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Biology-Oriented Synthesis

A Natural Product Inspired Tetrahydropyran Collection Yields Mitosis Modulators that Synergistically Target CSE1L and Tubulin**

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In biology-oriented synthesis (BIOS) biological relevance and prevalidation are employed as key criteria for the design and synthesis of focused compound collections for chemical biology and medicinal chemistry research.[1] The scaffolds of natural products are privileged, biologically relevant molecular frameworks and natural products, and analogues thereof have served as efficient probes in chemical research, for example, in the study of processes related to cancer, in particular mitosis.[2]

Tetrahydropyrans occur widely in nature (for examples, see Figure S1 in the Supporting Information) and are endowed with pronounced biological activities.[3] Their synthesis has received intense attention, in particular when a Prins cyclization is employed as the key step.[3b,4]

Here we report the synthesis of a natural product inspired compound collection based on the 4-hydroxytetrahydropyran scaffold. Evaluation in a phenotypic screen revealed structurally novel modulators of mitosis termed tubulexins which target the chromosome segregation 1-like protein (CSE1L, CAS, exportin-2) and the vinca alkaloid binding site of α/β-tubulin.

For the synthesis of a compound collection based on the 4-hydroxytetrahydropyran scaffold by means of the Prins cyclization as the key transformation[4,5] we envisioned a synthetic route in which the tetrahydropyran **1** is generated in one step from a polymer-bound aldehyde **2** and a readily available homoallylic alcohol **3** (Scheme 1 a). Immobilization and, thereby, limited exposure of the electron-rich hydroxyaromatic aldehyde to Lewis acids should reduce side reactions which have been encountered in related systems.[6]

For the solid-phase synthesis by means of the IRORI/MacroKan technology, reactors were loaded with chloromethyl-derivatized polystyrene resin **4** and heated with hydroxybenzaldehydes **5** (R^1 or $R^2 = OH$) for immobilization (Scheme 2, loading 1.05–1.51 mmol g^{-1}; see the Supporting Information).[7] Eleven homoallylic alcohols **3** were synthesized in solution by asymmetric allylation of the corresponding aldehydes with Brown's allylborane.[8]

The Prins cyclization carried out in the presence of $BF_3{\cdot}OEt_2$, TMSOAc, and acetic acid in dichloromethane or tetrahydrofuran at room temperature[6b] (Scheme 2) selectively yielded substituted 4-tetrahydropyranyl acetates **6** with all-equatorial substituents (see also below). The esters could readily be hydrolyzed to yield alcohols **9** for further transformation.

Protected tetrahydropyrans **7** were released from the solid support in high yields by treatment with trifluoroacetic acid/dichloromethane (1:1) in the presence of 0.4% thioanisol (cleavage conditions **A**) for 2 h at room temperature (Scheme 2, see Table S2 in the Supporting Information). Tetrahydropyrans with unprotected phenolic hydroxy groups were obtained by treatment of immobilized tetrahydropyrans **9** with freshly prepared 2-bromobenzo[*d*][1,3,2]dioxaborole (*B*-bromocatecholborane, **8**) in combination with $BF_3.OEt_2$ (cleavage conditions **B**).[9]

These methods yielded 54 4-tetrahydropyranyl acetates **7** in overall yields of 3–35% based on the polymer-bound aldehydes **2** (see Table S2, entries 1–54 in the Supporting Information). In general, the structure of the polymer-bound aldehyde appeared to influence the yield only to a minor extent. The yields of a doubly benzyl-protected homoallylic alcohol (**3e**) and a thienyl-substituted homoallylic alcohol (**3k**; see Table S1 in the Supporting Information) were low most likely because of undesired side reactions under the acidic conditions.

NOE spectroscopic investigation of a representative 4-tetrahydropyranyl acetate (see Table S1, entry 1 in the Supporting Information) proved the all-*cis* configuration of this product and equatorial position of the substituents. The results revealed that the axial 4-H proton in the tetrahydropyran is in proximity to the axial 2′-H and the axial 6′-H

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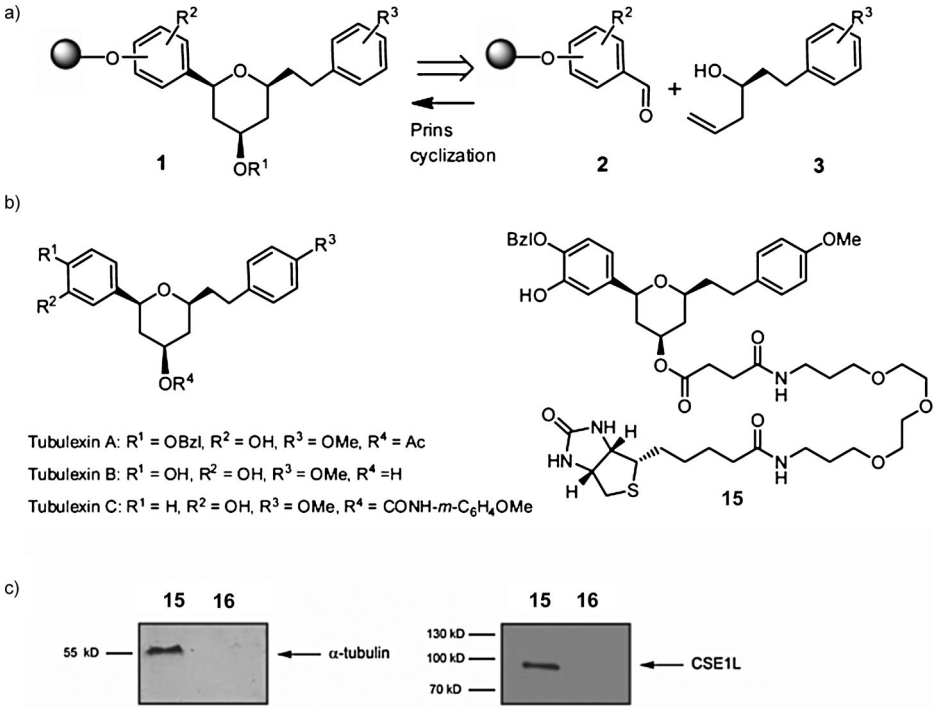


Scheme 1. Retrosynthetic analysis and structures of tubulexins, as well as an affinity probe for chemical proteomics. a) Retrosynthetic analysis for the synthesis of a 4-substituted tetrahydropyran collection on a solid support. b) Structures of tubulexins A–C and of tagged reagent **15** employed in the affinity pull-down experiments. c) Affinity isolation of α-tubulin and CSE1L by biotinylated tubulexin A. Affinity probe **15** was immobilized on streptavidin beads followed by incubation with HeLa cell lysate for affinity isolation of target proteins. Bound proteins were released by specific elution (tubulin) or by boiling (CSE1L), and were resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to immunoblotting by using specific α-tubulin and CSE1L antibodies. Results are representative of three independent experiments. Bzl = benzyl.

proton and that significant signal enhancements are observed upon irradiation (see the Supporting Information). Since the other tetrahydropyrans showed similar coupling patterns in the ^{1}H NMR spectra, their relative configuration was assigned by analogy. The absolute configuration was derived from the preset configuration of the stereogenic center introduced with the homoallylic alcohol.

For extension of the collection, the polymer-bound 4-tetrahydropyranyl acetates **6** were saponified on the resin and released as described above to yield 4-tetrahydropyranols **10** in overall yields of 4–44% based on the polymer-bound aldehydes **2** (Scheme 2, see also Table S2, entries 55–99 in the Supporting Information). Analysis of a representative selection of the 45 4-tetrahydropyranols **10** by HPLC on a Chiracel AD-H column proved no significant loss of enantiomeric purity, which may occur in Prins cyclization reactions.[6a] In addition, intermediates **9** were treated with 3-methoxyphenyl isocyanate **11** and converted into 14 carbamates **12** in overall yields of 2–22% based on the polymer-bound aldehydes **2** (see Table 2, entries 100–113 in the Supporting Information) and carbonates **14** were obtained in yields of 2–16% based on the polymer-bound aldehydes **2** (Table S2, entries 114–150 in the Supporting Information) as shown in Scheme 2.

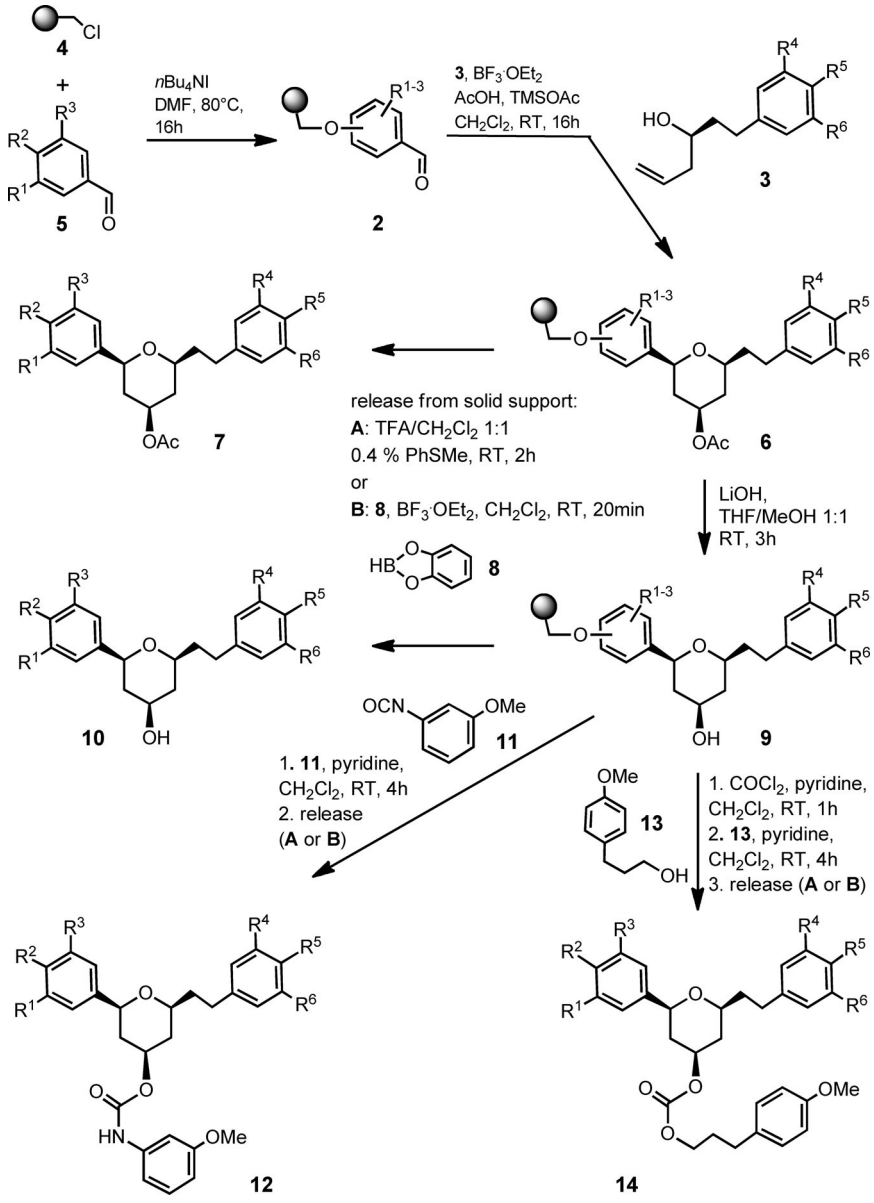
In total, the Prins cyclization with immobilized aldehydes **2** and the subsequent transformations yielded a 150-membered collection of 2,4,6-all-*cis*-substituted tetrahydropyrans in two to five steps on the polymeric carrier.

Phenotypic screening of the collection in BSC-1 cells at a concentration of 30 μM by monitoring changes associated with impaired mitosis[10] led to the identification of three compounds that induced accumulation of round cells with condensed DNA and membrane blebs, which are indicative of mitotic arrest and apoptosis (see Figure S2a in the Supporting Information). These compounds were termed tubulexin A, B, and C (Scheme 1b) because they target tubulin and CSE1L (chromosome segregation 1-like protein, also termed exportin-2, see below).

Subsequent DNA staining and fluorescence activated cell sorting (FACS) analysis revealed that treatment with tubulexin A arrests BSC-1, HeLa, and MCF-7 cells in the G2M phase (see Table S3 in the Supporting Information) at concentrations as low as 2 μM (see Figure S2b in the Supporting Information), whereas tubulexins B and C were less potent. Induction of apoptosis by tubulexin A was confirmed by the elevated activity of caspase-3 and caspase-7 in HeLa and BSC-1 cells (see Figure S2c and Table S4 in the Supporting Information) as well as by monitoring the caspase-3 substrate[11] PARP1 by immunofluorescence staining of HeLa cells treated with tubulexin A (see Figure S3 in the Supporting Information). In addition, staining with annexin V revealed increased localization of phosphatidylserine (PS) at the outer leaflet of the cell membrane[12] (see Figure S4 in the Supporting Information). Staining with trypan blue revealed that up to 30 μM tubulexin A resulted in no major necrosis occurring, but the proliferation rate was reduced to 50% compared to cells treated with DMSO (see Figure S2d in the Supporting Information).

Immunostaining of the cytoskeleton in BSC-1 cells showed that tubulexin A modulates the microtubule but not the actin network (see Figure S5 in the Supporting Information), with a lowest effective concentration of 2 μM. At 5 μM and 10 μM, microtubules were diffuse and the BSC-1 cells showed polynucleation, most likely because of disturbed mitosis.

The dynamics of the cellular microtubules was monitored by examining the cellular regrowth of microtubules in BSC-1 cells after depolymerization by cold treatment with ice. Microtubule organizing centers (MTOCs) already reap-



Scheme 2. Synthesis of a natural product inspired compound collection with a 4-hydroxytetrahydropyran scaffold by employing the Prins reaction as the key transformation. TMS = trimethylsilyl, TFA = trifluoroacetic acid.

peared in cells treated with DMSO after two minutes of repolymerization and the microtubule cytoskeleton had recovered after 15 minutes. In contrast, 10 μM tubulexin A completely inhibited microtubule regrowth (Figure 1). Tubulexins B and C were less potent (see Figure S6 in the Supporting Information).

These observations (see also Table S5 in the Supporting Information) indicated that the (4-methoxyphenyl)ethyl substituent common to tubulexins A–C is required for activity (see also Table S4 in the Supporting Information). A preference emerged for the phenyl substituent at C2 of the tetrahydropyranyl scaffold. The active tubulexins A–C contain a hydroxy group in the *meta* position, while the substituent at the *para* position varies (H, OH, OBz). Methylation of the *meta*-OH group (see Table S1, entries 1 and 8–12 in the Supporting Information) leads to loss of activity, while benzylation of the *para*-OH group increases the activity. The substituent at C4 of the tetrahydropyranyl scaffold can vary more widely, including an alcohol, ester, or a urethane. On the basis of this structure–activity correlation, biotinylated affinity probes **15** and control probe **16** (structure of the probe **15** see Scheme 1b; for the structure of control probe **16**, see Figure S7a in the Supporting Information) were synthesized. Biotinylated tubulexin A retained its biological activity in all relevant cellular assays (see Table S6 in the Supporting Information). Both biotinylated probes were immobilized on magnetic streptavidin-coated beads and employed in affinity pull-down experiments using the quantitative SILAC (stable isotope labeling by amino acids in cell culture) approach to identify potential target proteins (see the Supporting Information for details).[13] This method enabled the chromosome segregation 1-like protein (CSE1L, CAS, exportin-2) and α- and β-tubulin to be identified as potential target proteins with relevance to mitosis (see Table S7 as well as Figures S7 and S8 in the Supporting Information). The binding of tubulexin A to CSE1L and tubulin was confirmed by immunoblotting after the affinity pull-down experiment (Scheme 1c).

The binding of CSE1L was reversible, as shown by concentration-dependant competition between free and immobilized tubulexin A for binding to CSE1L (Figure 2a). Binding of CSE1L to immobilized **15** was monitored by an enzyme-linked immunosorbent assay (ELISA), which revealed an apparent K_d value of (0.87 ± 0.05) μM (Figure 2b).

Investigation of the in vitro polymerization of porcine tubulin in the absence of microtubule-associated proteins by means of turbidity measurement revealed that tubulexin A inhibits tubulin polymerization (see Figure S9a in the Supporting Information). Tubulexin A does not bind to the colchicine site (see Figure S9b in the Supporting Information),[14] but competes with a fluorescent-tagged analogue of vinblastine (BODIPY FL vinblastine)[15] for binding to the vinca alkaloid binding site on α/β-tubulin in a dose-dependent manner (Figure 2c). The EC_{50} values determined for vinblastine as a control $(2.95 \pm 0.63$ μM) and tubulexin A $(2.67 \pm 0.70$ μM) indicated stoichiometric competition with BODI-

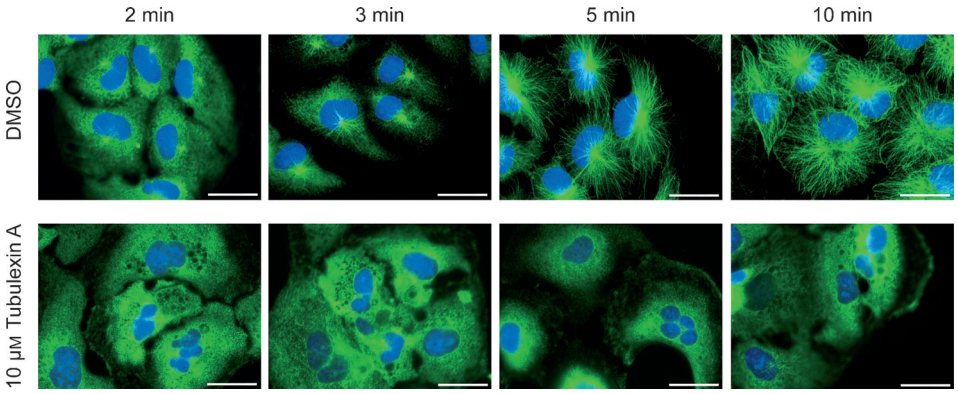


Figure 1. Tubulexin A inhibits the regrowth of microtubules in cells. Cells were incubated for 20 h with 10 μM tubulexin A or DMSO as a control. Microtubule repolymerization after cooling to 0 °C was detected at the given time points after rewarming, fixation with methanol, and staining with an anti-α-tubulin antibody and a secondary antibody coupled to Alexa Fluor488, as well as 4′,6-diamidino-2-phenylindole (DAPI) for visualizing the DNA. Scale bars: 20 μm.

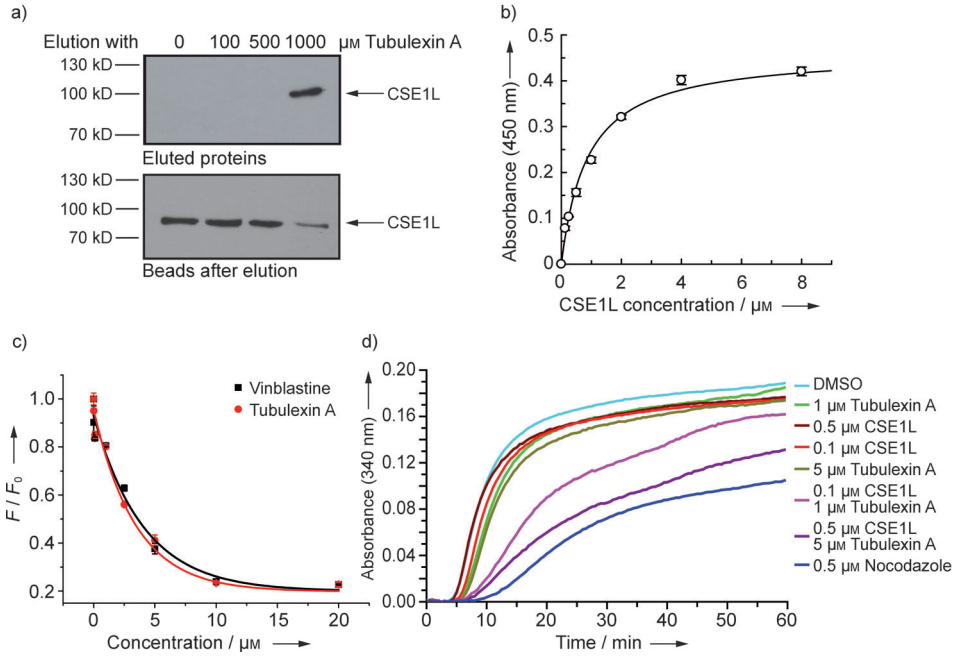


Figure 2. Tubulexins inhibit in vitro tubulin polymerization synergistically with CSE1L and bind to the vinca alkaloid binding site on tubulin. a) and b) Binding of CSE1L to tubulexin A. a) CSE1L was incubated with immobilized **15**. Bound protein was released by increasing concentrations of tubulexin A. Eluted protein and protein bound to the beads (and released by subsequent heating) were detected after immunoblotting using a CSE1L-specific antibody. b) Biotinylated tubulexin A **15** was immobilized on a streptavidin-coated plate and incubated with different protein concentrations. Binding was monitored by means of ELISA by using a CSEL-specific antibody and a secondary antibody coupled to horseradish peroxidase (HRP). c) Vinblastine competition assay: 2.5 μM α/β-tubulin was incubated with 2.5 μM BODIPY FL vinblastine for 20 min. Subsequently, tubulexin A or vinblastine and DMSO as controls were added. Mean values are shown in triplicates with standard deviations in relation to the DMSO control. Decrease in fluorescence indicates competition with BODIPY FL vinblastine for binding to the vinca binding site ($\lambda_{ex}/\lambda_{em}$ 470/514 nm). Data are shown as means ± SD with a sigmoidal fit. d) Synergistic mode of action of tubulexin A and CSE1L. In vitro polymerization of tubulin was monitored at 340 nm in the presence of tubulexin A and CSE1L or DMSO and 0.5 μM nocodazole as controls. The results are representative of three independent experiments.

PY FL vinblastine. The EC_{50} values for tubulexin B and C are slightly higher (see Figure S9c in the Supporting Information). Tubulin binding agents may influence the intrinsic GTPase activity of tubulin.[16] However, tubulexin A did not have any impact on the intrinsic GTPase activity of tubulin (see Figure S10 in the Supporting Information).

Investigation of the influence of tubulexin A on the assembly of microtubules in the presence of CSE1L (Figure 2d) revealed that 1–5 μM tubulexin A or 0.1 μM CSE1L only moderately decreased the polymerization of tubulin, but tubulin polymerization was inhibited in a synergistic manner in the presence of both tubulexin A and CSE1L in vitro.

The dual mode of action of the tubulexins might enable them to overcome the resistance mechanisms of cancer cells based on the binding of the vinca alkaloids.[17] Investigation of the influence of tubulexin A on the proliferation of HeLa cells and KB-V1 cells (a multidrug-resistant (mdr) subclone of the HeLa cell line KB-3-1)[18] by means of a WST-1 proliferation assay confirmed the resistance of the KB-V1 cells to vinblastine (254 fold increase in IC_{50} values for KB-V1 cells compared to HeLa cells, see Figure S11 in the Supporting Information). In contrast, tubulexin A inhibited the proliferation of KB-V1 cells ($IC_{50} = 3.75 \pm 0.1$ μM) and HeLa cells ($IC_{50} = 4.29 \pm 0.03$ μM) with nearly identical efficiency (see Figure S11 in the Supporting Information).

In conclusion, we have reported the synthesis of a natural product inspired compound collection based on the 4-hydroxytetrahydropyran scaffold which yielded novel modulators of mitosis that we have termed tubulexins. Tubulexin A defines a novel chemotype for the inhibition of tubulin polymerization, simultaneously targeting the chromosome segregation 1-like protein (CSE1L, CAS, exportin-2) and α/β-tubulin at the vinca binding site.

The dual mode of action of tubulexin A is unique among tubulin-binding small molecules and differentiates the tubulexins from other tubulin-destabilizing compound classes. Given the fact that modulators of tubulin assembly are among the most successful clinically used anticancer drugs, this finding may inspire novel medicinal chemistry approaches aimed at discovering treatments for cancer. The synergistic mode of action of tubulexin A and CSE1L may hint at a possible mechanism for the modulation of tubulin polymerization and microtubule growth by CSE1L. CSE1L is overexpressed in various cancers, associates with microtubules,[19] and binds to and stabilizes the α,β-tubulin heterodimer leading to enhanced formation of microtubules in cells.[20] This enhancement is linked to the prevention of tubulin phosphorylation through the binding of CSE1L to tubulin. Protection from phosphorylation, in turn, is thought to also prevent the disassembly of microtubules and thereby lead to an enhancement in the formation of microtubules.

The results of our microtubule polymerization assay in vitro demonstrate that CSE1L alone does not have a tubulin-polymerization-promoting but rather a weakly destabilizing influence. Thus, the prevention of tubulin disassembly in cells by protection from phosphorylation and not the direct interaction of CSE1L with tubulin itself may indeed be decisive for the enhanced assembly of microtubules.

Competition by tubulexin A could sequester protective CSE1L on tubulin polymers, thereby leading to the formation of unprotected α/β-tubulin polymer. Tubulexin A could then additionally bind to the vinca alkaloid binding site on the then unprotected polymer and induce depolymerization into α/β-dimers which would no longer be stabilized by CSE1L.

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