Drug Discovery

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Structural Basis of Microtubule Stabilization by Laulimalide and Peloruside A**

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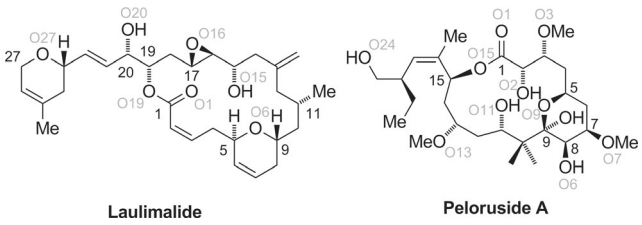
Abstract: *Laulimalide and peloruside A are microtubule-stabilizing agents (MSAs), the mechanism of action on microtubules of which is poorly defined. Here, using X-ray crystallography it is shown that laulimalide and peloruside A bind to a unique non-taxane site on β-tubulin and use their respective macrolide core structures to interact with a second tubulin dimer across protofilaments. At the same time, they allosterically stabilize the taxane-site M-loop that establishes lateral tubulin contacts in microtubules. Structures of ternary complexes of tubulin with laulimalide/peloruside A and epothilone A are also solved, and a crosstalk between the laulimalide/peloruside and taxane sites via the M-loop of β-tubulin is found. Together, the data define the mechanism of action of laulimalide and peloruside A on tubulin and microtubules. The data further provide a structural framework for understanding the synergy observed between two classes of MSAs in tubulin assembly and the inhibition of cancer cell growth.*

Taxane-site MSAs like paclitaxel and epothilones are one of the most successful classes of chemotherapeutic drugs against cancer.[1] However, their clinical application is hampered by toxicity and the development of resistance. The marine sponge products laulimalide[2] and peloruside A (peloruside)[3,4] are promising non-taxane-site MSAs (see Scheme 1). Both compounds potently inhibit the growth of multidrug-resistant cancer cells,[5,6] and act synergistically with paclitaxel or epothilones in the promotion of microtubule stability[7,8] and induction of cell death.[9,10] These properties make laulimalide and peloruside potentially attractive next-generation MSAs for use in combination chemotherapy with taxane-site ligands. How this class of MSAs promote tubulin assembly and microtubule stability, and how they synergize with taxane-site drugs is not well understood. Here, we set out to address these important unanswered questions by means of X-ray crystallography.



Scheme 1.

Using crystals of a protein complex composed of αβ-tubulin, the stathmin-like protein RB3 and tubulin tyrosine ligase (T_2R-TTL),[11,12] we determined the tubulin-bound laulimalide and peloruside structures at 2.1 and 2.2 Å resolution, respectively (see Figure S1 and Table S1 in the Supporting Information). The overall structure of tubulin in the two T_2R-TTL-MSA complexes superimposed well with the one obtained in the absence of the ligand[11] (root mean square deviation (rmsd) ranging from 0.21–0.28 Å over >1700 Cα-atoms), suggesting that binding of laulimalide or peloruside does not affect the global conformation of the protein. Both MSAs occupy a common pocket on β-tubulin that is formed by hydrophobic and polar residues of helices H9 and H10, and the loops H9-H9′ and H10-S9 (Figure 1a); this pocket is referred here to as the "laulimalide/peloruside-

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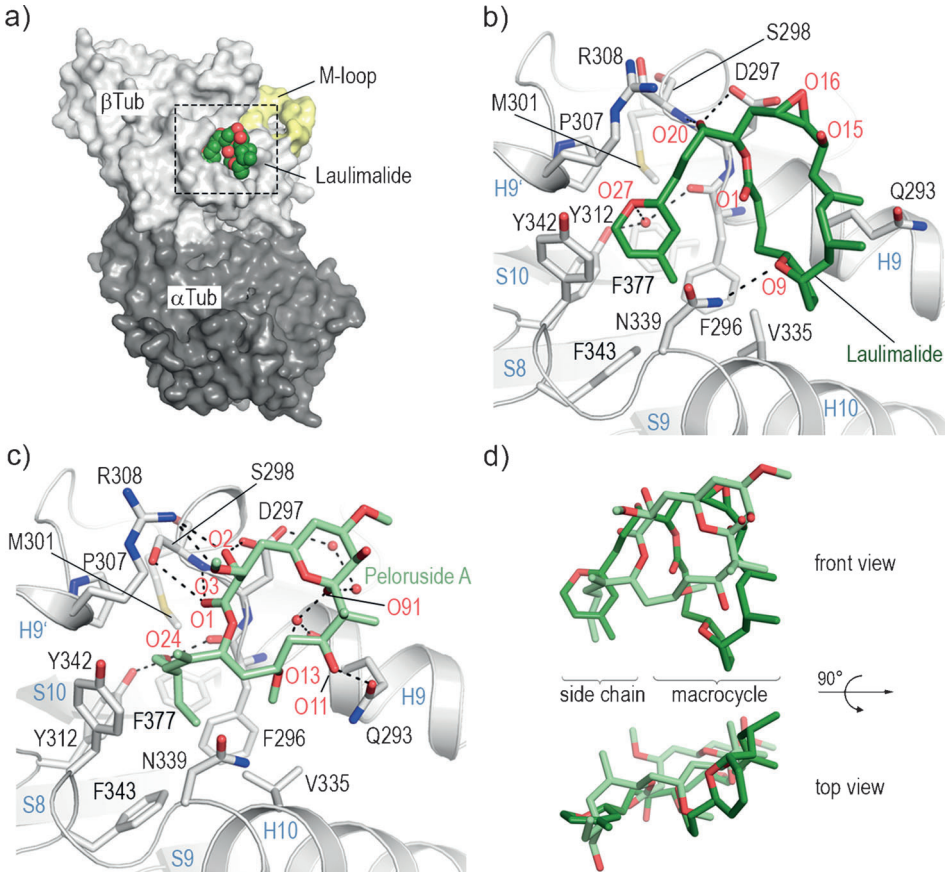


Figure 1. a) Overall view of the complex formed between tubulin and laulimalide. The dashed box depicts the area shown in more detail in (b, c, and d). b and c) Close-up views of the interaction network observed between β-tubulin and laulimalide (dark green; (b)) or peloruside (pale green; (c)). d) Close-up view of the superimposed laulimalide- and peloruside-binding sites. Only the ligands are shown for simplicity. Both laulimalide and peloruside are displayed in two different orientations using the same color code as in (b and c).

pocket". These results establish the tubulin-binding site for laulimalide and peloruside to be located on β-tubulin, consistent with predictions on the location of the site based on hydrogen–deuterium exchange coupled to mass spectrometry (HDX-MS) experiments.[13–15]

In the tubulin–laulimalide complex, the side chain and parts of the macrocycle of laulimalide are deeply inserted into a pocket formed by residues Gln293, Phe296, Pro307, Arg308, Tyr312, Val335, Asn339, Tyr342, and Phe343 of β-tubulin (Figure 1b). The O9 atom of the tetrahydropyran ring in the macrocycle and the O20 atom in the side chain form hydrogen bonds with the side chains of Asn339 and Asp297/Ser298, respectively; the O27 atom of the dihydropyran moiety in the side chain forms a water-mediated hydrogen bond to the side chain OH group of Tyr312 and the main chain carbonyl of Phe296. The binding mode of peloruside to β-tubulin is very similar (Figure 1c). In this case, hydrogen bonds are formed between the O1, O2, O3, O11, and O24 atoms of peloruside and the side chains of Ser298, Asp297/Arg308, Arg308, Gln293, and Tyr312 of β-tubulin, respectively. The comparison of the tubulin–laulimalide and tubulin–peloruside structures shows that both the side chains as well as the macrocycles of the MSAs superimpose well, demonstrating a common binding mode (Figure 1d). In α-tubulin, because of a tilting of the helix H10, Arg339 impedes access to the corresponding laulimalide/peloruside-pocket by contacting Asn293 and Glu297, thus explaining the selective binding of laulimalide and peloruside to β-tubulin (Figure S2a).

A feature of both the tubulin–laulimalide and tubulin–peloruside structures is the partial ordering/stabilization of the disordered loop S7-H9 of non-liganded β-tubulin (denoted the M-loop; Figure 2a and Figure S2b), most likely because of the stabilization of a helical conformation of segment Gln294-Phe296 at the C-terminus of helix H9. In the non-liganded state of the protein this segment switches between a helical and a turn-type III conformation (Figure 2b–d). In the latter state, Phe296 is stacked between the side chains of Met295 and Arg308, and fills the pocket formed by Pro307, Tyr312, Tyr342, and Phe343. Upon laulimalide or peloruside binding, segment Gln294-Phe296 is locked in the helical state, thereby extending the H9 helix by one turn. At the same time, the side-chain OE1 atom of Gln294 is shifted by 4.1 Å towards the N-terminal base of the M-loop, and forms a water-mediated hydrogen bond to the backbone amide group of Leu275 and to the ND2 side-chain atom of Asn300 (see also Figure S2c). In combination with the stabilization of additional structural elements in the vicinity of the M-loop, these changes offer an explanation for the partial ordering/stabilization of the M-loop in a conformation without any regular secondary structure upon laulimalide/peloruside binding. Because structuring of the M-loop is a critical molecular process involved in the establishment of lateral tubulin contacts between adjacent protofilaments in microtubules,[12,16,17] we denote the state in which the Gln294-Phe296 segment is in a helical conformation as the polymerization-competent, microtubule-stabilizing "on-state"; conversely, the turn-type III conformation of the Gln294-Phe296 segment is denoted as the "off-state" and is assumed to be characteristic of the assembly-incompetent form of tubulin.

Tubulin dimers in microtubules assume a "straight" structure;[18,19] whereas, a "curved" conformation is characteristic of unassembled tubulin.[20,21] To assess possible structural differences between the laulimalide/peloruside-pocket in unassembled tubulin and microtubules, we compared

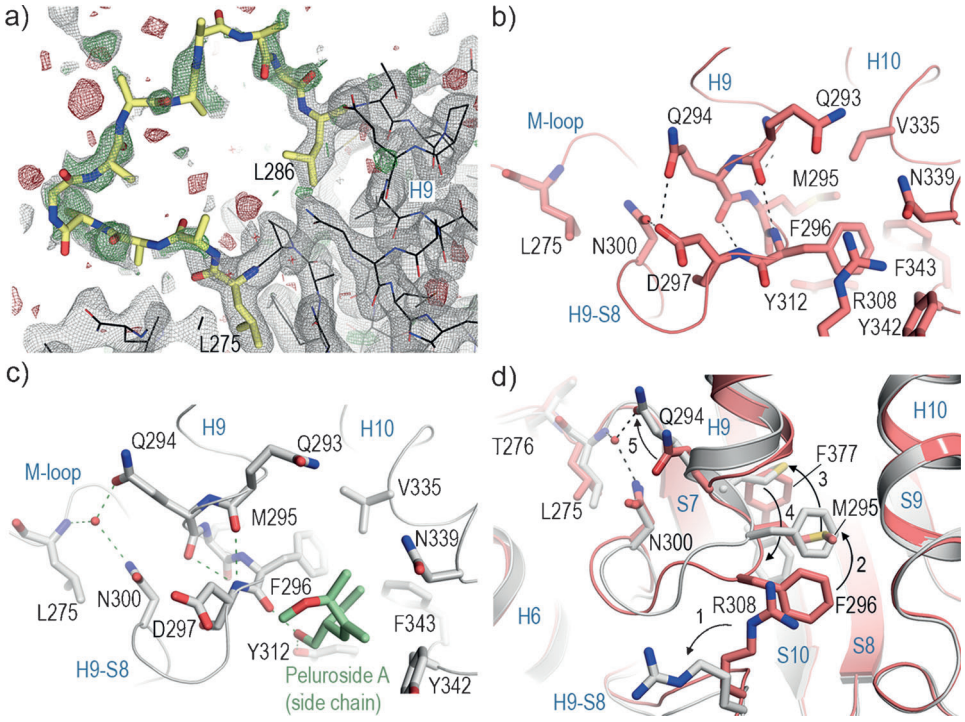


Figure 2. a) 2mFo-DFc (gray mesh, contoured at 0.9 σ) and mFo-DFc (green and red mesh, $\pm 2.5\ \sigma$) electron density omit maps of the region surrounding the M-loop (modeled as a polyalanine chain) of β-tubulin in the tubulin–peloruside complex. b and c) View into the non-liganded "off-state" (b; PDB ID 4DRX) and liganded "on-state" (c; tubulin–peloruside complex) laulimalide/peloruside pocket of β-tubulin. d) Superimposition of the liganded (tubulin–peloruside complex; gray; "on-state") onto the non-liganded (PDB ID 4DRX; salmon; "off-state") laulimalide/peloruside pocket. The ligand-induced structural rearrangements of Arg308 (1), Phe296 (2), Met295 (3), Phe377 (4), and Gln294 (5) are indicated.

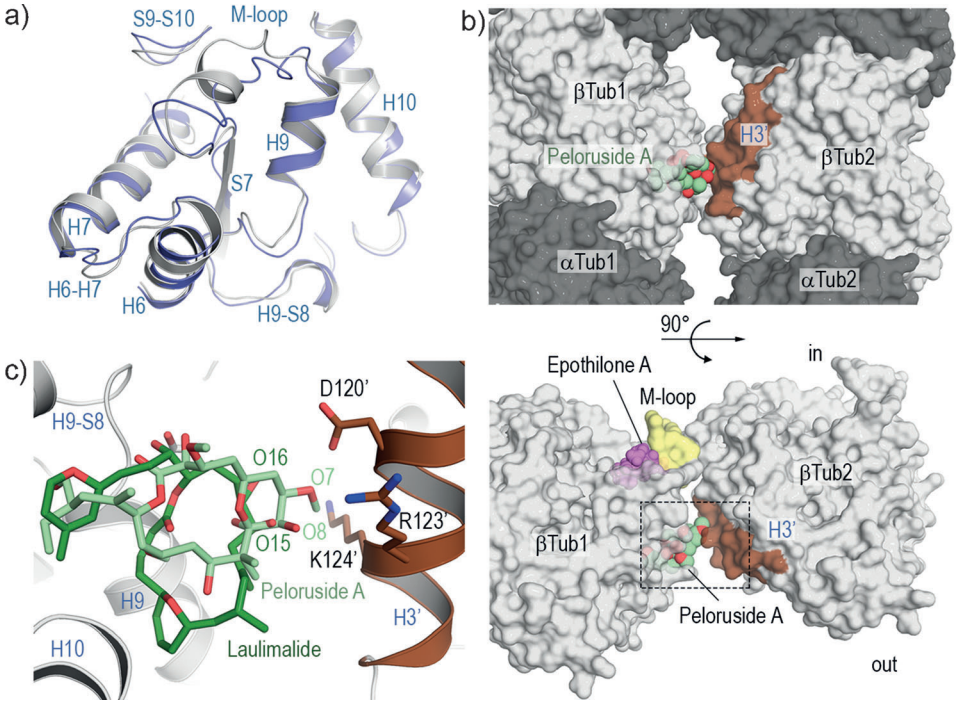


Figure 3. a) Superimposed laulimalide/peloruside and taxane pockets in the curved (tubulin–peloruside complex; gray) and straight (PDB ID 1JFF; blue) tubulin conformational states. b) Two different views of the tubulin–peloruside interaction in the context of the microtubule (PDB ID 2XRP). The M-loop and helix H3 are highlighted in yellow and brown, respectively. The dashed box in the lower part of the figure depicts the area shown in detail in (c). c) Close-up view of the inter-protofilament β-tubulin contact formed by laulimalide and peloruside in the context of the microtubule.

models of β-tubulin in the curved and straight conformational states. Superimposition of these structures revealed that the overall conformation of the laulimalide/peloruside-pocket is not significantly affected by the curved-to-straight structural transition (rmsd of 0.72 Å over 44 Cα-atoms). Simultaneous superimposition of the laulimalide/peloruside- and the adjacent taxane pocket in the curved and straight tubulin conformational states show that both sites superimpose well (rmsd of 0.90 Å over 98 Cα-atoms; Figure 3a). This analysis suggests that the two classes of MSAs can bind to both unassembled tubulin and microtubules. Notably, segment Gln294-Phe296 is stabilized in the helical "on-state" in the straight structure of tubulin (see above).

Next, we modeled the pose of laulimalide and peloruside in the context of the microtubule lattice. For this purpose, we used the straight tubulin structure[18] and cryo-electron microscopy reconstructions of microtubules at about 8 Å resolution.[17,22] In contrast to the taxane pocket that is located on the luminal side of the microtubule,[19] the laulimalide/peloruside-pocket is located on its outer surface (Figure 3b). Importantly, in our model, the macrocycles of both MSAs are oriented in a manner that allows for lateral interactions with helix H3 of a β-tubulin subunit from a neighboring protofilament: The O15 and O16, and the O7 and O8 atoms of laulimalide and peloruside, respectively, are in hydrogen-bonding distance from the side chains of Asp120′, Arg123′, and Lys124′ (Figure 3c).

Our structural analysis in combination with biochemical information[5,6] indicates that taxane-site ligands can bind to β-tubulin concurrently with lau-

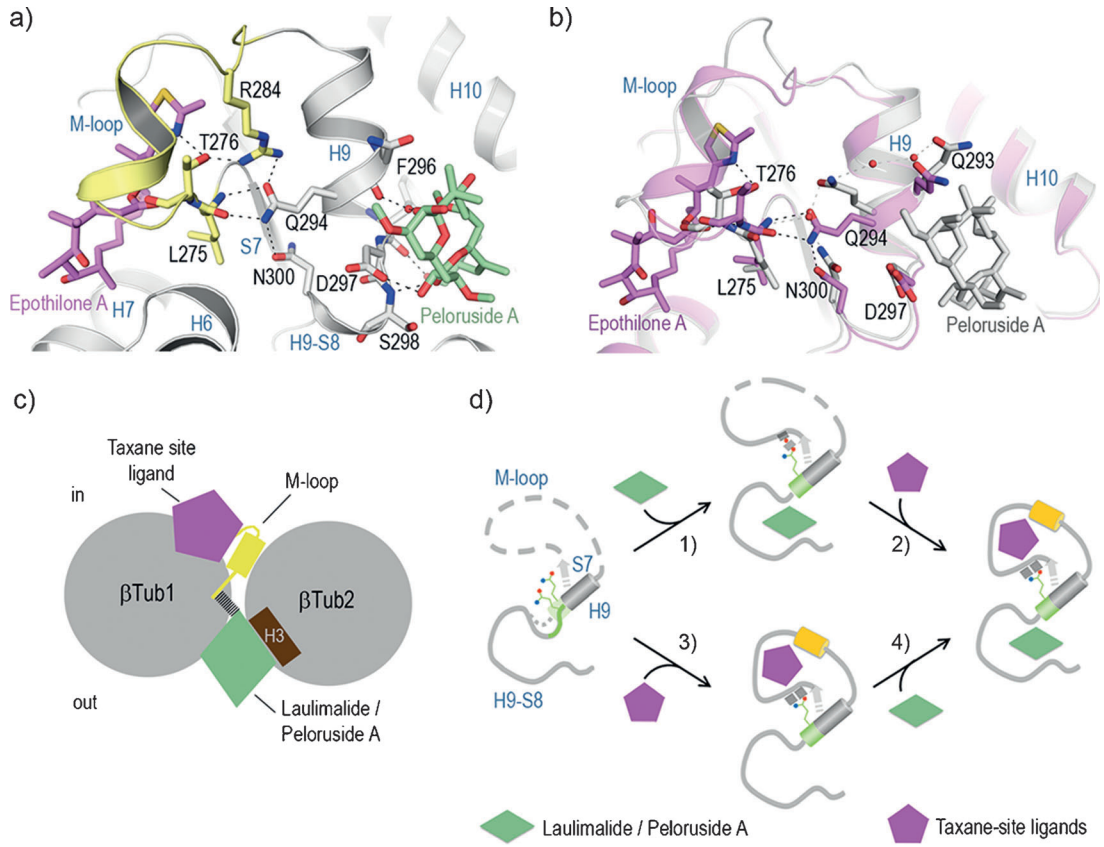
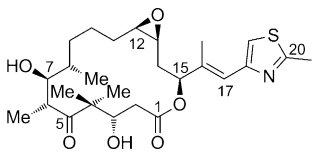


Figure 4. a) Close-up view of the ternary tubulin–peloruside–epothilone complex. Peloruside and epothilone are shown in green and pink, respectively. b) Close-up view of the superimposed tubulin–epothilone (pink; PDB ID 4I50) and tubulin–peloruside (gray) complexes highlighting the interaction network involving Gln294. c) Schematic representation of a top view of two adjacent β-tubulin subunits across protofilaments in the microtubule. The key contacts promoted by both the taxane- and the laulimalide/peloruside-site ligands are highlighted. The hatched black rectangle schematizes the stabilization of the N-terminal base of the M-loop upon ligand binding via Gln294 of β-tubulin. d) Schematic representation of M-loop stabilization by ligand binding to unassembled tubulin. In the absence of ligands the M-loop is disordered (dashed line, left-hand cartoon) and segment Gln294-Phe296 (green) switches between a turn ("off-state") and helical ("on-state") conformation. 1) Laulimalide/peloruside binding stabilizes segment Gln294-Phe296 in the helical "on-state" conformation. As a consequence, Gln294 shifts, interacts with the M-loop (black stripes) and stabilizes it. 2) The preorganization of the taxane pocket because of M-loop stabilization favors ligand binding. 3) Taxane-site ligands structure the M-loop and induce the formation of a short helix (yellow cylinder). As a consequence, the N-terminal base of the M-loop interacts with Gln294 and the segment Gln294-Phe296 is stabilized in the helical "on-state" conformation. The stabilized, extended helix H9 facilitates ligand binding in the laulimalide/peloruside pocket.

limalide or peloruside. We thus crystallized tubulin–laulimalide and tubulin–peloruside complexes in the presence of epothilone A (epothilone) and solved their structures at 2.4 and 2.5 Å resolution, respectively (Table S1 and Scheme 2). Both classes of MSAs were simultaneously present in the same tubulin dimer (Figure 4a). The epothilone molecule binds to the taxane pocket of β-tubulin that is formed by helix H7, β-strand S7, the M-loop, and loops H6-H7 and S9-S10. Its binding mode and conformation in the pocket are very similar to those previously described in the absence of laulimalide/peloruside[12] (Figure S3). The pose of laulimalide and peloruside in the ternary tubulin-laulimalide/peloruside-epothilone complexes is essentially identical to that obtained in the absence of epothilone. In the ternary complexes, the M-loop of β-tubulin is structured, with its conformation superimposing well with that of the binary tubulin–laulimalide/peloruside or tubulin–epothilone[12] complexes (Figure S3d). As reported previously,[12] the side chain of epothilone interacts with the M-loop and causes segment Arg278-Tyr283 of β-tubulin to assume a helical conformation.

To assess whether ligand binding to the taxane site induces structural changes in the laulimalide/peloruside-pocket, we carefully re-examined the structure of our previously reported binary tubulin–epothilone complex.[12] This analysis revealed that M-loop stabilization upon binding of this taxane-site ligand causes the backbone amide hydrogen and carbonyl oxygen of Leu275 to form two hydrogen bonds with the side chain of Gln294 (Figure 4b). As a result, segment



Epothilone A

Scheme 2.

Gln294-Phe296 of the laulimalide/peloruside-pocket assumes the same helical "on-state" conformation that is observed for laulimalide/peloruside binding in the absence of a taxane-site MSA (Figure 2b–d).

Our results provide detailed insights into the molecular mechanism of action of MSAs interacting with two distinct binding sites on β-tubulin. First, we found that the major consequences of laulimalide/peloruside binding, apart from some less well-defined global effects,[13,15] are 1) the stabilization of the β-tubulin M-loop in a conformation without any regular secondary structure and 2) the bridging of two adjacent tubulin dimers across protofilaments in microtubules (Figure 4c). These observations explain the promoting effect of laulimalide/peloruside on microtubule assembly and stability. Importantly, the simultaneous interaction of peloruside or laulimalide with two protofilaments offers a new perspective on how small molecules can stabilize microtubules; to the best of our knowledge, such a mechanism has not been implied for any known MSA so far. Second, our data suggest an allosteric crosstalk between the laulimalide/peloruside- and the taxane pocket in both the unassembled and assembled tubulin states (Figure 4c,d). It has been reported that ligand binding to the laulimalide/peloruside site in microtubules assembled from chicken erythrocyte tubulin stabilizes the conformation of the taxane pocket, including the M-loop, based on HDX-MS experiments.[15] Conversely, ligand binding to the taxane pocket has been found to stabilize structural elements forming the laulimalide/peloruside site in microtubules.[13,15] Our results offer a structural framework for understanding this crosstalk and thus the proposed synergistic effect between laulimalide/peloruside- and taxane-site ligands on tubulin assembly and the inhibition of cancer cell growth.[7–10]

Coordinates have been deposited at the Protein Data Bank (PDB) under accession numbers 4O4H (T_2R-TTL-laulimalide), 4O4J (T_2R-TTL-peloruside), 4O4I (T_2R-TTL-laulimalide-epothilone), and 4O4L (T_2R-TTL-peloruside-epothilone). Note that tubulin residue numbering is as defined in Löwe et al.[18]

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