimethoxyphenyl ring, are known to be potent cytotoxic agents and are 300 times more potent than colchicine in arresting cell division.[195,196] Chalcones like trans-1-(2,5-dimethoxy)-3-[4 (dimethylamino) phenyl]-2-methyl-2-propen-1-one (MDL) are strong antimitotic agents. They display rapid and reversible binding to the colchicine-binding site of β-tubulin at the interface with α-tubulin and cause inhibition of its assembly to microtubules. It has been shown that the binding of chalcones is inhibited by colchicine and podophyllotoxins, as the binding orientations of chalcones (MDL), colchicine, and podophyllotoxin are very similar on the tubulin subunit.[195,197–200] In silico docking models suggest that combretastatin A4 and colchicine bind to β-tubulin in a similar orientation, whereas chalcones and podophyllotoxins share a similar binding mode that is different from colchicine. This indicates that combretastatin A4 and colchicines belong to a different pharmacophore group compared with chalcones and podophyllotoxins.[197]

D. Kinder-Gentler Microtubule-Modulating Drugs: Noscapinoids

Noscapinoids represent an emerging class of novel microtubule-modulating agents that do not display the harsher effects of currently available chemotherapeutic agents because they leave the total polymer mass of tubulin unaffected. The parent molecule noscapine (Fig. 16), a phthalideisoquinoline alkaloid from opium plant, *Papaver somniferum*, has been in medicinal use as an antitussive drug in humans for decades,[201–203] and it is currently in Phase I/II clinical trials for the treatment of multiple myeloma. The identification of noscapine as a tubulin-binding antimitotic agent[10] was based upon a cell-based drug screen to scrutinize naturally existing compounds with structural similarity to other microtubule-binding drugs such as colchicine, podophyllotoxin, and MTC [2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one]. Noscapine binds tubulin stoichiometrically

and stalls the cell cycle of rapidly dividing cancer cells in mitosis.[9,10,203–207] Noscapine attenuates microtubule dynamics by lengthening an attenuated "pause" state in which growth or shortening is not detectable, without affecting the polymer/monomer ratio of tubulin even at high concentrations.[91,204–206,208]

In silico molecular modeling to facilitate rational design of better noscapine analogs has led to the identification and synthesis of a battery of more potent noscapine derivatives.[11,209–219] Essentially, these analogs set themselves apart from other tubulin-active molecules because they leave microtubule arrays intact and instead attenuate microtubule dynamics just enough to engage mitotic checkpoints without perturbing the total polymer mass of tubulin.[215,218] Because of their subtle effects on microtubule dynamics, they do not perturb the transport functions of microtubules in other types of post-mitotic cells such as neurons. Thus, noscapinoids do not cause any hemo-, immuno- or neuronal toxicity based upon their unique mechanism of action.[9,203,209,210,214,215,217] Another unique edge of noscapine over the currently available antimitotics lies in their oral bioavailability.[215,220] Based on their favorable attributes, noscapine and its analogs are collectively referred to as "kinder and gentler" microtubule-modulating agents.[11]

4. FUTURE DIRECTIONS

Although significant progress has been made in developing new antimitotic drugs, much work still remains to be carried out to truly understand their mechanisms of action. Although their effects on a cellular and microtubule-wide level have been characterized, the binding locations of the major drugs—taxane, vinblastine, and colchicine- on tubulin itself have all been determined using crystals made of the protein in a non-microtubule form, as microtubules themselves prove to be a difficult target for crystallization. As a consequence, it becomes inherently difficult to draw firm conclusions from these solved structures revealing the atomic binding states of the drugs within tubulin and the drugs' effects on the larger microtubule structure itself. However, certain trends are being revealed—a fraction of which are summarized in Table IV. Table IV summarizes the drug interactions with tubulin, listing the mode of binding and binding site, the mechanisms of action, and resulting microtubule dynamics, characteristics and structure. Trends in resultant structure and function activity with respect to drug type and binding mode are highlighted. This highlights obvious biological responses with respect to the binding sites. Taxol binds at the internal (lumenal) edge of b-tubulin, it does not affect the conformation of the dimer nor the GTP hydrolysis or exchange rate of the dimer, and thus supports microtubule growth and stability. Vinca drugs bind to the dimer, resulting in conformational changes of the monomers. More specifically, though, vinca drugs bind to the interdimer interface, altering the interaction between neighboring heterodimers, and thereby create a kink in the biological vector such that it no longer aligns with the straight microtubule axis. In addition, vinca drugs bind at a site near the hydrolyzable GTP, thus decreasing the GTP hydrolysis or exchange rate, and decreasing the polymerization of the microtubules. Multiple vinca-site binding drugs alter the structure of the tubulin polymers. Colchicine binds to the free heterodimer, at the intradimer interface, altering the conformation of the heterodimer, opening a wedge between the two monomers, and most significantly changing the biological vector from that of straight protofilaments. This kinked or peeling structure is allowed only at the ends of microtubules. Colchicine binds at a site neighboring the nonhydrolyzable GTP and allows an increase in GTP hydrolysis and exchange rate, thus decreasing polymerization and increasing depolymerization of the microtubules. To further understand the structure-function activity of these drugs, we propose a new perspective in assessing the effect of tubulin-binding drugs, focusing on how their binding modes alter internal vectors within the protein or microtubule structure. Initially, an internal vector connecting the nucleotides of the subunits of tubulin heterodimer (Fig. 17) has been monitored and discussed in this

review. Since only the straight GTP-bound tubulin conformation can be integrated into the microtubule lattice, any drug that supports a straight vector (such as taxol, green vector) will act as a polymerizer and aid microtubule growth, while any drug that interrupts this vector (such as vinblastine or colchicine, blue kinked vector) will instead lead to depolymerization and will restrict microtubule growth. Additional vectors will be studied to monitor other biological changes in structure upon drug binding. Even minute changes in the microtubule structure can translate into significant changes in the biological vector and thus its dynamics and biological function. When viewed on an atomic level, this perspective will hopefully lead to novel insights into the understanding of the actions of antimitotic drugs on the structure of microtubules, which will in turn inform the rational design of new and more effective chemotherapeutic agents.

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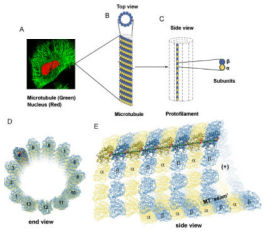

Figure 1.

A depicts a cervical cancer HeLa cell with individual microtubule filaments stained with FITC-labeled anti-α-tubulin antibody and nucleus stained red using the DNA binding dye, DAPI. **B**. Microtubules show a typical arrangement of 13 protofilaments (top view in **C**). Packaging of protofilaments adjacent to each other in a hollow cylinder forms the interacting surface of microtubule polymer. **D** shows an atomic-resolution model of a 13 protofilament microtubule built using coordinates of the refined electron crystallographic structure of αβ tubulin dimer at 3.5 Å,[221] which has been fit within the 8-Å resolution electron microscopy data of Li and Downing.[222]**E** shows the apical side view of this model, with the three-start seam seen at the bottom of the figure. One straight protofilament is shown in detail, with the nucleotides (GDP or GTP of each tubulin monomer) highlighted as space-filling molecular models. A straight axis of growth connecting the nucleotides of the protofilament is in green to show the vector of growth of the microtubule in the plus direction. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

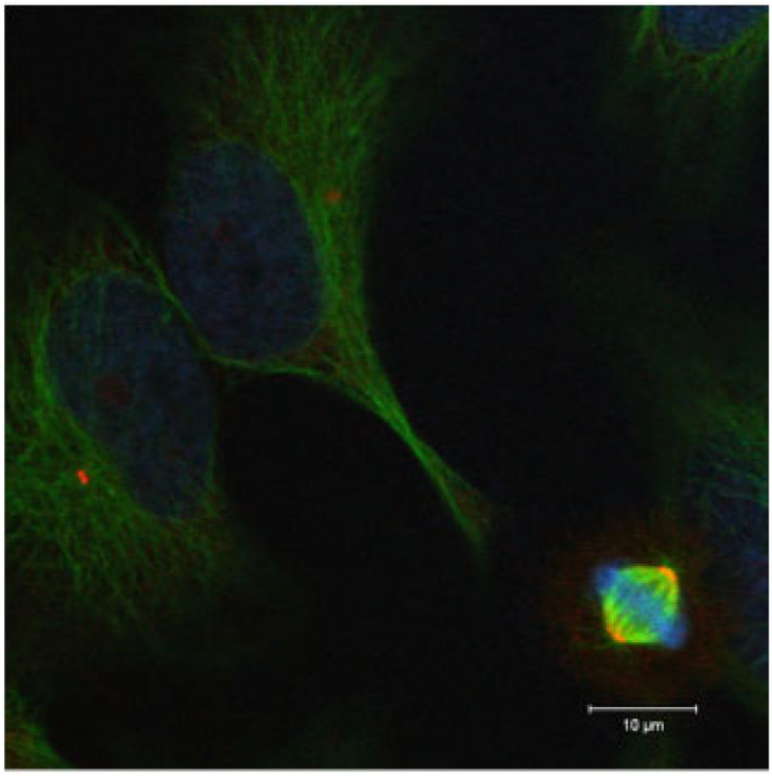

Figure 2.

Confocal immunofluorescence microscopy using HeLa cells showing two interphase cells and a mitotic cell (lower right). Microtubules are stained in green, the chromatin material (DNA) in blue and the arrow denotes the red “centrosome” stained using antibody against γ-tubulin.The centrosome is also referred to as the MTOC responsible for nucleating microtubular arrays. Scale bar = 10 μM. MTOC, microtubule organizing center. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

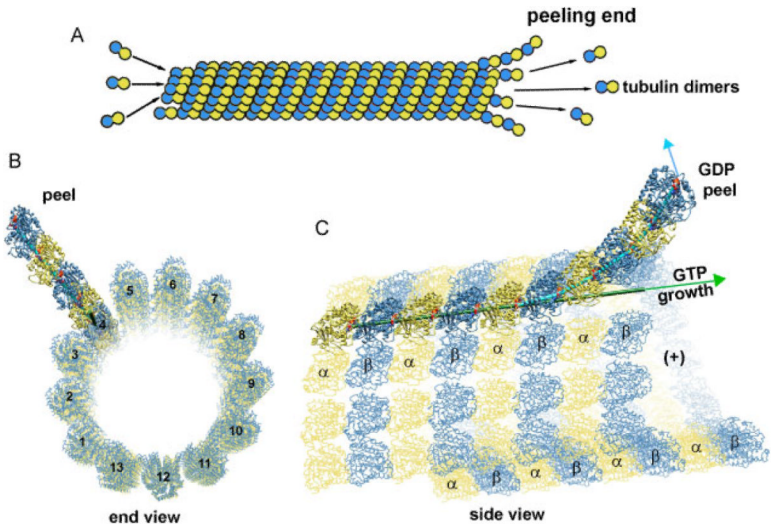

Figure 3.

A shows the treadmilling of a microtubule, whereby tubulin dimers are added to the positive end of the microtubule while peeling off the negative end. **B** shows the end view of atomic-resolution microtubule model (built as described earlier). Proto-filaments peel off of the microtubule or thogonally to the microtubule surface,[25] showing kinks of 12 and 18° at the intra and interdimer interface of tubulin, respectively, forming bending protofilament.[27]**C** shows the same figure from a side apical perspective. A green axis is shown connecting the nucleotides of tubulin subunits in the direction of straight protofilament growth while the cyan vector shows the direction of the protofilament peel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

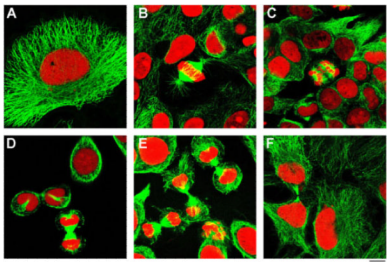

Figure 4.

Exquisite spatiotemporal regulation of microtubule dynamicity dictates cell division, a process by which a parent cell divides into two daughters. Panels show confocal immunomicrographs of HeLa cells with microtubules (green) and DNA (red) displaying hallmarks of a typical cell division process, in particular, interphase (**A**) showing distinct filamentous microtubular arrays; metaphase (**B**) showing a characteristic bipolar mitotic apparatus (featuring mitotic and astral microtubules) with all chromosomes (red) perfectly aligned at the metaphase plate; anaphase (**C**) visualizing the push-pull of spindle microtubules that govern accurate and precise portioning of the genomic material into two daughter cells; telophase (**D**) where the chromosomes have arrived at the poles of their respective spindles. Nuclear envelope reforms before the chromosomes decondense and the spindle fibers begin to disassemble; initiation of mid-body formation (**E**) to accomplish the cytokinetic process of splitting the daughter cells apart by formation of a cleavage furrow that pinches the two cells apart; cytokinetic abscission (**F**) showing the deposition of membrane between the daughter cells and sealing of the cytoplasmic bridge between them to complete their separation. The mid-body is usually inherited by one of the progeny cells. Finally, each daughter cell receives an identical complement of chromosomes. The microtubular arrays appear to be spreading again into interphase arrays and the chromosomes have completely decondensed. Scale bar = 10 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

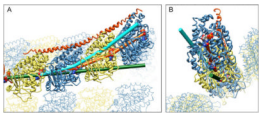

Figure 5.

A shows the structure of RB3-stathmin-like domain (orange helix) complexed with two tubulin subunits as solved by Dorleans et al.[87] superimposed onto the microtubule structure for reference. Orange vector connects sugar ring of the nucleotides in the tubulin subunits within the complex. Green and cyan vectors showing alignment of nucleotides in a straight and peeling protofilament, respectively, are included for reference. **B** shows the same structure from the axial perspective of the growing end of the microtubule. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

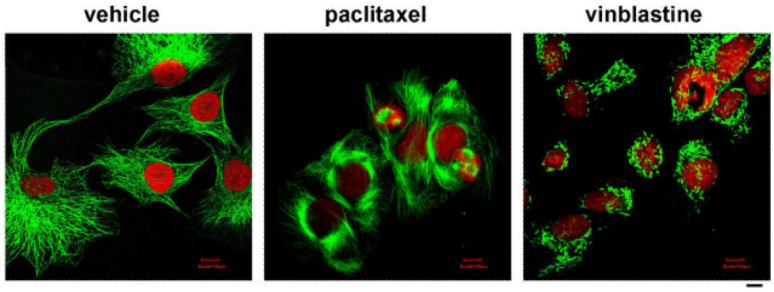

Figure 6.

Confocal micrographs showing dual color staining of microtubules (green) and DNA (red) for control untreated, paclitaxel-treated, and vinblastine-treated MCF-7 cells. Paclitaxel facilitates tubulin assembly resulting in highly resistant tubulin polymers with shorter and highly polymerized microtubules. Vinblastine binds free tubulin heterodimers, resulting in the formation of paracrystals, spirals, and tubules. Scale bar = 10 μM. [Color figure can be viewed inthe online issue, which is available at wileyonlinelibrary.com.]

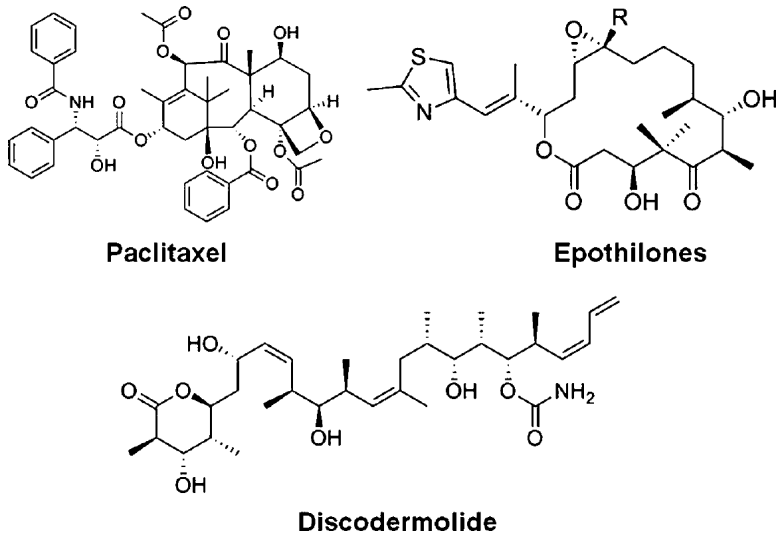

Figure 7.
Major taxane domain-binding drugs: Paclitaxel, Epothilone, and Discodermolide.

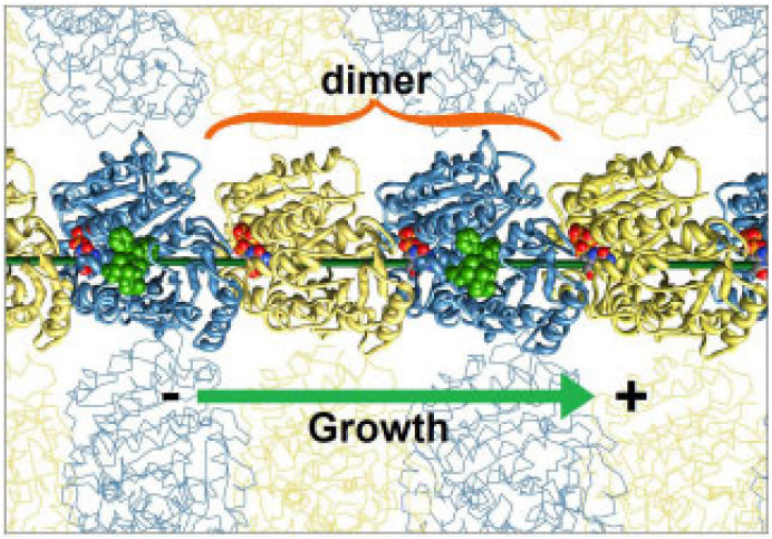

Figure 8.

Lumenal view of paclitaxel (green spacefill) bound to a straight protofilament of a microtubule as solved by Lowe et al.[221] Note that paclitaxel is bound completely in the β-subunit of the dimer. The green vector shows the direction of growth of a straight protofilament, to which the tubulin–paclitaxel vector is aligned. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

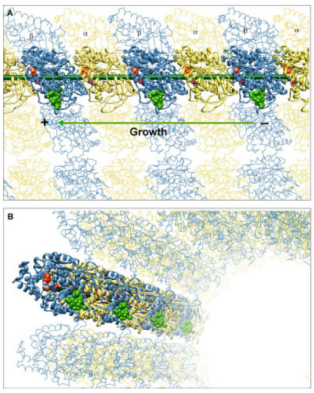

Figure 9.

A shows the lumenal view of taxol bound to a straight protofilament of microtubule as solved by Lowe et al.[221] Taxol supports the growth of microtubules by stabilizing the straight structure of protofilaments as illustrated by the green vector connecting the nucleotides of tubulin subunits along a straight axis. **B** shows the position of taxol from the perspective of the microtubule lumen. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

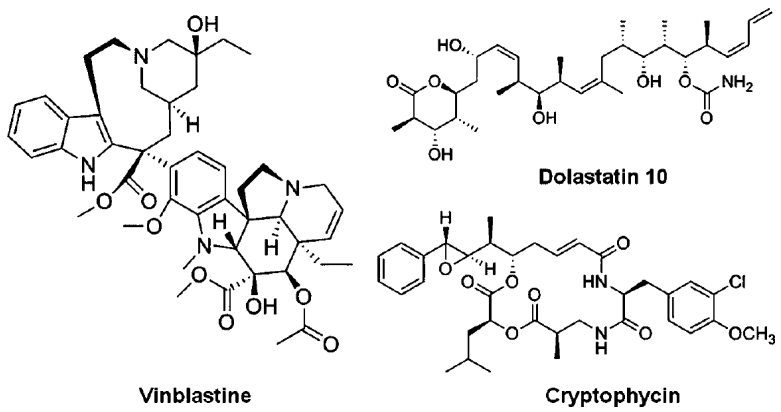

Figure 10.
Major vinca domain-binding drugs: Vinblastine, Dolastatin10, and Cryptophycin.

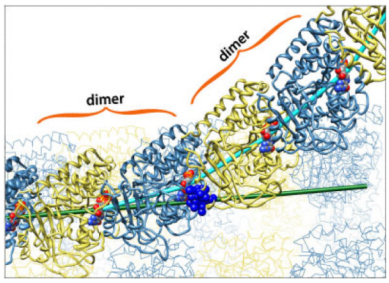

Figure 11.

Depolymerizing drug, vinblastine, is shown as space-filling model in blue, bound to the interdimer interface of the peeling protofilament as determined by Gigant et al.[223] As in previous figures, the green axis shows direction of growth of a straight protofilament while the cyan axis connects nucleotides of a peeling protofilament. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

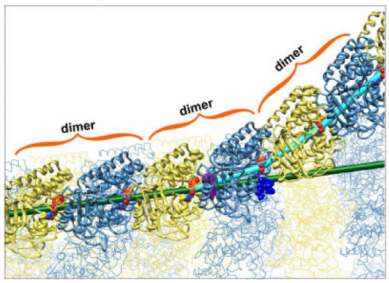

Figure 12.

Depolymerizing drug-binding positions on a peeling protofilament. Purple cylinder shows position of several colchicine-domain binding drug binders (colchicine, ABT751, and T138067) as determined by Dorleans et al.[87] at the intradimer interface of a peeling protofilament. Superimposed on this structure, Vinblastine is shown as space-filling model in blue, bound to the interdimer interface of the peeling protofilament as determined by Gigant et al.[223] As in previous figures, green axis shows direction of growth of a straight protofilament while cyan axis connects nucleotides of a peeling protofilament. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

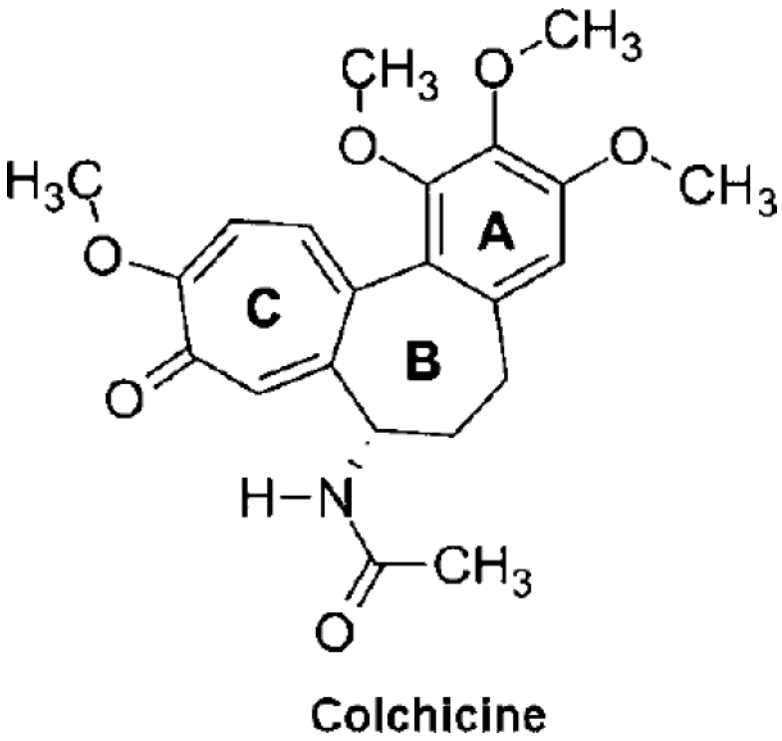

Figure 13.
Structure of colchicine showing the characteristic three hexameric rings (A, B, C).

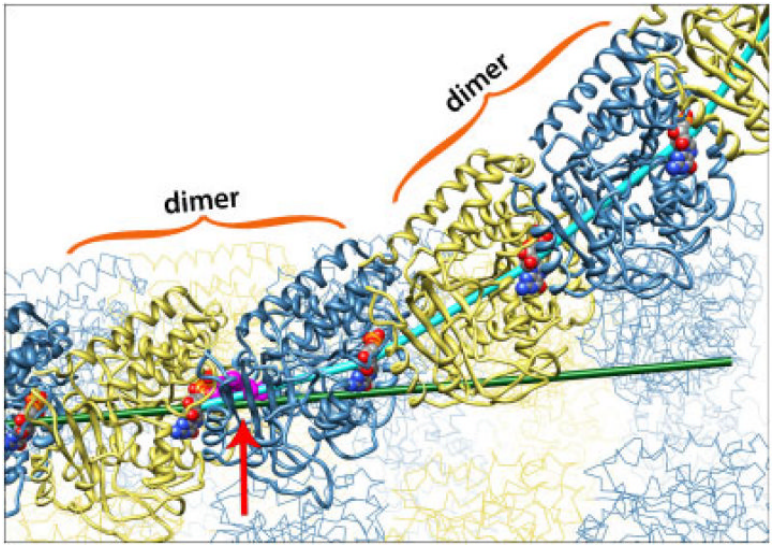

Figure 14.

Depolymerizing drug, Colchicine is shown as a space-filling model (magenta, highlighted with arrow) at the intradimer interface at the start of the peeling protofilament as determined by Ravelli et al.[86] As in previous figures, the green axis shows direction of growth of a straight protofilament while the cyan axis connects nucleotides of a peeling protofilament. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

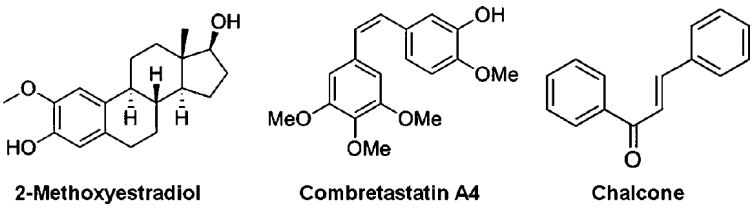

Figure 15.
Major colchicine-domain binding drugs: 2-Methoxyestradiol, Combretastatin A4, and Chalcone.

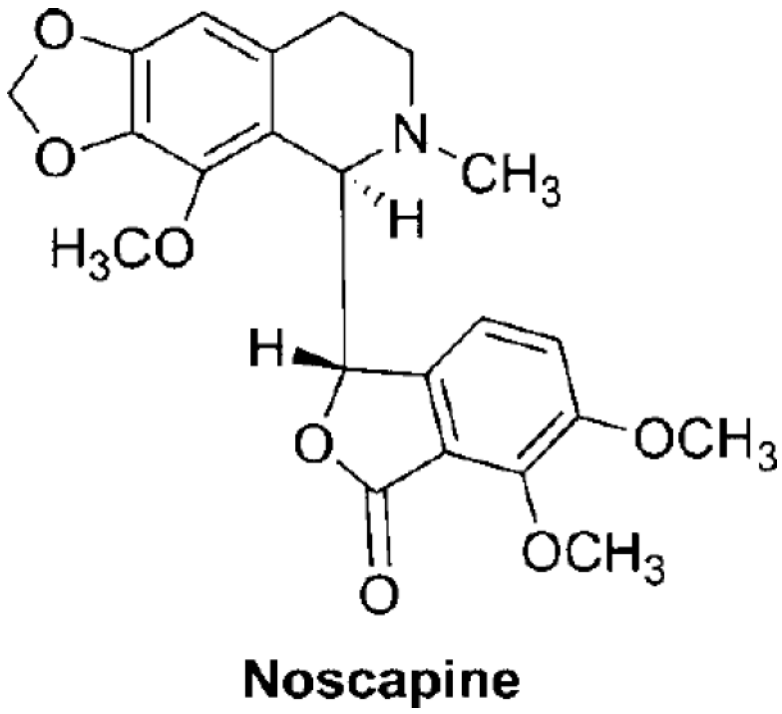

Figure 16.
Molecular structure of noscapine.

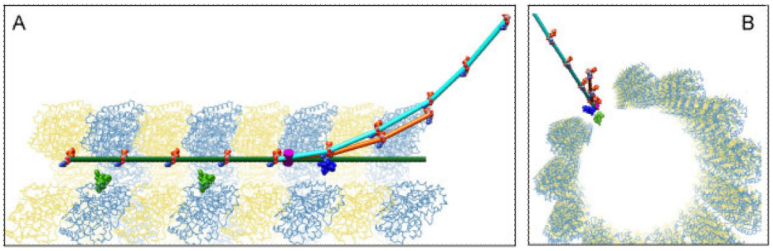

Figure 17.

Apical side view (**A**) and axial view (**B**) of biological vectors of three phases of tubulin polymerization shown in context of microtubule superstructure. All axes are made by connecting the nucleotides of sequential tubulin subunits to one another. Green represents straight protofilaments, which are integrated into microtubule structure and supported by the polymerizing drug taxol (shown as green space-filling molecule model).[221,222] Cyan axis shows directionality of peeling protofilament,[27] a microtubule depolymerizing action supported by the colchicine-domain drugs (binding domain shown as purple cylinder)[86,87] and the vinca-domain drugs (represented by the space-filling model of vinblastine shown in blue).[223] The orange vector showing the directionality of an RB3/stathmin-like protein and two tubulin complexes are shown superimposed on growing end of microtubule as a reference, as this structure has been used to determine the binding domain of several depolymerizing drugs.[86,87,223] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I

Diverse Origin of Taxane Domain-Binding Drugs

Origin	Drug	Source
Plant	Paclitaxel	*Taxus brevifolia* (Yew tree bark)
	Docetaxel	*Taxus baccata* (semi-synthetic)
	10-deacetylbaccatin III	*T. brevifolia* (Yew tree leaves)
Bacterial	Epothilones	*Sporangium cellulosum* (myxobacterium)
	Cyclostreptin	*Streptomyces* sp.
Marine	Discodermolide	*Discoderma dissolute* (marine sponge)
	Dictyostatin	*Spongia* (marine sponge)
	Laulimalide	*Hyattella* sp. and *Fasciospongia rimosa* (marine sponges)
	Peloruside	*Mycale hentscheli* (marine sponge)
Coral	Eleutherobin	*Eleutherobia* sp. (soft coral)
	Sarcodictyins	*Sarcodictyon roseum* (soft coral)

Table II

Diverse Origin of Vinca-Domain Binding Drugs

Origin	Drug	Source
Plant	Vinca alkaloids	
	Vinblastine	*Catharanthus roseus* (Vinca rosea)
	Vincristine	
	Vinorelbine	
	Vinflunine	
	Vindesine	
	Maytansinoids	
	Maytansine	*Maytenus ovatus*
	Ansamitocins	*Nocardia*
Marine	Dolastatin 10	*Dolabella auricularia*
	Dolastatin 15	
	Halichondrin B	*Halichondria okadai Kodata*
	Spongistatins	*Hyrtios altum*
Fungal	Rhizoxin	*Rhizopus chinensis*
	Phomopsin A	*Phomopsis leptostomiformis*
	Ustiloxin	*Ustilaginoidea virens*
Cyanobacterial	Cryptophycins	*Nostoc* sp.

Table III

Diverse Origin of Colchicine-Domain Binding Drugs

Origin	Drug	Source
Plant	Colchicine	*Colchicum autumnale*
	Combretastatins	*Combretum caffrum*
	Podophyllotoxin	*Podophyllum peltatum* (dried roots)
	Flavonols	
	Centauridin	*Polymnia fruticosa*
	Flavanol-2	*Zieridium pseudobtusifolium, Acronychia porteri, Polanisia dodendra, Polanisia tachysperma, Guttierrezia microcephala and Guttierrezia sarothra*
	Rotenone	*Lonchocarpus nicou* and *Derris elliptica*
	Steganacin	*Steganotaenia araliacea* (stems and bark)
	Sanguinarine	*Papaver somniferum* (seeds)
	RPR112378	*Ottelia alimoides*
	RPR115781	
	Chalcone	*Pityrogramma calomelanos* (ferns), and (evergreens) *Calythropsis aurea, Piper aduncum, Fissistigma lanuginosum*
Mammalian	2-methoxyestradiol	Derivative of primary estragenic hormone, β-estradiol
Bacterial	Curacin A	*Lyngbya majuscule* (blue-green cyanobacterium)
Fungal	Griseofulvin	*Penicilliurn griseofulvin*

Table IV

Drug Effects on Tubulin and Microtubules

Drug	Binding site and binding kinetics		Induced conformational change		Microtubule (MT) dynamics						
	Binding site	Binding site: competitive or non-competitive	Change conformation dimer upon binding	Dimer can incorporate in MT	MT nucleation, polymerization, and stability			GTP hyd/exch	MT depolymerization	Altered MT structure aggregates	References
					N	P	S				
Taxane site											95
Paclitaxel (PTX)			+	+	+	+	+		−	Bundles	95,96,98–102
Docetaxel			+		+	+	+				
Epothilone	~PTX	Comp			+	+	+			Bundles, ab spindle	105 – 107
Discodermolide	PTX	Comp			+	+	+			Short, bundles, ab spindle	115,116
Dictyostatin	PTX	Comp				+				Bundles	115,116
Laulimalide	LAU					+	+				112 – 114
Peloruside	LAU	Comp				+	+	+			113,114
Vinca site											
Vinblastine (VLB)			+	−	+	−	(+)	−	+	Spirals	2,8,117–120,121–124
Vincristine								−	+		118
Maytansinoid	~VLB/MAY	Comp	+			−	(−)	−	+	No spiral, no aggregates	128,130–133
Rhizoxin	~VLB/MAY	Noncomp					−	− −			127,134,135,137
Dolastatin 10	VLB/DOL	Noncomp	+		−		−	−	+	+	127,129,143–146
Dolastatin15	VLB/DOL	Noncomp						+		+	127,129,147
Spongistatin	~VLB/DOL	Noncomp	+		−		−	−	+	-Spindle	129,137,144,148,149
Halichondrin B	VLB	Noncomp	+	Ends		−		−	+/−	No aggregates	129,137,140
Phomopsin	VLB		+			−		−		Spiral, aggregates	129,137,139,143
Cryptophycin (peptide) *Colchicine site*	VLB	Noncomp	+			−		−	+	Rings, spirals	127,129,150,151,157
Colchicine site											
Colchicine (CN2)		Hi low aff	+	Ends		−		+	+	Shortened	86,118,158–161,164–169

Drug	Binding site and binding kinetics		Induced conformational change		Microtubule (MT) dynamics				
	Binding site	Binding site: competitive or non-competitive	Change conformation dimer upon binding	Dimer can incorporate in MT	MT nucleation, polymerization, and stability	GTP hyd/exch	MT depolymerization	Altered MT structure aggregates	References
Combretastatin	CN2	Comp			−				129,182–185,187
Podophyllotoxin	~CN2	Comp			−	+/−		ab spindle	129,190
Chalcone	CN2	Comp			−		+		195 – 200
2ME	~CN2	Comp	−	+	−		−	ab spindle	181,188,189
Noscapine			+		*		−	ab spindle	10,204,210,211,213

Binding Site: ~PTX, near paclitaxel site, not completely overlapping. Change conformation of dimer upon binding: 1, conformation does change; −, conformation does not change. Dimer can incorporated in Microtubule: 1, dimer can incorporate; −, dimer cannot incorporate; Ends, dimer can incorporate at the ends only, disallows continued microtubule growth. Microtubule Nucleation, Polymerization, and Stability: 1, increase of each; −, decrease of each; *, alters dynamics of MT, does not significantly promote or inhibit polymerization. GTP hydrolysis/exchange: −, inhibits GTP hydrolysis or GDP/GTP exchange; 1, increase GDP–GTP exchange. Microtubule depolymerization: 1, increases depolymerization; −, does not cause depolymerization. Altered Microtubule Structure: 1, altered microtubule structure; ab spindles, aberrant spindle structure, − spindle, reduces spindle structures. MT, mirotubule.