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Review

The Binding Sites of Microtubule-Stabilizing Agents

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Microtubules (MTs) are a highly successful target for anticancer therapy. MT-stabilizing agents (MSAs) bind to MTs, promoting their polymerization, blocking mitosis, and causing cell death. There are currently four clinically important MSAs, with many others in preclinical and clinical development. MTs have three binding sites for these compounds; however, the exact locations and drug-protein interactions of these sites are still controversial. This review will describe the possible binding sites, the compounds that bind to them, and the effect of this binding on MT function. The binding site of an MSA on tubulin is important for characterizing the compound as an anticancer agent and provides insight not only into possible synergistic interactions with other compounds but also on the MSA "pharmacophore." This information can aid in the design of novel MSAs with improved properties.

Microtubules

Microtubules (MTs) are essential in all eukaryotic cells as key components of the cytoskeleton. They are critical for a number of cellular processes, most importantly, cell division and mitosis. MTs are characterized by their dynamic behavior, constantly switching between periods of elongation and shrinkage, driven by the energy obtained from guanosine-5'-triphosphate (GTP) hydrolysis. Upon polymerization, tubulin assumes a straight conformation, and potential energy is stored within the MT lattice. Depolymerization releases this energy, and the released dimers resume a curved conformation (Nogales et al., 2003). The tubulin heterodimer is made up of an α and a β subunit, arranged in a polar head-to-tail fashion, and these heterodimers assemble into protofilaments that arrange in a parallel manner to form the MT. Each subunit contains a nucleotide binding site. The α subunit has a stable nonexchangeable nucleotide binding site (N-site) that binds GTP. The β subunit has an exchangeable nucleotide site (E-site) that binds GTP or GDP. Bound GTP at the E-site is hydrolyzed to GDP soon after polymerization. Assembly is favored when GTP is bound to the E-site; whereas, GDP favors disassembly. A polymerizing MT therefore has a "GTP cap" on its growing end that stabilizes the straight conformation of the MT lattice (Akhmanova and Steinmetz, 2008). Hydrolysis of GTP to GDP is not required for the assembly of the MT but is required for its dynamic instability (Jordan and Wilson, 2004).

GTP-bound dimers are able to form protofilaments; whereas, GDP-bound dimers are inactive, prone to disassembly, and form double rings in the presence of Mg^{2+}. Although it was originally thought that GTP-bound dimers were straight (allosteric model), it has now been shown in biochemical and structural studies that this is not the case. The lattice model predicts that unassembled tubulin is in a naturally curved state, regardless of the bound nucleotide, and that on polymerization, it takes on a straight or activated state. The γ-phosphate of GTP only lowers the free energy between the two different nucleotide dimer states. The dimers only differ in their flexibility for adopting a straight conformation that is driven by the lateral and longitudinal contacts occurring upon polymer assembly (Buey et al., 2006). This implies that all tubulin dimers, irrespective of nucleotide bound, are in a "MT incompatible" state, or an inactive curved state, and are driven to the straight conformation as a consequence of lateral contacts made during MT assembly. This has been confirmed by crystallography (Gigant et al., 2000; Nawrotek et al., 2011).

Microtubule-Targeting Drugs

Drugs that target the MT can be classified into two main groups based on their mechanism of action. MT-destabilizing agents (MDAs) promote depolymerization and prevent polymerization of tubulin, and MT-stabilizing agents (MSAs) promote polymerization of tubulin and stabilize the polymer, preventing depolymerization. These compounds are able to induce the formation of tubulin polymers even when GDP is bound in the E-site; whereas, normally under these conditions, tubulin does not assemble into MTs. Although many MT-associated proteins (MAPs) are known to be MT stabilizers, nonendogenous MSAs tend to bind more tightly, inhibiting the natural activity of tubulin by promoting near irreversible assembly (Amos, 2011). The exact mechanism of mitotic arrest and consequent apoptosis induced by an MSA is still not fully understood. It is however widely accepted that their antimitotic action occurs through interference with the spindle dynamics within the cell. Affected cells fail to pass mitotic checkpoints and arrest at the G_2/M phase of the cell cycle. G_2/M block is the hallmark of MTAs. Cells blocked in G_2/M subsequently undergo cell death (Jordan and Wilson, 2004). Both MSAs and MDAs have proven successful in the clinic in treating a number of different cancers. At high concentrations, these drugs have opposite effects on polymer mass; however, at low concentrations, these drugs have a similar effect, that of inhibiting the dynamic instability of MTs (Nogales, 2000; Jordan and Wilson, 2004). MTs are also required for endothelial cell migration, and thus some MT-targeting agents are able to inhibit angiogenesis as well as mitosis, inhibiting vascularization of tumors (Jordan and Wilson, 2004).

The majority of MSAs are natural products or synthetic derivatives of natural products. These compounds have most likely evolved as broad-spectrum toxins to target eukaryotic tubulin in predators and/or prey. In addition to cancer, some MSAs

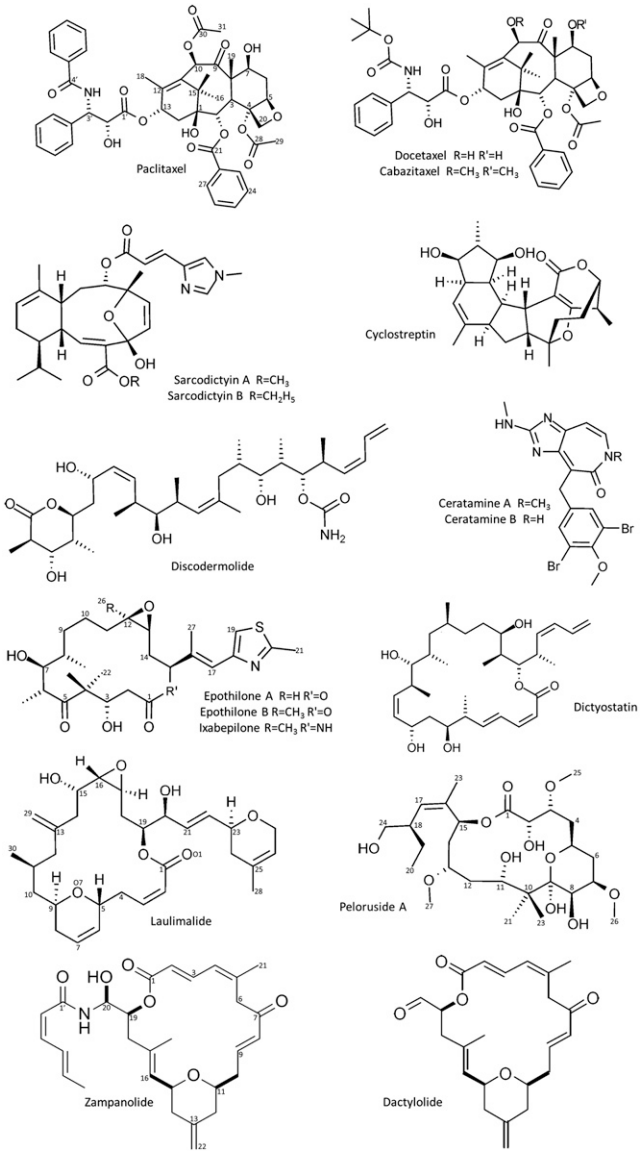

Figure 1. Structures of Microtubule-Stabilizing Agents
Stabilizing agents referred to in the text have been numbered using standard IUPAC numbering.

also show promise as treatments for other diseases (Crume et al., 2009; Brunden et al., 2009). The first agent to be identified as an MSA was paclitaxel (Taxol) (PTX) (Schiff et al., 1979). PTX received FDA approval in 1992 and has been clinically used against cancer since 1993, along with one of its semisynthetic analogs docetaxel (Taxotere) (DTX), which has been clinically available since 1996. These drugs are used to treat solid tumor malignancies such as breast cancer, ovarian cancer, nonsmall cell lung cancer, gastroesophageal cancer, germ cell tumors, and cancers of the head and neck. The taxanes can be used in neoadjuvant, adjuvant, and metastatic settings or in synergistic combinations with other drugs (Jordan and Wilson, 2004). More recently, cabazitaxel (Jevtana), another semisynthetic taxane, has been FDA-approved (Galsky et al., 2010). Cabazitaxel has poor affinity for the P-glycoprotein drug efflux pump that is involved in multidrug resistance and is effective in DTX-resistant cancers. It is currently used in the clinic to treat hormone-refractory metastatic prostate cancer. The epothilones (EPOs) are macrolide antibiotics, and like the taxanes, bind to and stabilize MTs (Bollag et al., 1995). The EPOs are considered easier to produce than the taxanes with one synthetic derivative currently used in the clinic: aza-EPOB (Ixempra) to treat metastatic breast cancer. A number of EPOs are currently in clinical development for different tumor types. New generation EPOs are able to penetrate the blood-brain barrier, giving potential for treatment of glioblastoma (Dumontet and Jordan, 2010). Other MSAs at various stages of development, preclinical and clinical, (reviewed by Amos, 2011) include discodermolide (DSC), the sarcodictyins, eleutherobin, the laulimalides (LAUs), the covalent binder cyclostreptin (CYC), the pelorusides (PELs), dictyostatin, ceratamines (Manzo et al., 2003), and the covalent binder zampanolide (ZMP) and its related enantiomer dactylolide (DAC) (Figure 1). DSC entered a phase I clinical trial, but the trial was discontinued due to significant pulmonary toxicity (Dumontet and Jordan, 2010). In preclinical tests in mice, despite showing promising results in cell-based and pharmacokinetic studies, LAU proved ineffective in preventing cancer xenograft growth and also presented with significant systemic toxicity and mortality (Liu et al., 2007). PEL has shown good efficacy in preclinical trials in mice. Three lung cancer xenograft studies in mice were carried out comparing PEL to PTX and DTX. Overall, PEL showed superior tumor growth inhibition, including some actual tumor regression, and it was better tolerated than the taxanes (Meyer et al., 2006). Given these initial encouraging results, further preclinical development of PEL was considered, but because a large-scale synthetic program has not been possible, no clinical trials have yet been entered into.

Recognizing the importance of MTs as a drug target, this review will provide a description of the possible small molecule binding sites on MTs, overview the different compounds that bind to them, and discuss the effects of this binding on MT function.

Microtubule-Stabilizing Agent Binding Sites

There are currently two well-known binding sites for MSAs on the MT—the well-characterized luminal taxoid site and the less well-characterized LAU/PEL site. There is also an external pore type I site proposed that is associated with the taxoid site. Thus, MSAs can be divided into two groups depending on the site on the MT where they bind. The larger group includes PTX and its biomimetics. These compounds bind to a site on the luminal surface of β-tubulin in the MT, the taxoid site (Nogales et al., 1998) and also may interact with the pore type I site (Díaz et al., 2003, 2005; Barasoain et al., 2010). Binding to the pore type I site on its own can cause MT stabilization, leading to cytotoxicity (Barasoain et al., 2010). Currently, all drugs that bind the taxoid site bind with 1:1 stoichiometry: one MSA molecule per heterodimer; thus, binding at these two sites must be mutually exclusive (Díaz and Andreu, 1993; Nogales et al., 1995). The second group of binding agents consists of LAU and PEL that target a not yet fully characterized binding site. This site was originally shown, however, to be biochemically distinct from the taxoid site (Pryor et al., 2002; Gaitanos et al.,

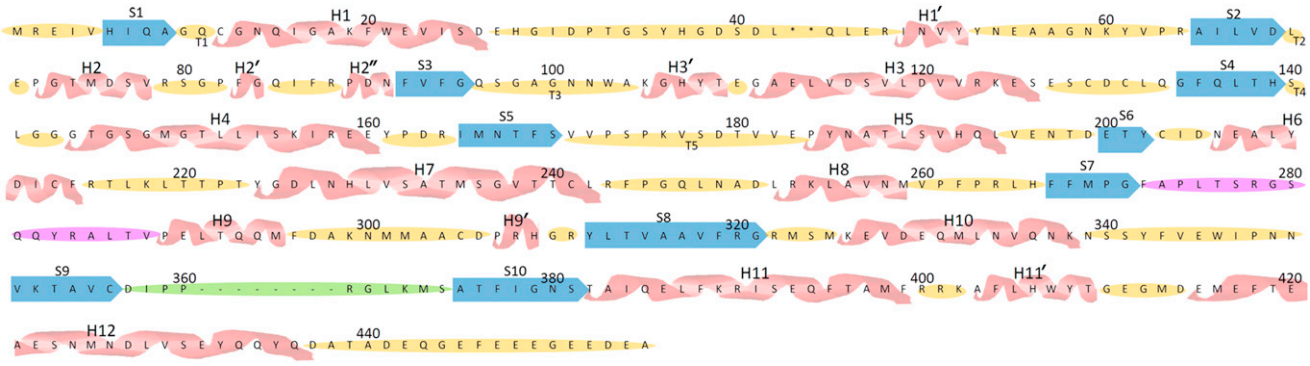

Figure 2. Sequence and Secondary Structure of β-Tubulin
Alpha helices are shown in pink, β sheets in blue, and joining loops in yellow. The M-loop is shown in purple and the S9-S10 loop in green. All segments are labeled and defined as in Löwe et al. (2001), protein data bank entry 1JFF.

2004). There are experimental data supporting the location of this site on both the α- and β-tubulin subunits, but most recent studies support an external β-tubulin site (see below).

The Taxoid Binding Site

The PTX binding site was first described 20 years ago by direct photoaffinity-labeling to be on the β-tubulin subunit (Rao et al., 1992), launching an active area of structural biology research. Over the years, the view of the PTX binding site has been gradually refined from an initial 6.5 Å model (Nogales et al., 1995) to 3.5 Å, to provide critical insights into this region (Nogales et al., 1998, 1999; Löwe et al., 2001; Snyder et al., 2001; Li et al., 2002). The early structural work confirmed that PTX binds with 1:1 stoichiometry and affects interactions between protofilaments. PTX was observed to bind in the intermediate domain, occupying a site on the luminal face of β-tubulin where eight extra residues are found in α-tubulin. The main interactions of the compound involve the taxane ring. The majority of known MSAs bind to the taxoid site and reversibly compete with PTX for binding.

In this review, we explore the key structural features that form the molecular basis for PTX /MT binding. We use the residue numbering based on the structure alignment between α and β subunits (Löwe et al., 2001) (1JFF in the Protein Data Bank); therefore, the numbering of specific residues may differ by two when referring to some of the original cited articles. Residues 361–368 (in the S9-S10 loop) are missing in the β-sequence because the α subunit has an extra eight residues in this region. Thus, the β-sequence numbering proceeds from β360 straight to β369 (Figure 2).

The PTX binding pocket is located at the luminal site in β-tubulin and is made up of the S9-S10 loop and parts of helices H1, H6 and H7 (the core helix of the β subunit), and S7 (Figure 2). The taxane ring of bound PTX sits on the N-terminal side of the MT-loop (M-loop, connecting S7 to H9) that protrudes from the protofilament and is an important secondary structure for stabilization of the MT. Protofilaments are connected primarily by interprotofilament interactions between the M-loops, H1′-S2 loops, and the H2-S3 loops in adjacent protofilaments (Nogales et al., 1998, 1999; Sui and Downing, 2010) where the most important interaction is with the H1′-S2 loop of the adjacent protofilament. PTX binding strengthens this contact by pushing the M-loop out toward the neighboring protofilament, resulting in more favorable contacts between adjacent protofilaments. This interaction is crucial for stabilization by PTX and its biomimetics because it increases the lateral protofilament interactions (Nogales et al., 1995, 1998, 1999; Li et al., 2002; Sui and Downing, 2010). The luminal site in β-tubulin has PTX facing the inside of the MT, with the equivalent area in the α subunit being occupied by eight extra amino acids (within the S9-S10 loop) (Nogales et al., 1998; Amos and Löwe, 1999). The extra residues in α-tubulin have a stabilizing effect on the M-loop, and it is thought that PTX and other taxoid site compounds may mimic this effect in the β subunit (Nogales et al., 1999; Löwe et al., 2001). This has recently been confirmed by X-ray crystallography (Prota et al., 2013).

The taxoid binding pocket lies in a "deep hydrophobic cleft" where three hydrogen bonds (H-bonds) and multiple hydrophobic contacts are made between PTX and tubulin (Snyder et al., 2001). A considerable number of amino acid residues have direct contacts with PTX when it is bound to tubulin (see Figure 1 for carbon numbering of PTX, Figure 3 for the interaction maps, and Table 1 for details about specific residues). In brief, the view of PTX binding that emerged from early studies was that PTX binds the β subunit in its second globular domain on the opposite side of the core helix from where the E-site is located and faces the inside of the MT (Amos and Löwe, 1999). Photoaffinity labeling identified amino acids within the β subunit that were important in the binding of PTX (Rao et al., 1994, 1995), and Arg284, a residue that is also mutated in some PTX-resistant cells, was later identified as important for the binding (Rao et al., 1999). Overall, the resolution of these structures, however, was not good enough to completely characterize PTX in its bound conformation, and the fact that the information was based on zinc-stabilized sheets rather than functional MTs meant that the interactions in vivo could differ to some extent. In 2001, Snyder and colleagues used computer docking based on crystallographic density analysis to construct a model of PTX in its bioactive conformation (Snyder et al., 2001). This model confirmed that the taxoid binding site was located within a deep hydrophobic cleft and involved three potential H-bonds and a number of hydrophobic interactions, supporting the previous research.

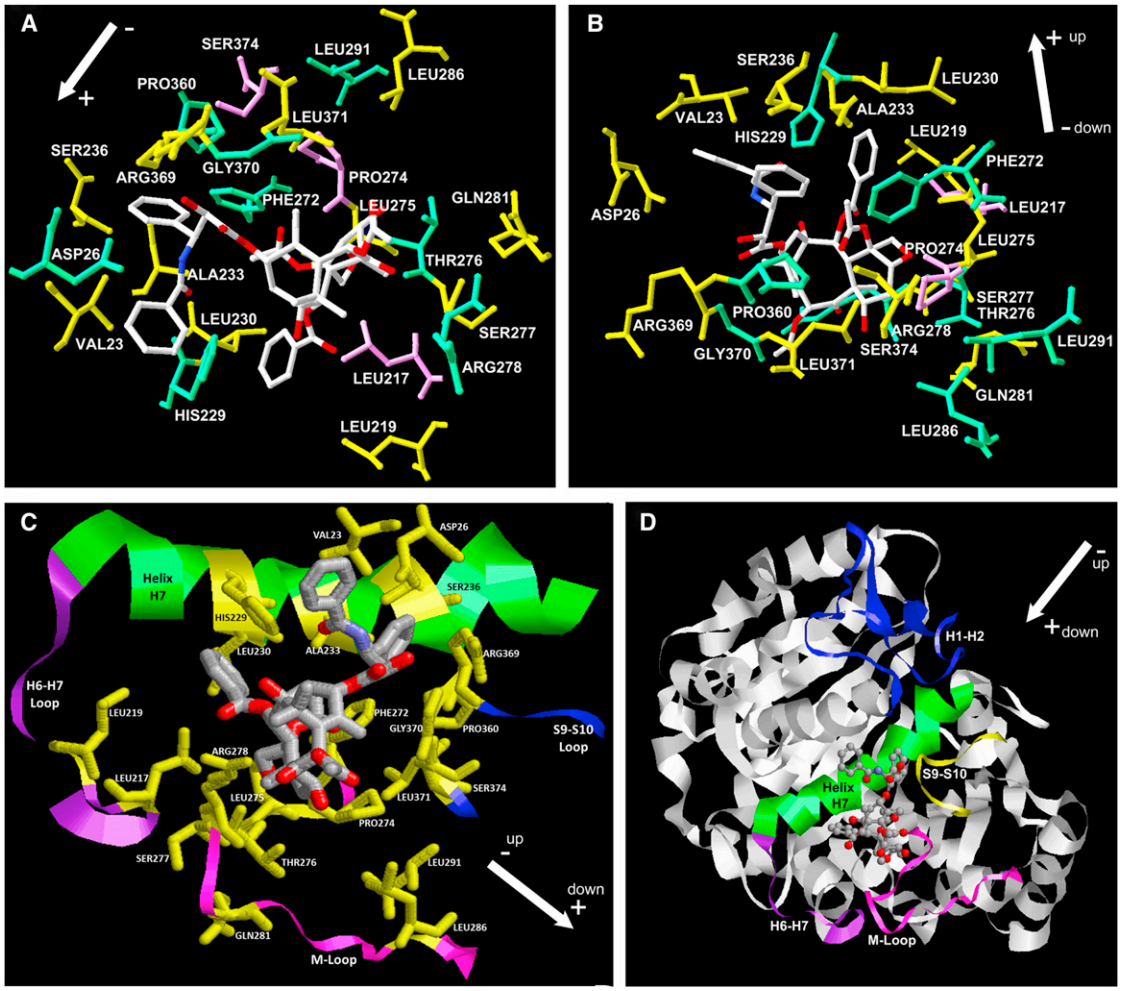

Figure 3. The Luminal Taxoid Binding Site

(A and B) PTX (CPK coloring) in its binding site with the interacting amino acids.

(C) Overall view of PTX (CPK) in its binding site showing all interacting residues and secondary structures (the core helix 7 is shown in green, the M-loop in magenta, the H6-H7 loop in purple, and the S9-S10 loop in blue).

(D) View of the β-tubulin subunit with PTX (CPK) in its binding site. The core helix H7 is shown in green, the M-loop in magenta, the S9-S10 loop in yellow, the H6-H7 loop in purple, and the H1-H2 loop in blue.

The location of the taxoid site is also supported by mutant cell data from the Giannakakou laboratory, who described PTX- and EPO-resistant cell lines (Giannakakou et al., 1997). PTX-resistant cells have single amino acid changes at Ala374 (this residue is a Ser in the bovine brain tubulin used in Figure 2) located in the S9-S10 stabilizing loop and at Phe272 at the start of the M-loop, both regions proposed to be in the binding site of PTX (Nogales et al., 1999) (Table 2). Two EPO-resistant cell lines also demonstrated cross-resistance to PTX. These cell lines have single amino acid mutations at positions Thr276 and Arg284, both amino acids having been shown to be directly involved in PTX and EPO binding (Giannakakou et al., 2000). A number of other mutant cell lines have also been described that induce resistance to some but not all of the PTX-site compounds, depending on how the MSAs interact with the pocket (see Table 2 for details). In general, these mutations occur at specific sites within structural features that surround the taxoid binding site or mediate contacts between two tubulin subunits in the context of the protofilament. A number of these mutations have been confirmed using random mutagenesis and a number of new mutations identified, all of which showed that mutations tended to cluster around the H6-H7 loop (Yin et al., 2012). However, mutations outside the binding site could still affect the ability of an MSA to bind due to allosteric or structural changes in tubulin. Tubulin mutations often cause changes in the stability of a MT, even without a drug, and thus the mutated amino acid may be completely outside the binding site of a particular compound.

The Pore Site: A Two-Site Binding Hypothesis

Rapid staining of MTs with the fluorescently-labeled PTX analog Flutax (FTX) led to speculation that PTX binds on the surface of the MT, with the C7 and C10 moieties facing toward the solvent (Evangelio et al., 1998). This is consistent with the finding that PTX rapidly induces flexibility in MTs assembled in vitro, indicating that access to the binding site is unhindered and fast (Dye et al., 1993). However, the taxoid binding site model

Table 1. Tubulin Regions and Amino Acid Residues Involved in PTX, EPOA, ZMP, LAU, and PEL Stabilization

Structural Features	Residues	Interactions	References
Paclitaxel			
H1	Gln15–Ser25	near C3′of PTX	Rao et al. (1994); Nogales et al. (1998)
H5 and H5-H6	Ile212–Pro222	near C2	Rao et al. (1995); Nogales et al. (1998)
M-loop	Leu275	main interaction point with taxane ring	Nogales et al. (1998)
M-loop	Arg284	C7 contact	Rao et al. (1999)
M-loop; H1′-S2	Arg284, Glu55	salt bridge (in PTX-bound MTs)	Mitra and Sept (2008)
M-loop	Pro274, Leu275, Thr276, Ser277, Arg278	interaction with taxane ring	Nogales et al. (1999); Löwe et al. (2001)
H1	Val23	hydrophobic contact with 3′ and 4′ phenyl rings	Löwe et al. (2001)
H1	Asp26	H-bond distance with nitrogen side chain	
H6-H7	Leu217, Leu219	hydrophobic contact with C2 phenyl ring	
H7	His229, Leu230	complete hydrophobic contacts with C2 phenyl ring	
H7	Ala233, Ser236	contact with 3′ phenyl ring	
S7 end	Phe272	hydrophobic contact with 3′ phenyl	
M-loop	Pro274, Leu275, Thr276	contact with oxetane ring	
S9-S10	Pro360, Arg369, Gly370, Leu371	binding pocket contacts	
H7	Leu230, Ala233	hydrophobic basin holding C4 acetate ring	Snyder et al. (2001)
M-loop	Phe274, Pro274, Thr276 (CH_3), Leu286		
H9	Leu291		
S9-S10	Pro360, Leu371		
S10	Ser374 (CH_2)		
M-loop	Thr276, Gln281	C8 methyl van der Waals contacts	
M-loop	Thr276	$O_2$1 weak electrostatic interaction	
S9-S10	Leu371	in proximity to C12	
M-loop	Ser277	side chain H-bond with C7 OH	Freedman et al. (2009)
Epothilone A			
H1	Asp226	OH7 H-bond with side chain oxygen	Prota et al. (2013)
M-loop	Thr276	O1 H-bond back bone NH_2 and N20 H-bond side chain OH	
M-loop	Gln281	OH_3 H-bond side chain amide nitrogen	
M-loop	Arg278–Tyr283	restructured into short well-defined helix	
Zampanolide			
H7	His229	C9 covalent bond with His side chain NH (major)	Field et al. (2012)
H7	Asn228	C3 covalent bond with side chain amide (minor)	
M-loop	Thr276	C20 OH and C1′ carbonyl H-bond with main chain carbonyl and NH_2	Prota et al. (2013)
M-loop	Arg278–Tyr283	restructured into a short well-defined helix	
Laulimalide (β subunit)			
H10-S9	Asn339	H-bond with C15 OH and defines cavity entrance	Bennett et al. (2010)
H9-H9′, H10-S9	Phe296, Arg308, Tyr342	side chains protrude into binding pocket and reorganize	

(*Continued on next page*)

Table 1. *Continued*

Structural Features	Residues	Interactions	References
H9-H9′, S8, H10, H10-S9	Phe296, Tyr312, Val335, Asn339, Ser341, Tyr342, Phe343, Ile347	residues with binding interactions in pocket; O1 close to Asn339 side chain NH, O3 epoxide to Tyr342 phenolic OH, C20 OH to backbone NH of Ser341 and Tyr342	Nguyen et al. (2010)
S8	Tyr312	side chain pyran O7 H-bond to Tyr312 backbone NH2	
H10-S9	Phe343 aromatic ring	side chain pyran group stacked	
H10-S9	Phe343 ring and Ile347 side chain	C28 methyl of pyran ring wedged between residues	
H10	Val335	pyran C5-9 packed against hydrophobic side chain	
H9-H9′, S8	Phe296, Tyr312 aromatic side chains	favorable binding surfaces with C30 CH_3 and C29 CH_2	
H10-S9	Undefined	C20 OH could H-bond with this loop and residues may form water-mediated H-bonds with LAU	
Peloruside A (β Subunit)			
Various	β288–293, 296–303, 304–316, 334–343	important in binding pocket	Huzil et al. (2008); Chen et al. (2008)
H9-H9′, H9′, H10-S9	Asp297, Ala298, Pro307, Arg308, Asn339, Tyr342	pocket holding PEL macrolactone	Huzil et al. (2008)
H9, H9-H9′, H9′ H10-S9	Gln294, Asp297, Arg308, Asn339, Val335, Try342	side chains involved in pocket holding macrolactone	
H9-H9′	Phe296	C26 wedged against side chain	Nguyen et al. (2010)
H9′	Arg308 guanidine side chain	C9 and C11 hydroxyl long-range electrostatic interactions or potential water-mediated H-bonds or potential intermittent H-bonds between 308 and OHs	
H9′	Arg308 side chain	hydrophobic binding surface for PEL; hydrophobic interactions with C10 and C12	
H9′	Arg308	C27 interacts with aliphatic side chain	
S8	Tyr312	hydrogen bond with C24 hydroxyl	
S8	Tyr312	C10 CH_3 groups wedged against side chain, C23 interacts with side chain	
H10	Val335	C25 hydrophobic interaction	

developed by Nogales et al. (1999) showed that the binding site was located in the lumen of the MT. Thus, it was proposed that taxanes and other taxoid site ligands reach their luminal binding site by diffusion through openings on the MT surface.

Openings in the MT wall were first observed in 1974 by Amos and Klug (1974) and later modeled at high resolution (Nogales et al., 1999). In the MT structure, two different types of pores have been described, type I and type II. The type I pore has the β subunit at its lower boundary (plus-end orientated upward), with the luminal taxoid site in close proximity; whereas, the type II pore has the α subunit at its lower boundary. The main differences between the two pores are changes in the H6-H7 loop and the degree of hydrophobicity (Díaz et al., 2003). The size of one of these pores in the MT wall is considered too small for passive diffusion of MSAs. Although pore size is comparable with ligand size, diffusion would be extremely slow, and larger openings are not consistent with the MT structure and kinetic data. Diffusion of ligands from the open MT end is highly unlikely. In addition, the binding of taxoids to the MT is slowed considerably when MAPs are bound to the outer surface of the MT. Further evidence against direct diffusion of MSAs to the taxoid site is that PTX reaches its binding site too quickly (Díaz et al., 2003), with the number of protofilaments changing within 1 min of PTX addition (Díaz et al., 1998). Also, as mentioned, PTX rapidly modifies MT flexibility (Dye et al., 1993), and FTX rapidly stains MTs (Evangelio et al., 1998). Other work has shown that the taxoid binding site is likely to be kinetically inaccessible as it is hidden from the exterior in MT models (Nogales et al., 1999). To explain the observed data in view of the structure of the MT, a fast binding theory for ligands that target the taxoid

Table 2. Mutations in β-Tubulin that Confer Resistance to MSAs

Resistance to	Cell Line	WT Residue	Mutated Amino Acid	Location	Reference
Paclitaxel	1A9-PTX10	Phe272	Val	M-loop	Giannakakou et al. (1997)
	1A9-PTX22	Ala374	Thr	S9-S10 loop	
	CHO	Leu217	His, Arg, Phe	H6-H7 loop	Gonzalez-Garay et al. (1999)
	CHO	Leu219	Arg, Asn, Pro	H6-H7 loop	Gonzalez-Garay et al. (1999)
	CHO	Leu230	His, Phe	H7	Gonzalez-Garay et al. (1999)
	KB-3-1	Asp26	Glu	H1	Hari et al. (2006)
Docetaxel	LNDCr	Phe272	Ile	M-loop	Hara et al. (2010)
Epothilone A	1A9-A8	Thr276	Ile	M-loop	Giannakakou et al. (2000)
	HeLa	Pro175	Ala	T5-loop	He et al. (2001)
Epothilone B	1A9-B10	Arg284	Gln	M-loop	Giannakakou et al. (2000)
	HeLa	Tyr434	Cys	H12	He et al. (2001)
	A549, CCRF-CEM, A549	Gln294	Glu	H9	He et al. (2001); Verrills et al. (2003)
					Yang et al. (2005)
	CCRF-CEM	Ala233	Thr	H7	Verrills et al. (2003)
	A549	Val62	Phe	H1′-S2	Yang et al. (2005)
Laulimalide	1A9-L4	308	His (70%); Cys (30%)	H9′	Kanakkanthara et al. (2011)
Peloruside	1A9-R1	Ala298	Thr	H9-H9′ loop	Kanakkanthara et al. (2011)
	1A9	Ala298	Ser	H9-H9′ loop	Begaye et al. (2011)
	1A9	Arg308	His	H9′	
	1A9	Asn339	Asp	H10-S9 loop	
	1A9	Tyr342	Ser	H10-S9 loop	

Mutations in β-tubulin that confer resistance to MSAs. The "resistance to" column gives the MSA with which the cells were selected. 1A9 cells (subclone of A2780), human ovarian carcinoma; CHO cells, Chinese hamster ovary; KB-3-1 cells, epidermoid carcinoma; LNDCr cells, human prostate cancer; A549 cells, human nonsmall cell lung cancer; HeLa cells, human cervical adenocarcinoma; CCRF-CEM cells, leukemia (desoxy-EPOB not EPOB used).

site was required, and thus it has been hypothesized that an external binding site exists. Pore type I describes the space between α and β subunits from neighboring heterodimers, specifically in the region of Phe214, Thr220, Thr221, and Pro222 (H6-H7) (Figure 4). In this model, binding to both sites is mutually exclusive, and both sites possibly share a "switching element" that accompanies the two different binding modes. This is supported by the 1:1 stoichiometry observed for taxoid site ligands. Díaz et al. (2005) demonstrated that fluoresceinated taxoids could be labeled by an antifluorescein antibody, and this would only occur if the molecule was bound at the surface, given that antibodies cannot diffuse into the luminal compartment of the MT. Additionally, doublecortin, a MAP known to bind to the MT in a position that covers the pore site, causes MT assembly (Moores et al., 2004); thus, a pore location for an assembly promoter is not a new concept. In order to facilitate this movement of an MSA from the outer site to the inner site, it is proposed that there are conformational changes involving residues in the external binding site moving in toward the luminal site and resulting in loss of the external site and formation of the internal site.

The H6-H7 loop in β-tubulin, which is flexible at Gly225 and highly hydrophobic, may play a role in the initial binding of PTX at the pore type I site, and this binding then assists in the translocation of PTX to the luminal site (Díaz et al., 2003). This loop is also involved in the luminal binding site, with Leu217 and Leu219 making hydrophobic contact with PTX (Löwe et al., 2001), and mutations in this loop are associated with PTX resistance (Gonzalez-Garay et al., 1999; Yin et al., 2012). Using computational analysis, it has been proposed that arrangement of the H6-H7 loop causes the formation of an external binding pocket large enough to accommodate an MSA. The same computational analysis was applied to the other pore site on the dimer, pore type II; however, no binding pocket was seen, indicating that the proposed external binding site is likely only found at pore type I (Magnani et al., 2009). This binding model describes taxoid site drugs binding to the temporary pore type I site on the surface of the MT with the H6-H7 loop acting to transport the ligand from the pore site to the luminal site (Díaz et al., 2003, 2005; Buey et al., 2007; Magnani et al., 2009). Thus, pore type I acts as a transition station for compounds passing through to the luminal PTX binding site. This hypothesis is supported by the fact that 7-Hexaflutax (fluorescent taxoid derivative), which only binds to the pore type I site, has similar MT-stabilizing activity to compounds that bind to the luminal site. This suggests that the pore type I site can be probed as a new druggable site on MTs (Díaz et al., 2005; Barasoain et al., 2010). The existence of this external site is also supported by indirect NMR evidence for a low affinity binding site for MSAs, in which measurements done with DTX and DSC support the idea that a site with much lower affinity, not the luminal site that has high affinity for taxoid site MSAs, is responsible for recognition of these MSAs (Canales et al., 2008, 2011).

Consistent with this external binding site hypothesis, a recently discovered MSA, CYC, the first MSA identified to bind

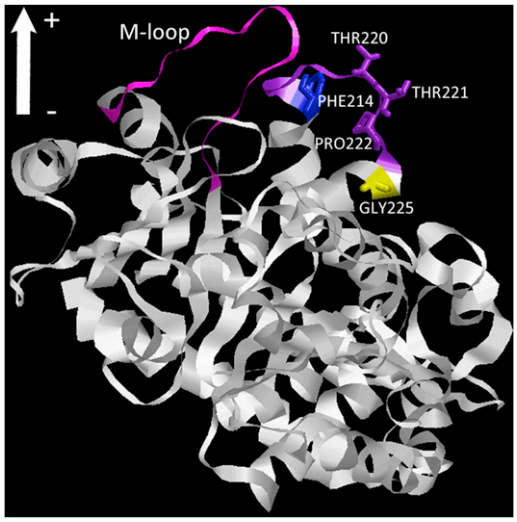

Figure 4. Pore Type I Site
Beta subunit showing the M-loop (magenta) and the H6-H7 loop (purple). Important residues are highlighted. GLY225 (yellow) is thought to act as a hinge in the movement of this loop between the pore site and the luminal binding site. Note that this image has been made from zinc-stabilized sheets, and therefore the H6-H7 loop is in its luminal binding site conformation rather than the pore site. Thus, this is not a true representative pore site image and only shows interacting components.

covalently, is able to alkylate both MTs and tubulin dimers. CYC irreversibly competes with taxoid site drugs and covalently modifies two amino acids in β-tubulin, Thr220, located in pore type I, and Asn228, located near the taxoid site with its side chain facing into the E-site. In unpolymerized tubulin, only Thr220 is labeled by CYC, indicating that the pore type I is in fact a binding site and suggesting that pore type I may be the only binding site for MSAs in dimeric tubulin (Buey et al., 2007).

To summarize, binding of MSAs to the taxoid site is proposed to occur in a two-step mechanism (Díaz et al., 2000), with the first step being binding to the pore type I site on the outside of the MT (Buey et al., 2007) from which the MSA dissociates with fast kinetics (Díaz et al., 2000). The second step is a slow reaction that involves a shift of the MSA to the internal binding site. These events would be reversed for dissociation of the MSA from the MT, with translocation from the luminal site to the external pore site being the slow step and the second step being the fast release of the ligand from the pore site. This hypothesis explains how PTX can reach the kinetically unfavorable site in the lumen of the MT. Freedman et al. (2009) tested this hypothesis using computational molecular modeling and confirmed that the H6-H7 loop in the pore acted as a hinge in the first binding step of PTX. This motion was stabilized by a H-bond with Ser277 located in the M-loop. This residue is an alanine rather than a serine in βIII- and βVI-tubulin isotypes (Freedman et al., 2009), and the fact that βIII-tubulin is less sensitive to PTX than βI-tubulin (see review by Burkhart et al., 2001) further supports the role of Ser277 and H6-H7 loop motion.

Arguing against the two-step binding hypothesis, Prussia et al. (2010) claim that a low affinity site on the exterior of the MT is not necessary to explain the available data. They base their conclusion on results of molecular dynamics and modeling work that suggests that the shape and lipophilic character of pore site I make it an unlikely binding site for MSAs. It is proposed that the pore simply slows MSA diffusion into the interior of the MT, acting like a "funnel" or channel without a distinct binding event occurring. It is further suggested that the fast binding kinetics of MSAs can be explained by a pause in the diffusion of the ligand traveling through the pore via transient interactions with specific residues, explaining the observed rapid binding. This would be consistent with CYC labeling of Thr220 because it is located in the narrowest part of the pore. Overall, it is proposed by Prussia et al. (2010) that the concept of a binding site at the pore is an artifact of the slow diffusion through the pore into the luminal site, with the pore being nothing more than an entry site.

In contrast to the Prussia model, the evidence supporting the pore type I binding site has been accumulating over the last few years, and includes the previously discussed 7-hexaflutax binding behavior and data on the binding kinetics of FTX-2, both of which suggest that the interaction is specific (Díaz et al., 2005; Barasoain et al., 2010). A recent study of CYC analogs has shown that two of the analogs also covalently modify Thr220 (Calvo et al., 2012), again supporting pore type I as an MSA binding site. The current overall consensus is that MSAs may bind either the luminal or pore site in unassembled tubulin; whereas, in MTs it is likely that they first bind pore type I (fast kinetics) and then move into the luminal binding site with the assistance of residues in the H6-H7 loop. This is consistent with and explains the majority of the data currently available in the field.

From the above studies, it is still not clear whether PTX and its biomimetics bind to only the inner, only the outer, or to both sites, even though it is known that taxoids bind in a 1:1 stoichiometry (Díaz and Andreu, 1993; Díaz et al., 2000) and that binding to either site on its own causes tubulin assembly (Barasoain et al., 2010). Both sites utilize the residues in the H6-H7 loop, indicating that when a ligand is bound at one site, binding to the other site is not possible. Thus, ZMP (which binds the luminal site) and 7-Hexaflutax (which binds pore site I) cannot bind at the same time (Field et al., 2012; Magnani et al., 2009). Therefore, binding to either site prevents binding to the other, and likely involves a switching element between the two sites, such as the H6-H7 loop. Cryo-electron microscopy of doublecortin-stabilized MTs indicates that the taxoid binding pocket is empty (Fourniol et al., 2010). It is therefore likely that the pore site is a transient binding site for taxoid site compounds on their way to their final destination in the lumen.

The Luminal Binding Site in Unassembled Tubulin

MSAs promote the assembly of tubulin heterodimers into MTs, shifting the equilibrium toward the polymeric state. MSAs were not originally thought to bind with measurable affinity to unassembled tubulin, because it was believed the taxoid binding site only existed in assembled MTs (Parness and Horwitz, 1981; Díaz and Andreu 1993). Interprotofilament contact was therefore assumed to be required for binding to the taxoid site. It is now accepted that MSAs can bind to dimeric tubulin because they are able to induce the formation of MTs in conditions unfavorable to MT assembly, conditions in which no MTs exist. This has now been confirmed by direct biochemical studies with ZMP and X-ray crystallography (Field et al., 2012;

Prota et al., 2013) and also by the fact that the taxoid site in zinc-stabilized sheets is similar to that in unassembled tubulin, with only small structural differences (Prota et al., 2013).

Ligand binding to dimeric tubulin is necessary to explain the way in which MSAs work. An MSA must have a binding affinity higher for the assembled species than the dimeric species (Díaz et al., 1993; Canales et al., 2011). Given this higher free energy of binding toward polymerized MTs, the equilibrium would displace toward the polymer (Wyman and Gill, 1990). Because MSAs can induce MT assembly in conditions in which no preformed MTs exist, it is reasonable to predict that a lower affinity site on dimeric tubulin starts the assembly process. The first experimental proof of this low affinity site was based on the detection of NMR signals with MSA binding (Carlomagno et al., 2003; Sánchez-Pedregal et al., 2006; Canales et al., 2008, 2011).

Until recently, there has been no direct biochemical evidence for the luminal site existing in dimeric tubulin. Nuclear magnetic resonance (NMR) and computational studies have now provided evidence of a low affinity site on dimeric tubulin (Canales et al., 2008, 2011) as well as low affinity binding of MSAs to non-homogeneous tubulin preparations (Carlomagno et al., 2003; Sánchez-Pedregal et al., 2006). From these studies, however, it is not possible to determine whether binding is at the pore site or at the luminal site. It has now been shown that ZMP and DAC covalently modify two amino acids within the luminal binding site of the isolated heterodimer, providing the first direct evidence that the luminal binding site exists in tubulin dimers (Field et al., 2012). Therefore, as previously proposed by Canales et al. (2011) and Reese et al. (2007), MSAs can bind to both the luminal site and the pore type I site in unassembled tubulin. The exception, of course, is CYC that can only bind the pore site in the dimer. The existence of the luminal site is also supported by the X-ray crystallographic structures of ZMP and EPOA bound to unassembled tubulin (Prota et al., 2013).

Binding of Other MSAs to the Taxoid Site

After the taxanes, the next most important MSAs are the EPOs, given that an analog (Ixempra) is currently approved for use in the clinic. The detailed binding interactions of EPOA with the MT were first solved to 2.9 Å resolution by electron crystallography using zinc-stabilized sheets (Nettles et al., 2004). This gave direct evidence that the taxanes and EPOs share the same binding region, as previously thought. Recently, the X-ray crystallography structure of EPOA bound to unassembled tubulin has been solved to 2.6 Å (Prota et al., 2013). This model contrasts with the earlier model, possibly because the X-ray crystallographic model is more defined, allowing for a clearer interpretation of the conformation and orientation of the bound ligand. In the model by Prota et al. (2013), EPOA forms four H-bonds with residues in the taxoid site (Table 1) that are all key participants in PTX binding. When EPOA is bound, it induces restructuring of the M-loop into a short and well-defined helix with residues 278–283 forming a number of hydrophobic and polar contacts with the side chain of EPOA and stabilized by intramolecular H-bonds between the M-loop and the H9. When no ligand is bound, the M-loop is relatively unstructured. This restructuring is now thought to be the underlying molecular basis of stabilization by MSAs (Prota et al., 2013).

DSC is a taxoid site MSA with a unique profile relative to the taxanes. DSC competes with PTX for binding to MTs (Hung et al., 1996) but can also synergize with PTX, suggesting differences in binding modes (Martello et al., 2000). Unlike other MSAs, DSC is a more flexible, linear compound (Figure 1). A photo-labeled DSC analog labeled residues that form part of S9 and the S9-S10 loop in β-tubulin (Xia et al., 2006) and are in close vicinity to the taxoid site (Löwe et al., 2001). Given the synergy seen between PTX and DSC, the two compounds presumably have distinct poses in the site. Using chicken erythrocyte tubulin, PTX was shown to bind to the luminal taxoid site, orientated toward the M-loop, stabilizing the β-tubulin side of the interdimer; whereas, DSC is orientated away from the M-loop—more toward the H1-S2 loop and stabilizing the α-tubulin side of the interdimer interface (Khrapunovich-Baine et al., 2009). In support of this distinct binding mode, DSC has previously been shown to be active in PTX-resistant human cells (Kowalski et al., 1997).

ZMP and DAC also target the taxoid binding site; however, they do so in a different manner to the traditional MSAs, covalently attaching to His229 (major product) and Asn228 (minor product). Solving the ZMP-tubulin structure by X-ray crystallography to 1.8 Å resolution has provided an accurate binding site for ZMP in unassembled tubulin (Prota et al., 2013). As the previous model proposed, ZMP covalently binds to His229 in the taxoid binding pocket; however, the placement of the ZMP side chain conflicts with the earlier modeling predictions. ZMP forms two H-bonds with M-loop residue Thr276. The ZMP side chain induces restructuring of the M-loop into a short, well-defined helix, much like the side chain of EPOA. Although their side chains superimpose well, their macrolide cores are oriented 90° to one another, held in the taxoid site by different sets of interactions. This induced helical structuring of the M-loop is explained by the extensive hydrophobic and polar contacts formed between the side chain of ZMP and M-loop residues, as seen with EPOA. This helical M-loop restructuring is now considered a "hallmark" of MSA binding and explains the effect of these MSAs on MT assembly and stabilization. It also explains why MSAs have higher affinity for MTs compared to unassembled tubulin, because the M-loop is already ordered in MTs (Prota et al., 2013).

The Laulimalide/Peloruside Binding Site

The second major MSA binding site on the MT is shared by LAU and PEL and is biochemically distinct from both the taxoid site (Pryor et al., 2002; Gaitanos et al., 2004) and the pore type I site (Buey et al., 2007). The location of the site was highly debated as there was evidence to support its location on both the α and β subunit, and structural studies using zinc-stabilized tubulin sheets have so far been unsuccessful because the sheets were unable to be formed with LAU (Thepchatri et al., 2005). The binding site for LAU was first localized to the S9-S10 loop region on the α-tubulin subunit (Pineda et al., 2004). This site was supported by NMR describing the bioactive conformation of PEL bound to the α subunit, with interactions centered on the αM-loop (Jiménez-Barbero et al., 2006). A more recent study showed that PEL analogs with substitutions at C24 lose their polymerizing activity (Pera et al., 2010). From the above studies, the LAU/PEL site appeared to be located on α-tubulin in a zone equivalent to that occupied by PTX in β-tubulin (Figure 5A).

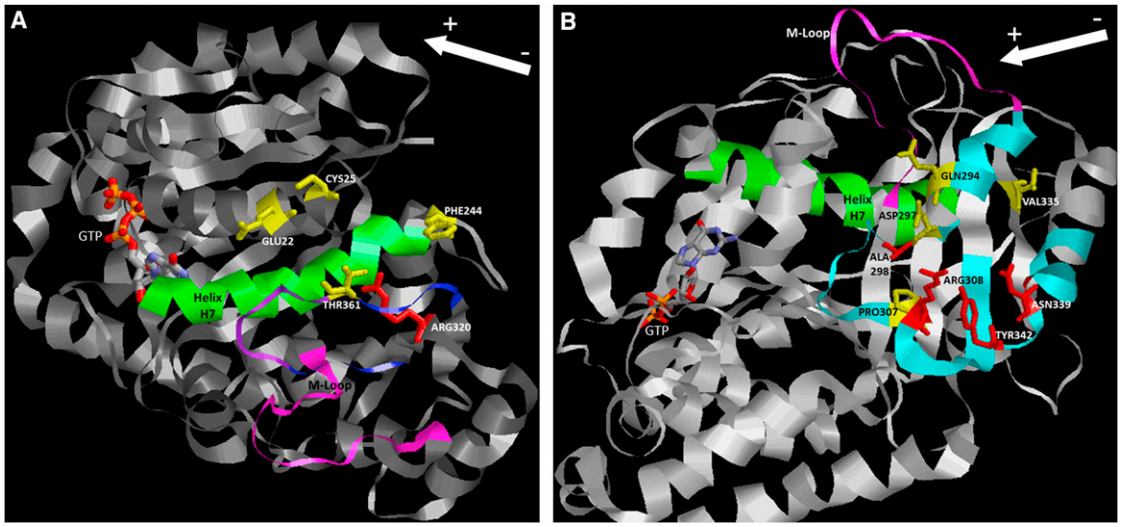

Figure 5. Models of the Proposed LAU/PEL Sites
(A) Model of the proposed site on α-tubulin showing the core helix (green), the M-loop (magenta), the S9-S10 loop (blue), and the eight extra amino acids of the S9-S10 loop (purple). Amino acids important in PEL binding to the α subunit are colored in yellow. ARG320 (red) H-bonds with PEL. GTP is colored in CPK.
(B) Model of the proposed binding site on β-tubulin. Secondary structures are colored as for (A), but note the missing purple residues in this subunit. The peptides shown in cyan are those identified by Huzil et al. (2008) using HDX-MS. Interacting residues are shown in yellow and red. Those in red are the amino acids that are mutated in the different LAU/PEL resistant cell lines.

A limitation with this model was that the αS9-S10 loop has eight extra amino acids (Figure 2). These residues are thought to occlude this site and cause stabilization of the αM-loop, giving extra strength to the lateral contacts between α subunits in different protofilaments (Nogales et al., 1999; Fourniol et al., 2010); thus, it seems unlikely an MSA would bind in this region.

Using hydrogen-deuterium exchange mass spectrometry (HDX-MS), Huzil et al. (2008) first proposed that the PEL binding site was located on the exterior surface of the β subunit, in a cavity near the αβ subunit interface. Although this β subunit site is relatively close to the taxoid site in sequence, it is structurally distant, with the macrolactone moiety of PEL held in a distinct pocket (Table 1; Figure 5B). Using digital signal processing, a technique that detects hot spots on the protein where binding occurs, a region nearly identical to that originally proposed by Huzil et al. (2008) was identified as the PEL binding site (Chen et al., 2008). This β subunit site was then independently supported by modeling and biochemical studies with [^{3}H]-PEL that provided a more rational structural basis for binding and a greater array of hydrophobic and electrostatic interactions with the β-site relative to binding at the α-site (Nguyen et al., 2010). Although this model proposed binding to the same site, PEL was orientated differently and generated more H-bonds to the model proposed by Huzil et al. (2008) (Table 1). Specifically, an intramolecular H-bond is formed that stabilizes the orientation and allows interaction of hydroxyl groups with Arg308, and the side chain of Arg308 provides an important hydrophobic platform for PEL. In the Nguyen et al. (2010) model, the macrolide cores of LAU and PEL lined up, and modification at C24 would reduce binding due to loss of a H-bond with Tyr312, consistent with the findings by Pera et al. (2010). Another study in the Schriemer lab (Bennett et al., 2010) used mass shift perturbation analysis and data-directed molecular docking simulations to show that LAU binds on the external surface of the MT. The site was located near the intradimer interface, just above the colchicine binding site, in the vicinity of the type II pore. This makes the LAU/PEL site unique by its being located on the outside of the MT near pore type II; whereas, all other MSA and MDA sites, except for the less characterized pore type I site, are located on the inner interfaces of the MT. Binding at this external site would cause reorganization of the side chains that protrude into the binding cavity, thus promoting the stabilization of this region. A more recent paper using HDX-MS supported the β subunit binding site, concluding that the α-site was unlikely for PEL and LAU binding (Khrapunovich-Baine et al., 2011).

Biological support for the above modeling was recently provided from mutation mapping following long-term exposure of cells to stepped concentrations of MSAs. Mutations to residues important for PEL and LAU binding impaired tubulin polymerization in the presence of either PEL (Ala298Thr) or both LAU and PEL (Arg308His/Cys), but not to taxoid site agents (Kanakkanthara et al., 2011). A subsequent study described four resistant cell lines, two that were identical to those previously described and two new mutations Tyr342Ser and Asn339Asp (Begaye et al., 2011). The four mutations all centered on a cleft that may interact with the side chain of PEL (Huzil et al., 2008), and all four mutations conferred resistance to PEL and showed cross-resistance to LAU (Table 2). Both of these cell-based studies supported the β-tubulin location of the binding site rather than α-tubulin, and both highlighted the importance of the side chain cavity. Residue Arg308 is essential in defining the entrance into the deep, narrow hydrophobic cleft in which the side chains of both compounds penetrate, and mutations in Arg308 caused the greatest resistance of the cells. LAU is thought to reorganize the side chains of amino acids that point into this cleft to cause stabilization of this region through stabilization of Arg308 via its interactions with Tyr342

(Bennett et al., 2010). However, this reorganization is not thought important in PEL binding, which instead may involve interactions between residues Arg308 and Ala298 (Begaye et al., 2011) (Figure 5B). At this stage, it is likely that LAU and PEL bind to the β-tubulin site first proposed by Huzil et al. (2008), but additional confirmation by X-ray crystallography is required to definitively prove this location.

Although it is possible that LAU and PEL may bind at both the described α and β subunits sites, stoichiometry studies giving 1:1 ratios of binding do not support simultaneous binding (Pera et al., 2010). Thus the α- and β-sites, if both exist, must be mutually exclusive, similar to the taxoid site and the pore type I site. Pera et al. (2010) suggest that, like the taxoid site drugs, LAU and PEL may have a two-step binding mechanism in which binding would involve an initial event, possibly an interaction with the β subunit, given the proximity of this proposed site to pore type II, followed by binding at the α-tubulin site. It is also possible that the initial binding site may be with low affinity on the α subunit, because this is the site that is picked up by NMR studies, with the final binding on the β-tubulin site. This alternative mechanism better explains the fact that mutations in resistant cell lines are only found in the β subunit, much like the resistant cell lines described for PTX that have mutations in the luminal binding site rather than the pore type I site (Giannakakou et al., 1997). It is not uncommon for drugs to have a two-step binding mechanism. For example, the MSD colchicine and a number of its analogs bind in a two-step mechanism in which the first step is slow and occurs with low affinity binding, followed by a conformational change causing high affinity binding (Skoufias and Wilson, 1992). The difference here, however, is that two distinct colchicine binding sites do not exist, but two subsites are found in a single region of β-tubulin.

Mechanisms of Stabilization

Thermodynamically, MSAs and MDAs are molecules that change the ratio between polymerized MTs and unassembled tubulin. They do this by binding preferentially to either tubulin polymers or tubulin dimers. Ligands binding preferentially to dimers inhibit assembly of MTs; whereas, ligands binding preferentially to MTs prevent disassembly (Díaz et al., 2009). This is due to thermodynamic linkage and is independent of where the binding site is or changes in structural dynamics on binding. A common thermodynamic mechanism of assembly induction can be proposed for reversible MSAs. Reversible MSAs bind the assembled form of tubulin tightly, but not covalently; whereas, they do not bind the unassembled form with measurable affinity (Díaz et al., 1993). The main thermodynamic driving force for their induced assembly is the difference in their binding constants for dimeric tubulin relative to assembled tubulin. For example, the binding constant of DSC for the dimer (Canales et al., 2011) is significantly lower than that for stabilized MTs (Buey et al., 2005). In structural terms, the difference in affinity and thus the mechanism of assembly induction should arise from a difference in binding site conformation. In unassembled tubulin, in the absence of an MSA, the M-loop is disordered; whereas, in MSA-bound unassembled tubulin, a short well-defined helix is formed, with the rest of the taxoid site binding pocket remaining unchanged (Prota et al., 2013). In unligated dimers, the M-loop has to undergo a structural change in order to accommodate the ligand, and the related free energy has to be subtracted from the overall free energy of the newly formed MSA/protein interactions. In MTs, this M-loop may already be in a helical structure, and no rearrangements are needed to bind the MSA. It is evident that the linked assembly binding process should have an energetic advantage of ~−30 kJ/mol over the assembly process even if the restructuring is energetically negative. These differences in binding constants would drive the reaction toward the assembled state.

The case would be entirely different for an irreversible binding compound such as ZMP. In this case there is no reaction equilibrium (no off-rate of covalently bound ZMP), and the reaction is only kinetically controlled by the formation of the covalent bond. The energy of restructuring would be irrelevant because there are two different molecules with different structures, tubulin and the ZMP-tubulin adduct. Once ZMP has bound irreversibly, the M-loop residues have a different equilibrium conformation as a result of new bonds forming that are different from the M-loop residues in the unligated protein. This conformational change of structuring, energetically unfavorable in the case of the unligated protein, is necessary to form the lateral contacts, and the difference in the equilibrium polymerization of free energy of both processes is a reflection of the energetic cost of M-loop restructuring. In ligated tubulin, restructuring has already occurred prior to the lateral contacts being made; thus, ligated tubulin assembly will have lower free energy than unligated tubulin assembly.

Taxoid Site-Binding Stabilization

The knowledge on how MSAs bind to and stabilize MTs has initially come from using PTX as a probe. Much more information is known about this mode of stabilization and very little about LAU/PEL stabilization, especially given that neither LAU nor PEL is commercially available. Binding of an MSA to the taxoid site strongly influences the interactions at three key regions within the tubulin molecule, the M-loop, H3, and S3 (Matesanz et al., 2011), all of which are known to be important for interprotofilament interactions (Nogales et al., 1998). This binding causes increased lateral contacts by facilitating the key interaction of the β subunit M-loop with the two loops (H1′-S2 and H2-S3) in a neighboring protofilament, resulting in stabilization of the MT (Amos and Löwe, 1999; Nogales et al., 1999; Sui and Downing 2010). PTX, however, is able to stabilize zinc-induced tubulin sheets that have an alternating and antiparallel arrangement to one another, and this orientation of the subunits limits this M-loop interaction (Amos and Löwe, 1999; Mitra and Sept, 2008). In addition, PTX also stabilizes individual protofilaments via stabilization of longitudinal contacts (Elie-Caille et al., 2007); thus, PTX must cause stabilization by another mechanism at the same time as it increases lateral protofilament interactions (Mitra and Sept, 2008). PTX sterically hinders the natural movement of the M-loop, having a significant effect on its conformation and causing displacement of the loop downward and away from H6. This results in increased interactions with the loops in an adjacent monomer via van der Waals forces and electrostatic and ionic interactions, increasing the dynamics of the H1-H2 loop and also affecting the H6-H7 loop to some extent (Mitra and Sept, 2008). Orientation and dynamics depend on which taxoid site ligand is bound to the site (Matesanz et al., 2011). Specifically, a stable salt bridge is formed between Glu55

(H1-S2 loop) and Arg284 (M-loop) in a different protofilament when PTX is bound. In the absence of PTX, a salt bridge is formed between Arg284 and a residue in the same monomer that leads to a change in the M-loop, causing a small opening of the taxoid binding site. This opening may assist in the binding of PTX (Mitra and Sept, 2008). MSA interactions at the taxoid site occur close to the interprotofilament region of the MT and dictate the number of protofilaments making up the MT. PTX decreases the number of protofilaments making up the MT from 13 to 12 in purified tubulin (Andreu et al., 1992); whereas, DTX binding has no effect on the number of protofilaments (Andreu et al., 1994). This suggests that different MSAs can alter the way in which protofilaments laterally interact, changing the number of protofilaments in a MT and the interprotofilament angle (see Matesanz et al., 2011). Increasing the strength and duration of interactions between protofilaments should cause overall stability of the MT, but it is questionable whether these interactions would be sufficient to promote assembly of MTs from GDP-bound dimers.

After nucleotide hydrolysis, the MT becomes unstable because of its straight, constrained conformation. However, in the presence of PTX, the MTs remain stable, even when GDP is bound to the E-site. This maintained stability is thought to be due to strong longitudinal and lateral binding between dimers in adjacent protofilaments. If certain bonds are weakened by hydrolysis, PTX must somehow compensate for this in such a way that the MT remains stable. This can occur by increasing the strength and duration of lateral bonds; however, there is controversy over this because PTX increases the flexibility within the MT (although the opposite has also been reported). Currently it is accepted that PTX increases MT flexibility (Sui and Downing, 2010), and it is possible that these lateral bonds may also be flexible. Although there is debate over the exact mechanism, it is likely that PTX causes changes in the nucleotide binding site that compensate for the hydrolysis and allow the straight conformation of the MT to remain stable (Amos and Löwe, 1999). This is supported by the fact that PTX has allosteric effects on the T1–T5 and H11 loops (E-site), and these effects cause an increase in flexibility, compensating for the GTP hydrolysis (Amos and Löwe, 1999; Mitra and Sept, 2008). This in turn counteracts the conformational change, thus promoting stability and leading to a straighter, less strained protofilament. These allosteric changes are thought to be the primary effect of PTX, with lateral interactions a secondary means of stabilization. Alternative hypotheses have been reviewed by Mitra and Sept (2008). X-ray crystallography has shown that the taxoid binding site is only slightly affected by the curved to straight conformational change, with only minor rearrangements of the residues shaping the pocket (Prota et al., 2013). Additionally, all crystal structures of unassembled tubulin with bound MSAs are in the curved conformation, as discussed above. The data presented in Prota et al. (2013) also confirm that MSAs activate tubulin via restructuring of the M-loop into a short and well-defined helix. This structural change may be the molecular basis of the curved-to-straight conformational change that is observed upon tubulin assembly and predicts that the loss of this helical structure would be an important feature of disassembly.

Insights into Laulimalide/Peloruside Site Stabilization

Although the taxoid site and the LAU/PEL site are biochemically distinct, binding at either site results in the same end point—stabilization of the MT. Interestingly, the taxoid site and the proposed β subunit site for LAU/PEL are located close to each other in the amino acid sequence with H9 involved in the LAU/PEL interaction, located C-terminal of the M-loop. Therefore, LAU and PEL binding at the proposed β-tubulin site may induce some level of stabilization of the M-loop, enough to cause enhanced protofilament contacts and a more stable MT structure, a mechanism similar to that of PTX and its biomimetics (Bennett et al., 2010).

A contrasting hypothesis is that LAU/PEL binding to the β-tubulin site causes stabilization of the intradimer interface (Bennett et al., 2010), returning the polymer to a more "GTP-like state." GTP bound at the E-site gives a stable form of tubulin, which is why there is always a GTP cap on a growing MT. Occupancy at the E-site changes the proposed LAU/PEL site, with GDP destabilizing the MT via residues β304–312. Interestingly, Jiménez-Barbero et al., (2006) have provided some evidence for docking of PEL at the E-site, but ruled this out as a true binding site; however, their results can be explained by the above observations. Thus, LAU could reduce the tension within the MT lattice by binding at the E-site (Bennett et al., 2010).

PEL and LAU were suggested to cause the same stabilization as taxoid site drugs via effects at the interdimer interface with contributions from the αβ-intradimer interface as well as protofilament contacts (Huzil et al., 2008; Bennett et al., 2010). In addition, however, it was suggested that the binding of PEL was complemented by relaxation of the intradimer interface and β-β interactions at the lateral interface—a distinct stabilization mode for PEL different to that of taxoid site drugs. Most MSAs cause a conformational change in the T5 loop (located adjacent to the E-site), promoting improved interactions across the interdimer interface and resulting in stabilization of this interface and possibly compensating for the lost stability when GTP hydrolysis occurs (Huzil et al., 2008).

Allosteric versus Matchmaker Mechanisms of Nucleation

When tubulin is at a critical concentration in which polymerization is favored over depolymerization, MTs assemble via noncovalent, nucleated condensation polymerization. These reactions are characterized by cooperative behavior (Díaz et al., 1993). Ligand-induced MT assembly also proceeds with a critical concentration that is less than in the absence of ligand and is concentration-dependent (Díaz et al., 2009). The disruption of MT polymerization dynamics appears to be the main mechanism by which MT-stabilizing drugs function, and they do so without a significant effect on polymer mass (Nogales, 2000). Given that there are at least two, and likely three, different MSA binding sites on MTs, the luminal taxoid site, the pore type I site, and the LAU/PEL site, and given the extreme structural diversity of MSAs that bind the MT, especially at the taxoid site, all MSAs are unlikely to have exactly the same structural effects at the molecular level (Díaz et al., 2009). It is well known that MSAs promote tubulin assembly in conditions where tubulin is unable to assemble on its own, such as when no GTP or glycerol is present in the buffer or when GDP is bound to the β subunit (Díaz and Andreu 1993; Parness and Horwitz 1981). Therefore, MSAs are involved in the nucleation-elongation step of MT assembly. MSAs are proposed to bind to unassembled tubulin with low

affinity and induce polymerization via two possible mechanisms. Either they promote a conformational change resulting in the activation of the dimer or they join two subunits together to form a high affinity site from two lower affinity sites. These two mechanisms are termed the allosteric and matchmaker processes, respectively (Díaz et al., 1993, 2009; Reese et al., 2007). The binding of an MSA to the dimer is the first in a series of events that leads to the MSA-induced stabilization of MTs (Sánchez-Pedregal et al., 2006). CYC modifies the pore site in dimeric tubulin (Buey et al., 2007), and ZMP modifies the luminal binding site (Field et al., 2012; Prota et al., 2013). Binding at the pore site in dimeric tubulin supports the matchmaker mechanism in which the full pore site is formed from two half sites. Binding at the luminal site in dimeric tubulin supports the allosteric mechanism of MSA-induced assembly in which the internal taxoid site is present in dimers but has low affinity for MSAs, and the high affinity site develops after assembly (Díaz et al., 1993). An alternative allosteric mechanism could involve binding to the luminal site to activate tubulin, leading to nucleation (Field et al., 2012). More recently, the allosteric model has been directly supported by X-ray crystallography of both EPOA- and ZMP-bound tubulin (Prota et al., 2013).

Future Investigations

Given the success of MSA chemotherapy in the clinic, the numerous compounds at different stages of preclinical and clinical development, and the novel agents still being discovered, the biochemistry of MSAs remains an expanding area of research. The next logical step in this exciting field would be to determine the exact molecular mode of action in which MSAs bind to the MT and cause stabilization. This is likely to take time, given the complexity of the interactions. It is also likely that taxoid site drugs bind via a two-step mechanism, interacting first at the surface of the MT followed by translocation into the lumen. It would be interesting to see how this occurs and if every ligand that targets the taxoid site follows the same two-step process. The exact location of the LAU/PEL site also needs to be determined. A two-step binding mechanism may also exist at this site, with the two separate sites displaying different affinities for LAU and PEL. A complete understanding of the molecular mechanisms underlying MT stabilization is essential for understanding the MSAs currently in clinical use. It is possible that LAU and PEL have advantages over the taxoid site ligands because they are poor substrates for the drug efflux pump and they are more polar. This suggests that unlike PTX they may not require a vehicle for delivery, thus decreasing toxic side effects. There is also evidence that PEL is better tolerated in vivo. It would therefore be worthwhile to develop these drugs or their analogs for translation to the clinic. Overall this field of research is exciting and has great potential for development of new drugs to treat solid tumors. Despite the focus on targeted treatments, chemotherapy-based cancer treatment still remains an important approach in the fight against cancer.

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