Microtubule-binding agents: a dynamic field of cancer therapeutics.

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Preface

Microtubules are dynamic filamentous cytoskeletal proteins that are an important therapeutic target in tumor cells. Microtubule binding agents have been part of the pharmacopoeia of cancer for decades, and until the advent of targeted therapy microtubules were the only alternative to DNA as a therapeutic target in cancer. The screening of a variety of botanical species and marine organisms has yielded promising new antitubulin agents with novel properties. Enhanced tumor specificity, reduced neurotoxicity, and insensitivity to chemoresistance mechanisms are the three main objectives in the current search for novel microtubule binding agents.

Introduction

Microtubules play several key roles that are important in cell proliferation, trafficking, signalling, and migration in eukaryotic cells. For this reason several microtubule binding agents have been developed with different aims, including as pesticides, antiparasitics and anticancer agents. In mammalian cells microtubules are present both in interphase cells and in dividing cells. In the latter, microtubules constituting the mitotic spindle are highly dynamic and exquisitely sensitive to therapeutic inhibitors. This explains why compounds altering microtubule function have proven to be highly active in patients with cancer. The vinca alkaloids, identified over 50 years ago [1] and the taxanes, first isolated almost 40 years ago [2,3] are currently administered in a large variety of indications including solid tumors and haematological malignancies [4-6]. They are most often integrated in combination chemotherapy regimens, including in some curative regimens, for example in patients with non-Hodgkin's lymphoma. Taxanes have become an essential component in the adjuvant and advanced setting of patients with breast cancer and are also extensively used in patients with ovarian cancer, non small cell lung cancer (NSCLC), and Kaposi's sarcoma[7,8].

A peculiarity of microtubule binding agents is their extreme structural diversity and, in many cases, structural complexity (Figure 1). It should be stressed that many agents were isolated from marine organisms or botanicals which are not cultivated, and in which they are present in minute amounts [9]. Many of the most active agents such as taxanes were difficult to develop in the clinic due to scarcity of their natural sources (Pacific yew bark in the case of taxol), a problem which was in some cases later solved by partial or total synthesis of the compounds of interest, although total synthesis has not proven to be the best option for some compounds such as taxanes [10]. This problem is still prevalent today for many of the novel microtubule binding agents, explaining, at least in part, the slow clinical development of many of the newer agents [11-13].

In the age of small molecule targeted therapies and therapeutic monoclonal antibodies it is noteworthy that extensive resources and scores of clinical trials are still being devoted to the identification and evaluation of microtubule-targeted agents including taxanes, epothilones, vinca alkaloids, halichondrins, maytansinoids, colchicine-site binding agents, and others. This is partly due to the extremely large untapped reservoir of potential therapeutic natural compounds which influence microtubule dynamics and also to our growing understanding of the role of the microtubule cytoskeleton in cancer cells. After briefly reviewing mechanisms of action of and resistance to anticancer microtubule binding agents, we will focus on novel agents, in particular those that have recently been approved or reached the stage of clinical trials. An increasingly important issue is that of toxicity, since many of these agents cause significant neurological toxicity.

Mechanisms of action

A large number of chemically diverse substances generally originating from natural sources bind to tubulin and/or microtubules (Table 1), altering microtubule polymerization and dynamics in diverse ways. A reasonable hypothesis is that plants and animals evolved this vast number of compounds

that mimic endogenous regulators of microtubule behavior in order to avoid predation. All of these compounds are antimitotic agents that inhibit cell proliferation by binding to microtubules and suppressing microtubule dynamics during the particularly vulnerable mitotic stage of the cell cycle (Figure 2). To document the suppressive effects of these agents on microtubule dynamics, most studies have used time-lapse microscopy to analyse interphase microtubules in live cells [14]. Spindle microtubule dynamics are more difficult to analyse because of microtubule density but may be indirectly evaluated by the study of centromere dynamics. [15,16] These studies have confirmed that inhibition of spindle and interphase microtubule dynamics occurred at the same concentrations as those inducing mitotic arrest (Box 1).

Depolymerizing vs. stabilizing agents

The microtubule-targeted antimitotic drugs are often classified into two major groups, the microtubule-destabilizing agents and the microtubule-stabilizing agents, according to their effects at high concentrations on microtubule polymer mass. The so-called "destabilizing" agents inhibit microtubule polymerization when present at high concentrations. Most of these agents bind in one of two domains on tubulin, the "vinca" domain and the "colchicine" domain (Table 1). Vinca site binders include the vinca alkaloids (vinblastine, vincristine, vinorelbine, vindesine, and vinflunine), the cryptophycins, the dolastatins, eribulin, spongistatin, rhizoxin, maytansinoids, and tasidotin. Colchicine-site binders include colchicine and its analogs, podophyllotoxin, combretastatins, CI-980, 2-methoxyestradiol, phenylahistins (diketopiperazine), steganacins, and curacins [17,18] . Some of the destabilizing agents, including the hemiasterlins, estramustine, noscapine, herbicides such as carbendazim, psychoactive drugs such as phenytoin, and food components such as sulforaphane found in cruciferous vegetables [19,20], bind to novel sites on tubulin. The "microtubule-stabilizing" agents enhance microtubule polymerization at high drug concentrations and include taxol (paclitaxel, Taxol™), docetaxel (Taxotere™), the epothilones, ixabepilone (Ixempra™) and patupilone, discodermolide, eleutherobins, sarcodictyins, cyclostreptin, dictyostatin, laulimalide, rhazinilam, peloruside A, certain steroids and polyisoprenyl benzophenones**.** Most of the stabilizing agents bind to the same, or an overlapping, taxoid binding site on beta tubulin which is located on the inside surface of the microtubule [21]. However, two of the agents, laulimalide and peloruside A, are not displaced by paclitaxel and for this reason are believed to bind to a novel site on tubulin [22,23]. Overall several hundred compounds have been reported to arrest mitosis by their effects on microtubules. In all cases where it has been investigated, they do so most potently by suppressing microtubule dynamics [24,25] .

Suppression of microtubule dynamics

Both classes of drugs, those that increase and those that decrease microtubule polymerization at high concentrations, potently suppress microtubule dynamics at 10 to 100-fold lower concentrations. The sensitivity of microtubule dynamics to regulation means that both kinds of microtubule-regulating drugs can kinetically stabilize the microtubules without changing the microtubule polymer mass. At a very basic mechanistic level, these two classes of drugs act similarly to block mitosis. Supporting this common mechanism of action is the finding that taxanes and vincas or estramustine can be combined clinically in chemotherapy regimens with no apparent antagonism [26-28]. In addition, combinations of taxanes with vincas, estramustine or colchicine analogs have shown synergism *in vitro* [29,30]. At high concentrations, there are clear differences in their cellular effects on microtubule mass [31]. However, to target cells as they enter mitosis in order to gain maximum therapeutic efficacy it may be important it may be more important to maintain a low drug concentration in the tumor cells or in their adjacent endothelial cells for a reasonably long duration than to achieve a brief pulse of high intracellular drug concentration [32].

Antiangiogenic and vascular-disrupting effects

The tumor vasculature is a superb therapeutic target as it is easily accessible to blood-borne drugs, and tumor cells generally die unless continually supplied with oxygen and nutrients from the blood.

The two approaches to inhibit vascular function are to inhibit angiogenesis (the formation of *new* blood vessels), and to destroy the integrity of *existing* tumor vasculature using vascular-disrupting agents [33]. Formation of new blood vessels involves both proliferation and migration of endothelial cells, and both of these processes appear to be extraordinarily sensitive to microtubule-targeted drugs [25,34]. It has been suggested that prolonged exposure times and frequent dosing of low concentrations of microtubule-targeted drugs, the so-called "metronomic" schedules, may favor the antiangiogenic properties of these agents but clinical confirmation of such an effect will require both randomized trials and the demonstration of an antiangiogenic effect in patients [32,35].

Since the late 1990's, the combretastatins and N-acetylcolchicinol-O-phosphate, compounds that resemble colchicine and bind in the colchicine domain on tubulin, have undergone extensive development as vascular-disrupting agents [36]. When combretastatin-A-4 phosphate (CA-4-P) is added to cultures of endothelial cells, microtubules rapidly depolymerize, cells become round within minutes, bleb and detach [37]. When administered to rodents, the bloodflow may drop by >95% in less than an hour, vascular permeability increases and haemorrhaging from peripheral tumor vessels occurs [38-40]. These vascular-disrupting agents appear to be fairly specific for tumor vasculature although the reasons for this specificity are not known. Since the targeted endothelial cells are non-tumor cells, a potential advantage of this approach is that the cells may be less susceptible to the development of resistance to these drugs than genetically unstable tumor cells. The development of these agents has also prompted novel methods aiming to evaluate changes in tumor perfusion, such as dynamic MRI measurements of gadolinium diethylenetriaminepentaacetate uptake and washout, and positron emission tomography of ^{15}O-labeled water or dynamic contrast enhanced magnetic resonance imaging. [40-42]

Several currently-used microtubule-targeted agents, such as the vinca alkaloids, damage tumor vasculature in animal models. It is our belief that the difference between these classical anti-mitotic anti-proliferative microtubule-targeted agents and the novel agents that are undergoing clinical testing as vascular-disrupting agents may rely on the fact that the effects of novel vascular-disrupting agents are more rapidly reversible, either because of the reversibility of their binding to tubulin, or their lack of long-term retention in cells. Those agents which exert depolymerizing effects over a short period of time may act best as anti-vascular agents while those that are retained and induce a long-term mitotic arrest may work best as antiproliferative agents.

Mechanisms of resistance

Understanding mechanisms of resistance to microtubule-binding agents is a key element in the development of novel, more potent microtubule-targeted compounds. Resistance to microtubule-binding agents can occur at several levels in the pharmacodynamics of these agents, including primarily cellular efflux of the anticancer agents, ineffective interaction with the target, and deficient induction of apoptosis. In addition, resistant tumors and cell lines show a multitude of changes in protein and microRNA expression whose relationship to the actions of microtubules is not always easy to discern.

ABC proteins and drug efflux

Membrane efflux pumps of the ATP binding cassette (ABC) family represent the primary resistance mechanism developed by tumor cells when these are exposed to microtubule binding agents *in vitro* [43]. While Pgp, the product of the mdr1 gene is responsible for the "classical multidrug resistant phenotype" (MDR) and actively effluxes both vincas and taxanes, thereby reducing their intracellular concentrations and cytotoxic activity, other transporters transport only some types of antitubulin agents. Vincas are actively transported by the MRP1 protein, taxanes are substrates for MRP2 and

MRP7, and epothilone B is transported by MRP7 [44-46]. Given the potential importance of these efflux pumps as mechanisms of resistance to chemotherapy, newer agents which are insensitive to active efflux have been identified and further developed (Table 1). The clinical relevance of ABC pumps in patients with cancer remains controversial, with limited data to support the routine study of these proteins in patients [47]. While the expression of these pumps in primary tumors often correlates with a lower response rate to therapy with microtubule-targeted agents, the presence and/or function of ABC proteins in clinical samples is not generally used to tailor therapy in individual patients due to difficulties in standardizing assays [48,49]. Attempts to reverse drug resistance by combining microtubule agents with inhibitors of drug efflux proteins have been disappointing [50]. Conversely, the fact that microtubule-binding agents constitute substrates for ABC efflux pumps significantly limits their diffusion inside the central nervous system, and constitutes an obstacle to their oral administration, suggesting that novel compounds which are less susceptible to transport by ABC proteins could possess original pharmacokinetic profiles [51].

Alterations in microtubules

A second level of resistance to antitubulin agents consists in alterations in the target of these agents, the tubulin/microtubule complex. Qualitative or quantitative modifications of microtubules which can influence drug binding or the effects of drug binding on tubulin conformation and/or GTPase activity are likely to influence sensitivity to microtubule binding agents. These microtubule-based mechanisms of resistance to microtubule binding agents are extremely varied, and concern either individual components of the microtubule array itself or regulatory proteins. A variety of proteins participate in tubulin protein folding, tubulin dimer sequestration, microtubule dynamics or interact with microtubules and tubulin and participate in their regulatory pathways. These include the proteins FHit, survivin, MAP2, MAP4, stathmin, STOP and survivin [24,52-57]. Alterations in the levels, intracellular localizations (nuclear or cytoplasmic), post-translational modifications and function of these proteins are likely to influence sensitivity to microtubule binding agents.

Microtubules are composed of at least 13 isotypes of α- and β-tubulin. The quantitative tubulin isotype composition of microtubules has been reported to influence sensitivity to microtubule binding agents. Most notably, increased levels of beta tubulin III is associated with reduced response rates to taxanes in several tumors including lung, breast and ovarian cancers [54,58]. In contrast, epothilones may be indifferent to beta III tubulin content [59]. In addition to beta III tubulin, increased levels of beta V and beta II tubulins have also been associated with taxane resistance [60-62]. In contrast, decreased expression of class III beta-tubulin and increased levels of MAP4 protein have been detected in vinca resistant cell lines along with increased microtubule stability in these resistant cells as identified by the high levels of polymerized tubulin [63]. However, in contrast, small interfering RNA-mediated knockdown of either betaII- or betaIVb-tubulin hypersensitized lung cancer cell lines to Vinca alkaloids [64]. It is worth noting that the role of beta III tubulin expression in cancer may extend beyond its role in drug resistance. Recent studies have found that beta III tubulin appears to be a “survival factor” that can increase the incidence and progression of cancer irrespective of drug treatments [65]. These preclinical data have been confirmed in the clinic since high levels of beta III tubulin have been found to be associated with worse prognosis and lower response rates in a variety of tumor types [58,66].

There are several reports of mutations in tubulin genes in cell lines resistant to microtubule binding agents [67-69]. However, confirmation of these observations in the clinic is currently lacking. In spite of early suggestions that mutations in the taxol binding site were found in patients with NSCLC [70], subsequent studies have found no evidence that polymorphisms in beta tubulin genes are frequent events in clinical samples [71,72].

Resistance due to deficient apoptotic signaling

A third mechanism of resistance to microtubule binding agents involves apoptotic signalling downstream of the microtubule insults to which tumor cells are exposed. Microtubules physically interact with a variety of cell organelles and various regulatory proteins (Box 2). An interesting case is that of P53 protein and sensitivity to taxol. High hopes were raised by the observation that inactivation of P53 — a common mechanism of resistance to anticancer agents — induced preferential sensitivity to taxol in normal human or murine fibroblasts [73]. However, later observations suggested that P53 status had little or no impact on sensitivity to taxanes[74,75]. Several studies have failed to establish P53 as a predictive factor of response to taxanes in the clinic[76,77]. p53 may influence sensitivity to microtubule binding agents by regulating microtubule composition and dynamics thereby suggesting that p53 is not only a guardian of the genome but also of the microtubule cytoskeleton as well [57]. Apoptotic regulators or effectors also influence sensitivity to taxanes, for example a small molecule inhibitor of BclXL sensitized tumor cells to paclitaxel [78].

It is also becoming clear that the balance of expression of proteins that have no currently recognized direct interactions with microtubules or tubulin can also play a role in resistance or sensitivity to microtubule-targeted drugs, possibly through a complex web of interactions with other proteins that are part of the recognized microtubule functions in transport, cell cycle, signalling, and apoptosis. Examples of these include prohibitin, glutathione-S-transferase π, α-defensins, inflammation, GTSE-1 (G(2) and S phase-expressed-1)-protein modulation of p21, and hypoxia and hypoxia-inducible factor 1 α {Bublik #249;Patel #250;Huang #251;Bauer #252;Townsend, 2003 #253}. Micro RNAs have also been found to contribute to resistance to microtubule-targeted drugs. For example miR-125b conferred resistance to paclitaxel by suppressing the pro-apoptotic BAK1 and miR-148a increased sensitivity to paclitaxel by decreasing expression of mitogen and stress-activated protein kinase MSK1 {Zhou #254;Fujita #255}.

Novel microtubule targeted agents and/or formulations

Microtubule-binding agents are unique among anticancer agents not only because of their original mechanisms of action but also because of their extreme structural diversity. In most cases natural agents with potent antitumor activity have led the way for original synthetic analogues. Surprisingly this remains true even for the vinca and taxane families, the first members of which have been in clinical use for decades (Table 2).

Vinca domain binding agents

Vinca alkaloids (vincristine, vinblastine, vindesine and vinorelbine), originally isolated from the periwinkle plant *Catharanthus rosea,* represent the oldest and to this day most diversified family (in terms of number of approved compounds within a given family) of microtubule targeted agents. Vinflunine (Javlor™), a novel fluorinated compound which was obtained by superacid transformation of vinorelbine in the presence of fluorhydric acid, has recently been approved for the second-line treatment of bladder cancer [86]. Also a liposomal formulation of the off-patent agent vincristine, which allows a prolonged and regular delivery of this active compound, is currently the object of clinical trials.

The dolastatin family, originally identified by isolation of marine peptides from the ocean shell-less mollusk *Dolabella auricularia*, includes dolastatin 10, cemadotin, tasidotin (ILX651), soblidotin, and malevamide E [87]. While dolastatin 10 itself was not active in patients with various tumors including advanced breast cancer or pancreaticobiliary cancers, its analog soblidotin jnduced minor responses in patients with NSCLC and a partial response in a patient with advanced esophageal cancer in a phase I trial but was not further evaluated in a phase II trial [88,89]. Romidepsin, a dolastatin 15 analog

which also possesses activity as an HDAC inhibitor, was recently found to be active in cutaneous T cell lymphoma, with a 34% objective response rate.[90]

Eribulin mesylate, a synthetic halichondrin derivative, was found to be active in patients with metastatic breast cancer relapsing after anthracyclines and taxanes. In a randomized phase III trial patients receiving single agent eribulin mesylate benefited from significant improvement in overall survival when compared to patients treated according to physician's choice [91].

Taxol domain binding agents

Besides paclitaxel (Taxol™) and docetaxel (Taxotere™), cabazitaxel (Jevtana™, XRP 6258, RPR116258, Sanofi-Aventis) has displayed promising results in patients with breast and prostate cancer and has recently been approved by the FDA for the treatment of hormone-resistant metastatic prostate cancer after failure of docetaxel [92,93]. Issues with currently available taxanes include their mode of administration, currently limited to the intravenous route, their poor water solubility, requiring the use of surfactants such as Cremophor and ethanol for intravenous administration, with an associated risk of hypersensitivity reactions [94,95], and the nearly universal recurrence of disease when patients are treated in the advanced setting. Some of the novel taxanes are poor substrates for ABC transport pumps and may in some cases be administered orally or pass through the blood-brain barrier, a particularly important property for the treatment of CNS metastases.

Conversely a phase II trial evaluating BMS 275183 given orally twice weekly in patients with relapsing NSCLC was terminated because of highly variable pharmacokinetics. Unpredictable individual pharmacokinetics is a major limitation in the development and use of orally administered anticancer agents.

Novel taxane formulations are being developed with the intent of reducing issues associated with poor solubility or hypersensitivity. In a phase III trial comparing nanoparticle albumin-bound paclitaxel (Abraxane™, nab-paclitaxel) and conventional docetaxel for the therapy of patients with metastatic breast cancer, nab-paclitaxel was associated with better outcome as well as with a lower rate of severe neutropenia and a similar rate of reversible sensory neuropathy [96]. Nab-paclitaxel has also demonstrated activity in other settings including melanoma, gynaecological tumors and prostate cancer [97-99]. Several novel generic formulations of paclitaxel and docetaxel aim to eliminate surfactants from current formulations, which may eventually lead to reduced hypersensitivity reactions [100].

Epothilones were originally isolated from the myxobacterium *Sorangium cellulosum*. They represent a promising novel family of agents for cancer treatment as they may retain activity against taxane-resistant tumors [101,102]. Epothilones are easier to produce than taxanes, display good water solubility and do not appear to be substrates for the Pgp efflux pump [103], allowing passage through the blood brain barrier [104]. Besides ixabepilone (Ixempra™), a semisynthetic analog of epothilone B, which is currently approved for the treatment of advanced taxane-resistant breast cancer in the United States, several other epothilones are currently being studied in clinical trials. These include patupilone [105], sagopilone [106-108] and KOS-862 (epothilone D) [109,110] which are being evaluated in various solid tumor types.

Colchicine domain binding agents

Combretastatins represent an exciting family of microtubule targeted agents as they are lead compounds of the vascular targeting or vascular disrupting agents, compounds which produce rapid disruption of tumor blood flow, probably by their effects on the microtubule cytoskeleton of endothelial cells. In phase I trials combretastatin A4 (CA4), isolated from the *Combretum caffrum* tree, induced unusual toxicities including tumor pain, ataxia and cardiovascular modifications, including prolonged QTc interval and ECG modifications consistent with acute coronary syndrome [111-113]. Fosbretabulin (CA4 phosphate) is currently being evaluated in combination trials in patients with

anaplastic thyroid cancer and with chemotherapy naïve lung cancer [114]. Other antivascular agents that have undergone clinical evaluation include ZD6126 [115], OXI4503 [116], ombrabulin (AVE8062A)[117], crinobulin (EPC2407)[118] as well as auristatin PE (TZT-1027, a dolastatin derivative) [119] which binds in the Vinca domain. A key issue for the approval of this family of agents will be the lack of significant toxicity on normal vasculature, as well as the mode of administration in combination with other agents.

Additional agents binding at or near the colchicine binding site of tubulin such as CI-980 and 1069C85 have been discontinued while ABT-751, and indibulin are currently in phase I [120]. 2-methoxyestradiol (ME2), displayed limited activity in patients with hormone-refractory prostate cancer [121], breast cancer [122] and multiple myeloma [123] leading to improved formulations consisting of nanocrystal colloidal solutions [124]. The lack of myelosuppression by ME2 has been attributed to the resistance of the hematopoietic-specific beta tubulin to this agent [125].

Other agents

Several other agents with original properties have undergone clinical evaluation. Cevipabulin (TTI-237) is an unusual agent which appears to bind the vinca site but promotes microtubule polymerization [126] Noscapine, which has the ability to cross the blood-brain barrier [127] is currently being evaluated in a phase I/II trial in patients with multiple myeloma (NCT00912899). A number of analogs with increased potency are under investigation.

Toxicity of microtubule targeted agents

The evaluation of some microtubule binding agents has been discontinued because of significant toxicity. This is exemplified by the discodermolides which are highly potent natural polyketide products isolated from the Caribbean sponge *Discodermia dissolute*, which appear to be synergistic with taxol [128,129]. A phase I trial of this compound (Novartis) initiated in 2004 was interrupted because of significant pulmonary toxicity. Dictyostatin is a structurally related compound for which the total synthesis has recently been obtained [130]. Cryptophycins were obtained from cyanobacteria or were prepared by total synthesis. While some disease stabilisation was observed in patients receiving cryptophycin 52 (LY355703), there were no responses in patients treated for advanced NSCLC in spite of significant neurological toxicity [131,132].

Neurological toxicity

A major limitation in the use of microtubule-targeted agents is the high rate of neuropathy induced by these compounds [133]. This potentially severe and dose-limiting side effect, which is dose-cumulative and more frequent in patients with preexisting neuropathy, be it due to chronic alcoholism or diabetes mellitus, usually manifests itself as a painful and debilitating peripheral axonal neuropathy for which there is currently no effective symptomatic treatment [134]. This has prompted the search for predictive factors such as neurologic function tests or biological markers such as myelin basic protein and gliofibrillar acid protein [135-137] Other manifestations include constipation or intestinal paralysis due to neurological toxicity against the autonomic nervous system. While symptoms tend to disappear a few months after the end of treatment, some patients retain significant sequelae several years after therapy. The preferential toxicity of these agents for the nervous system is not understood at a mechanistic level but can be partially explained both by the relative abundance of tubulin in neurons, and the importance of an intact, functional microtubule cytoskeleton for adequate nerve conduction.

Peripheral neuropathy has been a limiting factor in the development of several agents, leading, as in the case of cryptophycins, to termination of their development. In contrast, there have been few reports of central nervous system (CNS) toxicity with the currently administered agents, partly due to the fact that they are Pgp efflux pump substrates and thus do not cross the blood brain barrier. The

development of newer agents which are not substrates of Pgp might be associated with CNS toxicity, or with activity against tumors within the CNS [104].The question of neuropathy is particularly important when considering the combination of these agents with other potentially neurotoxic agents. Among the classical agents, the platinum compounds, which induce peripheral neurotoxicity to various degrees, are commonly used in combination with taxanes and vincas, in particular in patients with NSCLC or with germ cell tumors, in the latter case with a large proportion of long term survivors [138]. Among the more recently approved agents, several compounds, such as bortezomib or thalidomide, can also induce high grade peripheral neuropathy in a significant proportion of patients. The mechanisms of neurotoxicity have not been precisely determined for all of these compounds and may or may not be related to microtubules [139,140]. The combination of these agents with microtubule-targeted agents may therefore prove to be difficult and assays, quite likely based on genetic polymorphisms, predicting high grade sensory neuropathy in individual patients would be of great use.

A major difficulty in the screening of novel agents is the lack of adequate preclinical models of drug-induced peripheral neuropathy. Glial cell cultures are extensively used to analyse this type of toxicity *in vitro*, but animal models that reliably correlate with or predict neurotoxicity in patients remain imperfect [141-145]. The development of reliable predictive models would be of great use for the future development of novel agents and of neuroprotective compounds. Alternatively the identification of differences between the microtubule cytoskeleton in peripheral nerves and tumor cells could serve as a basis to design or select novel agents with reduced neuropathy. Eribulin induced no significant reduction in nerve conduction velocity or amplitude in caudal and digital nerves when administered to mice at the maximal tolerated dose [146]. Phase I and II clinical trials of eribulin demonstrated significant activity with only a low incidence of neuropathy and no grade 4 neuropathy [147]. Indibulin (ZIO-301/D-24851) has been reported to distinguish between mature neuronal tubulin and non-neuronal tubulin and has entered clinical evaluation as an oral formulation [148,149]. In a phase I study, ispinesib (SB-715992), a kinesin inhibitor was found to induce myelosuppression but no neurotoxicity [150]. Phase II trials evaluating ispinesib as a single agent have not yet demonstrated significant activity [151,152].

Other toxicities

Myeloid toxicity is frequently observed with microtubule-targeted agents, with subtle differences between compounds within the same family [153]. Neutropenia is often the most frequent and/or severe side-effect observed in combination regimens including these agents [70,86,154]. In several recent phase II studies neutropenia was one of the dose-limiting toxicities [88,155-158]. This toxicity, which is often added to similar toxicities of other agents used in combination regimens, is usually manageable. In contrast, some toxicities are relatively compound specific, such as fluid retention observed in patients receiving docetaxel or diarrhoea after patupilone therapy [159-161].

An intriguing issue concerns the possible mutagenic properties of microtubule binding agents and henceforth the risk that they may increase the risk of secondary tumors. Given the fact that cells exposed to these compounds can develop aneuploidy due to missegregation, there is a theoretical risk that these agents might increase the risk of iatrogenic leukemias and/or solid tumors. Chromosomal instability and an aneuploid-prone phenotype have been described to be correlated with response to taxanes [162,163]. Administration of paclitaxel to nude mice and to rhesus monkeys has caused prolonged aneugenicity and abnormal mitoses, respectively, but clinical confirmation of such an effect has yet to be demonstrated [163,164]. As these agents have been widely used in combination with alkylating agents, and the initial indications mostly concerned patients whose life expectancy was short, it has been difficult to establish whether these agents are potentially carcinogenic *per se*. As a result of the widespread use of these agents in the adjuvant setting, in patients whose prognosis may be globally favorable, the question of whether microtubule-targeted agents increase the risk of secondary neoplasms has become clinically relevant.

Improving therapy with microtubule-targeted agents

Microtubules represent a highly-validated target in cancer therapy, explaining the abundance of efforts to develop novel agents directed against this target. All of the currently approved compounds bind directly to tubulin, either to soluble tubulin or to tubulin that is polymerized into microtubules, although the binding occurs at different sites on the tubulin molecule or to different regions of the microtubule. Novel approaches aim to improve upon existing compounds either by selecting agents that are insensitive to resistance mechanisms, that increase tumor selectivity, that reduce side effects such as peripheral neuropathy or by targeting the numerous other components of the tubulin/microtubule complex.

Several promising agents have been reported in preclinical models. These include eleutherobin[165], laulimalide[166,167], hemiasterlins[168], peloruside A[22,169], taccalonolide[170], coumarins[171] and cyclostreptin[172] . Most of the novel agents have been selected because of their activity in models that show resistance to taxanes. Several of these novel agents are not substrates of efflux pumps such as Pgp or other ATP-Binding Cassette proteins. In some cases these agents are also insensitive to the presence of mutations in beta tubulin and/or to overexpression of specific tubulin isotypes, in particular tubulin βIII. This has led some investigators to identify either βIII-indifferent agents, or βIII-targeted agents [170,173]. The demonstration that tumor aggressivity and in some cases of sensitivity to chemotherapy is influenced by the content of βIII tubulin isotype suggests that the development of agents targeting this isotype would be of particular interest in patients with high risk disease due to high expression of this isotype. Such a strategy is corroborated by the reports that inhibition of tubulin III by oligonucleotides and by silencing RNA induced sensitization of tumor cells to various anticancer agents [62,64]. In this regard, secotaxoids, which are predicted to bind well to beta III tubulin isotype and retain activity in paclitaxel resistant preclinical models appeared to be particularly promising but have not been further evaluated in the scope of recent clinical trials[174]. Another attractive approach involves vectorisation of microtubule binding agents to the tumor cell using a monoclonal antibody. Maytansine conjugates are being studied in various indications, in particular in haematological diseases and breast cancer [175-177]. A recent trial of trastuzumab-DM1, a maytansinoid conjugated to the anti-HER2 therapeutic antibody trastuzumab, showed good efficacy in metastatic breast cancer and the CD-56 targeting antibody-maytansine conjugate, lorvotuzumab-mertansine, has shown promising results in solid and liquid tumors that express CD56 [178,179].

It is now clear that alterations in microtubule dynamics are the main mechanism of action of microtubule binding agents [24,180]. Given the multiple roles of microtubules, several proteins other than tubulin itself are likely to constitute therapeutic targets in cancer cells. These potential targets include proteins involved in the lifecycle of tubulin peptides and dimers as well as proteins involved in microtubule nucleation, dynamics, and interaction with chromosomes or cellular organelles. Of particular interest are the motor proteins such as kinesin Eg5 (for which the first inhibitors such as AZD4877are currently being evaluated [181]) and tau protein[182], a key microtubule-associated protein which has been correlated with outcome in patients with breast cancer. Another potential target is survivin[183], a protein that is intimately involved in spindle microtubule behaviour as well as apoptosis. Other potential targets include MCAK, a mitotic centromere-associated protein that regulates microtubule dynamics[184], and stathmin[185], an important regulator of the soluble tubulin dimer pool as well as dynamics.

Another important avenue for the optimization of microtubule binding agents is the identification of patient subsets most susceptible to respond to therapy or to develop significant toxicity, using tumor-related parameters or patient characteristics [186]. This approach is of particular interest in diseases such as lung cancer, in which there are several therapeutic alternatives, none of which has clearly proven to be superior [187]. A randomized trial is currently analyzing the potential benefit of ixabepilone in patients with βIII tubulin-positive lung cancer (NCT00723957). Analyses of targeted polymorphisms in patients receiving microtubule-binding agents has not yet allowed the

identification of patients with the highest chance of response or the highest risk of developing dose-limiting side effects of chemotherapy [188]. High throughput analyses of large patient cohorts and validation series will help establish personalized therapy with microtubule-binding agents.

Concluding thoughts

In light of the development of microtubule-targeted agents over the past decades, the recent approvals of a novel vinca alkaloid, a novel taxane and the first epothilone, and the recent advances in the understanding of the role of the microtubule cytoskeleton in cancer cells, the stakes are high that this family of anticancer compounds not only will still be in use years from now, but will also will be considerably enriched with less toxic and highly active molecules. The tremendous diversity of naturally occurring compounds interacting with mammalian microtubules represents a largely untapped source for future anticancer agents. A major aim in this very dynamic field will be to purify, screen and ultimately offer to the cancer patient the best of nature's gems.

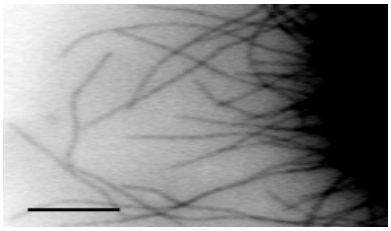
Legends to Figures

Figure 1. Chemical structures of microtubule binding agents according to binding domains
This figure shows the extreme chemical diversity as well as the complexity of many of these agents. The complex structure of certain natural compounds explains the difficulty encountered by chemists to perform total synthesis of these molecules.

Figure 2. Microtubule formation and binding sites of microtubule inhibitors
Fig 2A. Soluble tubulin dimers containing one alpha tubulin peptide and one beta tubulin peptide polymerize to form a "nucleus". Additional dimers are added head-to-tail and the resulting microtubules are highly dynamic structures containing a (+) end characterized by an exposed β tubulin peptide and a (-) end characterized by an exposed α tubulin peptide.
Fig 2B. Binding sites of microtubule inhibitors. While vinca alkaloids bind to microtubule ends, colchicine binds to soluble dimers which can be incorporated within the microtubules. Taxanes bind along the interior surface of the microtubules.

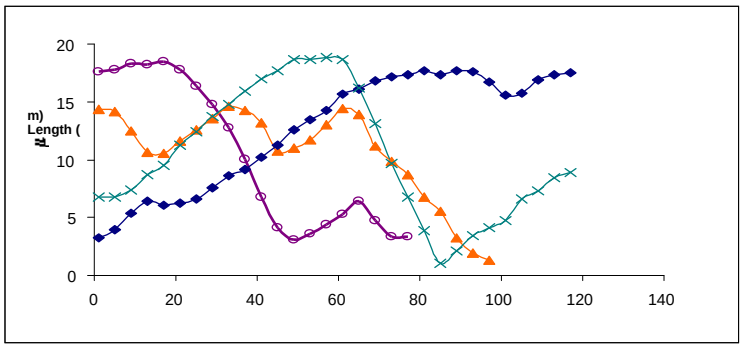
Box 1. Microtubule dynamics

Microtubules are dynamic structures composed of αβ-tubulin molecules that are constantly integrated or shed into the cytoplasm as the microtubules dramatically grow and shorten. Dynamics can be measured in live cells using fluorescently labelled tubulin (either labelled ex vitro and microinjected or using an expressed GFP-tag) and video-microscopy. Several parameters of dynamics can be assessed to determine the effects of microtubule targeted drugs on dynamics. These include the rates and durations of growing and shortening events and the mean frequency of rescue or catastrophe. Although these parameters are generally analysed on interphase cytoplasmic microtubules and not on spindle microtubules, systems using markers of the ends of spindle microtubules such as GFP-CENP-B have found that the suppressive effects of drugs on dynamics of interphase microtubules are very similar to their suppressive effects on mitotic microtubules.

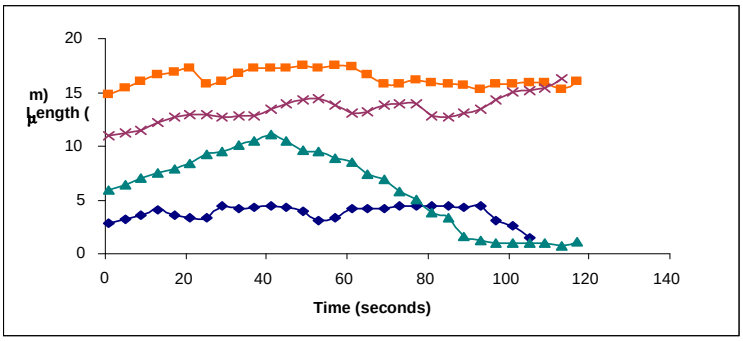


Box 1A:Time-lapse sequence analysis of microtubules, using fluorescent-labelled tubulin microinjected into human mammamy adenocarcinoma MCF7 cells

Control



7.5 nM paclitaxel



Box 1B: Reduced length changes of individual microtubules in the presence of taxol show suppression of microtubule dynamic instability by taxol

Box 2. Interactions of microtubules with other proteins and cellular organelles

Microtubules interact with a variety of intracellular components including mitochondria, the Golgi apparatus, the endoplasmic reticulum, and lysosomes. In the mitotic spindle microtubules allow the proper alignment of chromosomes during metaphase, followed by the equal distribution of chromatids to the two daughter cells during anaphase. This phenomenon relies on the physical interaction between microtubule (+) ends of the microtubules and the kinetochores.
A number of key proteins involved in cell cycle and/or apoptosis have also been shown to physically interact with microtubules. P53 is physically associated with dynein, a microtubule motor protein. Bcl2, survivin and several other proteins that play a role in cell survival also colocalize with microtubules although it is not clear whether microtubules serve as molecular scaffolds for these proteins to exert their activity or whether the proteins are sequestered by microtubules and therefore functionally inactive.

Glossary

Adjuvant therapy: a treatment which is administered to patients with minimal or no detectable sign of disease, in order to prevent disease recurrence
Catastrophe: the switch of a growing or stable microtubule end to rapid shortening
Dynamics: the nonequilibrium dynamic behaviors of microtubules in cells which are crucial to their functions. The two kinds consist of “dynamic instability" in which the ends of individual microtubules randomly switch between phases of growth and shortening and “treadmilling” which consists of net growth at one microtubule end and a balanced net shortening at the opposite end resulting in a flow of tubulin subunits through the microtubules. Microtubule dynamics are much faster during mitosis than in interphase and are crucial to cell division, making mitotic cells highly susceptible to microtubule-targeted drugs. They are also important in the trafficking of elements within the cell and for cell migration; their suppression is thought to impair cell metastasis.
Kinetochore: the complex assemblage of proteins at the chromosome centromere to which dynamic mitotic spindle microtubules attach, ultimately producing equal segregation of chromosomes to the daughter cells.
Microtubule-associated proteins (MAPs): a number of proteins bind very tightly to microtubules and can be purified along with the microtubules. The most famous of these are tau and Microtubule-associated proteins 2 and 4. In addition, many proteins can bind less tightly to microtubules in cells and regulate their behaviour.
Microtubule binding agents: drugs and endogenous regulators of microtubule dynamics can bind selectively to several sites on a microtubule. They can bind preferentially to one or both microtubule ends (vincas, eribulin, cryptophycins, maytansinoids and others) or to the sides of the microtubule (taxanes, epothilones). They may also copolymerize into the microtubule with the tubulin (colchicines).
Tubulin dimer: the heterodimeric protein subunit that polymerizes into microtubules. Each subunit is composed of one α-tubulin and one β-tubulin molecule.
Tubulin isotype: there are at least 13 different isotypes of α- and β-tubulin. The tubulin isotype composition of cells varies between cell types within the same tissue and between tissues. For example, brain cells contain high amounts of βIII-tubulin, but non-neuronal cells generally contain only low amounts of this isotype. Isotype content also differs between tumor cells and the non-tumor cells of the same tissue. The complement of tubulin isotypes can be induced to change in response to treatment by many drugs.
Rescue: the switch of a shortening microtubule end to growth or to a state of stable microtubule length

Agent	Sensitivity to ABC efflux pumps	Sensitivity to β-tubulin content	references
Vincas	MDR sensitive MRP sensitive	Sensitive to βIII-tubulin content	[44,189-191]
Cryptophycins	MDR insensitive	n.a.	[192,193]
Dolastatins	MDR sensitive	n.a.	[194]
Taxanes	MDR sensitive MRP2 and MRP7 sensitive	Sensitive to βIII-tubulin content	[45,46]
Epothilones	MDR sensitive	No	[103,195]
Discodermolides	MDR sensitive MRP1 sensitive	Sensitive to βIII-tubulin content	[196,197]
Cyclostreptin	MDR insensitive	n.a.	[172]
Laulimalides	MDR insensitive	n.a.	[198]
Taccalonolide	MDR insensitive	More active if high beta III content	[170]
Peloruside	MDR insensitive	n.a.	[169]
Hemiasterlin	MDR insensitive	n.a.	[168]
Combretastatins	MDR insensitive	Yes	[199-201]
2 methoxyestradiol	MDR insensitive	Inactive against beta I	[125,202]

Table 1. Characteristics of microtubule binding agents
MDR: multidrug resistance; ABC: ATP Binding Cassette transport pumps; n.a.: not available

Binding domain	Family	Agent	Approved Indications *	Clinical trials	Comments
Vinca	Vincas	Vincristine	ALL, lymphomas Various solid tumors	Various tumor types	Natural compound Generic Parenteral administration
		Vinblastine	Lymphomas Various solid tumors	Various tumor types	Natural compound Generic Parenteral administration
		Vinorelbine	Breast, NSCLC	Various tumor types	Semi-synthetic Generic Oral and parenteral administration
		Vindesine	ALL, lymphoma Lung cancer	Various tumor types	Semi-synthetic Generic Parenteral administration
		Vinflunine (Javlor®, Pierre Fabre)	Bladder	Breast in combination with Herceptin	Semi-synthetic Parenteral administration
		Liposomal vincristine	-	leukemia melanoma, myeloma, sarcoma	Prolonged and regular delivery
	Dolastatins	soblidotin (TZT-1027)	-	Phase I in advanced solid tumors No ongoing trials	Responses in NSCLC and esophageal cancer
		romidepsin Istodax® Gloucester Pharmaceuticals	Cutaneous T cell lymphoma	Myeloma, lymphoma, solid tumors	Dolastatin 15 analog
		brentuximab vedotin (SGN 35)	-	Phase III trial recruiting in Hodgkin's disease	Antibody-vectorized agent directed against CD30 positive malignancies
	Cryptophycins	Cryptophycin 52 LY355703	-	Phase II NSCLC Terminated	Caused significant neurological toxicity
	Halichondrin	Eribulin (E7389, NSC 707389)	-	Phase III in advanced breast cancer	Improved OS when compared to treatment of physician's choice
	Hemiasterlin	E-7974	-	Phase I	Hematological MTD
	Maytansinoids	Mertansine immunoconjugates (BT-062, IMGN388, BIIB015)	-	Head and neck, oesophagus, advance HER2 positive breast cancer, myeloma	Phase II and III underway
	Folate vectorized vinca alkaloid	EC-145		Ovarian, endometrial, lung cancer	Folate-targeted vinca alkaloid conjugate
Taxane	Taxanes	Paclitaxel Taxol®	Ovarian, breast, NSCLC	Various solid tumor types	May induce hypersensitivity reactions
		Docetaxel Taxotere®	Breast, NSCLC, prostate, stomach, head and neck	Various solid tumor types	May induce hypersensitivity reactions
		cabazitaxel (XRP6258) Jevtana®	Metastatic hormone-resistant prostate		Approved June 2010
		Milataxel (MAC-321, TL-139)	-	Phase II mesothelioma	Active in preclinical models of resistance to taxanes [203,204]
		Larotaxel (XRP9881)	-	Phase III pancreatic	Active in preclinical models of resistance to taxanes, poor MDR substrate [205-207].
		Ortataxel IDN-5109 BAY 59-8862	-	Phase II taxane-resistant tumors	Active in Pgp-expressing models [208]
		Tesetaxel	-	Phase II gastric	Oral administration

		DJ-927		Phase II colorectal Phase II melanoma	Is not transported by Pgp [209,210]
		BMS 275183	-	Phase II NSCLC Terminated	Oral administration Unpredictable pharmacokinetics
		TPI 287 (ARC-100)	-	Phase II prostate cancer Phase I pediatric CNS cancers	Investigated in neurological tumors in combination with temozolomide
		Nab-paclitaxel (ABI-007) Abraxane® Abraxis Bioscience Nab-docetaxel (ABI-008)	Breast cancer	Various solid tumors Prostate cancer	Shorter infusion times than paclitaxel Does not require premedication
		NKTR-105		Phase I	PEGylated formulation of docetaxel ; pre-treatment with corticosteroids not required
	Epothilones	Ixabepilone Ixempra® Bristol Myers Squibb	Breast cancer	Solid tumors	Several ongoing trials in solid tumors Is not a substrate for Pgp
		Patupilone (epothilone B)	-	Brain metastases in breast cancer, ovarian, melanoma, other solid tumors	Penetrates in the CNS Is not a substrate for Pgp Possesses radiosensitizing properties
		Sagopilone	-	Glioblastoma, prostate, lung cancers	First fully synthetic epothilone Penetrates in the CNS
		KOS 1584 (epothilone D)	-	NSCLC Phase II	Investigated in breast and prostate cancer
	Discodermolide	-	-	Phase I Terminated	Pulmonary toxicity
Colchicine	CI-980	-	-	Phase II trials Terminated	No responses observed in sarcoma or colorectal cancer [211,212]
	2 methoxy-estradiol (ME2) Panzem® EntreMed	-	-	Phase II in prostate, myeloma, glioblastoma	Endogenous metabolite of estradiol with no affinity for estrogen receptor Side effects : DVT and increased transaminases
	1069C85	-	-	Phase I Terminated	Oral administration [213]
	ABT 751 E7010	-	-	Phase II in various solid tumors No ongoing trials	Orally bioavailable sulfonamide Neurotoxicity [214]
	Indibulin	-	-	Phase I/II in metastatic breast cancer	Discriminates between neuronal and non-neuronal tubulin [148,215]
	Combretastatins	Fosbretabulin (CA4 phosphate)	-	Phase II in lung and thyroid cancer, glioma	Vascular disrupting agent
		Verubulin		Phase II glioblastoma	Vascular disrupting agent
		Crinobulin	-	Phase I	Vascular disrupting agent
		Plinabulin		Phase I	Vascular disrupting agent
		Ombrabulin	-	Phase III in sarcoma	Vascular disrupting agent
Other	Noscapinoids	Noscapine	-	Phase II multiple myeloma	Oral opium alkaloid used as antitussive
	Estramustine	-	Prostate cancer	Combination with taxanes, vincas, ixabepilone in prostate cancer	Generic Binds to microtubule associated protein

Table 2. Selected microtubule-binding agents which have been approved or have undergone clinical evaluation

ALL: acute lymphoblastic leukemia; CNS: central nervous system; DVT: deep vein thrombosis; MTD: maximal tolerated dose; NSCLC: non small cell lung cancer; OS: overall survival; Pgp: P glycoprotein
Data in this table have been obtained from clinicaltrials.gov, Pubmed, ASCO, company sites and the Thomson Pharma Partnering database.

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Further information

Initiation of a phase I/II study of oral indibulin in breast cancer patients by ZIOPHARM
http://ir.ziopharm.com/releasedetail.cfm?ReleaseID=457504