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Taxol and other microtubule-interactive agents

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Introduction

Among anticancer agents, drugs targeting tubulin or microtubules are among the most, if not the most, effective class of agents. The list of compounds which bind to tubulin or the microtubules is large and continues to expand. The overwhelming majority are natural products, and their chemical structures are remarkably diverse. Clinical drug development saw the introduction of the vinca alkaloids in the 1950s. Initially represented by vincristine (oncovin), this class was eventually expanded to include vinblastine (velban) and vinorelbine (navelbine). Although the vinca alkaloids were shown to be useful in a wide range of malignancies, including both leukemias and solid tumors, interest in developing new agents targeting microtubules declined, until the introduction of paclitaxel (taxol) into clinical oncology. Arguably the most effective agent introduced since cisplatin, paclitaxel's remarkable activity in a broad range of malignancies ignited intense interest in tubulin and microtubules as chemotherapeutic targets. This review will attempt to cover a broad range of agents targeting tubulin, which have collectively been referred to as microtubule-targeting agents (MTAs) or microtubule-interactive agents (MIAs). We will attempt to provide a brief, but hopefully helpful, background and then devote the rest of the chapter to describing and contrasting these compounds, placing special emphasis on paclitaxel. A large number of excellent recent reviews covering the different aspects included herein are available [1,2,3•,4•]. The reader is encouraged to consult these for more depth.

Tubulin and microtubules: A brief primer

Microtubules are cytoskeletal protein polymers critical for cell growth and division, motility and signaling. The basic subunit of the microtubule is 'tubulin', a heterodimer composed of two related polypeptides, α- and β-tubulin. Microtubules are not simple equilibrium polymers, they exhibit complex polymerization dynamics, important for many microtubule-dependent processes in cells.

Microtubules are polymers built by the self-association of α/β-tubulin dimers. The polymerization process involves two types of contacts between tubulin subunits: head-to-tail binding of dimers results in protofilaments that run along the length of the microtubule, and lateral interactions between parallel protofilaments complete the microtubule wall. Addition of tubulin monomers can occur at either end of a microtubule. The addition of tubulin monomers is reversible and noncovalent, but requires energy. GTP is bound exchangeably to tubulin monomers, and is irreversibly hydrolyzed as a tubulin monomer is added to a microtubule. Tubulin monomers can be removed from either end of a microtubule, a process which does not require energy. This ability to add and remove monomers from either end is very important, because it renders microtubules intrinsically dynamic.

Microtubule dynamics can be manifested as 'treadmilling' and 'dynamic instability'. 'Treadmilling' refers to the net growth of microtubules at one end and the net shortening at the opposite end. The net effect of 'treadmilling' is to effectively move individual tubulin subunits along the length of a microtubule. Thus, for example, a tubulin subunit located in the center of a microtubule will effectively move to one end of the microtubule (the shortening end) as one end grows and the other shortens. 'Dynamic instability' refers to the stochastic switching between an extended phase of growth and an extended shortening phase at either end of a microtubule.

Because of their dynamic nature, microtubules are constantly exchanging their tubulin subunits with the pool of soluble tubulin. In interphase, exchange of the 'interphase microtubule network' occurs with a half-life of ~3 min to several hours. By contrast, the microtubules which comprise the mitotic spindle are 10 to 100-fold more dynamic. They exchange their tubulin with half-times of about 15 s [5-8]. These differences in dynamics reflect the differing functions. The dynamics of spindle microtubules must be rapid so as to build the spindle and move chromosomes accurately in a short time. Dynamic instability is required to build the spindle and establish attachments to kinetochores. Once attached, treadmilling provides tension and allows for movement of chromosomes. In contrast, the less dynamic interphase microtubule network provides a more stable framework on which intracellular trafficking can occur.

In interphase cells, microtubules are organized into a single array, with their minus ends associated with the microtubule organizing center (MTOC), located near the nucleus in the center of the cell. Their plus ends radiate towards the cell periphery near the plasma membrane, giving the cell a defined polarity. The microtubule-based motor proteins kinesins and dyneins utilize this polarity, by moving to the plus and minus ends of microtubules, respectively. Thus, microtubules provide the cell with a system for the directional flow of information. In addition, because of their abundance, microtubules provide a large surface area that can be used for protein-protein interactions.

In addition to the tubulin monomers, other proteins, most notably microtubule-associated proteins or MAPs, are essential for the formation of microtubules.

Agents targeting tubulin and microtubules

Agents which target tubulin and microtubules interfere with the function of the mitotic spindle, and block cells at the metaphase/anaphase junction [9,10••]. For convenience, they are often divided into two groups: those that destabilize microtubules (see Figure 1) and those that stabilize microtubules (see Figure 2). Destabilizing agents include: (1) the vinca alkaloids, (vincristine, vinblastine and vinorelbine); (2) colchicine; (3) nocodazole; (4) cryptophycins, and the (5) hemiasterlins. Stabilizing agents include: (1) the taxanes (paclitaxel and docetaxel); (2) the epothilones (epothilone A and B); (3) discodermolide; (4) the eleutherobins/ sarcodictyins, and (5) laulimalide.

The division into these two major classes is based on their effects on microtubule polymerization and the mass of microtubule polymers. At high drug concentrations, destabilizing agents decrease the mass of cellular microtubules and inhibit tubulin polymerization, while stabilizing agents increase the polymer mass, stabilize microtubules and induce formation of microtubule bundles in cells. However, it is now recognized that at low concentrations, the effects of destabilizing and stabilizing agents are very similar. Thus, at low drug concentrations, destabilizing agents stabilize microtubules and suppress microtubule dynamics with little or no accompanying microtubule depolymerization; while stabilizing agents suppress microtubule dynamics without increase in the polymer mass or formation of microtubule bundles (for review see [3]).

Destabilizing agents

Vinca alkaloids

At nanomolar concentrations, the vinca alkaloids suppress both dynamic instability and treadmilling, without affecting the microtubule polymer mass [11,12•,13]. This suppression of microtubule dynamics occurs preferentially at plus ends, while the rates at minus ends are unchanged. Thus, binding of approximately one molecule of vinblastine per microtubule results in 50% inhibition of treadmilling with a negligible effect on microtubule polymer mass.

Vinblastine binds preferentially to tubulin monomers. Binding to tubulin monomers inhibits microtubule assembly, indirectly resulting in depolymerization and reduction in the microtubule polymer mass. Vinblastine can also bind directly to microtubules, without first binding a soluble tubulin monomer. Binding to microtubules can occur at either high affinity sites located near the plus ends of microtubules, or at low affinity sites located throughout the microtubule surface. Binding to the high affinity sites is likely responsible for the stabilizing effect on microtubule dynamics, seen at low drug concentrations. Binding to the low affinity sites likely results in depolymerization of microtubules [14-16]. Both of these effects may be important for cell proliferation, and can manifest differently. Thus the block or slowing of mitosis at the metaphase/anaphase transition seen after the addition of vinblastine may result from either: (a) microtubule depolymerization at high drug concentrations or (b) suppression of microtubule dynamics. In the former, vinblastine binding to tubulin monomers results in inhibition of microtubule assembly, so that following the dissolution of the nuclear envelope the mitotic spindle is not formed. In the latter, one sees morphologic changes characteristic of a blocked spindle [9,13].

Colchicine

As with the vinca alkaloids, colchicine's effect is dependent on drug concentration. At low concentrations colchicine also inhibits tubulin dynamics without affecting microtubule polymer mass. At higher concentrations, it inhibits microtubule polymerization, so that the mitotic spindle dissociates or is not formed. However, unlike the vinca alkaloids, colchicine cannot bind directly to microtubule ends, or does so only with very poor affinity. It does, however, bind soluble tubulin, and is in turn incorporated at microtubule ends, with a resultant attenuation or inhibition of microtubule polymer elongation [17,18,19]. Colchicine is not widely used for cellular studies because its binding to tubulin is slow and not easily reversible.

Nocodazole

Nocodazole binds in the colchicine-binding domain of tubulin and shares with colchicine the property of increasing the GTPase activity of tubulin in the absence of polymerization (for review see [1]). This property is distinct from the drugs that bind at the vinca-binding domain of tubulin that in general suppress GTPase activity in parallel with inhibition of microtubule polymerization. Nocodazole has largely replaced colchicine and colcemid in studies of microtubules, in part because of colchicine's poor reversibility. From the point of view of chemotherapeutics, however, this property may prove valuable. It will be interesting to follow the development of a chemotherapeutic agent targeting the 'colchicine binding site'.

Cryptophycins

Cryptophycins are another recently-discovered class of remarkably potent natural products that are active against human solid tumors in murine xenografts. Cryptophycins bind tightly to tubulin, and they inhibit tubulin polymerization at high drug concentrations, while they slow or block mitosis at picomolar concentrations [20]. Cryptophycin 1, in particular, binds at or near the vinca binding site on tubulin, suppressing microtubule dynamics more potently than vinblastine or paclitaxel without inducing net microtubule depolymerization [21,22].

Hemiasterlins

Hemiasterlin A and hemiasterlin B are newly isolated cytotoxic tripeptides from the sponge genus *Auletta* with potential as antitumor drugs. At the nanomolar concentrations at which they are cytotoxic, the peptides induce arrest in mitotic metaphase. Hemiasterlin A produces abnormal mitotic spindles like those produced by paclitaxel, nocodazole and vinblastine at low concentrations. At high concentrations hemiasterlin A causes microtubule depolymerization [23]. Hemiasterlin inhibits non-competitively the binding of vinblastine to tubulin, stabilizes the colchicine binding activity of tubulin, and induces the formation of stable tubulin oligomers even at low drug concentrations [24].

Figure 1. Microtubule-destabilizing agents.

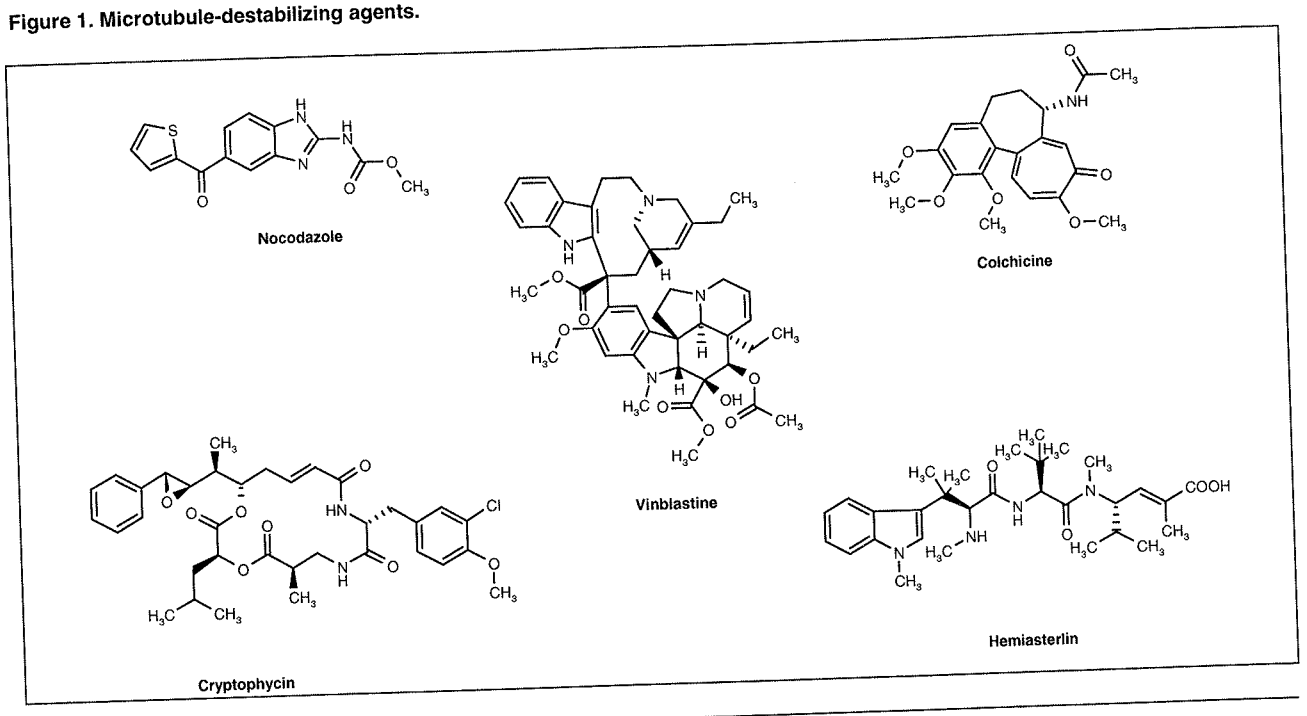
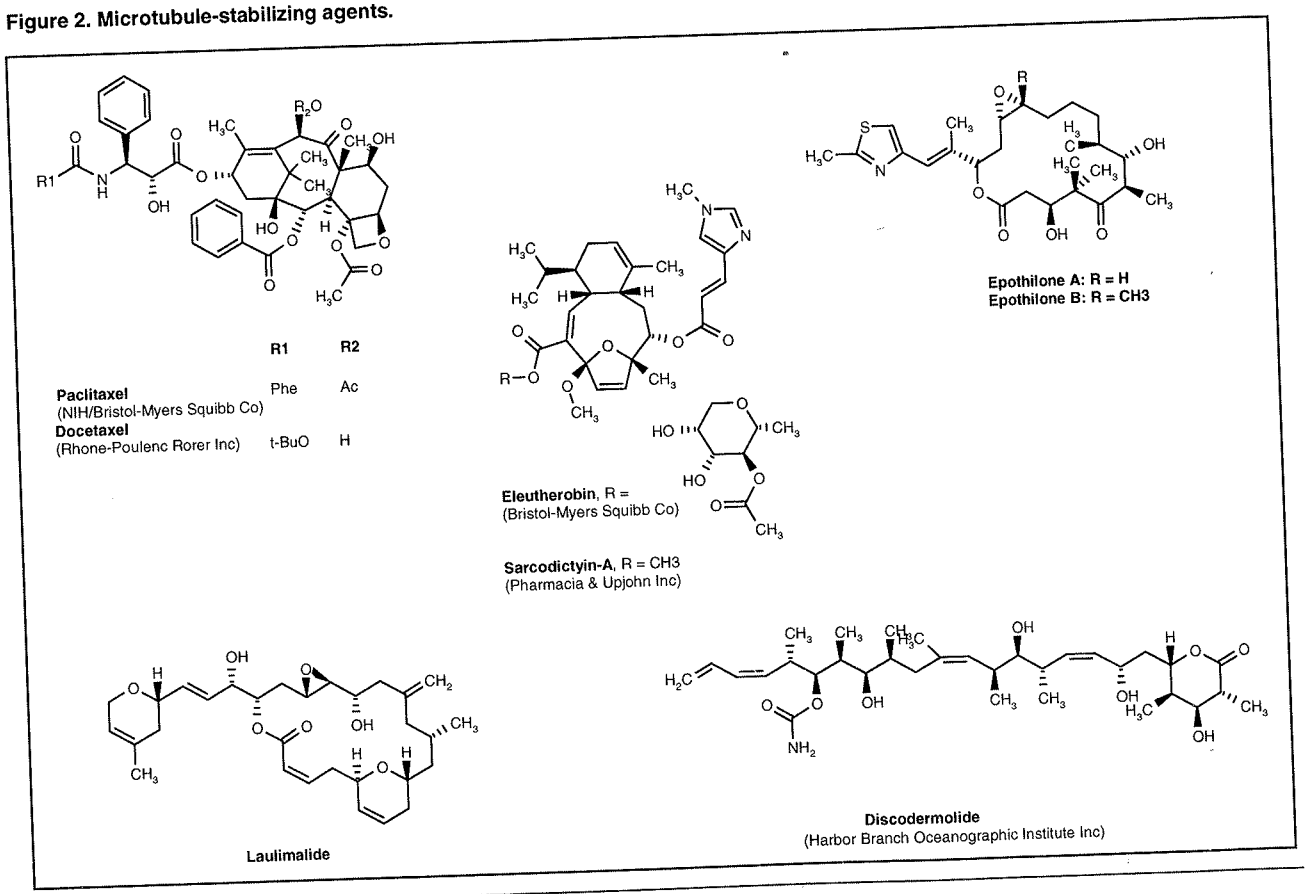


Figure 2. Microtubule-stabilizing agents.



Stabilizing agents

Taxanes

Paclitaxel (taxol) is the prototype of microtubule-stabilizing agents. Paclitaxels' antitumor activity *in vitro* was first described in 1971, following its isolation from extracts of the bark of the pacific yew tree *Taxus brevifolia* [25•]. Paclitaxel, however, despite its novel structure and antitumor activity, generated only modest enthusiasm for clinical development, until the elucidation of its unique mechanism of action as a microtubule-stabilizer [26••,27]. In cells, paclitaxel binds directly to microtubules. *In vitro*, paclitaxel binds reversibly to microtubules with high affinity [28]. Paclitaxel can also bind soluble tubulin subunits, but with a markedly reduced affinity [28,29,30]. Direct photoaffinity labeling has shown preferential paclitaxel binding to the β-tubulin subunit of microtubules [31••]. The paclitaxel binding site on microtubules is specific, and distinct from the binding site of other drugs as evidenced by the fact that paclitaxel binding does not inhibit the binding of other MT-destabilizing drugs, including vinblastine, colchicine, or podophyllotoxin [32,33].

Like other microtubule-stabilizing agents, paclitaxel enhances microtubule polymerization *in vitro*, promoting both the nucleation and elongation phases of polymerization and reducing the soluble tubulin concentration at steady state [32, 33,34]. The microtubules formed in the presence of paclitaxel are very stable [32,33,34]. Paclitaxel effects on microtubule dynamics vary with the concentration of paclitaxel. Low paclitaxel concentrations can significantly reduce the rate and extent of microtubule shortening at plus ends, without significantly affecting microtubule minus ends [35] [36]. Similar to low concentrations of depolymerizing agents, these concentrations act principally by suppressing microtubule dynamics, leading to arrest at the metaphase/anaphase transition in a large majority of cells. All of these changes occur without a significant increase in the microtubule polymer mass, consistent with a predominant effect on microtubule dynamics. At intermediate concentrations of paclitaxel, the rates of growing and shortening are suppressed equally, resulting in a 'pause', while at high concentrations, the microtubule polymer mass increases as tubulin is recruited into microtubules. At these high concentrations, paclitaxel treatment leads to the appearance of large and dense asters containing prominent bundles of stabilized microtubules. It is interesting that while at high concentrations paclitaxel binding to microtubules saturates at a ratio of 1 mole of paclitaxel per mole of tubulin, the 'occupancy' rate at lower concentrations is substantially less. Thus, at low paclitaxel concentrations (10 to 100 nanomolar), only one paclitaxel molecule is bound every 270 tubulin dimers. Since mitotic arrest is observed under these conditions, it is assumed that microtubule shortening occurs until a bound paclitaxel molecule is reached, at which point, shortening stops.

While the principal cellular target for paclitaxel is the tubulin/microtubule system, an increasing number of non-microtubule effects have also been reported. Numerous studies have demonstrated activation of a variety of signal transduction pathways following the addition of paclitaxel. However, these effects must be interpreted with caution. Some effects may be cell line-specific; while others can be demonstrated only at high concentrations of paclitaxel. The latter often exceed the concentrations that can be achieved clinically, and are thus of uncertain significance [37•]. What is less clear is the extent to which these effects are independent of microtubule binding and the resultant mitotic arrest. Putting aside these considerations, one can summarize the myriad of effects on signal transduction pathways observed following paclitaxel treatment as follows: (1) Paclitaxel activates c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) through both Ras and apoptosis signal regulating kinase (ASK1) pathways. Both dominant negative Ras and dominant negative Ask1 prevent JNK activation. The activation requires microtubule binding. JNK activation does not occur in cell lines harboring mutant tubulins which are insensitive to paclitaxel [38]. Others have suggested that paclitaxel-induced gene expression and cell death are both mediated by the activation of JNK/SAPK [39]. (2) Paclitaxel has been shown to phosphorylate Shc, and in paclitaxel-treated murine macrophages formation of Shc-/Grb2 complexes have been demonstrated [40]. (3) Paclitaxel treatment has been shown to activate Raf-1 [38,40-43]. (4) Paclitaxel activates extracellular signal regulated kinase (ERK) [38]. (5) Paclitaxel treatment results in activation of p38 in human breast cancer cells [44].

Numerous studies have also reported the effect of paclitaxel on proteins involved in apoptosis [41••,42,45,46,47-49••,50•]. Together with early evidence indicating that Bcl-2 could be inactivated by phosphorylation, the observation that Bcl-2 was phosphorylated following paclitaxel treatment provided a potential explanation for how paclitaxel could bring about apoptosis. Serine 70 in Bcl-2 has been identified as one of the critical residues; however, the requirement of Bcl-2 phosphorylation for paclitaxel cytotoxicity is still in dispute [48•]. Studies are underway to determine the kinase(s) responsible for these effects. It must be stressed, however, that this effect, as well as other paclitaxel effects may be simply a reflection of paclitaxel-induced mitotic arrest. Ample evidence exist to indicate that serine protein phosphorylation of numerous proteins occurs during meiosis and mitosis. Consequently, arresting cells at this stage in the cell cycle may result in widespread serine protein phosphorylation [37•].

Paclitaxel can cause both mitotic arrest and apoptotic cell death [51]. Paclitaxel can also cause cell death independent of apoptosis. Paclitaxel-induced cell death may be secondary to its interaction with the tubulin/microtubule system, or may occur via pathways that are independent of cell cycle arrest. The bulk of the evidence suggests that cell death following paclitaxel is a consequence of mitotic arrest. However, at least in some cells, cell death can occur independent of mitotic arrest. In A549 cells, for example, paclitaxel-mediated cell death has been proposed to result from two different mechanisms. At paclitaxel concentrations below 9 nM, apoptosis occurs without a G_2/M block, while at higher concentrations, cell death follows mitotic arrest [43].

Because many of the paclitaxel effects reported to date are likely to be a consequence of paclitaxel's interaction with microtubules and the resultant mitotic arrest, it is likely that

these effects will be observed with other MIAs. However, one effect that appears to be paclitaxel-specific is its ability to mimic LPS activity [52]. Epothilone B, for example, which like paclitaxel stabilizes microtubules, does not show paclitaxel-like endotoxin activity [53]. Paclitaxel has also been shown to activate 'early-response genes', including transcription factors and enzymes that modulate inflammation and apoptosis [54]. Moreover, it has been concluded that these effects are independent of microtubule stabilization since a high concentration of paclitaxel (10 μM) was used, which is much higher than the concentration needed for microtubule stabilization. One must interpret the significance of these observations cautiously, since both the LPS-effect and the 'early-response gene' activation have been observed only at very high paclitaxel concentrations, which are not clinically relevant.

Other stabilizing agents

The clinical successes of the taxanes, paclitaxel and the semisynthetic derivative docetaxel (taxotere), has stimulated a worldwide search for new agents with a similar mode of action but improved characteristics. The low aqueous solubility of paclitaxel and the development of clinical drug resistance, mediated by both the overexpression of P-glycoprotein (Pgp) and the presence of β-tubulin mutations [55••], are factors that could potentially hamper its clinical applicability. This search has resulted in the identification of four non-taxane chemical classes of natural products: the soil bacteria-derived epothilones A and B [56•], the marine sponge-derived discodermolide [57•,58], the coral-derived eleutherobins/sarcodictyins [59•,60] and the marine sponge-derived laulimalide and isolaulimalide [61•].

These four classes of natural products stabilize microtubules and three out of the four classes have been shown to competitively inhibit the binding of paclitaxel to tubulin polymers (for laulimalide such data do not yet exist), indicating overlap of binding sites [56•,57•,58,62,63•].

Epothilones

The epothilones A and B were first discovered in 1993 (for review see [64•]). These compounds were isolated from the cellulose-degrading myxobacterium *Sorangium cellulosum* (Myxococcales) strain So ce90, first found in soil collected from the banks of the Zambesi river in South Africa, and were initially found to exhibit a narrow antifungal spectrum against the fungus *Mucor hemialis* only [65]. Based on this finding, the epothilones were first tested as potential antifungal and pesticide agents, but field experiments proved the epothilones to be too phytotoxic. In the mean time, others had independently isolated epothilones A and B and furthermore they discovered that these compounds kill tumor cells through a mechanism of action similar to that of paclitaxel, namely through induction of tubulin polymerization and stabilization of microtubules [56•]. In the latter report, the biological effects of the epothilones were compared to those of paclitaxel, and epothilones were shown to be equipotent and exhibit kinetics similar to paclitaxel in inducing tubulin polymerization *in vitro* and in stabilizing microtubules in cultured cells. In addition, epothilones were shown to competitively inhibit [^{3}H]-paclitaxel binding to microtubules, with a similar 50% inhibitory concentration and slope to unlabeled paclitaxel [56•,58]. Epothilones also cause cell cycle arrest at the G_2/M transition, leading to cytotoxicity, similar to paclitaxel.

In contrast to paclitaxel, however, epothilones retain a much greater toxicity against P-glycoprotein-expressing multiple drug resistant (MDR) cells [56•]. This characteristic suggests that epothilones as antineoplastic agents could provide an important advantage over paclitaxel. In addition, *in vivo* experiments in nude mice bearing human tumors showed that although epothilones performed similarly to paclitaxel in sensitive tumor xenografts (CCRF-CEM human lymphoblastic T cell leukemia, MX-1 human mammary and HT-29 colon tumor), their effects were clearly superior against MDR tumors (CCRF-CEM/paclitaxel human lymphoblastic T cell leukemia and MCF-7/ADR human mammary adenocarcinoma) [66•,67,68,69].

Epothilones were also found to retain activity against a panel of human ovarian carcinoma cell lines, resistant to paclitaxel due to acquired β-tubulin mutations which impair paclitaxel/tubulin interaction [58,70••,71••]. This result strongly suggests that although epothilones competitively inhibit paclitaxel binding to microtubules, the mutations identified in these paclitaxel-resistant cell lines, at residues $\beta270^{Phe\rightarrow Val}$ and $\beta364^{Ala\rightarrow Thr}$, are not as important for epothilones' binding as they are for paclitaxel binding. Indeed, the recently identified atomic model of α/β-tubulin with bound paclitaxel [72•] and its docking into a 20 Å map of the microtubule [73••] shows that $\beta270^{Phe}$ is located in the paclitaxel binding pocket and its side chain is stacked against the C3' phenyl group of paclitaxel. The crystal structure of epothilones bound to tubulin, however, has not yet been elucidated.

A recent report provides additional insight into the nature of epothilones' binding onto tubulin, by identifying β-tubulin mutations which confer resistance to epothilones and impair their binding to tubulin [74••]. In addition, in the same report, a common pharmacophore shared between taxanes and epothilones is identified, despite their apparent structural dissimilarity, and modeling of epothilone binding onto tubulin is achieved [74••].

Soon after the recognition of the biological importance of the epothilones, the total synthesis of the natural epothilones was achieved [67,71••]. This synthesis paved the way for the chemical synthesis of a large number of designed epothilones for chemical biology studies. Thus, the design and chemical synthesis of epothilone libraries allowed the biological evaluation of a large number of epothilone analogs providing invaluable information regarding the structure-activity relationships of these promising compounds.

In addition to the chemical synthesis of the epothilones, a novel method of obtaining sufficient amounts of natural epothilones has been recently reported [75,76•]. In these reports, the gene cluster responsible for epothilone biosynthesis in *Sorangium cellulosum* has been cloned and sequenced and its heterologous expression in a surrogate microbial host (*Streptomyces coelicolor*, CH999) provided a plentiful supply of epothilones. The availability of the

cloned epothilone gene cluster and a plasmid-borne expression system should facilitate the generation, by combinatorial biosynthesis approaches, of designer epothilones with superior characteristics.

Discodermolide

Discodermolide is a potent antitumor compound derived from the Caribbean deep-water sponge *Discodermia dissoluta*. Discodermolide was isolated and characterized in 1990 [77] and was originally described to have immunosuppressive activity [78,79]. The microtubule-stabilizing activity of discodermolide was discovered through studies using computational methods to identify novel compounds with structural analogy to colchicine site inhibitors [57•]. Discodermolide is more potent than paclitaxel and competitively binds at the same site on tubulin. Discodermolide retains activity against paclitaxel-resistant cells that either overexpress Pgp or harbor β-tubulin mutations [62,70••]. Interestingly, although discodermolide competitively inhibits paclitaxel binding on tubulin, a recent report demonstrates that paclitaxel and discodermolide represent a synergistic drug combination in four different human cancer cell lines [80•] (for comments see [81]). This finding, together with observations that discodermolide retains activity against paclitaxel- and epothilone-resistant cell lines [70••,74••], harboring distinct β-tubulin mutations that impair paclitaxel- or epothilone-interaction with tubulin, suggest that the mechanisms of action of the two drugs, namely paxlitaxel and discodermolide, are distinguishable.

Eleutherobins/Sarcodictyins

Eleutherobins and Sarcodictyins are marine natural products isolated from the corals *Eleutherobia aurea* and *Sarcodictyon roseum*, respectively [82,83]. Eleutherobins and sarcodictyins, like paclitaxel, stabilize microtubules, induce microtubule bundle formation in cells and arrest cells at mitosis [59•,84]. Pgp-expressing resistant cell lines are cross-resistant to eleutherobins and sarcodictyins. Similarly, eleutherobins retain cross-resistance against epothilone-resistant cells due to acquired β-tubulin mutations, while sarcodictyins show increased activity in these resistant cells compared to sensitive parental cells [74••]. This finding suggests once more, that microtubule-active agents with similar mechanisms of action possess slightly different properties, which could allow their effective combination even in cases where drug-resistance emerges.

Laulimalide

Laulimalide and isolaulimalide are marine natural products isolated from the sponge *Cacospongia mycofijiensis*, collected in Indonesia, Vanuatu and Okinawa. Both compounds are 18-membered macrocyclic lactones, with isolaulimalide being a laulimalide rearrangement product. Both compounds were initially isolated on the basis of their toxicity, but their mechanism of action was not elucidated [85,86]. A number of international research centers are currently investigating these agents. A recent report showed that laulimalide and isolaulimalide are paclitaxel-like stabilizers of microtubules that cause alterations in both interphase and spindle microtubules [61•]. In addition, they are poor substrates of P-glycoprotein. Among the five groups of known MIAs with a paclitaxel-like mechanism of action, laulimalide most closely resembles the epothilones. This similarity seems to translate to similar activities.

Resistance to drugs which target tubulin and microtubules

Several mechanisms of resistance to drugs which target microtubules have been proposed, including (1) overexpression of MDR-1/P-glycoprotein; (2) altered expression of β-tubulin isotypes; (3) intrinsic or acquired mutations in β-tubulin, and (4) expression of novel genes.

Increased expression of MDR-1/P-glycoprotein as a mechanism of resistance has been extensively documented [87]. Numerous studies have demonstrated that both the vinca alkaloids and the taxanes are 'good P-glycoprotein substrates' [88,89,90] and cell lines selected for resistance to these agents have been shown to overexpress P-glycoprotein [91,92•]. In addition, overexpression of the multidrug resistance protein (MRP) can confer resistance to the vinca alkaloids, but not the taxanes, while expression of the sister gene of Pgp (sPgp) has been implicated in paclitaxel resistance [93,94,95].

Altered expression of β-tubulin isotypes has also been advanced as a potential mechanism of drug tolerance, most prominently for paclitaxel. However, despite extensive circumstantial evidence, the importance of functional differences among β-tubulin isotypes is uncertain. Whether differential expression of different isotypes can modulate microtubule function and a cell's response to drug is even less certain. For paclitaxel, this latter question has been addressed most aggressively, motivated largely by several studies that reported acquired changes in β-tubulin isotype expression in paclitaxel-resistant cells and in paclitaxel-resistant ovarian tumors [2,96-99]. While the jury is still out on this important question, *in vitro* evidence suggests that isotype composition may affect paclitaxel sensitivity. Paclitaxel was shown to suppress the dynamics of microtubules composed of purified αβIII- and αβIV-tubulin 7-fold less strongly than control microtubules, suggesting that overexpression of βIII and βIV isotypes may lead to paclitaxel resistance [100•]. In addition, it has been reported that tubulin lacking the βIII isotype assembles *in vitro* into microtubules twice as rapidly as does normal brain tubulin [101].

Accumulating evidence indicates that mutations in β-tubulin can also confer resistance. As with the studies examining the expression of β-tubulin isotypes, the data is more extensive for paclitaxel. However, similar data has been obtained for the epothilones, and indirect evidence exists for other agents. The most straightforward mutations described involve acquired mutations at the paclitaxel binding site in β-tubulin, namely mutations at residues β270, β274, and β282 [70••, 74••]. According to the atomic model of α/β-tubulin with bound paclitaxel [73••] and its docking into a 20 Å map of the microtubule [72•], these residues have been localized to the taxane binding site, where they confer resistance to the taxanes, and also to the epothilones, albeit to varying extent. Supporting and further extending these findings in the clinic, preliminary clinical data suggests that

β-tubulin mutations may also confer paclitaxel resistance in patients with non-small cell lung cancer [55••]. Mutations have also been described at sites other than the taxane binding site, leading to the interesting proposal that resistance in these cases is a result of alterations in microtubule stability [102, 103•]. According to this model, such mutations can result in either a 'hypostable' or 'hyperstable' microtubules with differential sensitivity to MT-destabilizing or stabilizing agents.

Finally, recognizing the complex nature of drug interactions, it is likely that as yet undefined mechanisms of resistance will be identified. The identification of genes whose expression is altered in drug-resistant cell lines represents a first step in this process [104, 105].

Conclusion

The clinical success of microtubule-interactive agents has allowed us, in the last fifty years, to learn what nature figured out long ago: tubulin and microtubules are excellent drug targets. For no other single target, including DNA, has a more diverse group of agents been successfully developed. The questions we must then ask are: [1] 'Why are tubulin and microtubules such good targets?' and [2] 'Can other agents be found to target microtubules, and will they add anything to our existing armamentarium?'.

Why are tubulin and microtubules such good targets? The simple answer is that they are involved in critical cellular functions, the most dramatic of which, especially for cancer cells, is cell division. However, one can argue that a myriad of other proteins are similarly involved in cell division, yet to date 'natural products' targeting these have not been identified, suggesting that at least 'nature' has not considered them as essential. We believe several properties make tubulin/microtubules ideal targets: [i] A lack of redundancy. Unlike many other proteins/systems for which redundancy exists, cells do not have a redundant microtubule system. Consequently, interference with microtubule function is likely to result in a cellular effect. In contrast, enzymes often have overlapping activities, or alternate pathways can be utilized. [ii] Microtubules are intrinsically dynamic and this is essential for their function. This 'dynamism' is best manifested in mitotic cells, where the rapid turnover of the tubulin/microtubule pools provides an excellent opportunity to interfere with this process. In this regard it is not surprising that the principal effect of MIAs is most likely a result of interfering with turnover. An important corollary of this is that the percent of target sites occupied need not be large for a maximum effect to be observed. When one is dealing with a protein whose 'structure' can turn over in as little as 15 s, it is sufficient to have a drug on one of every 200 potential target sites, as occurs with paclitaxel. This is in contrast to an enzyme, for example, where inhibition of 1 in every 200 molecules would be inconsequential; indeed where anything less than near complete inhibition would be insufficient. [iii] Several sites on microtubules are valid targets. Unlike enzymes, for example, which in many cases require the active site to be the target, the binding sites on tubulin and the microtubules differ greatly among the various classes of agents. [iv] Microtubules are involved in many basic cellular functions. Consequently, interfering with microtubules results in a myriad of other effects. In dividing cells, cell division is impaired, while in resting cells intracellular trafficking and the supporting structure are impacted.

Can other agents be found to target microtubules, and will they add anything to our existing armamentarium? One can safely predict that additional agents targeting tubulin will be discovered and that some will likely become part of our clinical armamentarium. For example, an agent targeting the colchicine site has never been successfully developed as an anticancer agent, and it is likely that one will. It is also possible that new agents can be 'designed' to target microtubules, especially interphase microtubules. While concentrations which can alter the microtubule mass can be achieved clinically, interference with the mitotic spindle appears to be the principal effect of these agents. Indirect evidence for this includes observations both *in vitro* and in patients, that these agents are active principally against actively dividing cells. Although this may reflect a cell's ability to tolerate interference with the interphase functions of microtubules, it more likely reflects the less dynamic nature of interphase microtubules. Treadmilling and dynamic instability are not as crucial during interphase, as evidenced by the fact that exchange of the tubulin pool can sometimes take several hours. During this phase of the cell cycle, a stable microtubule on which intracellular trafficking can occur is likely to be more important. In this case, a low occupancy of target sites is less likely to be effective or even ineffective. We may come to understand that in interphase cells, targeting the microtubule-motor proteins which mediate microtubule-based trafficking, may be more effective. Such an approach may help make these agents active against less rapidly dividing cells, albeit at the risk of greater normal tissue toxicity.

In summary, the clinical success of paclitaxel has led to a wealth of new scientific knowledge regarding the importance of the tubulin/microtubule system as a target for cancer chemotherapy as well as the need to identify novel tubulin-active agents. While we remain in awe of nature's exquisite molecular engineering abilities, we hope that as we gain more knowledge and insight into the molecular mechanisms of action of microtubule-interactive compounds, we will be able to design 'better' novel chemotherapeutic agents or find 'better' ways to use the existing ones.

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