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REVIEW ARTICLE

Microtubule drugs: action, selectivity, and resistance across the kingdoms of life

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Abstract Microtubule drugs such as paclitaxel, colchicine, vinblastine, trifluralin, or oryzalin form a chemically diverse group that has been reinforced by a large number of novel compounds over time. They all share the ability to change microtubule properties. The profound effects of disrupted microtubule systems on cell physiology can be used in research as well as anticancer treatment and agricultural weed control. The activity of microtubule drugs generally depends on their binding to α- and β-tubulin subunits. The microtubule drugs are often effective only in certain taxonomic groups, while other organisms remain resistant. Available information on the molecular basis of this selectivity is summarized. In addition to reviewing published data, we performed sequence data mining, searching for kingdom-specific signatures in plant, animal, fungal, and protozoan tubulin sequences. Our findings clearly correlate with known microtubule drug resistance determinants and add more amino acid positions with a putative effect on drug-tubulin interaction. The issue of microtubule network properties in plant cells producing microtubule drugs is also addressed.

Keywords Microtubule drugs · Tubulin mutations · Paclitaxel · Colchicine · Dinitroanilines · Vinblastine

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Introduction

Microtubule drugs (also known as mitotic poisons) constitute a variable group of compounds targeted against the microtubular cytoskeleton, an intricate intracellular system of hollow cylindrical polymers composed of αβ-tubulin dimers. They have been intensely studied for their actions in animal cells (reviewed in Jordan and Wilson (2004); Ganguly and Cabral (2011)), and some of them are widely used in anticancer treatment. Although several compounds have been exploited in agriculture for their herbicidal effects, their actions on plant cells are known to a lesser extent. Molecular effects of these compounds on fungi and protozoa have also been rarely studied.

The principal building blocks of microtubules are α- and β-tubulin subunits. Knowledge of their structure is crucial to understanding the binding of microtubule-active drugs. Both α- and β-tubulins are globular proteins consisting of approximately 450 amino acids each (Downing and Nogales 1998a) with ~40 % amino acid identity among α- and β-tubulins of each species, as determined from a wide array of tubulin sequences (Little and Seehaus 1988). Their 3D structures consist of two internal β-sheets surrounded by several α-helices (Nogales et al. 1998). This compact model of a tubulin subunit can still be dissected into several domains with diverse functions. The N-terminal domain, made of several closely packed α-helical and β-sheet units, binds GTP. The amino acid chain continues by a smaller intermediate domain. This is then followed by two antiparallel α-helices running along the two domains and thus forming an additional C-terminal domain. The C-terminal domain is the most variable part of the tubulin molecule and plays an important role in tubulin interactions with other proteins (Downing and Nogales 1998b; Lefèvre et al. 2011; Amos 2011).

Tubulin genes are present in all known eukaryotes, often in multiple copies (isotypes). Plants may have as many as 13 α-

and 20 β-tubulin genes (Breviario et al. 2013), while there are 8 functional α- and 7 β-tubulin genes in humans (Verdier-Pinard et al. 2009). Both animal and plant tubulin isotypes are differentially expressed in various tissues (Radchuk 2009; Leandro-García et al. 2010).

α- and β-Tubulin subunits form stable heterodimers in the cell. In favorable conditions, the heterodimers assemble into microtubules, long hollow cylinders of 24 nm in diameter. They consist of a variable number of protofilaments—often 13 in mammals, but microtubules with 8–19 protofilaments have been observed in other organisms (Chrétien et al. 1992; Breviario et al. 2013). The protofilaments are arranged in a circle and run lengthwise from one end of the microtubule to the other. The C-terminal tubulin domains face the outer microtubule surface, whereas the N-terminal GTPase domains are exposed to the inner space and GTP is always bound between the neighboring subunits (Downing and Nogales 1998a; Amos 2011). One end of microtubules (called the "minus end") is typically anchored, while the plus end grows into the surrounding cytoplasm by adding subunits with bound GTP. Periods of growth alternating with periods of rapid shortening constitute the basis for microtubule dynamics (Jordan 2002).

The overall arrangement of microtubular cytoskeleton inside eukaryotic cells is very diverse. Animal microtubules emanate from the centrosome, a microtubule organizing center (MTOC) where minus ends of microtubules are typically anchored. Vascular plants lack a centrosome, having instead a rather dispersed system of microtubules attached to various membranes (Wasteneys 2002; Brown and Lemmon 2007). The centrosome is also missing in cells of many fungi, as well as unicellular eukaryotes, i.e., protists (Adl et al. 2005). Protists also possess many intricate microtubule-based structures such as flagella, cilia, or axostyles. These structures have a precisely arranged heritable system of microtubules and often contain unusual types of tubulin (Gull 2001). Despite the differences, the overall structure and function of microtubules remain the same. Common and distinct features of various microtubular systems are reflected in their affinity towards an array of tubulin-binding compounds.

Microtubule drugs and mechanism of their action

The list of microtubule-active natural compounds is enormous and still continues to grow. The canonical drugs used in medicine include colchicine, paclitaxel (and related taxanes), and vinblastine (or other vinca alkaloids), but several hundred of other similarly acting compounds have been discovered, such as podophyllotoxin, cryptophycins, dolastatins, epothilones, discodermolides, halichondrin, and many others. Moreover, a significant proportion of known herbicides directly or indirectly act on microtubules (Vaughn and Lehnen 1991). This group is chiefly represented by dinitroaniline herbicides (oryzalin, trifluralin, benefin, ethylfluralin, pendimethalin, and prodiamine) and pyridine-based compounds such as dithiopyr and thiazopyr (Mallory-Smith 2003). Despite their diverse chemical structures (Fig. 1), most of them bind directly to a common substrate, tubulin, or microtubules. General characteristics of key microtubule drugs are shown in Table 1.

The compounds fall into two major groups according to their origin. Oryzalin and trifluralin are synthetic compounds of the dinitroaniline group, formerly manufactured as dyes in chemical industry. However, many other microtubule drugs are naturally occurring compounds, produced by plants, animals, or microbes to mimic endogenous microtubule regulators. They have probably evolved to prevent predation or herbivory (Dumontet and Jordan 2010) or to defend the organisms against parasites and pathogens (Wagner 1994). For instance, vinblastine was isolated from Madagascar periwinkle (*Catharanthus roseus*, formerly known as *Vinca rosea*), colchicine was obtained from meadow saffron (*Colchicum autumnale*), while paclitaxel originates from the bark of Pacific yew tree (*Taxus brevifolia*). Because of its efficiency, microtubule poison-based strategy of defense is a widespread phenomenon and can serve as an example of evolutionary convergence (Goodin et al. 2004).

The mechanism of action of microtubule drugs has been a hot topic of cell biology for decades. Still, the majority of our knowledge is derived from studies in animals or animal cell cultures. The compounds usually show affinity to one of three principal binding sites of tubulin (Fig. 2). The luminal side of β-subunit binds taxanes; the interdimer space between the β-subunit of one dimer and α-subunit of the following dimer binds vinca alkaloids; and the intradimeric space between the α- and β-subunits of one heterodimer, adjacent to the GTP of the α-subunit, binds colchicine (Jordan and Wilson 2004). Podophyllotoxin binds to the colchicine-binding site or at least overlaps with it (ter Haar et al. 1996; Sharma et al. 2010). The binding site of dinitroanilines (such as oryzalin) likely resides on the α-subunit, close to the dimer-dimer interface, although the exact location remains unknown (Dempsey et al. 2013). Drug binding typically leads to intricate conformational changes in the tubulin molecule and/or whole microtubules, as reviewed in Stanton et al. (2011).

Microtubule drugs are thought to act on cells by increasing (e.g., paclitaxel) or decreasing (e.g., colchicine and vinblastine) the mass of polymerized tubulin (Dumontet and Jordan 2010). However, as reviewed in Correia and Lobert (2001), microtubule drugs often also decrease duration and rate of microtubule growth or shortening and often increase the time that microtubules spend in the pause state, which reduces the microtubule dynamics. Importantly, the effects on microtubule dynamics generally occur at even very low drug concentrations at which the polymer mass remains largely unchanged

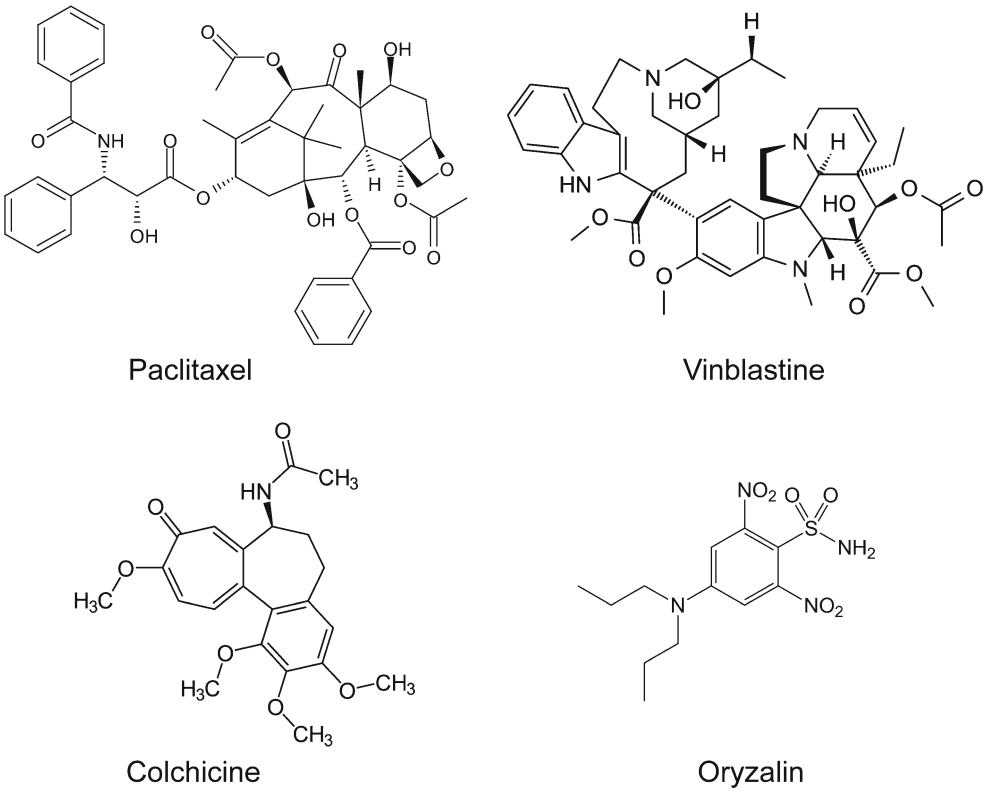

Fig. 1 Chemical formulae of four representative microtubule drugs. The image clearly illustrates the variety in the microtubule drug structure

(Jordan et al. 1992). The relevance of these two effects to the actual drug potency is a matter of debate (Jordan and Wilson 2004; Ganguly et al. 2010), and both mechanisms may apply.

Spindle microtubules are thought to be the ultimate target of these compounds, and investigators generally concentrate on the effects in proliferating cells such as those in tumors. On the other hand, only a small percentage of cells are dividing at any time in the tumor, calling the true target of microtubule drugs into question. Several scenarios have been presented to explain the discrepancies (Komlodi-Pasztor 2011; Mitchison 2012). Additionally, some studies point to a structural similarity between certain domains of tubulin and Bcl2 protein and show that paclitaxel may have more than one binding partner in the cell (Rodi et al. 1999; Ferlini et al. 2009), a phenomenon that could have functional consequences because Bcl2 is an important member of the apoptotic pathway. Whether such knowledge acquired from animal cells (and especially from cancer studies) is transferable to other kingdoms of life is an open question with no experimental data available.

Kingdom-specific effects: plants, animals, fungi, and protozoa

Both α- and β-tubulins are highly conserved proteins, and the evolutionary stability of their genes best resembles the conservative nature of histones. Tubulins are so conservative that a mix of tubulin heterodimers isolated from a chick brain and *Chlamydomonas reinhardtii* flagella easily copolymerizes into microtubules in vitro (Binder et al. 1975; Little et al. 1982). This would suggest that the effects of microtubule-binding drugs will be uniform in organisms belonging to distinct evolutionary groups. In reality, though, the ability to induce effects differs among the eukaryotic kingdoms (Table 2). The

Table 1 Microtubule drug origin and their binding sites on tubulin. The table also shows the main drug-producing species for each compound. Please note that production of paclitaxel is controversial. See text for details and references

Drug	Origin	Producing species	Binding site
Paclitaxel	Plant	*Taxus brevifolia*	Paclitaxel-binding site
Epothilones	Bacterial	*Sorangium cellulosum*	
Vinblastine	Plant	*Catharanthus roseus*	Vinca-binding site
Halichondrin	Animal	*Halichondria okadai*	
Colchicine	Plant	*Colchicum autumnale*	Colchicine-binding site
Podophyllotoxin	Plant	*Podophyllum peltatum*	
Dinitroanilines	Synthetic	–	Other

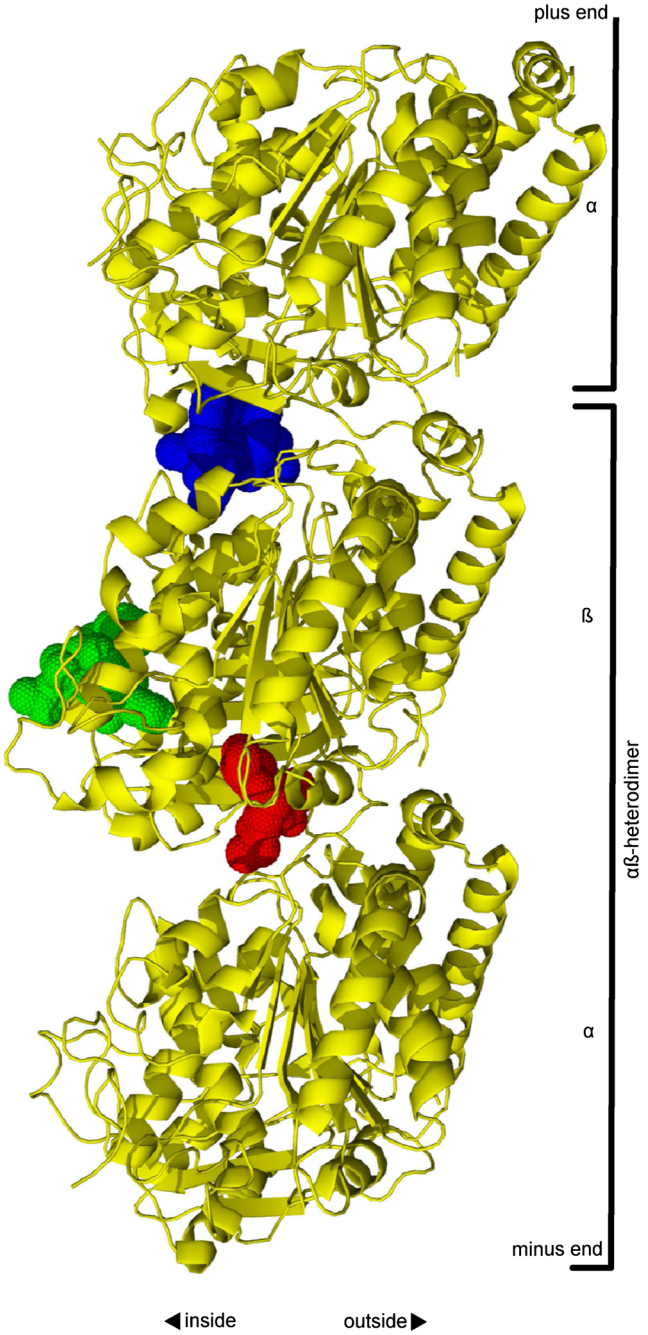

Fig. 2 Binding sites of colchicine (*red*), vinblastine (*blue*), and paclitaxel (*green*) on a fragment of a microtubule (one αβ-heterodimer and an adjacent α-subunit). *Lateral view*, *inside*, and *outside* of a hypothetical microtubule marked. Note that simultaneous binding of colchicine, vinblastine, and paclitaxel to one heterodimer is purely theoretical. The scheme is based on a superposition of tubulin structures 1SA0 (Ravelli et al. 2004), 1Z2B (Gigant et al. 2005), and 1JFF (Löwe et al. 2001)

sensitivity of various eukaryotic groups towards particular microtubule drugs can be mapped on a cladogram of eukaryotes (Fig. 3).

Resistance to microtubule drugs is attributable to many biological and biochemical phenomena. These include alterations in apoptotic pathways and changes in interactions with microtubule-associated proteins (MAPs) or tubulin isotype expression as well as an increased efflux of microtubule drugs by transmembrane proteins from the family of ATP-binding cassette (ABC) transporters, which are often associated with multidrug resistance (Fojo and Menefee 2005). The role of conserved tubulin posttranslational modifications—such as acetylations, phosphorylations, or glutamylations—in the resistance to microtubule drugs has not been evaluated to our knowledge. These modifications have a strong influence on microtubule stability (Wloga and Gaertig 2010), an important factor in resistance to microtubule drugs, but the data regarding tubulin posttranslational modifications among eukaryotic kingdoms are rather insufficient. Finally, mutations in tubulin sequence constitute an important cause of resistance (Fojo and Menefee 2005). The relevance of such mutations and their connection to effects of microtubule drugs in various kingdoms of eukaryotic organisms are summarized below.

Colchicine

Colchicine is one of the oldest known and most notorious microtubule drugs, effectively blocking microtubule assembly in animal cells. Upon administration, the disrupted microtubular cytoskeleton leads to a mitotic arrest, decreased cell motility, and impaired exocytosis and endocytosis. Accumulation of pathological effects can result in multiorgan failure and death (Finkelstein et al. 2010). Unfortunately, colchicine's anticancer action is difficult to achieve in safe dosage. However, the drug is prescribed for the treatment of gout—its antiinflammatory action has been explained by inhibition of both neutrophil migration and superoxide production (Nuki 2008). Why colchicine's action on microtubules often leads to such harmful effects on health, compared with other safer anticancer microtubule drugs, remains an open question.

In plants, colchicine working concentrations are generally much higher than in animals (Table 2). These have enabled scientists to use colchicine for the induction of polyploidy in plant cells, although sometimes in concentrations as high as 6 mM (Berger and Witkus 1943; Siddiqi and Marwat 1983). The drug is reported to be effective on plants at 250 μM or even higher (Kramers and Stebbings 1977; Gunning and Hardham 1982; Morejohn and Fosket 1984; Caperta et al. 2006). However, lower concentrations (25–250 μM) were shown to affect plant cell viability in a dose-dependent manner; weakly induce mixoploidy (Ascough et al. 2008); and, in one case, change the normal phenotype of mitotic cells (Schmit and Lambert 1988). Protozoa and fungi are also highly resistant to colchicine, with affinity constants ~10,000× weaker than in animals (Bode et al. 2002; Banerjee et al. 2007). One hundred micromolar colchicine did not inhibit growth of *Physarum polycephalum* amoebae (Quinlan et al. 1981), and *Tetrahymena pyriformis* tubulin

Table 2 Differential sensitivity towards microtubule drugs. The table reviews minimal effective concentrations of selected drugs in the representatives of five eukaryotic kingdoms. The values reflect differences in tubulin and microtubule structure, as well as uptake, accumulation, metabolism and/or efflux of the compounds. ND=exact concentration not determined, see text for details

	Animals (μM)	Plants (μM)	Fungi (μM)	Excavata (*Trypanosoma*)(μM)	Chromista (μM)	
Colchicine	0.1	250–1,000	>10,000	200	10,000	(Rosenbaum and Carlson 1969; Haber et al. 1972; Williams and Williams 1976; Hart and Sabnis 1976; Kramers and Stebbings 1977; Filho et al. 1978; Gunning and Hardham 1982; Morejohn and Fosket 1991; Caperta et al. 2006)
Paclitaxel	0.002–0.008	0.01	>10	1	0.001–0.1	(Baum et al. 1981; Molè Bajer and Bajer 1983; Jordan et al. 1993; Liebmann et al. 1993; Wagner 1994; Kovács and Csaba 2006)
Vinblastine	0.02	1	ND, resistant	3	0.05	(Kramers and Stebbings 1977; Dhamodharan et al. 1995; Grellier et al. 1999; Kiso et al. 2004; Kovács and Csaba 2006)
Oryzalin	>50	0.1	ND, resistant	ND	6	(Bajer and Molè-Bajer 1986; Kobayashi et al. 1997; Fennell et al. 2006; Lyons-Abbott et al. 2010)

shows very low binding affinity towards colchicine (Kovács and Csaba 2006). Colchicine is apparently selectively directed against animal predators.

The relatively high affinity of colchicine towards animal tubulin has been associated with two animal-specific amino acid residues in the colchicine-binding region—Ala 248β and Pro 268β. They are substituted by Ser 248β and Val or Ile 268β in plants, fungi, and protists (α and β symbols denote positions in α- and β-tubulin, respectively). These substitutions probably lead to complex rearrangements in the tubulin molecule that are thought to disrupt hydrogen bonding of tubulin with colchicine (Banerjee et al. 2007). Protozoan β-tubulin was also modeled using *Leishmania* sp. sequence aligned to a known crystallographic structure, indicating that the colchicine-binding site is hindered by an extension and torsion of an α-helix and a displacement of a β-sheet, preventing colchicine access (Luis et al. 2013).

Taxanes

Taxanes are a group of microtubule drugs best represented by paclitaxel (Taxol) and several related semisynthetic compounds with a common mechanism of action. Paclitaxel disrupts microtubular cytoskeleton in animal cells by decreasing microtubule dynamics and (in higher concentrations) by causing tubulin to assemble into superfluous stable structures (Jordan 2002). Treatment with paclitaxel has been successful in several types of cancer although the action on tumor is associated with several adverse effects. Neutropenia (a decrease in the number of neutrophils, a subset of rapidly dividing white blood cells) is symptomatic of the antiproliferative effects of paclitaxel (Mitchison 2012). Similarly, peripheral neuropathy has been linked to disruption of microtubule-associated transport in sensory neurons and neuronal cell death in the tissues of cancer patients (Komlodi-Pasztor 2011).

Paclitaxel differs from many other microtubule drugs because it is almost equally effective in plant and animal cells (Vaughn and Vaughan 1988). It is claimed that the paclitaxel-binding site is more conserved among all eukaryotes than the colchicine-binding site (Morejohn and Fosket 1984). A compelling explanation for this observation is that paclitaxel is an "unknown" molecule for both animal and plant cells because this compound is synthesized by the endosymbiotic fungi, such as *Taxomyces* sp., colonizing the bark of Pacific yew tree (Stierle et al. 1993). Indeed, several species of fungi (representatives of basidiomycetes, ascomycetes, and deuteromycetes) were found to be resistant to taxane treatment (Wagner 1994). Wildtype *Saccharomyces cerevisiae* tubulin does not bind paclitaxel either (Foland et al. 2005). However, a thorough metabolic assay found no traces of taxanes in the *Taxomyces* sp. cells, although paclitaxel biosynthesis genes were initially detected in their genome (Staniek et al. 2009), possibly by means of horizontal gene transfer. *Taxomyces* sp. genome was later completely sequenced, and an extensive search yielded no paclitaxel biosynthesis genes whatsoever (Heinig et al. 2013). In the light of these studies, the proposed explanation for the paclitaxel-binding site conservation does not seem valid anymore.

It is noteworthy that *Phytophthora* sp. and *Pythium* sp. oomycetes were found to be sensitive to paclitaxel (Wagner 1994; Mu et al. 1999) in times when oomycetes were considered to be a class of fungi. The current placement of oomycetes in the kingdom Chromista, far from fungi, creates a more logical image of paclitaxel selectivity. Otherwise, trypanosomatid protozoa, such as *Leishmania* and *Trypanosoma*, are now known to be susceptible to paclitaxel in micromolar concentrations and in a dose-dependent manner (Baum et al. 1981; Kapoor et al. 1999; Havens et al. 2000).

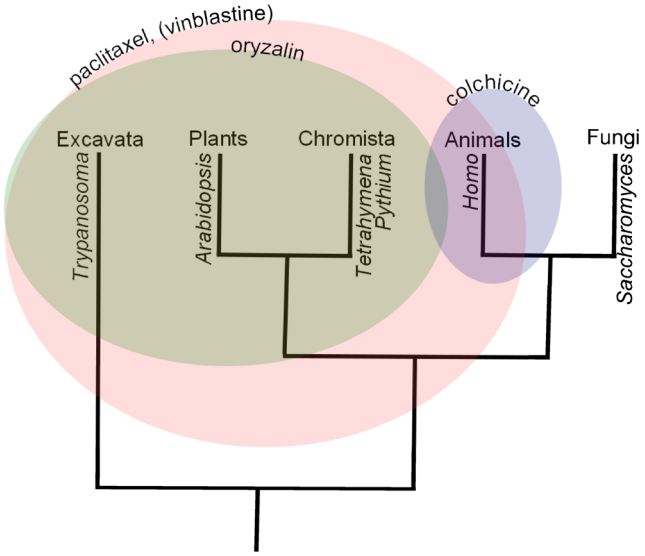

Fig. 3 Sensitivity of various eukaryotic groups towards microtubule drugs mapped in a cladogram. Only most sensitive groups are mapped. *Vinblastine in parenthesis* indicates lower quality of available experimental data

A closer look on *S. cerevisiae* β-tubulin led to important insights concerning its resistance to paclitaxel. By comparing fungal and animal β-tubulin, five key amino acid residues were found that could confer tubulin resistance towards paclitaxel: yeast residues Ala 19β, Thr 23β, Gly 26β, Asn 227β, and Tyr 270β; they all locate to paclitaxel-binding regions. When these residues were artificially replaced by their animal counterparts (Lys 19β, Val 23β, Asp 26β, His 227β, and Phe 270β), the yeast became paclitaxel-sensitive (Gupta et al. 2003). All five substitutions were later discussed in terms of their effect on paclitaxel binding; some of them cause a loss of contact between paclitaxel and tubulin, and others prevent the structural distortions of tubulin that are required for binding of paclitaxel (Das et al. 2012). A molecular modelling study by Akbari et al. (2011) came to similar conclusions and stressed residue 227β as a key to fungal resistance to paclitaxel.

Dinitroanilines

Dinitroanilines represent a group of potent microtubule drugs used as preemergent herbicides, applied to seeds before germination. Oryzalin and trifluralin are the most commonly used compounds from this class.

Oryzalin (Surflan) has been called the "colchicine of the plant kingdom" (Bajer and Molè-Bajer 1986) because its effects on plants are similar to those of colchicine, decreasing the mass of polymerized microtubules. Plant bodies and tissues show a characteristic response to mitotic disruption, symptomatic of microtubular damage. In *Arabidopsis thaliana* treated with oryzalin, cell division is inhibited and deposition of cellulose is impaired, leading to malformation in the proliferating shoot and root meristem regions. Both effects are known consequences of microtubule disruption: inhibition of mitosis results from interference with mitotic spindle, and deposition of cellulose requires microtubules to achieve correct orientation of microfibrils. Interestingly, the cells do not enter apoptosis after oryzalin treatment and rather continue to increase their volume (Corson et al. 2009). This is unlike the typical behavior of similarly treated animal cells. On a subcellular level, chromosomes enter a prometaphase arrest in a so-called colchicine (C) mitosis (Vaughn and Lehnen 1991), reminiscent of the mitotic arrest seen in animal cells. Oryzalin treatment, like that with colchicine, can elicit polyploidy in plants in vitro (Yemets and Blume 2008).

Oryzalin acts mainly on grasses and a few broad-leafed plants (Altland et al. 2003); therefore, it can be safely applied to control weeds in field nurseries or in certain crops such as soybean. Soybean (*Glycine max*) is approximately 110× more resistant to oryzalin in the seed germination test than oat (*Avena sativa*) and 10× more resistant than ryegrass (*Lolium* sp.), a weed commonly eliminated by oryzalin. Systematically, monocots are the most sensitive to oryzalin while rosids (including cucumber, soybean, cabbage, and other crops as well as many wild plants) are usually the most resistant (Feutz 1992). These differences still wait for a satisfactory explanation; they might be attributed to shifted expression ratio of plant tubulin isotypes or to subtle changes in their sequence. Position 253α is believed to play an important role, influencing the strength of dimer-dimer contacts. Asn 253α of sensitive plants is often substituted by Thr 253α in resistant plant species, animals, and fungi. Positions 16α, 136α, 239α, and 252α are other examples of residues which follow this distribution pattern distinguishing sensitive and resistant species (Délye et al. 2004).

While oryzalin is generally a potent inhibitor of plant microtubules (10 nM oryzalin is enough to affect the course of anaphase in *Haemanthus* sp. endosperm cells), *Xenopus* endothelial primary culture was found to be highly resistant to oryzalin—concentrations as high as 50 μM caused only slight disturbances to the normal cell physiology and proliferation (Bajer and Molè-Bajer 1986; Dow et al. 2002). Rust *Melampsora lini* was observed to be also resistant to oryzalin, compared to its host plant (Kobayashi et al. 1997). Fungi, close relatives of animals (Baldauf and Palmer 1993), are generally believed to be resistant to oryzalin (Lyons-Abbott et al. 2010; Lopes et al. 2012). In contrast, many protozoa are sensitive to oryzalin treatment such as *Plasmodium falciparum* (Fennell et al. 2006; Dempsey et al. 2013), a plastid-carrying parasite that is evolutionarily closer to plants than it is to animals (Cavalier-Smith 2010). Similarly, oomycetes, which are now classified into Chromista, are susceptible to oryzalin (Utkhede 1982; Walker and Morey 1999).

The mechanism of oryzalin susceptibility and resistance has been covered in several studies. One of the first explanations was proposed by Anthony et al. (1998) who discovered a point mutation in a strain of goosegrass (*Eleusine indica*) responsible for oryzalin resistance. Nowadays, a number of point mutations have been identified which cause the acquisition of oryzalin resistance in plants or protozoa. These mutations sometimes localize to the proposed dinitroaniline-binding site (e.g., 136α Leu→Phe) or the GTPase activating domain (252α Val→Leu). Importantly, some of the known important substitution sites can elicit the same effect in both plants and protozoa. Moreover, 29 amino acid residues were found to be characteristic for both trypanosomatid and plant α-tubulins. These residues are not conserved in animal tubulins and thus could potentially be implicated in sensitivity (Traub-Cseko et al. 2001; Lyons-Abbott et al. 2010).

Trifluralin is the second most studied dinitroaniline compound currently in use as a herbicide. Like oryzalin, it causes microtubule disassembly in the cells, disrupts the correct course of mitosis, and inhibits root growth. In *Chlamydomonas* sp. algae, it blocks the regeneration of flagella (Hess and Bayer 1977). The drug has also been shown to be effective against various protozoan parasites such as *Toxoplasma*, *Trypanosoma*, *Leishmania*, and *Cryptosporidium* (Traub-Cseko et al. 2001). Cross-resistance to oryzalin has been reported from trifluralin-resistant strains of various organisms (Vaughn et al. 1987; Stokkermans et al. 1996). Conversely, animal cells are generally immune to the antimicrotubule effects of trifluralin; it does not bind to animal tubulin nor does it affect the cell culture growth in a saturated solution of trifluralin (Hess and Bayer 1977). However, trifluralin toxicity has been reported in various animals including carp (Poleksić and Karan 1999) and millipedes (Merlini et al. 2012). Mechanism of this toxicity remains unknown and there is no evidence of trifluralin binding to animal tubulin. Trifluralin effects on the growth of fungi have not been studied yet.

Vinca alkaloids

Vinca alkaloids (vinblastine, vincristine, vinflunine, and others) are natural or semisynthetic compounds first discovered in periwinkle *C. roseus*. They have profound effects on mammalian microtubules, inhibiting their dynamics and, in higher concentrations, promoting the microtubule disassembly (Ngan et al. 2000). Only limited information is available regarding the action of vinca alkaloids in nonmammalian organisms. They were shown to have antiparasitic effects against *Trypanosoma cruzi*, inhibiting its mitosis and affecting its cell shape (Grellier et al. 1999). The compounds also bind to *Leishmania mexicana* tubulin and interfere with its assembly at low micromolar concentrations (Werbovetz et al. 1999) and affect growth of *T. pyriformis* ciliates (Kovács and Csaba 2006). Plants are, to a very limited extent, susceptible to the effects of vinca alkaloids (Degraeve and Gilot-Delhalle 1972; Hillmann and Ruthmann 1982), but there is a general lack of more recent reports on the subject. Root tip cells of garden cress (*Lepidium sativum*) and broad bean (*Vicia faba*) show metaphase arrest at 100 μM concentration of vinblastine (Kramers and Stebbings 1977; Hillmann and Ruthmann 1982). *Aspergillus nidulans* mutant with hyperstable microtubules was not rescued by vinblastine, indicating a lack of activity in fungi (Kiso et al. 2004). Vinblastine elicits no effects in *S. cerevisiae* either (Bode et al. 2002).

Novel compounds

Traditional classes of microtubule drugs have been reinforced by many newer compounds of synthetic or natural origin (reviewed in Kingston (2009)). The latter include epothilones discovered in bacterium *Sorangium cellulosum*, combrestatins isolated from Cape Bushwillow tree (*Combretum caffrum*), and dolastatins from a sea slug *Dolabella auricularia* (Bollag et al. 1995; Pettit et al. 1989; Pettit et al. 1981). Additionally, bodies of marine sponges are a rich source of novel compounds, including discodermolide from *Discodermia dissoluta*, halichondrins from *Halichondria okadai*, and hemiasterlins from *Hemiasterella minor* (Gunasekera et al. 1990; Hirata and Uemura 1986; Talpir et al. 1994).

Very little information is available on the activity of these compounds apart from their antiproliferative effects on cancer cells. Some of them may have exploited new target positions on the tubulin molecule. Other novel compounds share their binding site with the traditional classes of microtubule drugs—such as epothilones, targeting the paclitaxel-binding region (Akbari et al. 2011). Still, epothilones have markedly different binding properties. In contrast to paclitaxel, epothilones bind to yeast *S. cerevisiae* tubulin and promote its assembly (Bode et al. 2002). This is explained by the fact that the two compounds are chemically very different even though they share a common binding site. Key residues for epothilone binding are conserved in fungi, and epothilone can withstand certain substitutions as its 3D structure is more flexible than that of paclitaxel (Akbari et al. 2011).

As more knowledge is acquired, the relative affinity of these compounds in diverse eukaryotes will prove enormously interesting. Notably, many of the compounds are of animal origin but act potently on growth of animal cancer cells. Such high potency of the compounds on representatives of "their own" kingdom is not seen in colchicine or vinblastine but can be compared to that of paclitaxel.

Tubulin comparisons: shedding light on differences in drug action

Microtubule drugs show distinct activity patterns among the various systematic groups of eukaryotes. To understand these differences, it is useful to compare amino acid sequences of α- and β-tubulin proteins of animals, plants, fungi, and various protists and search for outstanding substitutions. These often constitute the underlying cause of drug resistance.

Many tubulin mutations conferring resistance to microtubule drugs have been described to date, and two general mechanisms have been proposed to explain their impact. Firstly, the amino acid substitutions can localize to the binding sites of the microtubule drugs or to their immediate proximity. Alternatively, resistance to microtubule drugs can be caused by any mutation which alters the microtubule dynamics: for instance, drugs which stabilize microtubules are likely to be less potent in mutants with intrinsically higher microtubule dynamics. The spatial distribution of resistance-conferring mutations in tubulin was reviewed by Nyporko and Blume (2009). However, these are almost exclusively mutations conferring drug resistance in representatives of taxonomical groups that are normally sensitive to it—exceptions to the rules, so as to say.

Literature dealing with kingdom-specific differences in tubulin sequences, i.e., mutations typically yet exclusively found in some eukaryotic kingdoms, is much more limited. Older articles, using comparisons of electrophoretic mobility of tubulins, can be traced back to the pregenomic era (Little et al. 1981; Little et al. 1982; Little et al. 1984). Intriguingly, one of these articles was describing a fundamental difference between chromist and plant tubulin on one hand and animal tubulin on the other hand (Little et al. 1982). Fungi were not included in these comparisons. Nowadays, phylogenetic data can be used together with the known location of several drug-binding sites: high-resolution model paclitaxel-binding site (Löwe et al. 2001) or paclitaxel-, colchicine-, and vinblastine-binding regions defined by 6-ångström (Å) radiuses around the bound drug molecules (Huzil et al. 2006) as well as the most recent estimation of the dinitroaniline-binding site (Nyporko et al. 2009). Sequence analysis has elucidated several kingdom-specific mutations in tubulin (Traub-Cseko et al. 2001; Banerjee et al. 2007; Luis et al. 2013) which were mentioned in the previous chapter.

Hunting for the kingdom-specific tubulin mutations

Thanks to the progress in sequencing, virtually all important groups of eukaryotes are now represented by at least a partially known genome sequence in the databases. The ever-growing list of sequenced tubulins can be used to extract the outstanding substitutions from their sequences "across" the eukaryotic kingdoms. A data set of 88 α-tubulin and 73 β-tubulin polypeptide sequences (partially adapted from Banerjee et al. (2007)) was carefully aligned in search for common features and differences. These can subsequently be visualized with a sequence logo, which is, in its simplest design, a graphical representation of amino acids present in the polypeptide positions. The size of amino acid symbols in the figure reflects the frequency of the corresponding amino acids at the specific position.

In the analyzed collection of sequences, approximately half of all amino acid positions are identical in at least 95 % (225 amino acid residues in α-tubulin and 248 in β-tubulin). Conserved regions are distributed along the whole length of tubulin. They include, among others, the residues in the proximity of a bound GTP or a magnesium ion and at the longitudinal contacts between subunits. β-Tubulin is generally richer in long unbroken conserved regions than α-tubulin. The most prominent variable region is the acidic C-terminal domain. The unique conservativeness of tubulin has been explained by the immense functional constraints inflicted on the tubulin molecule, which has evolved to bind GTP and assemble into intricate dynamic polymers (Ludueña 2013).

Not all positions are conserved and a careful analysis of the sequences yields a number of kingdom-specific mutations. A total of 69 α-tubulin and 51 β-tubulin kingdom-specific substitutions were recovered (see Electronic Supplementary Figs. 1 and 2), including both conservative and nonconservative substitutions as even a change in one methyl group of a residue can influence the binding properties of tubulin to microtubule drugs (Burns 1992). Some of these changes can be so consistently conserved that they provide hints to specific evolutionary events; for instance, a closer look on positions 22β or 248β reveals that substitutions probably occurred in the common ancestor of subdivision Pezizomycotina, the crown group of ascomycete fungi which does not include "lower" ascomycetes such as *Saccharomyces* or *Pneumocystis*.

The recovered substitutions include not only many previously published positions but also some new ones which might deserve further investigation. Several examples are shown in Fig. 4. Among those already known are positions 19β, 23β, and 26β implicated in *S. cerevisiae* resistance to paclitaxel (Gupta et al. 2003); 248β and 268β responsible for colchicine affinity in animals (Banerjee et al. 2007); and 313β and others, hypothesized to confer resistance to colchicine in kinetoplastid protozoa such as *Leishmania* or *Trypanosoma* (Luis et al. 2013). Residue 268α was also proposed to be responsible for the selective action of dinitroanilines by (Anthony et al. 1998). Many others were reported by Traub-Cseko et al. (2001) to account for the selectivity pattern of dinitroanilines. Noticeably, position 248β has been described in three of the articles mentioned above (Traub-Cseko et al. 2001; Banerjee et al. 2007; Luis et al. 2013); it may indeed

prove to be a hallmark of microtubule drug sensitivity in general. In contrast, a putative importance of residues 227β and 231β for fungal resistance to paclitaxel (Akbari et al. 2011) can be questioned as *S. cerevisiae* substitutions 227β His→Asn and 231β Ala→Ser are not kingdom-specific in any way. All kingdom-specific positions described in the cited literature are marked yellow in the Electronic Supplementary Figs. 1 and 2.

A few kingdom-specific positions reside in the vicinity of the proposed drug-binding sites, offering the most straightforward way of explaining selectivity of microtubule drugs. These include positions 23β and 26β in the paclitaxel-binding site (Löwe et al. 2001; Gupta et al. 2003), potentially responsible for fungal resistance to paclitaxel; the same may be true for 279β and 280β glutamines (shown in Fig. 4), located in the 6-Å area around the bound paclitaxel. Residue 232β is also a part of the 6-Å area around paclitaxel. Residues 238β, 248β, 257β, 350β, 351β, and 352β are located in the colchicine 6-Å area, but only 248β is specific for animals and thus could be accounted for colchicine selectivity. Residues 221β and 222β are found in the 6-Å vicinity of bound vinblastine (Huzil et al. 2006). Finally, 252α and 253α probably form a part of the proposed dinitroaniline-binding site (Nyporko et al. 2009). Previously undescribed kingdom-specific positions which reside in drug-binding sites or their surroundings include 252α (dinitroaniline site); 22β, 279β, and 280β (paclitaxel site); and 221β (vinblastine site). These have been marked blue in the Electronic Supplementary Figs. 1 and 2.

Some of the discovered kingdom-specific differences coincide with tubulin mutations known to cause resistance to microtubule drugs in various species of animals, plants, fungi, and protists (reviewed in Nyporko and Blume (2009)). Those could potentially be the sites which allowed drug-producing organisms to target the drug to their predators. Namely, it is the case of 268α Met→Thr mutation recorded in goosegrass and accounting for its resistance to dinitroanilines (Yamamoto et al. 1998), 383α Ala→Val in Chinese hamster cells leading to cross-resistance to vinblastine and colcemid (Hari et al. 2003), 26β Asp→Glu in KB-3-1 human cell line conferring resistance to taxanes (Hari et al. 2006), and 350β mutated Lys→Glu in *C. reinhardtii* and conferring resistance to colchicine and dinitroanilines (Lee and Huang 1990) or 350β Lys→Asn in human cell lines leading to resistance to indanocine, a synthetic colchicine site binding agent (Hua et al. 2001). None of these mutations simulate precisely the known kingdom-specific differences, but it is conceivable that resistance depends on a mere presence/absence of a key residue or chemical group. Overall, new point mutations rarely reside in the kingdom-specific positions, suggesting that the resistant lineages rarely "choose" from the pool of kingdom-specific sites. One notable exception are mutations in α-tubulin of *Toxoplasma gondii* lines which were selected for resistance to oryzalin; out of 17 identified mutation sites (Morrissette et al. 2004), 7 coincided with kingdom-specific mutations.

The outstanding conservation of tubulin, together with the presence of kingdom-specific substitutions, constitutes a living proof of the protein's crucial role in the life of eukaryotic organisms. A gradually emerging concept will once be able to map regions of the protein that are important for resistance to various microtubule drugs.

Drug-producing species: a taste of their own medicine

Several plants produce their own antimicrotubule compounds in order to protect themselves from their herbivores, and these plant species are thus naturally subjected to high concentrations of their "own" poisons. In any case, the producer plants clearly must have a mechanism to escape the effects of microtubule-active drugs. Considering the importance of this phenomenon which might help us develop more powerful or more selective drugs, it comes as a surprise that the understanding of this topic is fragmentary and only a handful of articles have been published to date.

First, relatively little is known about the kinetics and dynamics of microtubule-active compounds inside the plant bodies. Endogenous alkaloids are produced in a certain plant organ or structure (seeds, bark, leaves) but can sometimes be transported to another organ where they often accumulate in the cell vacuoles. Some alkaloids can freely pass the vacuolar membrane (tonoplast), but, once inside this acidic compartment, they are protonated and effectively trapped (Shitan and Yazaki 2007). While this may apply to vinblastine and other alkaloids, paclitaxel has no ionizable side groups (Mahoney et al. 2003) and cannot be easily trapped inside the vacuole. High amounts of paclitaxel are therefore found in the plant cell wall (Choi et al. 2001).

The first mitotic poison to be discovered—and subsequently studied—was colchicine. Very soon, scientists began to wonder why *Colchicum* is unaffected even though the plant itself contains as much as 0.4 % colchicine by dry weight (Blakeslee 1939). Blakeslee compared the situation to the "snake and snake's venom" conundrum and expressed a belief that *Colchicum* contains an antidote, which, although theoretically possible, has never been found in the plant. Further studies have shown that the resistance to colchicine is very specific—*Colchicum* is sensitive to acenaphthene, a different mitotic poison (Levan 1940). The biochemical mechanism for *Colchicum* resistance to colchicine is not known (Vaughn and Vaughan 1988), and no relevant publications have been published to date.

Several instances show that the plant resistance to its poison can be conferred by pharmacokinetic and pharmacodynamic characteristics of the drug inside the tissues of drug-

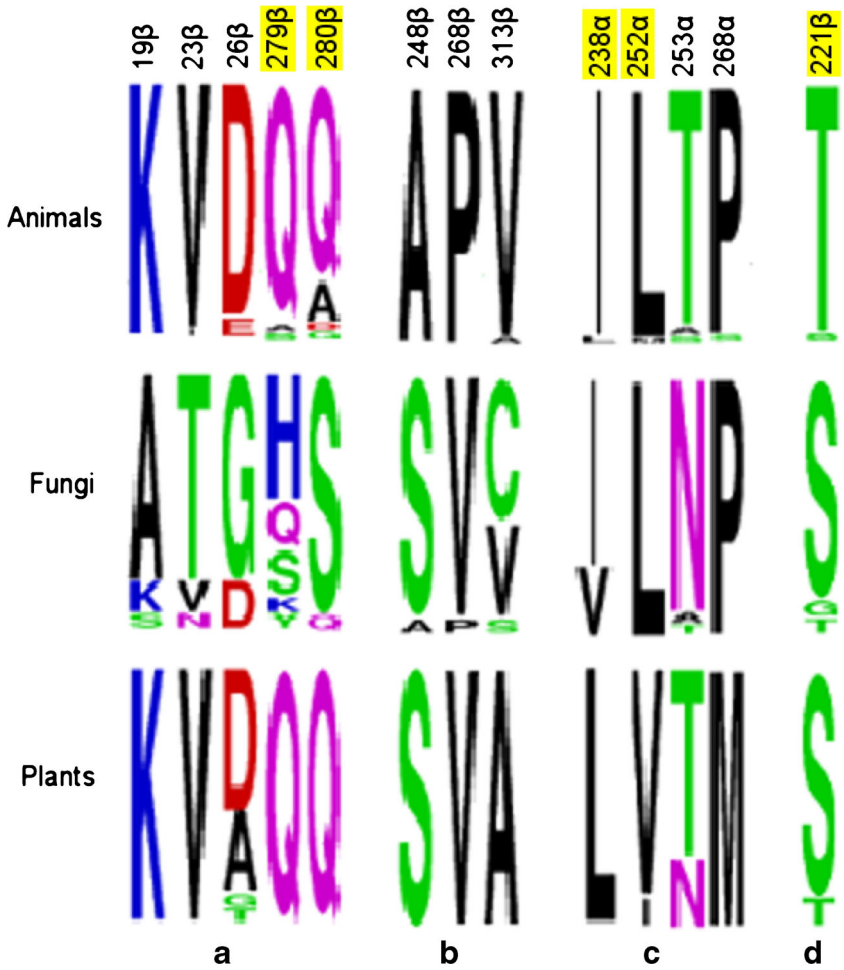

Fig. 4 Sequence logos for animal, plant, and fungal tubulin residues showing kingdom-specific differences located in the vicinity of drug-binding sites for **a** paclitaxel, **b** colchicine, **c** dinitroanilines, and **d** vinblastine. Some of these positions have already been mentioned in literature (see text); the unpublished ones are indicated with *yellow labels*. The scheme was generated by WebLogo (Crooks et al. 2004). Only selected residues, considered most interesting on basis of their kingdom specificity and location in the drug-binding sites, are shown

producing plants. In the case of vinblastine-producing *C. roseus*, which is at least 100–1,000× less sensitive to its drug vinblastine than a control species (garden cress—*Lepidium*, family Brassicaceae), it is hypothesized that intracellular vinblastine is in its inactive form, which is secreted from the cells, and, during or after secretion, modified to become the active vinblastine molecule (Kramers and Stebbings 1977). This hypothesis, however, fails to explain why the active vinblastine would not reenter the cells. A similar yet more sophisticated scenario has been suggested for the resistance of *Podophyllum peltatum* to its microtubule-binding drug podophyllotoxin. This compound is present in the plant vacuoles as an inactive glucoside (podophyllotoxin-4-*O*-β-D-glucopyranoside) and thus cannot harm the plant. At the same time, however, a highly specific β-glucosidase is present in the cytosol but is practically inactive because its pH optimum lies in acidic values (5.0, compared with pH ~7.0 in the cytosol). Only when insects feed on the *Podophyllum* plant, the cells are damaged, vacuolar membrane is disrupted and the enzyme comes into contact with its substrate. The acidic pH of the mix leads to rapid deglucosidation and activation of the podophyllotoxin, effectively poisoning the herbivorous insects (Dayan et al. 2003).

There are other solutions to being resistant to one's own poisons. One of them is to alter the molecular target of the drug and thus escape its deleterious effects. One of the possible explanations for *C. roseus* resistance to vinblastine is that the vinca-binding site in tubulin is mutated to prevent the vinblastine from binding to it (Kramers and Stebbings 1977). This has, however, never been proven for vinblastine or, until recently, for any other tubulin-binding compound. As a part of the 1000 Plants Initiative (1KP), European yew tree (*Taxus baccata*) transcriptome was sequenced and its tubulin gene has recently been analyzed to allow comparison with other tubulins. Results show that the yew tree tubulin is highly mutated in the paclitaxel-binding region when compared with a homologous human tubulin gene. Sixty-five percent of all substitutions in α- and β-tubulins are located on the surface of β-tubulin. Moreover, 95 % of these are substitutions on the luminal side of β-tubulin, where paclitaxel-binding site is found. Some of the mutations are located adjacent to the nanopores which form in the microtubule wall and allow

diffusion of paclitaxel towards the lumen of the microtubule, but subsequent analysis has shown that the size of these openings is not significantly altered by these substitutions (Tuszynski et al. 2012). Large-scale analyses are currently conducted on other plants such as *Colchicum* sp. (J. A. Tuszynski, University of Alberta, personal communication).

The resistance of the drug-producing marine sponges (Porifera) is as enigmatic as it is neglected in research. Thousands of new and promising compounds have been isolated from these marine invertebrates (Sipkema et al. 2005). Microtubule drugs constitute only a fraction of these, suggesting that their ability to produce drugs—while not causing harm to themselves—is a more general phenomenon. The production of toxic compounds by marine sponges may relate to their unusual body composition: up to 50–60 % of the sponge biomass is composed of endosymbiotic microorganisms such as actinobacteria and fungi. These microbes have sometimes been made responsible for the actual production of bioactive compounds (Thomas et al. 2010; Waters et al. 2010). In soil, actinobacteria are known to produce diverse bioactive compounds to compete with other microbes while being completely resistant to them (Hopwood 2007). Similar phenomenon might well explain the production of microtubule drugs by microbes living in the crowded sponge microenvironment. It will be very interesting to determine whether the compounds, produced by these endosymbionts, exert their activity against fungi or other competitors of drug-producing microbes inside marine sponges.

While marine sponges are presumably resistant to their toxins, their tubulin displays typical animal features including its sequence in the predicted drug-binding sites. Most importantly, the vinca-binding site of *Halichondria* sp., where halichondrin binds (Bai et al. 1991), is almost identical to the tubulin of vertebrates (unpublished data). It still remains a mystery why these compounds do not kill their host sponges while remaining active against human cancer cells—especially when taking into account that such drugs can often be produced in one cell and elicit their effect elsewhere (Waters et al. 2010).

Concluding remarks

We have summarized available information on two kinds of interspecies differences responsible for variable potency of microtubule drugs: changes specific for the various kingdoms of life ("kingdom-specific") and changes in drug-producing organisms. Although it was shown that both phenomena can be connected to changes in general behavior of microtubule drugs (spatial separation, pharmacokinetic parameters), they have usually been attributed to differences in tubulin sequence. These phenomena enable us to inspect pharmacologically interesting processes. The nature of changes, altering the sensitivity to drugs, offers an inspiration for a more precise drug delivery and selectivity and might provide hints at the problem of drug resistance. The data may thus prove relevant to anticancer therapy and herbicide research.

Why some of the differences in tubulins are so conserved and kingdom-specific remains an open question. Tubulin has very similar functions in all the kingdoms of eukaryotes, and adaptive mutations posing an evolutionary advantage for their carrier are presumably rare. Conceivably, some mutations could be adaptations to the need of more dynamic or, conversely, rigid microtubules in various kingdoms of life. Some of the known differences on the surface of tubulin might well be explained by the presence of specific MAP-binding sites. In contrast, it is highly unlikely that the observed interkingdom differences are adaptations to microtubule drugs: these drugs did not exist at the moment of divergence of all the major eukaryotic clades. Rather, the selective nature of many microtubule drugs suggests that the drugs themselves evolved to exploit the preexisting differences in tubulins. This was achieved by, for instance, mimicking MAPs to bind to ancient binding sites as there was a selective pressure on compounds that would harm the natural enemies but not the producing species. This takes us to the examination of tubulins in drug-producing organisms which should be especially tolerant to high concentrations of their microtubule drugs. We have seen that, apart from altering the tubulin structure, they have also exploited other ways of achieving resistance.

Many differences in sensitivity to microtubule drugs could potentially be explained by the evolution of tubulin paralogs, i.e., tubulin isotypes. Information on the relative importance, distribution, and function of tubulin isotypes in various eukaryotes is, to a large extent, missing. Some aspects of drug sensitivity and resistance could hypothetically be attributed to differences in drug efflux (expression of drug transporters) or metabolism. There is also a complete lack of data on many drug-producing plants and their unexplained resistance to their own toxins. Several plant stories might be uncovered soon, but other microtubule drug-producing organisms, such as marine sponges (Porifera) should not be missed out of focus either.

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