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REVIEW

Recent progress with microtubule stabilizers: new compounds, binding modes and cellular activities

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Covering: late 2008 to August 2013

Nature has yielded numerous classes of chemically distinct microtubule stabilizers. Several of these, including paclitaxel (Taxol) and docetaxel (Taxotere), are important drugs used in the treatment of cancer. New microtubule stabilizers and novel formulations of these agents continue to provide advances in cancer therapy. In this review we cover recent progress in the chemistry and biology of these diverse microtubule stabilizers focusing on the wide range of organisms that produce these compounds, their mechanisms of inhibiting microtubule-dependent processes, mechanisms of drug resistance, and their interactions with tubulin including their distinct binding sites and modes. A new potential role for microtubule stabilizers in neurodegenerative diseases is reviewed.

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1 Introduction

Microtubule stabilizing compounds are a group of chemically diverse molecules isolated from an extensive range of natural sources including microorganisms, sponges, and higher plants. New microtubule stabilizers continue to be isolated, new mechanisms of action and differences among microtubule stabilizers are being identified, and structural biology studies have localized the interactions and orientations of these diverse microtubule stabilizers within their corresponding binding sites on microtubules. This review will focus on the recent developments in the field of microtubule stabilizers over the past 5 years (late 2008 to 8/2013).

Microtubule stabilizers are a subclass of microtubule-targeting agents that stimulate the assembly of purified tubulin and increase the density of cellular microtubules by

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shifting the equilibrium of tubulin polymer from the soluble to the polymerized form (Fig. 1). In contrast, microtubule depolymerizers initiate the loss of interphase microtubules, and are represented by many other natural products, but will not be covered in this review.

In cancer therapeutics, microtubule stabilizers are of particular interest because of the significant anticancer activities of the taxanes, paclitaxel (Taxol) and docetaxel (Taxotere). While major advances in the treatment of cancer have been made in the past decade and numerous targeted therapies are available for most common adult solid tumours, the importance of cytotoxic therapies has not changed. Microtubule stabilizing drugs continue to play an important role in cancer chemotherapy for adult solid malignancies and new drugs with improved properties including ixabepilone (Ixempra), cabazitaxel (Jevtana) and nab-paclitaxel (Abraxane) provide effective options for cancer therapies. The clinical success of the taxanes in first-line treatment of cancer, and the diversity of chemical structures and natural sources of microtubule stabilizers has sustained the interest of the natural products community in the discovery of new agents of this class. Novel structural classes of microtubule stabilizers continue to be discovered from nature and while the clinical development of some of these new classes continues, others have been discontinued for a variety of reasons.

New taxane analogues and formulations of paclitaxel have expanded the clinical spectrum of activity and provide more treatment options for patients. Mechanistically, microtubule stabilizers have been characterized for decades as mitotic poisons but new compelling evidence suggests that they also impact non-mitotic, microtubule-dependent processes and these effects may be central to their anticancer actions.[1] Significant progress in understanding the molecular, cellular and anticancer mechanisms of action of diverse microtubule stabilizers has been made in the past 5 years. Studies describing new mechanistic information on the interphase effects of microtubule stabilizers and the implications for cancer cell survival will be reviewed. Recent progress in mapping the distinct microtubule stabilizer binding sites will also be covered. Together, this information might help identify how these diverse agents can best be used for cancer therapy and potentially in the treatment of neurological diseases.

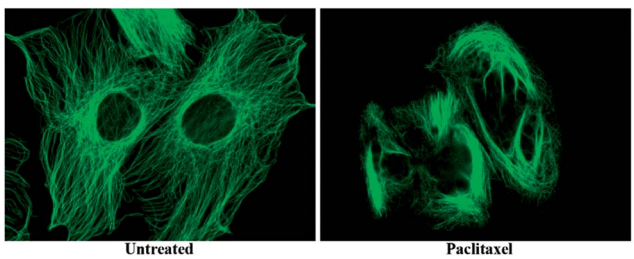
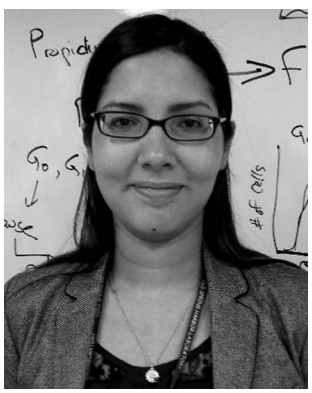


Fig. 1 Effects of microtubule stabilizers on the cellular interphase microtubule network of A-10 cells.

2 Microtubule structure and cellular function

Microtubules are dynamic, intracellular hollow filaments composed of αβ-tubulin heterodimers. These αβ-tubulin heterodimers are formed during protein synthesis by the actions of molecular chaperones. In mammals 6 α-tubulin and 8 β-tubulin isotypes have been identified that are expressed differentially in a tissue-specific manner.[2] The αβ-tubulin heterodimers assemble into protofilaments in a specific head-to-tail orientation that gives microtubules an innate polarity. Microtubules assemble into tubules with 13 protofilaments and a diameter of approximately 25 nm. The α-tubulin subunit is localized



Cristina Rohena was born in 1983 in Puerto Rico and received her B.S. degree in Biology from Cornell University in Ithaca, N. Y. in 2006. She received her Ph.D. in Biomedical Sciences in the field of Cancer Biology from the University of Texas Health Science Center at San Antonio in 2013, under the supervision of Dr Susan L. Mooberry. Her dissertation focused on elucidating both the mitotic and interphase specific molecular mechanisms of action of the taccalonolides as compared to other classes of microtubule stabilizers. She is currently conducting postdoctoral studies on chemically diverse microtubule targeting agents focusing on their effects on interphase, microtubule-dependent processes.

Susan L. Mooberry is a Professor of Pharmacology at the University of Texas Health Science Center at San Antonio (UTHSCSA) and co-leader of the Experimental and Developmental Therapeutics Program of the Cancer Therapy & Research Center at UTHSCSA. Dedicated to the discovery of more effective cancer therapies, she directs a drug discovery program that seeks to identify new anticancer agents from diverse natural products, including plants, marine organisms and fungi. Using a cell-based phenotypic screen, she identified the cryptophycins, laulimalides and taccalonolides as new microtubule disrupting agents. Her laboratory has expertise in elucidating the molecular mechanisms of drug action and in preclinical testing, with a goal of identifying clinical lead candidates.

towards the (−) end at the centrosome and the β-tubulin subunit, containing the exchangeable GTP site, is exposed at the (+), dynamic end of the microtubule, which often extends towards the cell periphery.[2,3] Microtubules are key components of the cytoskeleton and play crucial roles in cellular metabolism and intracellular transport. They help maintain cell shape, intracellular organization and are the structures along which intracellular vesicle trafficking occurs with microtubule motors carrying cargos as diverse as mRNAs and mitochondria. Microtubules are instrumental in interphase homeostasis as well as in mitosis and cell division.[2]

An important property of microtubules is dynamic instability, which describes the intrinsic nature of microtubules to rapidly shift between growth and shrinkage. This dynamicity is a process that is tightly regulated by multiple posttranslational modifications and by microtubule interacting proteins that bind directly to tubulin heterodimers or at the ends of microtubules.[2] The dynamicity of microtubules allows rapid and spatially localized changes which are needed, for example, during cell migration but that can also respond to local cellular needs.

At the onset of mitosis, the entire microtubule network undergoes rearrangement from the interphase microtubule array to specialized highly dynamic mitotic spindles nucleated from the centrosomes or the kinetochores. These specialized microtubule structures are responsible for guiding the sister chromatids toward the poles of the new daughter cells during mitosis, ensuring a complete genetic content for each. Mitotic spindles are highly susceptible to the actions of microtubule disrupting compounds including microtubule stabilizers, and these effects, measured in tissue culture models of rapidly dividing cells, led to the initial identification and characterization of these agents as anti-mitotic drugs.[2,3] In both mice and human tumours the growth rate of cancer cells is much slower than in culture, which has led to the hypothesis and supporting data that the actions of these drugs on interphase microtubule-dependent processes are important in their antitumour and anticancer activities.[1,4]

3 Natural sources of microtubule stabilizers

3.1 Plants

Taxol was first isolated from the bark of *Taxus brevifolia* in 1966 by Wani and Wall.[5] Its mechanism of action, the first of its kind, was identified by Horwitz and co-workers in 1979.[6] The original name, taxol was subsequently trademarked and Bristol Myers Squib (BMS) provided paclitaxel to the scientific community as the new generic name. The clinical success of paclitaxel and the second generation taxane, docetaxel, revolutionized the treatment of adult solid malignancies and led to the discovery and clinical development of numerous other classes of microtubule stabilizers from microorganisms and the marine environment. To date two other natural classes of microtubule stabilizers have been identified from plants, the taccalonolides, and rhazinilam, a biologically active microtubule stabilizing degradation product from plants of the family Apocynaceae.

3.1.1 Taxanes. The discovery and early development history of taxol have been reviewed by others.[7,8] Importantly, the excellent preclinical and clinical activities of taxol fostered creative solutions for the seemly insurmountable obstacles that occurred along its development path, including compound supply, which was solved by plant tissue culture and semi-synthesis from 10-deacetyl-baccatin III, an abundant plant precursor. While docetaxel and paclitaxel have been of substantial value in the treatment of solid tumours, side effects associated with their use are often dose limiting and include neutropenia and peripheral neuropathy. The identification of second-generation taxanes with superior properties including the ability to overcome drug resistance mechanisms including the expression of P-glycoprotein (Pgp), an ATP-dependent drug efflux pump, has been intense (Table 1). Cabazitaxel (Jevtana, XRP6258) (Fig. 2) is a semi-synthetic derivative of docetaxel that was selected for clinical development from approximately 450 taxane derivatives based on its microtubule stabilizing effects, *in vitro* activity against docetaxel resistant cell lines and anti-tumour efficacy in docetaxel resistant murine models.[9] The methyl substitutions on C7 and C10 resulted in improved activity of cabazitaxel against docetaxel resistant cell lines that express Pgp and the βIII isotype of tubulin (Fig. 2).[9] The extensive preclinical studies conducted with cabazitaxel, including evaluations of efficacy *in vitro* and *in vivo* in multiple drug resistant cell lines, were recently published and can serve as a model for the preclinical activities needed to advance a successful taxane derivative.[9] The anticancer actions of cabazitaxel in prostate cancer patients with docetaxel-resistant hormone refractory disease led to its FDA approval in 2010 for this indication. Many other taxane analogues have been evaluated in clinical trials and a list and development status is presented in Table 1.

Structure activity relationship (SAR) studies have provided valuable information about key moieties of the taxane skeleton involved in microtubule stabilizing activities and drug resistance and this information has been reviewed recently.[10] Both paclitaxel and docetaxel are metabolized by cytochrome P450 CYP3A4. Ojima and colleagues recently designed and synthesized a series of 3′-difluorovinyl taxoids with C10 modifications with and without additional modifications at C2 to specifically slow CYP3A4 metabolism.[11] Modifications of the C2 benzoate moiety in the *meta* position affected the potency and the ability of the analogues to overcome Pgp-mediated drug resistance with potency of the series $F < Cl \leq MeO < N_3$. The C10 modifications had little effect on the ability to circumvent Pgp-mediated drug resistance (Fig. 2).[11] Eight analogues had potency superior to paclitaxel with IC_{50}s in the picomolar range. Importantly, in metabolic stability studies few metabolites were detected, suggesting that the designed changes impeded metabolism.[11] Further evaluations of the role of the 2′-hydroxyl group in the C13 side chain of paclitaxel with 2 analogues and molecular modelling helped explain the role of this moiety in the biological actions of the parent molecule.[12] 2′-Deoxy-paclitaxel had 100 times lower affinity for microtubules than

Table 1 Clinical development of microtubule stabilizers

Drug	Clinical development
Novel Taxanes	
Cabazitaxel (Jevtana, Sanofi) A dimethoxy derivative of docetaxel.	Approved in US and Europe for hormone refractory prostate cancer.
Tesetaxel (DJ-927, Genta, Inc) Novel oral taxane derivative	Phase I and II trials are ongoing.
Larotaxel (XRP9881, Sanofi) A semi-synthetic taxane derived from 10-deacylbaccatin III	Phase I, II and III trials were completed in 2011.
BMS-184476 (Bristol Myers Squib) A 7-methylthiomethyl ether of paclitaxel	Phase I and II trials were completed in NSCLC and other solid tumours in 2007.
TPI-287 (Archer Biosciences) Paclitaxel derivative with a 5-membered baccatin ring, crosses blood brain barrier	Phase I and II trials ongoing in glioblastoma, neuroblastoma, medulloblastoma and breast cancers with brain metastasis.
New Taxane Formulation	
Nab-paclitaxel (Abraxane, Celgene) serum albumin conjugated paclitaxel	Approved in US and Europe for metastatic breast cancer and in the US for NSCLC and pancreatic cancer.
Xyotax (Opaxio, paclitaxel polyglumex (PPX) Cell Therapeutics, Inc.) paclitaxel conjugated to α-poly-L- glutamic acid	Phase III trials completed in ovarian cancer, phase I/II trials in many other solid tumours ongoing.
EndoTag-1 (Medigene AG) Cationic liposomal paclitaxel	Phase I and II trials completed in 2013 in breast, pancreatic, liver and head and neck cancers.
Genexol-PM (Samyang Gene, South Korea) Polymeric micellar paclitaxel formulation	Phase I, II trials completed. Phase III trials ongoing for the treatment of metastatic breast cancer.
DHA-paclitaxel (Taxoprexin, Protarga, Inc.) DHA-conjugated paclitaxel	Phase I and II trials completed in 2009 in melanoma, liver, NSCLC and prostate cancers.
Epothilones	
Ixabepilone (Ixempra, Bristol Myers Squib) An epothilone B lactam	Approved in the US for metastatic breast cancer.
Patupilone (EPO906, Novartis) Natural epothilone B	Phase I and II trials completed. Phase III trials completed in 2012 in ovarian cancer failed to show significant survival advantage.[110]
Sagopilone (ZK-EPO, Bayer Schering AG) Fully synthetic allyl derivative of epothilone B	Phase I and II trials completed in metastatic melanoma, ovarian cancer, prostate cancer, NSCLC, and breast cancer with brain metastasis in 2013.
KOS 862 (Kosan Biosciences; BMS-241027, Bristol Myers Squib) Natural epothilone D	Phase I and II trials completed in 2009. Phase I trials for Alzheimer's disease ongoing.
KOS 1584 (Kosan Biosciences) Epothilone D derivative	Phase I and II trials completed in 2011.
Other taxane binding agents	
Discodermolide (Novartis)	Failed in phase I trials due to pneumotoxicities.[40]

paclitaxel and had only slightly higher affinity than baccatin III, which lacks the C13 side chain.[12] Molecular modelling suggests that the 2′-OH is responsible for 80% of the binding free energy of the C13 side chain, illustrating the importance of this site in taxoid analogues. Molecular dynamic simulations confirmed expected hydrogen binding between the 2′-OH and tubulin at D26,[12] a site mutated in drug resistant cell lines.[12] The authors propose that the importance of the rest of the side chain is to appropriately position the 2′-OH for optimal hydrogen binding to tubulin. Interestingly, *N*-debenzoyl- 2′-deoxy-paclitaxel, which is missing both the 2′-OH and 3′-benzoyl groups was totally inactive in tubulin polymerization and cell-based cytotoxicity assays. Molecular simulations suggest that the loss of the 3′- benzoyl group prevents optimal anchoring interactions with tubulin, by changing the C13 side conformation to preclude optimal binding (Fig. 2).[12] Nicolaou and Valiulin explored reactions of 10-deacetylbaccatin III with diethylaminosulfur trifluoride (DAST) under various conditions, which yielded multiple new fluorinated and non-fluorinated C13-keto taxoid analogues.[13] Further reductions of the C13-keto group resulted in a series of 13α-hydroxy taxoid derivatives. The esterification of 13α-hydroxy group with the docetaxel side chain was used to produce an array of docetaxel analogues and other related compounds.[13] Three of the docetaxel analogues were highly active in the NCI-60 cancer cell panel with broad efficacy and potency with GI_{50} values less than 5 nM.[13]

In addition to new taxane derivatives substantial efforts have been directed at different formulations of the taxanes. Several approaches have been utilized and at least 5 different formulations of paclitaxel have been evaluated in clinical trials, including nab-paclitaxel (Abraxane), paclitaxel poliglumex (Opaxio, Xyotax), cationic liposomal paclitaxel (endoTAG-1), paclitaxel-loaded micelles (Genexol-PM) and a prodrug approach with DHA-paclitaxel to increase tumour uptake (Taxoprexin) (Table 1). A comprehensive review on these different formulations was published in 2012.[14] Many of these approaches have not yielded positive results in clinical trials, but nab-paclitaxel a nanoparticle human serum albumin stabilized paclitaxel, has proven to be superior to paclitaxel in some tumour types. This formulation of paclitaxel was approved in the United States in 2005 for treatment of metastatic breast cancer that was unresponsive to anthracyclines and in 2012 for the treatment of non-small cell lung cancer. Most

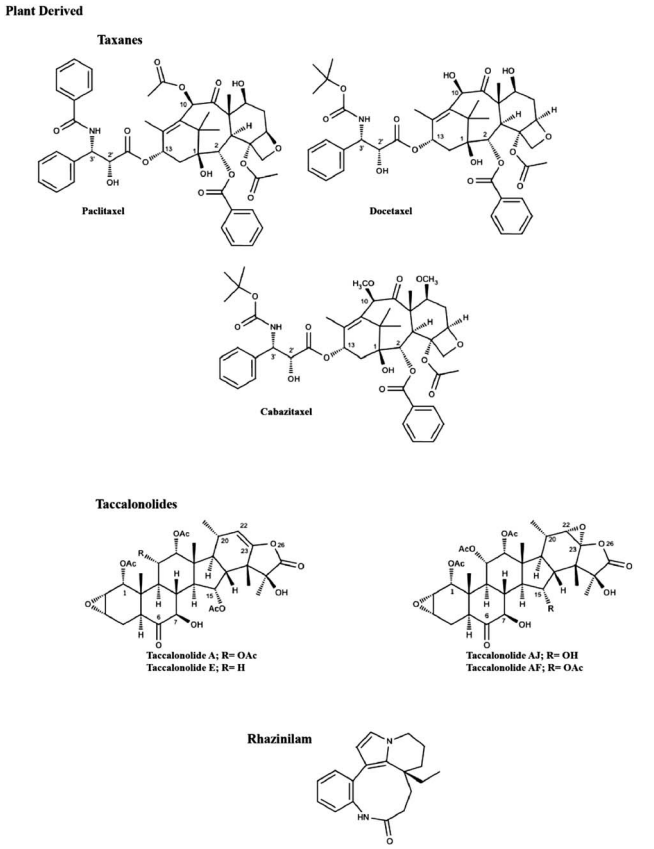


Fig. 2 Chemical structures of selected plant-derived microtubule stabilizers.

recently, in September 2013, nab-paclitaxel was approved in the United States for the treatment of advanced pancreatic cancer. The Phase III MPACT clinical trial showed that nab-paclitaxel in combination with gemcitabine led to a median survival of 8.5 months in patients with advanced pancreatic cancer *vs.* a 6.7 month median survival seen in patients treated with only gemcitabine.[15]

3.1.2 Taccalonolides. Plants of the genus *Tacca* yielded the taccalonolides, highly acetylated pentacyclic steroids with microtubule stabilizing activities. Scheuer first isolated a 6-membered ring constituent from the tubers of *T. leontopetaloides* in 1963,[16] and the structures of the taccalonolides (Fig. 2) were solved in 1987 from *Tacca plantaginea* by Chen and colleagues.[17–19] The microtubule stabilizing properties of the taccalonolides were first identified in 2003.[20] While the taccalonolides A and E cause cellular effects characteristic of microtubule stabilizers, including increased density of microtubules, microtubule bundles and formation of aberrant mitotic spindles leading to mitotic accumulation and initiation of apoptosis, a direct interaction with tubulin was not detected with these relatively low potency taccalonolides. The taccalonolides remained interesting because they overcome drug resistance mediated by the expression of Pgp, mutations in the paclitaxel binding site, multidrug resistant protein 7 and the βIII tubulin isotype *in vitro*.[21,22] Additionally, taccalonolides A and E were highly potent and effective antitumour agents against murine models of cancer, including a mammary tumour model that expresses Pgp and is insensitive to paclitaxel or doxorubicin.[21,22] The therapeutic window of these taccalonolides was narrow. Not unexpectedly, taccalonolide A had additive cytotoxic effects in combination with γ-radiation in cancer cells,[23] but it had an unexpectedly high degree of cellular persistence.[24] The effects of taccalonolide A are virtually irreversible, even following a short exposure time, and differ in this regard from paclitaxel and laulimalide.[24] The isolation of the potent taccalonolide AF and semi-synthetic generation of its closely related analogue, AJ, demonstrated for the first time a direct interaction of the taccalonolides with purified tubulin.[25] The taccalonolides AF and AJ stimulate tubulin polymerization to a degree comparable to paclitaxel but with different kinetics. In the presence of paclitaxel tubulin polymerizes immediately, yet a notable lag period was observed with either taccalonolide AF or AJ,[25] suggesting the possibility of a subtly different interaction with tubulin. Recently, it has been shown that the taccalonolides bind covalently to tubulin, which likely explains many of their distinct properties compared to other classes of microtubule stabilizers, including their high persistence and potent *in vivo* activity.[26]

Prior to 2011, 25 taccalonolides (A–Y) had been isolated from multiple *Tacca* sp. That year, 3 novel taccalonolides, designated taccalonolides Z, AA and AB were isolated from *T. chantrieri* and *T. integrifolia* with potencies of 120, 32 and 2,800 nM, respectively.[21] For the first time, a taccalonolide with activity in the low nanomolar range was identified. Additionally, the activities of the known taccalonolides R and T were determined to be >13 000 and 335 nM, respectively. The potency of taccalonolide T provided interesting SAR, showing that a bulky isovalerate group at C1 afforded greater potency compared to the acetyl group on taccalonolide A.[20] Subsequently, 5 additional natural taccalonolides, AC–AF and H2 were isolated from *T. plantaginea*.[25] While taccalonolide AF was isolated in very small quantities, a semi-synthetic route was employed to generate it from taccalonolide A by epoxidation of the C22–C23 double bond to an epoxide group with dimethyldioxirane.[25] Taccalonolide AJ was generated using the same reaction with taccalonolide B as starting material. Taccalonolides AF and AJ showed low nanomolar potency against cancer cell lines. This simple epoxidation of taccalonolide A increased the potency over 200-fold and the epoxidation of taccalonolide B to AJ provided a 750-fold increase in potency. The importance of this region on the taccalonolide skeleton was further demonstrated with taccalonolide AC, which has a hydroperoxyl group at C20 instead of a hydrogen like other taccalonolides shown in Fig. 2, and was biologically inactive.[25]

More recently, optimization of hydrolysis reactions were described to identify and characterize five new taccalonolides.[27] Biological evaluations of these modified taccalonolides led to further enhancement of SAR. A keto-enol tautomerization between the C6 ketone and C7 hydroxy groups on taccalonolide B yielded taccalonolide I, along with a 15-fold decrease in potency.[27] Additionally, hydrolysis of the acetoxyl group at C1 of taccalonolide N yielded taccalonolide AN and a 5.7-fold increase

in potency. Taccalonolides AK and AO each have a lactone ring between C26 and C15. This rearrangement of the lactone ring resulted in a complete loss of biological activity.[27] Initial SAR studies have identified moieties responsible for optimal potency and preclinical studies to identify a potential taccalonolide lead for clinical development are underway. The complex structural features of the taccalonolides have thus far precluded any total synthesis.

3.1.3 Rhazinilam. (−)-Rhazinilam is a microtubule stabilizer originally thought to be a natural product isolated from plants of the Apocynaceae family. However, studies showed it was a degradation product formed during isolation.[28] In cells (−)-rhazinilam stabilized microtubules, but had mixed effects in tubulin assays;[29] at low concentrations it inhibited tubulin polymerization and at high concentrations it enhanced tubulin polymerization. Tubulin spiral formation was noted with (−)-rhazinilam at high concentrations *in vitro*.[29] More recently it was reported that this phenotype was GTP dependent.[30]

3.2 Marine sources

Of all natural sources, marine organisms have provided the largest number of new microtubule stabilizing compounds, including the eleutherobins, sarcodictyins, discodermolide, the dictyostatins, laulimalides, pelorusides, zampanolide and dactylolide. The majority of these compounds bind to the taxane site on microtubules, albeit in different manners and poses within the binding site. The exceptions are laulimalide and peloruside A, which bind to a non-overlapping binding site on microtubules. The most recently identified marine-derived microtubule stabilizers, zampanolide and dactylolide bind covalently within taxane binding site.

3.2.1 Discodermolide. Discodermolide is a polyketide isolated from the marine sponge *Discodermia dissoluta*. It was first identified in 1990 by Gunasekera and Longley at the Harbor Branch Oceanographic Institution,[31] and the microtubule interacting properties first detected based on a computer assisted search for putative tubulin-binding agents.[32] Discodermolide was unexpectedly found to be a potent microtubule stabilizer[32] that binds with high affinity to the taxane site on microtubules and overcomes Pgp-mediated drug resistance.[33,34] Of all the marine microtubule stabilizers identified to date, discodermolide is the only one that is not a macrolide (Fig. 3). The yield from the sponge was extremely low (0.002%), but the biological activities were sufficient to initiate interest in clinical development (Novartis). A major synthetic undertaking produced 60 g of material, sufficient to begin clinical trials.[35–39] However, severe lung toxicities halted the Phase I clinical trials and clinical development of discodermolide was discontinued.[40] Improvements in aquaculture show that *D. dissoluta* can be successfully cultivated from sponge fragments and discodermolide yield improved.[41] Discodermolide's chemical structure consists of a flexible chain that can adopt infinite numbers of conformations both in solution and with tubulin. A study by the Horwitz laboratory, using hydrogen deuterium exchange showed that unlike paclitaxel, which interacts mainly with the M-loop of tubulin, discodermolide orients towards the

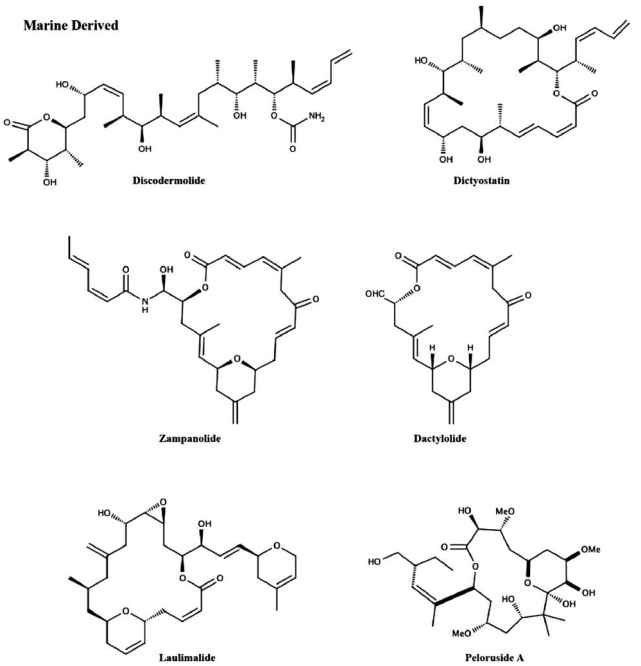


Fig. 3 Chemical structures of selected marine-derived microtubule stabilizers.

N-terminal H1-S2-loop.[42] The successful synthesis of a fluorescently labelled discodermolide that retains biological activity will facilitate further studies of its binding interactions.[42] Biologically, discodermolide has several interesting properties including the ability to initiate cellular senescence dependent on the translation repressor protein, 4E-BP1.[43] A new strategy based on differential binding interactions of paclitaxel and discodermolide within the taxane binding pocket has led to the design, synthesis and biological evaluation of 5 analogues with potencies greater than discodermolide. These studies pave the way to identify hybrids with selectivity for different β-tubulin isotypes.[44]

3.2.2 Dictyostatin. Dictyostatin is a 22-membered marine macrolide (Fig. 3) first isolated from a sponge in 1994 by Pettit.[45] The potent microtubule stabilizing activities were identified from material isolated from a deep-sea sponge by the Wright laboratory. They additionally demonstrated the ability of dictyostatin to overcome drug resistance mediated by Pgp in human cell lines.[46] The total synthesis of dictyostatin was completed in 2004 by 2 independent groups.[47,48] Several total (and fragment/analogue) syntheses were reported and were reviewed recently.[49] The early synthesis routes were not amenable to commercial scale-up and significant efforts have been underway to simplify the synthesis and identify new analogues with potential for clinical development. Curran and colleagues were the first to synthesize dictyostatin from 3 fragments followed only by fragment couplings.[50] This increased convergency made this, at the time, the shortest synthesis of dictyostatin with 36–40 total steps.[50] This same group later[51] presented a streamlined total synthesis of 2 analogues of dictyostatin; 25,26-dihydrodictyostatin and 25,26-dihydro-6-*epi*-

dictyostatin. These syntheses were based on 3 complete carbon fragments coupled by Horner–Wadsworth–Emmons reaction sequences and an esterification.[51] These reactions produced dictyostatin in 7–8% yield with the synthesis of each fragment taking 10 steps or less, with 10 additional steps to couple the fragments. These new analogues retained the low nanomolar potency of dictyostatin, and are effective in paclitaxel and epothilone-resistant cells lines with mutations in the taxane binding site or that express Pgp.[52] A step-economical synthesis using a "Roche ester strategy" with the longest linear sequence of 14 steps was reported in 2013 by the Leighton laboratory. This step-efficient, fragment-based approach yielded multi-gram quantities of each of 3 fragments prepared with 4–5 steps each, including in some cases, one-pot procedures.[53] The fragments were coupled and synthesis completed in a total of 9 steps with high yields, 61–91%, at each step.[53] Progress in simplifying the synthesis of dictyostatin and in identifying its binding pose within the taxane binding site[54] increase the likelihood that a dictyostatin may be advanced for clinical development.

3.2.3 Laulimalide and pelorusides. The laulimalides/fijianolides are marine-derived polyketides (Fig. 3) that were first isolated individually by the Crews and Moore/Scheur/Paul laboratories in 1988.[55,56] Their low nanomolar microtubule stabilizing activities were first reported in 1999.[57] 6 additional fijianolides (D–I) have been isolated from different sources and they vary on the oxidation state and/or oxidation pattern of the C20 side-chain.[58] Laulimalide has been shown to synergize with paclitaxel and other taxane-binding agents and has activity in multidrug resistant cells that overexpress Pgp or have mutations in the taxane binding site.[59–61] The microtubule stabilizing activities of laulimalide led many laboratories to complete the total synthesis of the natural product and numerous analogues.[62] The total synthesis of neolaulimalide and isolaulimalide and a highly efficient route to laulimalide[63] was published by the Mulzer laboratory in 2009. Neolaulimalide was also synthesized in 21 steps with a 3% yield and this group confirmed its activity as a microtubule stabilizer.[63] Isolaulimalide was synthesized in 24 steps with a 2% yield. Their route to laulimalide began with economical starting materials and took 20 linear steps with a 7% yield.[63] Another recent synthesis of laulimalide was reported by the Trost laboratory combining the northern and southern fragments of laulimalide.[64] Subsequently, a second-generation route that provides a more concise synthesis of laulimalide was reported.[65] Laulimalide has been evaluated *in vivo* in multiple tumour models and while it provided an indication of antitumor effects in HCT-116 tumours in SCID mice,[58] more detailed studies with 5 doses each in 2 different nude mice tumour models showed no significant tumour growth inhibition within the limits of acceptable toxicities. This lack of *in vivo* activity precluded continued clinical development.[66] Further mechanistic studies with laulimalide showed that it has effects on mitotic signalling pathways different from either paclitaxel or the taccalonolides.[67] Additionally, the effects of low concentrations of laulimalide on interphase and mitotic microtubules were studied and compared with docetaxel.[68] Both laulimalide and docetaxel at 30 nM increased the presence of acetylated microtubules, an indication of post-translation modifications of mature, stable microtubules, within 2.5 h. Microtubule bundles were seen in the docetaxel-treated cells, however, laulimalide-treated cells had the capacity to generate mature, acetylated microtubules without microtubule bundling. Thus microtubule stabilization does not require microtubule bundling.[68] Laulimalide also had different effects from docetaxel on centrosome fragmentation and on kinetochore tension, demonstrating multiple differences in the mechanisms by which these microtubule stabilizers disrupt mitosis.[67,68]

In 2000 Northcote and West isolated peloruside A from the New Zealand marine sponge *Mycale hentscheli.*[69] It is a novel 16-membered macrolide (Fig. 3) that displayed cytotoxic activity. The mechanism of peloruside A as a microtubule stabilizer was identified in 2002 by Miller and colleagues.[70] Peloruside A was first synthesized by the De Brabander laboratory in 2003[71] and peloruside B, a 3-des-*O*-methyl variant of peloruside A (Fig. 3) was later isolated from the same sponge in 2010.[72] Peloruside B also had microtubule stabilizing effects. The total enantioselective and convergent synthesis of peloruside B was accomplished using Sharpless dihydroxylation, Brown's asymmetric allylboration reaction, reductive aldol coupling and Tamaguchi macrolactolization reactions.[72] Taylor and Zhao subsequently presented an efficient synthesis of peloruside A analogues in 18 steps from commercially available material that involved an esterification-based fragment coupling and a late stage ring-closing metathesis reaction.[73] The biological effects of peloruside A in cells in culture have been described in numerous publications, but to date no published papers have described *in vivo* antitumour effects and clinical development does not appear to be progressing. While peloruside A and laulimalide have not advanced to clinical trials, they have been of significant value in defining a new microtubule stabilizer binding site localized on the exterior surface of the microtubule.

3.2.4 Zampanolide and dactylolide. (−)-Zampanolide was first isolated in 1996 by Tanaka and Higa from the Okinawan sponge *Fasciospongia rimosa*.[74] Zampanolide has unique molecule architecture with a largely unsaturated 20 membered macrolactone core that includes a *syn*-2,6-disubstituted tetrahydropyran ring and an *N*-acyl hemiaminal side chain (Fig. 3). In 2009 the Northcote and Miller laboratories identified zampanolide as a microtubule stabilizer with low nanomolar potency that retains activity in Pgp-overexpressing cells, likely due to its ability to bind covalently to microtubules.[75] Zampanolide is an attractive synthetic target because it has only 4 stereogenic centers. Dactylolide is a structural analogue of (−)-zampanolide (Fig. 3) that was isolated in 2001 by Riccio and coworkers from a marine sponge of the genus *Dactylospongia*, collected off the north coast of the Vanuatu islands.[76] Later the configuration was demonstrated to be (+)-dactylolide.[77] (+)-dactylolide and (−)-zampanolide have common macrocyclic cores that share enantiomeric relationships (Fig. 3). Dactylolide has much lower potency than zampanolide with antiproliferative activity in the low micromolar range.[76] The confirmation of the microtubule stabilizing activities and modest potencies of the unnatural (−)-dactylolide and 3

analogues were shown in 2010 by the Altmann laboratory.[78] This indicates that the differences in potency between zampanolide and dactylolide relates not to the stereochemistry of the macrolactone ring but to the presence of the hemiaminal side chain.

The total syntheses of zampanolide and dactylolide were completed by several groups and more recently several enantioselective synthetic approaches have been described. Smith and co-workers completed the first total synthesis of (+)-zampanolide and (+)-dactylolide in 2002.[77] Refining these synthesis strategies Jennings' group reported the total synthesis of (−)-dactylolide and the formal synthesis of (−)-zampanolide *via* a targeted β-*C*-glycoside formation.[79] SAR studies helped illuminate the critical importance of the *N*-acyl hemiaminal side chain of zampanolide.[79] In 2011 an enantioselective synthesis of (−)-zampanolide was reported[80] that utilized an intramolecular oxidative cyclization reaction. This included a cross-metathesis reaction to make a trisubstituted olefin followed by a ring closing metathesis reaction to form the highly functional macrolactone and a chiral phosphoric acid catalysed stereoselective *N*-acyl aminal formation.[80] A further refinement, using an oxidative intramolecuar cyclization strategy was described in 2012.[81] More recently Altmann presented a new total synthesis of (−)-zampanolide, the non-natural (−)-dactylolide and 9 novel analogues using high-yielding Horner–Wadsworth–Emmons reactions to generate the macrolide core and the formation of the *syn*-2,6-disubstituted tetrahydropyran ring with a Prins-type cyclization.[82] Their results confirm the potent activity of (−)-zampanolide and the substantially lower potency of (−)-dactylolide and thus the role of the side chain in biological potency. Interestingly, one analogue that is devoid of the entire tetrahydropyran ring, yet maintains the hemiaminal side chain retains biological activity.[82] The ability of zampanolide to bind covalently to microtubules was identified in a collaborative effort led by Diaz[83] and is described in detail below in comparison with other microtubule stabilizers and their interactions with tubulin binding sites.

3.2.5 Ceratamines, eleutherobins and sarcodictyins. Eleutherobin was isolated from the soft coral *Eleutherobia* sp. and was found to have microtubule stabilizing activity.[84,85] Despite being synthesized[86–88] no recent developments have been made with this compound. The ceratamines are heterocyclic alkaloids isolated from extracts of the marine sponge *Pseudoceratina* sp.[89] Their mechanism as microtubule stabilizers was described in 2005 and are different from other marine stabilizers in that they cause ring-like bundling of interphase microtubules around the nucleus.[90] They were synthesized by two different groups, but no other progress has been reported.[91–93] Sarcodictyin A was first isolated from the Mediterranean stoloniferan coral, *Sarcodyctyon reseum* and subsequently from the South African soft coral *Eleutherobia aurea.*[94] The total synthesis has been completed,[95,96] but there is no indication of clinical development.

3.3 Microorganisms

Microtubule stabilizers isolated from microorganisms have also proven to be highly effective cytotoxic compounds with clinical utility. Two major classes have been identified from various species including the epothilones and cyclostreptin. Of these two, the epothilones have shown excellent clinical effects leading to FDA approval of the epothilone B analogue, ixabepilone (Ixempra). The other class of compounds isolated from microorganisms, cyclostreptins, were the first class of compounds to show covalent binding to microtubules.

3.3.1 Epothilones. The epothilones are macrolide compounds (Fig. 4) first discovered in 1987 from the fermentation of the soil myxobacterium *Sporangium cellulosum*.[97] The major natural products isolated were the epothilones A and B.[97] Their microtubule stabilizing activities were discovered by scientists at Merck in 1995 and they represented the first microtubule stabilizers isolated since paclitaxel.[98] Their robust microtubule stabilizing effects, efficacy against Pgp expressing multidrug resistant cell lines, the novelty of their mechanism of action and accessible supply from fermentation led to the successful clinical development of several epothilones. While many epothilones advanced to clinical trials (Table 1), only the epothilone B analogue ixabepilone has been approved for use in patients in the United States for refractory metastatic breast cancer. Structurally, the epothilones are 16-membered macrolides that are less complex than the taxanes. The only structural difference between epothilone B (patupilone) and epothilone A is a methyl group at C12 (Fig. 4). This difference provides key SAR because epothilone B is more hydrophilic than epothilone A and is twice as potent at stabilizing microtubules. Epothilone A was dropped from clinical development due to structural instability in animal plasma.[99] An excellent, comprehensive review of the chemistry and biology of semi-synthetic epothilones was published in 2011 by Altmann and colleagues[100] and only a few new studies are described here. Lin and co-workers[101] demonstrated the efficient and total synthesis of epothilone B that resulted in an 8% yield in 10–11 steps. This synthesis featured a bissiloxane-tethered ring closing metathesis reaction to approach a trisubstituted double bond. These reactions formed the basis for further development for the supply of epothilone B and ixabepilone. Although ixabepilone was approved in part due to its activity in taxane and anthracycline-

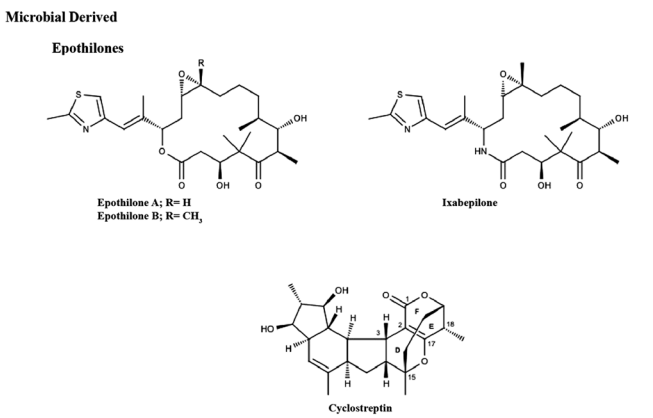


Fig. 4 Chemical structures of selected microbial-derived microtubule stabilizers.

resistant breast cancers, thought to be due to its inability to be exported by the Pgp drug efflux pump, a recent study showed that ixabepilone is a Pgp substrate but not a substrate for the breast cancer resistance protein (BCRP/ABCG2).[102]

3.3.2 Cyclostreptins. Cyclostreptin (FR182877) was first isolated from the fermentation broth of the bacterium *Streptomyces* sp. 9885 at the Fujisawa (Astellas) Pharmaceutical Co.[103–105] and shown to have antimitotic and microtubule assembly-promoting activities.[104] Cyclostreptin was shown to have microtubule stabilizing activity, but with low potency in biochemical assays as compared to paclitaxel.[104,106] In cells, however, cyclostreptin had low nanomolar potency and showed *in vivo* antitumour activity against a P388 mouse model.[104] Cyclostreptin has a hexacyclic structure with 12 contiguous stereogenic centres and a highly distorted and reactive push–pull alkene (Fig. 4).[105] Cyclostreptin was first synthesized in 2002 and was later found to be the first microtubule stabilizer to bind covalently to β-tubulin.[107,108] The covalent binding of cyclostreptin to microtubules provided the advantage of overcoming drug resistance mediated by Pgp. However, its chemical instability and low potency precluded any clinical development. Nevertheless, cyclostreptin and its derivatives have served to further map the binding process of taxane-site compounds.[109] Recently the Nakada group, which had previously completed asymmetric total synthesis of cyclostreptin, evaluated the biological activity of the highly strained DEF-ring moiety of cyclostreptin (Fig. 4)[65] and reported the asymmetric and highly selective stereoselective synthesis of this moiety *via* an inverse-electron-demand intramolecular hetero-Diels–Alder reaction. The straining of the DEF-ring moiety is due to the ethylene bridge between C15 and C19, making the C2–C17 alkene highly reactive. It can be oxidized to its epoxide in ambient air, making the molecule highly unstable.[106]

4 Identification of molecular mechanisms of action of microtubule stabilizers: interruption of cellular signalling

4.1 Mitosis

Microtubule targeting agents are often referred to as mitotic poisons because of their ability to interrupt the formation of a bipolar spindle leading to cell death due to mitotic failure.

However, the mechanisms by which microtubule stabilizers lead to aberrant mitotic spindle formation followed by cell death is poorly understood. Many studies evaluating the effects of microtubule stabilizers on mitotic pathways have involved genome-wide association studies or arrays. One study used gene expression profiling combined with pathway analysis to examine the effects of sagopilone, an epothilone analogue that advanced to clinical trials (Table 1), in a series of well-characterized primary NSCLC patient-derived xenografts.[111] An increase in the basal expression of genes involved in cell adhesion/angiogenesis was found in tumours that did not respond to sagopilone.[111] In those that did respond, they saw an increase in genes related to mitotic arrest. They also showed that mutations in the *TP53* gene or low *TP53* mRNA levels, which codes for the tumour suppressor gene p53, led to increased sagopilone responses.[111] p53, which normally regulates the transcription of genes related to the induction of cell cycle arrest, apoptosis, senescence, and DNA repair, is one of the most mutated genes in cancer. This study shed some light into some of the factors that might be associated with sagopilone responses in patients. However, no functional studies were done to confirm the array results. Another study used an siRNA-based drug modifier screen of 300 genes to investigate the mechanism behind sagopilone-induced mitotic arrest.[112] The results show that sagopilone-induced mitotic arrest could be enhanced by inhibition of MCAK (a kinesin like protein that promotes microtubule depolymerization). However, the activity of sagopilone was reduced in combination with an Eg5 inhibitor (a microtubule associated motor protein involved in centrosome separation). Another study comparing the effects of 3 classes of diverse microtubule stabilizers, paclitaxel, taccalonolide AJ and laulimalide, showed that these stabilizers caused marked differences in the expression of key mitotic kinases involved in the formation and maintenance of the mitotic spindle.[67] This work highlighted for the first time the distinct effects of each of these compounds on Aurora A and Plk1 dependent pathways, both of which are critical for formation and function of the mitotic spindle. The taccalonolides are the only stabilizers that cause centrosome disjunction defects, most likely through inhibition of Plk1 signalling.[67] Interestingly, each of these agents caused very different effects on the phosphorylation of Eg5, with taccalonolide AJ causing a dramatic inhibition of phosphorylation. This highlights the fact that while all microtubule stabilizers lead to aberrant mitosis they do so in distinct ways. While sagopilone requires centrosome separation to cause aberrant mitosis, taccalonolide AJ inhibits centrosome separation as part of its effects on the mitotic spindle. While microtubule stabilizers can no longer be referred to purely as mitotic poisons due to their effects on interphase cells described below, it remains true that when cells enter mitosis in the presence of these agents they are unable to properly complete mitosis, resulting in cell death. Therefore delineating the mitotic mechanisms of microtubule stabilizers continues to be of importance.

4.2 Interphase

The clinical success of the microtubule stabilizers and their classification as mitotic poisons spurred the development of targeted small molecule inhibitors of mitotic kinases. The goal behind these efforts was to identify compounds with the clinical efficacy of the taxanes and epothilones while avoiding some of the dose-limiting toxicities associated with tubulin targeting agents. However, while many of these agents showed great promise in preclinical models, clinically, none of these new tubulin independent antimitotics showed the efficacy of the taxanes. One clue as to why targeted antimitotic agents work more effectively in culture than in patients is the fact that patient tumour doubling times range from 114–391 days, whereas cancer cells *in vitro* divide roughly every 1–5 days.[11]

Therefore, mechanisms other than inhibition of mitosis (Fig. 5) must exist to fully explain the anticancer effects caused by paclitaxel. One of the challenges in studying the mechanisms of action of microtubule targeting agents is the high cell-to-cell variability observed. The Mitchison laboratory developed high-resolution *in vivo* microscopy to visualize cellular events in single cells in murine xenograft tumours.[113] As expected, treatment with paclitaxel caused tumour regression but interestingly, the peak mitotic index in the xenografts was much lower than what was observed with the same cell line in culture. These data suggest that tumour regression is likely occurring through mechanisms other than mitotic arrest.

To fully understand these alternative mechanisms of action, it is useful to consider the multiple cellular roles of microtubules, including intracellular transport. The innate polarity of microtubules is used by molecular motors to move cargo throughout the cell. Dynein moves cargo along microtubules towards the (−) ends, toward the nucleus. Kinesins, on the other hand move along microtubules towards the (+) ends of microtubules, toward the plasma membrane. Cargos transported along microtubules include secretory vesicles, signalling molecules and components essential for cellular growth. In recent years, numerous studies have demonstrated that microtubule stabilizers impact processes and signalling events that are dependent on intact microtubule structures.[114–117] Clearly, the molecular mechanisms of microtubule stabilizers are not simple, because all cells contain tubulin but not all cancers respond to microtubule targeting agents. It is likely then, that microtubule stabilizers act to interrupt microtubule-dependent signalling processes that are essential for cancer cell survival (Fig. 5). Moreover, these pathways will differ among cancers and even within cancer subtypes.

The ability of paclitaxel to inhibit epidermal growth factor receptor (EGFR) internalization and endocytic trafficking was evaluated using quantum dot tracking in A549 lung cancer cells.[118] EGFR is a receptor tyrosine kinase and has been implicated in driving the oncogenic phenotype of a variety of cancers, including lung cancer. EGFR's hyperactivation leads to uncontrolled cell proliferation. Single cell analysis showed that paclitaxel suppressed perinuclear, endocytic trafficking of EGFR and promoted EGFR's degradation in lysosomes. This begins to shed some light into the possible mechanisms of the taxanes in the treatment of lung cancers. Work by the Giannakakou laboratory found that microtubule integrity and dynamics are involved in HIF1α translation.[116] They showed that HIF1α mRNA traffics along dynamic microtubules during active translation and that paclitaxel stalls this transport and releases HIF1α mRNA from polysomes, suppressing its translation.[116] This work provided evidence for the first time that suppression of microtubule dynamics can regulate protein translation. A subsequent study by the same laboratory showed that HIF1α protein can also associate with microtubules and is carried into the nucleus by dynein.[117] Treatment with paclitaxel impaired this nuclear localization. Additionally, in renal cell carcinoma, a cancer where HIF1α is overexpressed due to mutations in the VHL gene, this microtubule-dependent regulation of HIF1α nuclear transport is lost.[117] Therefore, microtubule stabilizer treatment could no longer prevent nuclear accumulation of HIF1α. It is interesting to note that microtubule stabilizers are not clinically effective in patients with renal cell carcinoma.

The most studied of the pathways affected by microtubule stabilizers is the androgen receptor pathway. The taxanes are one of the few chemotherapy options available for the treatment of hormone refractory prostate cancer. In fact, docetaxel was the first chemotherapeutic agent that increased the survival of patients with hormone refractory prostate cancer and the subsequent approval of cabazitaxel in 2010 reinforces the efficacy of microtubule stabilizers in this tumour type. However, the mechanism behind this activity was poorly understood. Huang and colleagues showed that paclitaxel or docetaxel treatment of 22RV1 prostate cancer cells led to a decrease in the expression of androgen receptor activated genes like Nkx3.1 and increased expression of the androgen receptor repression genes, maspin and FOXO1.[114] This was the first study to reveal a previously uncharacterized, FOXO1-mediated androgen receptor inhibitory effect of paclitaxel in castration resistant prostate cancer cells, which may also be playing a role *in vivo*.[114] A subsequent study by Kyprianou *et al.* compared tissue microarrays from docetaxel or untreated prostate cancer patients and found that docetaxel-treated tumours had lower levels of nuclear androgen receptor.[119] These results were validated *in vitro* and further studies demonstrated that the androgen receptor associates directly with microtubules, suggesting microtubules act to sequester the androgen receptor in the cytoplasm preventing its transcriptional activation.[119] The Giannakakou laboratory then reported that the taxanes can inhibit the nuclear translocation of the androgen receptor and that this was prevented in cells with β-tubulin mutations that result in paclitaxel resistance.[115] The study was corroborated by analysis of circulating tumour cells from hormone refractory prostate cancer patients receiving taxane treatment. Significant correlation between response rates and the ability of a taxane to inhibit androgen receptor nuclear accumulation was shown. This suggests that the taxanes are effective against prostate cancer, at least in part by preventing the nuclear accumulation and transcriptional activity of the androgen receptor.[115]

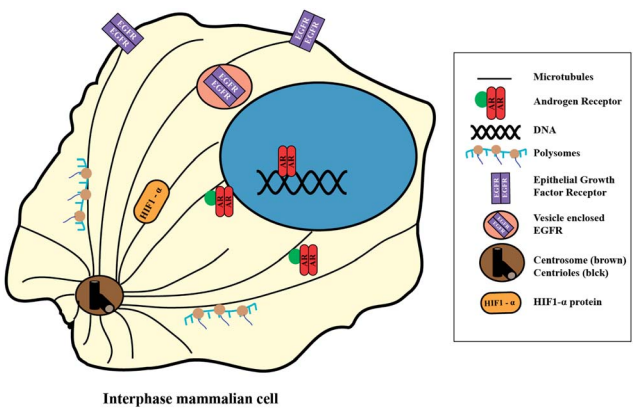


Fig. 5 Cellular processes dependent on the interphase microtubule network and shown to be disrupted by microtubule stabilizers.

These studies, demonstrating the ability of microtubule stabilizers to disrupt microtubule-dependent signalling events that drive cancers, highlights the interest in identifying whether diverse microtubule stabilizers differentially inhibit key dysregulated signalling pathways in cancer. If this is the case, it may lead to more personalized therapy based on the ability of a microtubule stabilizer to inhibit specific pathways that contribute to a patient's cancer.

5 Binding sites and molecular effects of microtubule stabilizers: structural insights into the binding and downstream effects on lateral and longitudinal associations of tubulin heterodimers

The vast majority of microtubule stabilizers have been shown to bind within one of two distinct, non-overlapping sites on microtubules. The classical taxane binding site has been mapped to β-tubulin on the interior lumen of the microtubule (Fig. 6). The second, microtubule stabilizer binding site on β-tubulin is known as the laulimalide/peloruside A site, which is found on the exterior of the microtubule (Fig. 6). More recently, another low-affinity taxane binding site has also been identified on the outside of the microtubule,[120–122] which facilitates the entry of taxane-site binding agents into the microtubule lumen (Fig. 6). A recent review by Fields and colleagues[123] details the efforts made in identifying the three distinct microtubule stabilizer binding sites.

5.1 Taxane binding: pore and luminal sites

Taxane site agents are so named for their ability to bind β-tubulin at the paclitaxel site in the interior lumen of the microtubule.[124] A variety of methods have been used to identify residues R284, H229 and V25 on β-tubulin as some of the specific residues involved in taxane binding within the microtubule lumen.[124–127] Microtubules are usually composed of 13 protofilaments, although this can vary. While docetaxel and paclitaxel differ in structure at two sites, they induce the formation of microtubules with 12 and 13 protofilaments respectively.[128] This finding demonstrates that even nearly identical compounds, which bind within the same pocket on β-tubulin can exert different effects on the lateral associations between protofilaments that impact global microtubule structure. Recently, a set of modified taxanes was used to study the structural mechanisms of differential microtubule stabilization.[129] Small angle X-ray scattering was employed to evaluate how modifications in the size and shape of taxane substituents affected changes in protofilament number and angles. These studies found that modifications at C7 and C10 of the taxane backbone influence elements involved in protofilament lateral interactions: the M-loop, the S3 β strand, and the H3 helix.[129] Modifications at C2 caused a rearrangement of the ligand in the taxane binding site, which changed the interaction of C7 with the M-loop.[129] The finding that differences at C2 affect regions known to be involved in lateral protofilament interactions explains differences in the structures of microtubules formed in the presence of paclitaxel or docetaxel. These differences may be responsible for lack of cross-resistance clinically.

In addition to the classical taxane-site binding agents, other microtubule stabilizers that bind within or near the taxane pocket have been identified, including discodermolide. The Horwitz laboratory utilized hydrogen deuterium exchange

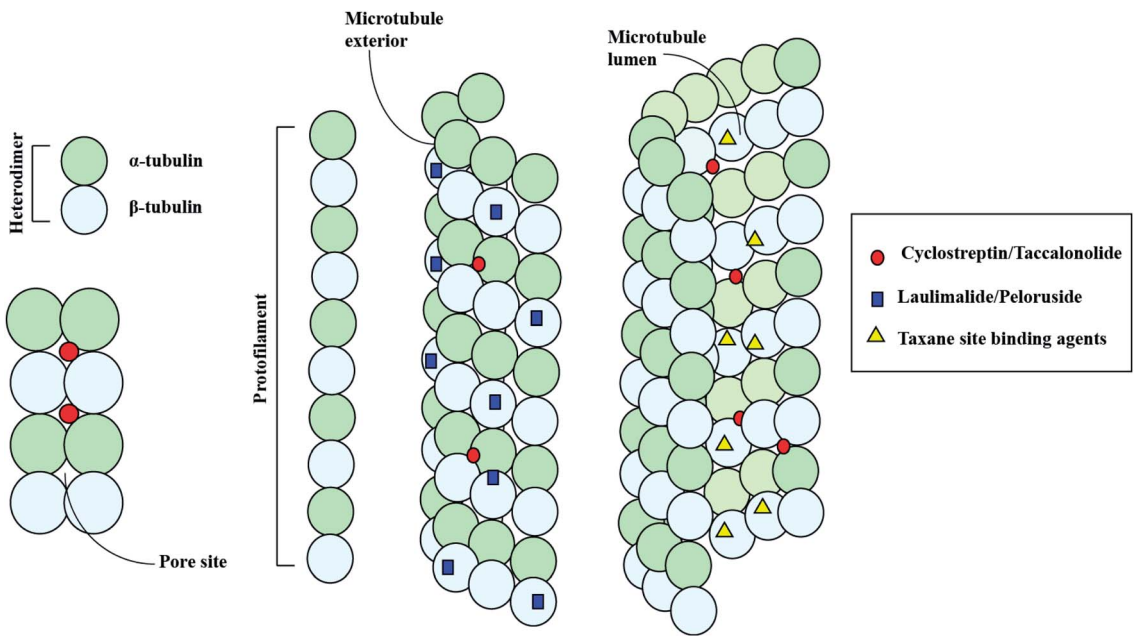


Fig. 6 Microtubule structure and the binding sites of microtubule stabilizers.

combined with mass spectrometry to determine that discodermolide binds to the taxane pocket in the microtubule lumen by way of a distinct binding mode.[130] Like paclitaxel, discodermolide led to stabilization of the intradimer interface, including Helix H10 and the H10-S9 loop in β-tubulin, however discodermolide was less effective than other taxane-site binding agents.[130] Both discodermolide and paclitaxel enhance the stability of microtubules by strengthening protofilament-protofilament interactions on the interdimer surface, albeit through distinct mechanisms. While paclitaxel stabilized lateral protofilament interactions through its interaction with the M-loop of β-tubulin, discodermolide instead interacted with the N-terminal H1-S2 loop, stabilizing microtubules mainly by affecting the interdimer contacts on α-tubulin and to a lesser extent on the interprotofilament contacts between adjacent β-tubulin subunits.[130] A series of hybrid discodermolide-paclitaxel molecules was synthesized and found to bind more tightly within the taxane pocket, leading to improved biological activities compared to discodermolide.[44]

Zampanolide and its less potent analogue dactylolide represent another class of microtubule stabilizers that interact with the taxane binding site in a manner distinct from the classical taxane-site binding agents due to their ability to covalently bind β-tubulin. Both compounds were shown to bind covalently to the N228 and H229 residues of β-tubulin in a stoichiometric ratio within the taxane pocket.[131] In spite of this distinct mode of binding, the allosteric effects imparted by zampanolide binding are similar to classical taxanes in that they interact with and stabilize the M-loop of β-tubulin to impart interprotofilament stability.[131] Interestingly, zampanolide was able to interact with these same residues in both dimeric tubulin as well as in intact microtubules, demonstrating that the taxane site is accessible in dimeric tubulin, which likely plays a role in the increased microtubule nucleation observed in the presence of taxane-site binding agents. This is consistent with previous suggestions that taxane-site agents, including docetaxel and discodermolide, can bind to and enhance the nucleation of dimeric tubulin.[132] Recently, high resolution crystal structures of α/β-tubulin heterodimers in the presence of zampanolide or epothilone A have been achieved.[131] Both compounds were deeply buried in the taxane pocket and caused helical structuring of the M-loop, which plays a critical role in the microtubule stabilization elicited by these and most other taxane-site binding agents.

While it has long been established that taxanes bind β-tubulin with high affinity on the interior lumen of the microtubule, additional data suggested they are able to access this site only after initially binding to a low-affinity site on the exterior of the microtubule.[133,134] A clue to how the taxanes might gain entry to the microtubule lumen was uncovered with the identification of the cyclostreptin binding sites on microtubules. Cyclostreptin covalently binds to the luminal taxane binding site at the N228 residue of β-tubulin, inhibiting the binding of taxanes to microtubules.[120] However, cyclostreptin has also been shown to covalently bind to residue T220 on β-tubulin, which is located in a small pore in the microtubule wall in close proximity to the taxane site.[109,120] It is believed that this pore site is a transient, low-affinity binding site for taxane-site agents that facilitates their entry into the lumen of the microtubule, which was only readily identifiable through characterization of a substrate like cyclostreptin that became covalently bound and "trapped" on its way through the pore. Interestingly, cyclostreptin can bind to the pore site in unpolymerized tubulin heterodimers, although the luminal site is only bound in the intact microtubule.[120] This finding has led to speculation that the weak binding of taxanes to the pore site in tubulin heterodimers may play a role in their ability to enhance microtubule nucleation.[109,135] However, it is interesting to note that cyclostreptin, which binds exclusively to the pore site in unpolymerized tubulin, is a particularly weak nucleator of microtubules compared to other taxane-site binding agents.[109,135] Interestingly, a third class of microtubule stabilizers, the taccalonolides, have recently been demonstrated to covalently bind to the 212–230 peptide on β-tubulin that contains both the luminal and pore taxane binding sites.[26] Unfortunately technical limitations prevented a determination of the exact residue(s) bound by this class of microtubule stabilizers. Similarly to cyclostreptin, the taccalonolides do not efficiently nucleate microtubules in biochemical preparations. However, their covalent binding to microtubules results in the formation of strikingly stable microtubules that are highly resistant to depolymerisation due to a marked lateral interprotofilament stability that is independent of the M-loop stabilisation observed with most other classes of microtubule stabilizers.[26] Further studies detailing the specific residues impacted by taccalonolide binding to microtubules are ongoing. The manner in which microtubule stabilizers, including the taxanes and cyclostreptin, interact with the microtubule pore site has been extensively studied both experimentally and by molecular modelling and a variety of distinct models have been put forth. Calvo[109] and colleagues conjugated thiol-reactive chloroacetyl groups onto cyclostreptin to allow for additional covalent linkages and the potential to further refine the binding site of cyclostreptin on microtubules. Each of the three analogues reacted with a distinct subset of β-tubulin residues, inducing the same T220 pore site and the N228 luminal site modified by the natural product. In addition, the thiol-reactive group on two of the analogues reacted with C241, which is near the luminal taxane binding site.[109] Although C241 is in close proximity to the taxane pocket, this residue was previously thought to be shielded from taxane-site binding agents by the B9–B10 loop of tubulin. The authors propose that binding of cyclostreptin proceeds from the T220 pore site to the N228 residue inside the microtubule lumen and finally to the extended luminal site at C241, although the ability of non-modified cyclostreptin to interact in this extended pocket has not been determined. Interestingly, C241 is a known reactive residue in β-tubulin as it has been shown to bind to modified colchicines, which destabilize microtubules.

Freedman and colleagues proposed that drug binding at the pore site involves binding of the taxane core to β-tubulin subunits in two adjacent protofilaments while the taxane side chain binds to a single α-tubulin subunit.[121] Magnani *et al.* however, proposed that the taxane core is only bound to a single

β-tubulin subunit with the side chain bound to α-tubulin.[136] Diaz aimed to better characterize the interactions of taxane-site binding agents within the pore site[122] using hexaflutax, a fluorescent taxane derivative that binds to the external pore site on microtubules with no observable modification of the interior luminal site. The kinetic techniques used to measure the interaction of hexaflutax with the pore site revealed it does so in two distinct ways, likely due to different rearrangements of taxane and fluorescein binding; one in which interactions are made only with β-tubulin subunits and another in which the interactions are made with both α and β-tubulin subunits.[122] This work showed that both binding interactions proposed separately by Freedman and Magnani are indeed possible. Hexaflutax led to the characteristic microtubule bundling and mitotic arrest seen with other microtubule stabilizers, albeit with much lower potency than cyclostreptin, suggesting that binding at the pore site may be sufficient for microtubule stabilization.[122] This work has led to the hypothesis that the pore site on microtubules may be a new pharmacological target for microtubule stabilization.

With the identification of two distinct taxane-binding sites on microtubules, much work has been done to try to understand the role of each site in drug binding and resulting microtubule stabilization. However, in spite of these data, there still remains controversy regarding the function of the pore site in taxane binding and its role in microtubule stabilization. Snyder and colleagues proposed that the microtubule pore functions simply as a funnel to slow down microtubule stabilizers' diffusion through the pore without any specific binding.[135] They argue that the majority of cyclostreptin binding occurs at the pore site, which leads to a weak effect on tubulin polymerization in biochemical assays, suggesting that although binding to this low-affinity site may be important for taxanes to gain access to the microtubule lumen, binding to the pore site may not be involved in their mechanism of microtubule stabilization.[135] It will be interesting to further characterize the role of both the pore and luminal taxane-binding sites in microtubule nucleation, stability and overall dynamicity.

5.2 Laulimalide/peloruside site

Early synergism and binding studies suggested that laulimalide and peloruside A shared a binding site that was distinct from that of the taxanes.[60,61,137–139] However, the electron crystallographic methods used to identify the taxane binding site were unsuccessful in the identification of the laulimalide binding site.[140] Initial modelling studies suggested peloruside A bound to α-tubulin in a location equivalent to the paclitaxel site on β-tubulin.[129,141] However, other studies using hydrogen–deuterium exchange mass spectrometric techniques in combination with data-directed computational strategies were used to propose that peloruside A binds to β-tubulin on the exterior surface of the microtubule.[142,143] Like epothilone A and docetaxel, peloruside A was found to stabilize microtubules through strengthening of the longitudinal interactions at the interdimer surface but, in addition, it was also able to stabilize interactions at the intradimer interface.[142]

To further map the binding site of peloruside A/laulimalide on microtubules, laulimalide and peloruside A resistant 1A9 ovarian cancer cell lines were generated.[144] The resistant cell lines showed mutations in R306H, R306C or A296T in the β-I isotype of tubulin, for the first time providing cell-based evidence for peloruside A and laulimalide binding to β-tubulin.[144] Shortly afterward, a separate study showed that peloruside A resistant ovarian cancer cell lines contained R306H, Y340S, N337D and A296S mutations in the β-I isotype of tubulin.[145] These results support a binding site for laulimalide and peloruside A on β-tubulin on the exterior of the microtubule surface in accordance with that proposed by the Schriemer laboratory.[143]

5.3 Mechanism of microtubule stabilization by drug binding

Two alternative models for microtubule nucleation in the presence of microtubule stabilizers have been proposed. Compounds like the epothilones, taxanes and discodermolide, which do not bind covalently, bind much tighter to formed microtubules than they do to unassembled tubulin.[83] The higher energy of binding to the formed microtubule shifts the equilibrium towards microtubule polymerization independent of any structural effects caused by the drug on the polymerized microtubule. On the other hand, compounds that bind covalently to microtubules displace this equilibrium through a structural allosteric effect in which the modified tubulin has a higher affinity towards polymerized microtubules than the unmodified fraction of tubulin.[83]

Hydrogen deuterium exchange studies were performed with a diverse group of microtubule stabilizers including epothilone B, ixabepilone, laulimalide and peloruside A to determine their allosteric effects on microtubule conformation.[146] This work showed that all 4 microtubule stabilizers led to significant conformational effects on the C-terminal H12 helix of α-tubulin.[146] From these results, it was proposed that the major mode of microtubule stabilization by these agents involves longitudinal interactions at the interdimer interface. However, another study showed that the effects on lateral interactions were distinct between stabilizers that bind to the peloruside A/laulimalide site *vs.* those that do not. Therefore, although each of these microtubule stabilizers exert similar effects on microtubule structure and stability, they also have subtle differences that likely lead to the synergistic actions observed between microtubule stabilizers that bind to the distinct sites on tubulin.

6 Mechanisms of drug resistance

While microtubule stabilizers have had great success in the clinic, both innate and acquired resistance limits their clinical utility. A variety of mechanisms have been linked to microtubule stabilizer resistance, including overexpression of the multi-drug resistance 1 gene (*MDR-1*), which encodes the Pgp drug transporter, mutations in drug binding sites on tubulin or alterations in tubulin isotype distribution, altered expression of

microtubule regulatory proteins, aberrant expression of miRNAs and impairment of apoptotic pathways.

6.1 β-tubulin isotypes and mutations

Tubulin isotypes share a high degree of homology; they are primarily distinguished from one another by their divergent carboxy terminal tails. The 6 α-tubulin and 8 β-tubulin isotypes are expressed in a tissue specific manner. βI and βIVb are expressed constitutively in all tissues. βII, βIVa and βIII isotypes are exclusively expressed in the brain. The βV isotype is expressed at low levels in all tissues and βVI is expressed specifically in hematopoietic tissues. Mutations in βI are often observed *in vitro* when cells become resistant to microtubule stabilizing agents. Studies have shown that cancer cell lines with βI mutations seen in cancer patients become resistant to paclitaxel,[147] however, there is no evidence that these mutations are clinically relevant as they are rarely seen in patients. Regardless, identification of tubulin mutations that lead to drug resistance has been useful in mapping microtubule stabilizer binding sites.

While βIII-tubulin is normally specifically expressed in neuronal tissues, its aberrant expression in tumours has been associated with clinical taxane resistance in lung, ovarian, breast, prostate and pancreatic cancer. Additionally, high levels of βIII-tubulin expression correlate with a worse prognosis in breast, ovarian and lung cancers and with tumour aggressiveness in prostate cancer patient tumour samples.[148] Most strikingly, βIII-tubulin expression was found to be an independent marker of biochemical recurrence after docetaxel treatment as well as an independent predictor of lower overall survival in castration resistant prostate cancer patients receiving docetaxel.[149] These findings were validated *in vitro* in a panel of prostate cancer cell lines, which suggested a role for βIII as a candidate biomarker to predict response to docetaxel chemotherapy. Expression of βIII-tubulin has also been shown to be a marker for poor overall survival in ovarian cancers and uterine serous carcinomas after taxane chemotherapy.[150–152] Surprisingly, cell lines derived from these same primary tumour samples were particularly sensitive to epothilones,[152] potentially identifying a subset of individuals that would benefit from a course of treatment that includes an epothilone. Low expression of βIII-tubulin was shown to be associated with increased sensitivity of NSCLC cells to paclitaxel without affecting microtubule dynamics.[153] Additionally another study showed that low βIII expression reduced anchorage independent growth of NSCLC cells and decreased the incidence of tumour progression in xenograft models. This study provided the first evidence specifically linking βIII levels directly to the regulation of anchorage-independent growth of cancer cells.[154] A more recent study of βIII expression in breast cancers of different histological grade suggested a role for βIII as a predictive biomarker for response in neoadjuvant chemotherapy for ER negative breast cancers.[155] This is the first study to show a positive association of high βIII levels with better prognostic factors, but only in patients with ER negative breast cancers and not those that had ER positive breast cancers. This work shows that tubulin isotype composition, in particular expression of βIII, can serve as either a negative or positive predictive biomarker of response, which is both cancer and subtype specific.

6.2 Microtubule associated proteins

Microtubules are highly dynamic structures and this dynamicity is tightly controlled by a variety of microtubule associated proteins that stimulate or inhibit microtubule polymerization. Therefore, aberrant expression of microtubule associated proteins could impact the sensitivity to microtubule stabilizers. It has been shown that when the microtubule destabilizing protein stathmin is overexpressed in BT549 breast cancer cells it results in a 29% decrease in microtubule dynamics and a 44% decrease in paclitaxel sensitivity.[156] The effects on paclitaxel sensitivity are not due to an effect on the cell's doubling times or mitotic index and instead are proposed to be due to altered protein expression. The expression of over 30 proteins was decreased in BT549 paclitaxel resistant-stathmin over-expressing cells.[156] A link between reduced levels of the intermediate filament protein vimentin and resistance to microtubule targeting agents has also been shown. While the mechanism underlying this link was not explored, the authors proposed that decreased vimentin, which plays an important role in cellular structure and organization in combination with microtubules, could lead to attenuation in signalling pathways required for cell survival and apoptosis.[157] Overexpression of the microtubule associated protein tau, which leads to tubulin stabilization, has also been shown to be associated with microtubule stabilizer resistance. The efficacy of both paclitaxel and ixabepilone is reduced with tau overexpression, but has no effect on peloruside A or laulimalide efficacy in mouse neuroblastoma cells.[158]

A direct link between paclitaxel treatment and apoptosis was demonstrated by the discovery that paclitaxel-stabilized microtubules serve as a scaffold for pro-caspase 8, which concentrates this protein, leading to proximity activation of an apoptotic cascade.[159] Additional studies showed that the paclitaxel resistance observed upon knockdown of the BRCA1 tumour suppressor was due to increased microtubule nucleation and dynamics that decreased the association of paclitaxel and therefore caspase 8 with microtubules.[160] These studies provide an important link in understanding the mechanism of interphase mediated cell death after paclitaxel treatment.

6.3 miRNA expression

In recent years the role of miRNAs in the regulation of a variety of processes critical to cancer cell survival and sensitivity to microtubule targeted agents has become evident. The role of miRNAs in microtubule stabilizer resistance has been recently reviewed by Kannakanthara and co-workers[161] and will only be discussed briefly. The microRNAs miR-125b, miR-221, miR-222 and miR-293 were found to be upregulated in paclitaxel resistant breast cancer cell lines.[162] Further investigations revealed that the pro-apoptotic protein Bak1 is a direct target of miR-125b.[162] Recently, upregulation of miR-106a and

downregulation of miR-591 were both found to be associated with paclitaxel resistance in ovarian cancer cell lines as well as in human tumour samples.[163] The authors proposed that the chemoresistance and poor patient survival associated with miR-106a is a result of BCL10 and caspase 7 inhibition, which are the direct targets of this miRNA. Additionally, they demonstrated that the role of miR591 in paclitaxel resistance is likely due to its suppression of ZEB1, a transcriptional repressor involved in inducing EMT.

6.4 Overcoming P-glycoprotein mediated resistance through covalent binding

The resistance of cancer cell lines and human tumours to a variety of microtubule targeted agents as a result of either innate or acquired expression of the Pgp drug export protein has been extensively studied.[164] Therefore, one of the most important considerations for new microtubule targeted drugs entering the clinic involves the ability to circumvent this common mode of resistance. One strategy employed to avoid Pgp-mediated resistance includes generating analogues that are poor substrates for the Pgp transporter. Compounds with these characteristics include TPI-287, cabazitaxel and sagopilone. However, another method of avoiding Pgp-mediated drug resistance in preclinical settings has been shown to involve covalent attachment of the drug to its target. This is the case for zampanolide, cyclostreptin and the taccalonolides which have demonstrated efficacy in Pgp expressing cell lines.[22,75,120] Although the possibility remains that these drugs may be substrates for Pgp, their covalent linkage to microtubules effectively inhibits their ability to be exported from the cell once they are bound. Interestingly, this irreversible binding may also help to circumvent other forms of drug resistance that involve decreased drug binding affinity. Although there may be unexpected negative consequences associated with covalent attachment of microtubule targeted agents to microtubules, it will be interesting to determine whether this strategy of circumventing drug efflux by covalent binding will be effective in clinical settings. As we learn more about the mechanisms involved in microtubule stabilizer-mediated resistance, it will inform the optimal use of these microtubule stabilizers in the clinical setting.

7 Possible new indications for microtubule stabilizers

7.1 Neurodegenerative diseases

The nerve damaging effects of microtubule stabilizers are well known and are responsible for many of their dose limiting toxicities. Microtubule stabilizer-induced axonal degeneration occurs most often in peripheral sensory neurons causing peripheral neuropathy. An excellent review of the nerve-damaging effects of paclitaxel and potential remedies was published in 2013.[165] It was of some surprise, given these effects of paclitaxel and other microtubule stabilizers on peripheral nerve damage that these compounds are now being evaluated in the treatment of neurodegenerative diseases. Neurons are the longest cells of the body and in humans can reach over a meter in length for the nerves that innervate the extremities. The cytoskeleton, and microtubules in particular, play an essential role in maintaining cell structures and in axonal transport of material to and from the cell body (Fig. 7). In axons, microtubules form polarized linear arrays with their (+) ends towards the synapses and their (−) ends towards the cell body (Fig. 7). Microtubules and the associated kinesins and dynein transport vital cellular materials, including nutrients, proteins and organelles throughout the length of the neuron (Fig. 7). As has been noted by others,[1] it is interesting that the peripheral neuropathies caused by microtubule targeting drugs occur in the distal extremities, the hands and feet, the longest distances for axonal transport. The microtubule associate protein, tau, is expressed in nerves and found most predominantly in axons. Functionally, tau stabilizes microtubules and its microtubule binding capacities are controlled by phosphorylation. Abnormal filamentous hyperphosphorylated tau is implicated in numerous neurodegenerative, including Alzheimer's and Parkinson's diseases and others, which are designated tauopathies. The intracellular neurofibrillary tangles found in Alzheimer's disease patients are composed of filaments of hyperphosphorylated tau. The exact mechanisms of tau-induced neurodegeneration are under investigation, and some evidence suggests that tau-induced loss of fast anterograde transport occurs when excess tau binds to microtubules, which displaces kinesins, thus inhibiting kinesin-mediated transport.[166] The hyperphosphorylation of tau prevents its association with microtubules, leading in some cases to microtubule severing and destabilization.[167] These effects might contribute to axon degeneration, a key characteristic of tau-initiated pathologies.[167] Axon degeneration often precedes cognitive defects[166,167] and a mechanism to prevent axon loss could slow the cognitive defects associated with these diseases. Multiple therapeutic strategies are being employed, including reduction of tau levels, aggregates or hyperphosphorylation.[166] Another approach has been to evaluate the efficacy of microtubule stabilizers as a mechanism to recapitulate the normal microtubule stabilizing functions of tau. First proposed almost 20 years ago,[168] several studies have evaluated the ability of microtubule stabilizers to reverse tau loss of function. Two limitations include the inability of most microtubule stabilizers to cross the blood brain barrier and the potential for neurotoxicity.

Epothilone D (BMS-241027) is one of the few microtubule stabilizers that can cross the blood brain barrier.[169] A transgenic mouse model of tau-pathology was used to evaluate the effects of weekly, long term (3 month), administration of 1 or 3 mg kg^{-1} of epothilone D. The results showed dose-dependent loss of dystrophic axons and the restoration of microtubule density, axonal trafficking and cognitive defects.[170] The activity of epothilone D was also evaluated in an MPTP-induced model of Parkinson's disease.[171] MPTP is a neurotoxin commonly used because it caused permanent symptoms of Parkinson's disease in animal models. This neurotoxin caused axonal impairment in the dopaminergic neurons together with posttranslational modifications in α-tubulin and an increase in the levels of the

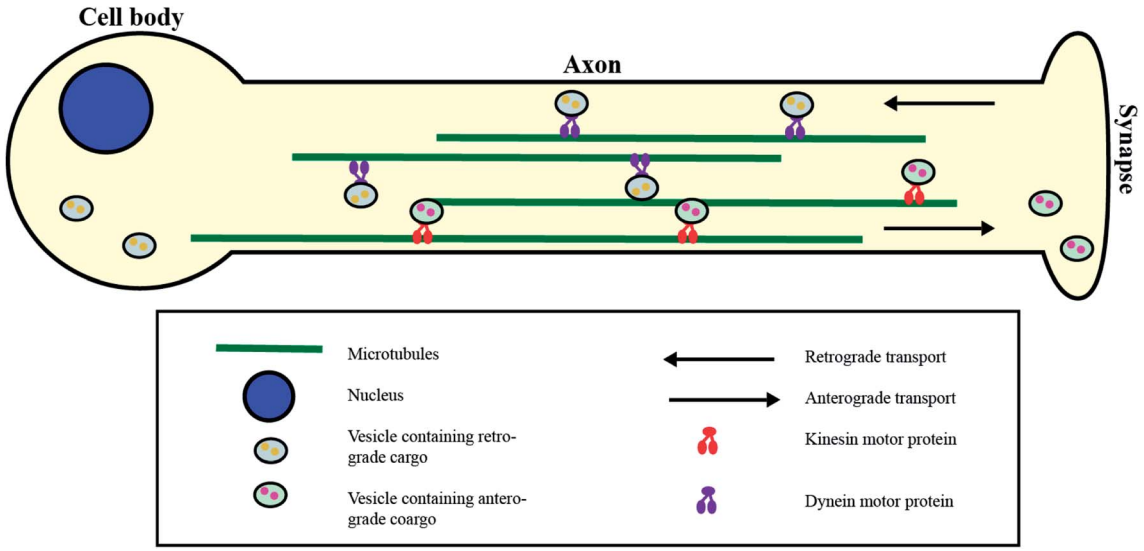


Fig. 7 Microtubules are key components of neurons.

βIII isotype of tubulin.[171] Epothilone D rescued these microtubule defects, and inhibited neuronal degeneration. An *in vivo* deuterium-labelling technique was used to evaluate brain cortical microtubule dynamics in transgenic mouse tau-pathology models.[170] In these models the dynamicity of the microtubules increased with age in a tau-dependent manner. Low dose epothilone D (1 and 10 mg kg^{-1} weekly for 3 months) was able to restore hyperdynamic microtubule turnover to baseline levels. Neurofibrillary tangles were reduced in the 1 mg kg^{-1} treatment group and neurocognitive defects were alleviated.[170] Although the clinical trials evaluating epothilone D for anticancer indications were discontinued in 2007 due to poor efficacy and severe neurological side effects, these preclinical studies led to a phase I clinical trial (NCT01492374) of low dose epothilone D in Alzheimer's patients.[172]

Studies have examined the ability of paclitaxel to rescue or prevent damage to *Aplysia* (mollusk) neurons with human mRNA-induced-tau pathologies. Exposure to a low concentration of paclitaxel (10 nM) prior to the microinjection of human tau mRNA rescued the neurons from tau-initiated neuronal degeneration degradation.[173] However, a higher concentration of paclitaxel, 100 nM, was not able to prevent the neuronal degeneration. Additionally, timing of paclitaxel administration was crucial as exposure to paclitaxel after the neurons had been damaged by tau expression was not able to reverse the damage.[173] These *in vitro* studies are of interest, but have limited clinical application, since paclitaxel cannot cross the blood brain barrier. More recently, Das and Miller investigated the ability of peloruside A to prevent an okadaic acid induced model of tauopathies in rat neurons in culture.[174] Pretreatment with peloruside A prevented the effects of okadaic acid on axonal outgrowth and branching and rescued neurons from growth cone collapse. Peloruside A did not inhibit the okadaic acid-initiated phosphorylation of tau, but it did restore the levels of acetylated tubulin and reversed the repression of growth associated protein, GAP43 which regulates axonal growth.[174] These studies demonstrate that the use of low concentrations of microtubule stabilizers, much lower than are used for anticancer actions, might have efficacy in preventing or slowing tau-induced pathologies, based on the ability of these microtubule stabilizers to correct defects causes by tau hyperphosphorylation. This remains an exciting area of active investigation.

8 Conclusions

Microtubule stabilizers from diverse natural sources continue to be of value in the treatment of cancer and significantly augment newer generation targeted therapies. All indications suggest that these drugs will continue to be of value in the treatment of cancer in the future. The discovery and development of new generation microtubule stabilizers and new formulations may overcome some of the limitations of the first and second-generation drugs. As we learn more about the mechanisms of action, binding sites and mechanisms of resistance of the different microtubule stabilizers, we might be able to further understand patient responses, cross-resistance or lack thereof to similar drugs and why not all types of cancers respond to these drugs. Thus, the continued discovery of new microtubule stabilizers from nature will continue to be important.

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