Inhibition of CPAP–tubulin interaction prevents proliferation of centrosome-amplified cancer cells

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Abstract

Centrosome amplification is a hallmark of human cancers that can trigger cancer cell invasion. To survive, cancer cells cluster amplified extra centrosomes and achieve pseudobipolar division. Here, we set out to prevent clustering of extra centrosomes. Tubulin, by interacting with the centrosomal protein CPAP, negatively regulates CPAP-dependent peri-centriolar material recruitment, and concurrently microtubule nucleation. Screening for compounds that perturb CPAP–tubulin interaction led to the identification of CCB02, which selectively binds at the CPAP binding site of tubulin. Genetic and chemical perturbation of CPAP–tubulin interaction activates extra centrosomes to nucleate enhanced numbers of microtubules prior to mitosis. This causes cells to undergo centrosome de-clustering, prolonged multipolar mitosis, and cell death. 3D-organotypic invasion assays reveal that CCB02 has broad anti-invasive activity in various cancer models, including tyrosine kinase inhibitor (TKI)-resistant EGFR-mutant non-small-cell lung cancers. Thus, we have identified a vulnerability of cancer cells to activation of extra centrosomes, which may serve as a global approach to target various tumors, including drug-resistant cancers exhibiting high incidence of centrosome amplification.

Keywords CCB02; centrosome activation; centrosome clustering; centrosomes; CPAP-tubulin module

Introduction

Centrosomes are the major microtubule organizing centers (MTOC) of mammalian cells (Zheng et al, 1995; Nigg, 2004; Nigg & Raff, 2009). Each centrosome consists of a pair of centrioles surrounded by peri-centriolar material (PCM), from which spindle and astral microtubules emanate (Bettencourt-Dias & Glover, 2007; Conduit et al, 2010). In healthy cells, strict regulation of centrosome duplication ensures the formation of only two functional centrosomes, which assemble bipolar spindles to avoid chromosomal aberrations in mitosis. In contrast, many cancer cells harbor extra centrosomes that perturb CPAP-tyrosine and nucleate enhanced numbers of microtubules prior to mitosis. This causes cells to undergo centrosome de-clustering, prolonged multipolar mitosis, and cell death. 3D-organotypic invasion assays reveal that CCB02 has broad anti-invasive activity in various cancer models, including tyrosine kinase inhibitor (TKI)-resistant EGFR-mutant non-small-cell lung cancers. Thus, we have identified a vulnerability of cancer cells to activation of extra centrosomes, which may serve as a global approach to target various tumors, including drug-resistant cancers exhibiting high incidence of centrosome amplification.
accompanied by chromosomal instability (Nigg, 2002). Thus, centrosome amplification and its structural aberrations is a hallmark of human cancers and has direct consequences on chromosomal instability and cancer cell invasion (Ganem et al., 2009; Godinho et al., 2014; Ganier et al., 2018; Marteil et al., 2018).

Intriguingly, centrosome loss in normal cells leads to irreversible cell cycle arrest, whereas in cancer cells, proliferation can still continue (Wong et al., 2015). This raises the possibility that cancer cells with extra centrosomes are fundamentally different from normal cells and use extra centrosomes for the benefit of cellular invasion. Consequently, there is a need to identify unique centrosomal properties of cancer cells, which can be exploited for conceptually new strategies to counteract cancer cell proliferation.

Amplified extra centrosomes should in theory cause multipolar mitosis, leading cancer cells to undergo mitotic catastrophe and cell death. However, cells with extra centrosomes achieve a pseudobipolar spindle via centrosome clustering, a key mechanism by which cancer cells cluster their extra centrosomes to circumvent mitotic catastrophe (Basto et al., 2008; Kwon et al., 2008; Leber et al., 2010; Pannu et al., 2015; Chavali et al., 2016). Indeed, when cells failed to cluster extra centrosomes in mitosis, they have been shown to undergo multipolar divisions and enter apoptosis (Ganem et al., 2009; Leber et al., 2010; Kawamura et al., 2013; Mathiens et al., 2013; Mason et al., 2014). Thus, inhibiting centrosome clustering to induce multipolar divisions has been proposed as a strategy to counteract tumors with high incidences of centrosome amplification (Ogden et al., 2012). An allosteric inhibitor of HSET, a kinesin motor protein required for centrosome clustering in mitosis, in fact induced multipolar spindles in cells containing extra centrosomes (Watts et al., 2013). Accordingly, direct interaction between the PCM protein CEP215 and HSET was shown to be required for centrosome clustering, highlighting an essential role for PCM in centrosome clustering (Chavali et al., 2016).

While these studies reveal the advantages of targeting centrosome clustering in cancer cells, how extra centrosomes can be manipulated to prevent them from clustering remains largely unknown. Microtubule nucleation by centrosomal PCM is spatiotemporally regulated to be minimal during interphase and increases as cells enter mitosis (Conduit et al., 2010, 2014; Avidor-Reiss & Gopalakrishnan, 2013). Accordingly, compared to metaphase centrosomes, interphase centrosomes remain relatively inactive displaying reduced to moderate level of microtubule nucleation (Piehl et al., 2004; Pannu et al., 2014; Sabino et al., 2015). Thus, we wondered that activating extra centrosomes to nucleate an enhanced level of microtubules before they cluster in mitosis could potentially lead to centrosome declustering. Although this rationale may differ from current view of centrosome-declustering mechanisms (Kwon et al., 2008; Fielding et al., 2011; Kramer et al., 2011; Watts et al., 2013), it may represent as one of the alternative mechanisms linking microtubule-nucleating activity and centrosome declustering.

Our studies in Drosophila have shown that cytoplasmic-free tubulin negatively regulates the microtubule-nucleating activity of centrosomes through its direct interaction with Sas-4 (CPAP in humans; Gopalakrishnan et al., 2012). Mutated Sas-4, which cannot interact with tubulin, activates interphase centrosomes to nucleate robust microtubules by recruiting increasing amounts of PCM proteins (Gopalakrishnan et al., 2012). Thus, to induce extra centrosomes to nucleate an enhanced level of microtubules prior to mitosis, we developed a proof-of-principle experiment in cells with extra centrosomes by genetically perturbing CPAP–tubulin interaction. Based on this experiment, we established AlphaScreen, a proximity-based protein–protein interaction assay that identified CCB02, a selective inhibitor of CPAP–tubulin interaction. Nuclear magnetic resonance (NMR) experiments and cellular pull-down assays have identified CCB02 as a tubulin binder that competes for the CPAP binding site of β-tubulin, a previously uncharacterized site that has not been occupied by known tubulin binders. CCB02 but not conventional tubulin binders activated extra centrosomes to nucleate an enhanced level of microtubules prior to mitosis and prevented them from clustering. Finally, via mouse xenograft experiments, we found that CCB02 has an anti-tumor activity.

Results

CPAP–tubulin interaction as a target to prevent proliferation of cells with extra centrosomes

To identify unique centrosomal properties that can be targeted for cancer-selective chemotherapy, we analyzed centrosomes in a spectrum of cancer cell lines. In contrast to normal cells containing only a pair of centrosomes, the tested cancer cell lines invariably displayed extra centrosomes (Appendix Fig S1Ai and ii). Highly increased centrosome numbers were observed in TKI-resistant non-small-cell lung cancer cell lines (NSCLC) carrying somatic and activating mutations in EGFR (H197T/K790M and HCC827-GR; Engelman et al., 2007; Guo et al., 2008; Pagliarini et al., 2015; Ahsan, 2016). Extra centrosomes cluster during interphase and mitosis (Gergely & Basto, 2008; Kwon et al., 2008; Leber et al., 2010; Pannu et al., 2014). Interestingly, we noticed that clustered interphase centrosomes in these cancer cells remained inactive with reduced microtubule nucleation (Pannu et al., 2014; Appendix Fig S1Aii). Thus, we sought to identify a mechanism that could activate extra centrosomes to nucleate enhanced microtubules prior to mitosis. We hypothesized that such a manipulation as one of the mechanisms that may perturb clustering of extra centrosomes in metaphase leading to multipolar spindles and eventual mitotic catastrophe (Appendix Fig S1B and C).

We previously showed that perturbing cytoplasmic tubulin from interacting with the centrosomal protein Sas-4 (the Drosophila homologue of CPAP) could activate interphase centrosomes to nucleate an elevated level of microtubules by recruiting increasing amounts of PCM proteins (Gopalakrishnan et al., 2012). Our recent CPAP-tubulin crystal structure revealed that CPAP binds β-tubulin at the microtubule outer surface via its conserved PN2-3 C-terminal loop–helix, thereby forming a high-affinity complex requiring CPAP’s Phe375 (Sharma et al., 2016; Zheng et al., 2016). Importantly, this CPAP binding site of tubulin is not occupied by so-far-studied tubulin binders (Pryor et al., 2002; Prota et al., 2013).

Building on these studies, we introduced CPAPF375A, a mutant with significantly reduced tubulin interaction into MCF10A cells, which were engineered to amplify centrosomes via doxycycline-induced overexpression of Plk4 (Godinho et al., 2014). For clarity, we term CPAPF375A as CPAPΔT, a CPAP variant with diminished interaction with tubulin.
To test whether introducing CPAP-WT or CPAP-D inhibits extra centrosomes to nuclear enhanced levels of microtubules, we performed live cell imaging experiments to identify events occurring during centrosome clustering or declustering in real time. Expressing CPAP-WT or CPAP-D in two centrosomes-containing MCF10A cells (without doxycycline induction; +Dox, two centrosomes) did not cause centrosome amplification or delay in mitosis (Fig 1Ai and ii, and Movie EV1A and B).

We then monitored extra centrosomes-containing MCF10A cells (with doxycycline induction; −Dox, extra centrosomes) expressing CPAP-WT or CPAP-D. In CPAP-WT-expressing cells, clustered centrosomes initially dispersed with a minimal microtubule nucleation, which appears to be G2 phase (from 1:55 to 2:10th minutes of Movie EV1C). These dispersed centrosomes eventually re-clustered to form bipolar metaphase (from 2:10 to 2:35th minutes of Movie EV1C). We speculated that activating these dispersed extra centrosomes to nucleate an enhanced level of microtubules could prevent them from re-clustering. CPAP-D expression indeed caused an enhanced level of microtubules at the dispersed state of G2 (from 1:35 to 2:20th minutes of Movie EV1C). As a result, these cells failed to re-cluster centrosomes and consumed much longer time in mitosis leading to multipolar metaphase (from 2:20 to 3:35th minutes of Movie EV1C). Overall, CPAP-D-expressing cells resided in mitosis ~ 4 times longer than control cells and apparently underwent cell death (Fig 1Aiii and iv, Appendix Fig S2A, Movie EV1C and D). We observed similar effects of multipolar mitosis when we introduced CPAP-D into extra centrosome-containing breast cancer (MDA-MB-231) and NSCLC (H1975 T790M) cells (Fig 1B). The multipolar mitosis is accompanied by 231) and NSCLC (H1975 T790M) cells (Fig 1B).

Identification of CCB02, a specific inhibitor of CPAP–tubulin interaction

In order to identify a small molecule that can perturb CPAP–tubulin interaction, we initiated a high-throughput compound screen based on the AlphaScreen assay technology (Schorpp et al., 2013). A library of 25,000 compounds was tested for their ability to disrupt the interaction between the conserved PN2-3 domain of CPAP (amino acids 319–394) and free tubulin (Appendix Fig S3A). The PN2-3 domain of CPAP harbors a tubulin-binding site and interacts with tubulin to form a non-polymerizable 1:1 complex (Hsu et al., 2008; Cormier et al., 2009). To exclude frequent hitters, we applied an algorithm, which resulted in a total of 49 initial hits (Schorpp et al., 2013; Table EV1, and Appendix Fig S3B and C). Subsequent cell-based assays evaluating centrosome-declustering activity further confirmed HTS1 as a compound that perturbs CPAP–tubulin interaction (Appendix Fig S3D and E). To increase the solubility of HTS1, we performed structural optimization, replacing the alkylamino residue at the C-3 position of the benzo[b][1,6]naphthyridine system with a methoxy group. This led to CCB02, which inhibits CPAP–tubulin interaction with an IC50 value of 0.689 μM as estimated by our AlphaScreen assay (Fig EV1A and B). This finding is further supported by our PN2-3 CPAP-GST pull-down assay in the presence of CCB02. We noticed that CCB02 could inhibit CPAP–tubulin interaction with an approximate IC50 value of 0.441 μM (Fig EV1C and ii). Finally, a similar PN2-3 CPAP-GST pull-down assay using cellular extract in the presence of CCB02 revealed that CCB02 could perturb interaction between CPAP and tubulin (Fig EV1Cii).

To exclude the off-target effects of CCB02 on kinases, we screened a panel of kinases and determined that CCB02 does not significantly inhibit the tested kinases, which include cell cycle- and centrosome-related kinases (Table EV2 and Fig EV1D). To further validate that CCB02 does not affect the tested cell cycle- and centrosome-related kinase activities in cells, we performed Western blots using phospho-specific antibodies that recognize substrates phosphorylated by Aurora A, Plk1, Plk2, CDK2, and CHK1. We identified that CCB02 does not affect these kinase activities (Fig EV1D, right panel).

CCB02 binds at the CPAP binding site of β-tubulin to perturb CPAP–tubulin interaction

To dissect how CCB02 perturbs CPAP–tubulin interaction, we performed 1D-1H NMR spectroscopy of CCB02 in the presence of tubulin and identified CCB02 as a tubulin binder (Fig 2A).
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A CPAP WT (-Dox, two centrosomes)

i Interphase to mitotic onset

Mitosis to cytokinesis

ii CPAP ΔT (-Dox, two centrosomes)

iii CPAP WT (+Dox, extra centrosomes)

Interphase to mitotic onset

Mitosis to cytokinesis

iv CPAP ΔT (+Dox, extra centrosomes)

B MCF10A (+Dox, extra centrosomes)

Dox+CPAP

Interphase

Mitosis

Dox+CPAP ΔT

C MDA-MB-231

CPAP

Interphase

Mitosis

CPAP ΔT

D H1975

CPAP

Interphase

Mitosis

CPAP ΔT

E

% of multipolar mitotic cells

MCF10A

MDA-MB-231

H1975

(+Dox, extra centrosomes)

F

i Tumor volume (mm^3)

Control

CPAP ΔT

ii Tumor volume (mm^3)

Control

CPAP ΔT

Days after cancer cell implantation

Figure 1.
INPHARMA experiments were then performed to identify the binding site of CCB02 using a CPAP-derived peptide (residues 375–386), which binds to the microtubule outer surface on β-tubulin with $K_D = 3.56 \mu M$ for tubulin (Sanchez-Pedregal et al., 2005; Orts et al., 2009). To obtain comparable binding affinities of the CPAP peptide and CCB02 to tubulin, we used CCB02.1, a CCB02 derivative that prevents CPAP–tubulin interaction with an IC$_{50}$ value of 6.94 μM (Appendix Fig S4A). We then generated NOESY spectra of a mixture of CPAP peptide (400 μM), CCB02.1 (200 μM), and tubulin (6.5 μM) at different mixing times (40, 70, 100, 150 ms) in D$_2$O. Importantly, we ensured that tubulin at 6.5 μM used in these NMR analyses is folded properly and not forming aggregates (For details, see Appendix Fig S4B).

We observed 10 intermolecular NOE peaks with a mixing time of 40 ms, while 50 peaks were observed at 70 ms (Fig 2B). Plotting NOE build-up curves of six non-overlapping intermolecular NOE peaks with mixing times of 40, 70, 100, 150 ms (Fig 2B).
peaks revealed a damped parabolic-shaped curve, characteristic of inter-ligand NOE cross peaks arising from protein-mediated spin diffusion. This observation suggests that the CPAP peptide and CCB02.1 have the same binding site on tubulin (Fig 2B and Appendix Fig S5C). The NOEY spectra showed strong inter-ligand NOEs from the H6/H7 protons of CCB02.1 to the aromatic side chain of Phe385 on the CPAP peptide although weaker NOEs were observed throughout the peptide at higher mixing times (Fig 2B and Appendix Fig S4D). In a control experiment, no intermolecular NOEs were observed when the CPAP peptide and CCB02.1 were mixed at a 1:1 ratio in the absence of tubulin.

*In silico* docking models combined with the NMR data suggest that CCB02.1 can occupy both the Phe385/Phe375 binding pockets on tubulin, with preference for the Phe385 pocket, which occupies the microtubule outer surface of β-tubulin (Appendix Fig S5C). Finally, we performed isothermal titration calorimetry (ITC) to validate specific interaction between CCB02 and tubulin. Under our optimized condition, we were able to capture a titration curve (light blue curve, Appendix Fig S5B) that displayed a fitted binding KD of 2.2 μM with ΔH of −6.1 kcal/mol and ΔS of 4.7 cal/mol/deg, which showed a binding trend for specific interaction between CCB02 and tubulin.

Taken together, these results indicate that CCB02 is a novel tubulin binder whose binding site overlaps with the CPAP peptide at the microtubule outer surface of β-tubulin (Sharma et al, 2016; Zheng et al, 2016). Importantly, some of the known conventional tubulin binders do not occupy this CCB02 binding site (Appendix Fig S5C; Pryor et al, 2002; Ravelli et al, 2004; Gigant et al, 2005; Lu et al, 2012; Prota et al, 2013). Via this mode of tubulin binding, CCB02 could perturb CPAP binding to tubulin in cells. To evaluate whether the major binding partner of CCB02 is cellular tubulin, we performed a CCB02-Biotin pull-down assay using cellular extracts and identified that CCB02 pulls down cellular tubulin (Appendix Fig S5D–iii). Indeed, mass spectrometric analysis of CCB02 complexes identified tubulin as the most significant binding partner of CCB02 (Appendix Fig S5E). In summary, these experiments substantiate that CCB02 is a specific tubulin binder in cells.

### CCB02-mediated inhibition of CPAP–tubulin interaction impairs proliferation of cells with centrosome amplification

We then tested CCB02’s effect on a spectrum of cancer cells exhibiting extra centrosomes compared to normal cells containing two centrosomes. A 72-hr exposure of CCB02 prevented cancer cell proliferation with IC50 values between 0.86 and 2.9 μM (Fig EV2A).

Importantly, correlating the percentages of extra centrosomes-containing cancer cells to their respective IC50 values revealed that cells with higher percentages of extra centrosomes have inversely proportional IC50 values for CCB02 (Fig EV2B). If this were true, we would expect that a long-term CCB02 treatment would selectively eliminate extra-centrosome-containing cells, but allowing the survival of two centrosomes-containing cells. To test this aspect, we exposed MDA-MB-231, HCC827-GR, Calu6 and Plk4-overexpressing MCF10A cells to CCB02. After 14 days of exposure, we observed that the CCB02-treated cultures mostly contained two centrosomes-containing cells as compared to vehicle-treated cultures, indicating that cells with higher levels of centrosome amplification are more sensitive to CCB02 (Fig 3A).

We then analyzed the effect of CCB02 on extra centrosomes. Similar to genetic perturbation, chemical perturbation of CPAP–tubulin interaction has also activated extra centrosomes to nucleate an enhanced level of microtubules prior to mitosis, resulting in the formation of multipolar spindles in mitosis (Appendix Fig S3B and Fig EV2C–I). To further verify that CCB02’s action occurs prior to mitosis and to make sure that we score similar stages of cells between treatment and control groups, we profiled CCB02-treated MDA-MB-231 cells using cyclin A staining, a bona fide G1–S transition marker (Hochegger et al, 2008; Gabriel et al, 2016). In vehicle-treated groups, cyclin A-positive cells mostly exhibited clustered (G1/S phase) or dispersed (G2 phase) centrosomes with less or no microtubule nucleation. In contrast, CCB02-treated cells exhibited clustered centrosomes with enhanced microtubule nucleation (Appendix Fig S6A and B).

To better capture CCB02-mediated effects, we performed live imaging of CCB02-treated MCF10A (+Dox, extra centrosomes) and HCC827-GR cells. Similar to CPAPAT expression, CCB02-treatment prevented extra centrosomes from clustering leading to multipolar mitosis with an apparent cell death (Fig 3C and D, and Movie EV2A–D). Importantly, cells with extra centrosomes exhibited much longer mitotic duration than two centrosomes-containing cells (Appendix Fig S6C and D).

We then tested whether CCB02-induced mitotic delay causes spindle assembly checkpoint (SAC) activation. To do this, we performed experiments using antibodies against Bub1 and Mad1 proteins. These components are known to accumulate on...
Figure 3.
unattached kinetochores (Shah & Cleveland, 2000; Kim et al., 2015; Musacchio, 2015). First, we verified that cells (regardless of two- or extra-centrosome-containing cells) normally showed an accumulation of Bub1 and Mad1 proteins in prophase (Fig EV3A, prophase panel). This is due to unaligned chromosomes to kinetochores at this stage of cell cycle (Johnson et al., 2004; Bolanos-Garcia & Blundell, 2011). As also expected, bipolar and pseudobipolar metaphase cells did not show a detectable accumulation of these proteins (Fig EV3A, metaphase panel). We then analyzed CCB02-treated MCF10A (−Dox, two centrosomes), MCF10A (+Dox, extra centrosomes), and MDA-MB-231 cells. Importantly, we have also used MCF10A (+Dox, extra centrosomes)-expressing CPAPAT as a control where CPAP–tubulin interaction is genetically perturbed. In both cases, we observed an accumulation of Bub1 and Mad1 proteins in prophase (Fig EV3B and C, prophase panels). However, in contrast to bipolar metaphase cells as observed in Fig EV3, these proteins are still accumulated in multipolar metaphase cells where CPAP–tubulin interaction is perturbed either by CCB02 treatment or CPAPAT overexpression (Fig EV3B and C, metaphase panels). These observations suggest that perturbing CPAP–tubulin interaction could activate the spindle assembly checkpoint in extra-centrosome-containing cells.

Finally to dissect the observed effect of CCB02 is CPAP dependent; we depleted CPAP in MDA-MB-231 and MCF10A cells harboring extra centrosomes for 48 h and analyzed a fraction of cells that still contained extra centrosomes (Fig 4A–C). We noticed that CPAP depletion did not prevent centrosome clustering (Fig 4D). We then treated these cells with CCB02 and identified that CCB02 treatment did not induce declustering of centrosomes or cell death (Fig 4E and F). These results suggest that CCB02-treatment specifically impairs proliferation of extra-centrosome-containing cells and the effect induced by CCB02 is CPAP dependent.

**Inhibiting CPAP–tubulin interaction by CCB02 enhances PCM recruitment to centrosomes**

We next sought to identify mechanisms by which CCB02 activates extra centrosomes for an enhanced microtubule nucleation. PCM recruitment to centrosomes is required for microtubule nucleation (Oegema et al., 1999; Nigg & Raff, 2009; Gopalakrishnan et al., 2011; Lee & Rhee, 2011). Enhanced microtubule nucleation of CCB02-treated centrosomes prior to mitosis suggests that these centrosomes recruit enhanced levels of PCM. To test this, we estimated the amounts of Cep152, PCNT, and CDK5RAP2 recruitment to interphase centrosomes of CCB02-treated two centrosomes-containing MCF10A cells. Notably, both human and *Drosophila* CPAP interacts with these proteins to form the S-CAP complex (Gopalakrishnan et al., 2011; Conduit et al., 2015; Chou et al., 2016). High-resolution imaging and heat map intensity analyses revealed that interphase centrosomes recruit enhanced amounts of these proteins compared to vehicle-treated cells (Fig 5A and B).

Furthermore, biochemically fractionated centrosomes from CCB02-treated cells revealed the presence of elevated levels of CPAP-interacting proteins (Appendix Fig S7A and B). Finally, we tested whether CCB02 could prevent tubulin binding to CPAP in cells and simultaneously enhance the ability of CPAP to bind its interacting proteins. To do this, we immunopurified CPAP complexes from cytoplasmic extracts of MCF10A cells in the presence of CCB02 and identified that CCB02 specifically perturbs tubulin binding to CPAP, thereby allowing CPAP to bind enhanced amounts of its interacting proteins (Fig 5C). We also observed a similar finding when we used cytoplasmic extracts prepared from interphase-synchronized HeLa cells (Appendix Fig S7C).

To validate that the enhanced recruitment PCM could cause microtubule nucleation, we performed a three-time point microtubule regrowth assay with MCF10A (−Dox, two centrosomes), MCF10A (+Dox, extra centrosomes), and MDA-MB-231 cells (Sankaran et al., 2005; Choi et al., 2010; Fig 6). CCB02 treatment caused centrosomes to nucleate an enhanced level of microtubules already at 1.5 min after induction of regrowth. Importantly, these centrosomes recruited significantly higher levels of γ-tubulin (Fig 6). This finding is in agreement with our live imaging experiments where CCB02-treated cells display centrosomes with robust microtubule nucleation (Movie EV2B and D, and Fig 6).

To exclude that the observed effects of CCB02 are not due to centrosome fragmentation, we analyzed MDA-MB231 and MCF10A
Figure 4.

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Figure 5.

A 

i 

CPAP and its heat map | Cep152 and its heat map

Vehicle | CCB02 (2 μM)

CPAP | Cep152 | Merge

ii 

CPAP and its heat map | PCNT and its heat map

Vehicle | CCB02 (2 μM)

CPAP | PCNT | Merge

iii 

CPAP and its heat map | CDKSRAP2 and its heat map

Vehicle | CCB02 (2 μM)

CPAP | CDKSRAP2 | Merge

B 

Proteins in interphase centrosomes

Relative intensity (a.u.)

0 1 2 3 4 5 6 7 8

Vehicle | CCB02 (2 μM)

CPAP | Cep152 | PCNT | CDKSRAP2

C 

i 

CCB02 (μM)

CPAP | α-TUB | γ-TUB | Cep152

ii 

Intensity (a.u.)

0 10 20 30 40

CCB02 (μM)
cells (+Dox, extra centrosomes) before and after CCB02 treatment. We did observe fragmented centrosomes in MDA-MB231 and MCF10A cells (+Dox, extra centrosomes) as determined by PCNT-negative centrin dots (centrin-3; Kohlmaier et al, 2009; Lawo et al, 2012; Godinho & Pellman, 2014; Karki et al, 2017). The intact centrosomes are determined by centrin colocalization with PCNT. Importantly, we did not observe any increase in the frequencies of fragmented centrosomes indicating that CCB02 does not induce centrosome fragmentation (Fig EV4A–C). Together, these results suggest that chemical inhibition of the CPAP–tubulin interaction could enhance the recruitment of CPAP-interacting proteins to interphase centrosomes. This finding is similar to what was observed when Sas-4-tubulin interaction was genetically perturbed in flies (Gopalakrishnan et al, 2012).

Effects of CCB02 in cells differ from the effects of known tubulin-binding agents

Most tubulin-binding agents act on spindle microtubules and thus prevent mitosis non-specifically (Kavallaris, 2010). To evaluate whether the effects of CCB02 are specific to CPAP–tubulin interaction and not due to general effects caused by known tubulin binders, we compared CCB02 to known tubulin binders such as taxol, bactulin III, docetaxel, and vinblastine. Except CCB02, neither of the tested tubulin binders could perturb CPAP–tubulin interaction, enhance PCM recruitment to interphase centrosomes, and prevent extra centrosomes from clustering (Fig 7A–C and Appendix Fig S8A–E). Finally, to test that CCB02 does not alter microtubule in vitro and live cells, we performed microtubule plus end-tracking assay using GFP-tagged EB1 and EB3, respectively. CCB02 at 1, 2, and 5 μM did not detectably influence various parameters of microtubule dynamics (Fig 7E and F, and Movies EV3 and EV4). Taken together, these results suggest that most effects of CCB02 differ from the effects of known tubulin-binding agents.

CCB02 impairs the invasiveness of NSCLC cells in 3D-organotypic cultures and has anti-tumor activity in vivo

Centrosome amplification triggers cellular invasion in 3D cultures (Godinho et al, 2014; Ganier et al, 2018). To test whether CCB02-mediated effects could impair the invasive behavior of NSCLC cells, we used 3D-organotypic cultures of H1975T790M and HCC827-GR exhibiting resistance to EGFR-TKIs. In contrast to 5 μM erlotinib treatment, which is a known TKI, CCB02 at 5 μM was sufficient to prevent cellular invasion emerging from 3D spheres (Fig 8A, and Appendix Fig S8F and G). This could be due to the lack of proliferation and cellular death mediated by CCB02 treatment. Indeed, we noticed cell rounding, characteristics of prolonged mitotic arrest with concomitant cell death as revealed by activated caspase-positive cells in H1975T790M spheroids (Appendix Fig S8G). As a result, CCB02-treated spheroids did not grow further from its original size (Fig 8B). To corroborate this finding and further to test whether CCB02 treatment could prevent the migration of extra centrosome-containing cells, we performed a wound-healing assay using MDA-MB-231 cells (Wang et al, 2012). CCB02 treatment significantly inhibited the migration of cells as determined by prolonged duration in wound closure (Fig EV5A and B). Together, these data suggest that CCB02 has the ability to impair the invasion and migration behavior of cancer cells in vitro.
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Figure 6.
Figure 7. Vulnerability of extra-centrosome-containing cancer cells to centrosomal activation.
Discussion

Since centrosome amplification and clustering is a hallmark of cancer cells, perturbing centrosome clustering to induce mitotic catastrophe has been proposed as a selective strategy for tumors with a high incidence of centrosome amplification (Ogden et al., 2012; Chavali et al., 2014). Mechanisms to manipulate centrosomal activities in living cells have not been thoroughly investigated.

At interphase of the cell cycle, the centrosome contains a basal level of PCM (Piehl et al., 2004; Wiese & Zheng, 2006; Nigg & Stearns, 2011; Roostalu & Surrey, 2017). However, as cells enter into mitosis, centrosomes recruit PCM and increase in size. The process of PCM recruitment enabling centrosomes to nucleate robust microtubules in mitosis is termed as centrosome maturation (Conduit & Raff, 2010; Conduit et al., 2010, 2014). However, how the timing and amount of PCM recruitment is determined is yet to be critically analyzed.

Our earlier biochemical studies in Drosophila have identified that tubulin negatively affects Sas-4’s (in humans, it is CPAP) ability to form cytoplasmic protein complexes. Drosophila expressing a Sas-4 variant that does not bind tubulin (Sas-4-AT) exhibited abnormal PCM recruitment (Zheng et al., 2016). In Sas-4-AT flies, the major PCM protein Cnn, normally detected only in mitotic centrosomes, was observed in interphase centrosomes, while mitotic centrosomes recruited at least twice the amount of Cnn as control centrosomes (Rusan & Peifer, 2007).10 These results suggested that tubulin present in wild-type Sas-4 complexes spatiotemporally regulates the amount of PCM recruitment; i.e., tubulin can function as a molecular switch in regulating Sas-4/CPAP-mediated PCM recruitment. From this, we hypothesized that perturbing CPAP–tubulin interaction could be used as a tool to enhance the microtubule-nucleating activities of centrosomes prior to mitosis.

In this work, we tested the significance of perturbing CPAP–tubulin interaction in cells, which harbor extra centrosomes. Indeed, our live imaging experiments revealed that CPAPAT-expressing cells exhibited an enhanced level of microtubule nucleation prior to mitosis starting from interphase itself. Importantly, the microtubule nucleation persisted over a longer period of time and prevented them from clustering leading to multipolar mitosis (Fig 1Aiv, Appendix S2A, and Movie EV1D).

To chemically inhibit CPAP–tubulin interaction, we identified CCB02, a selective inhibitor of CPAP–tubulin interaction, which caused similar effects as also caused by genetic perturbation of CPAP–tubulin interaction (Fig EV1). To dissect the mode of action, we identified that CCB02 binds β-tubulin at the microtubule outer surface, thereby perturbing its interaction with CPAP. This enhances recruitment of CPAP-interacting proteins to interphase centrosomes, which simultaneously activates centrosomes to nucleate an enhanced level of microtubules prior to mitosis (Fig 5 and Appendix Fig S6). Thus, the primary effect of perturbing CPAP–tubulin interaction is to activate centrosomes prior to mitosis.
Figure 8.

C Xenograft experimental scheme

D

Tumor volume (mm³)

Days after tumor cell implant

Relative tumor volume (%)
When analyzing mitosis, we noticed that both genetic and chemical perturbation of CPAP–tubulin interaction specifically in extra centrosome-containing cells causes prolonged activation of spindle assembly checkpoint (SAC; Fig EV3B and C). This could be due to at least two reasons. First, mitotic delay caused by centrosome-declustering activity. Second, CCB02 as a tubulin binder could have an additional effect on spindle microtubules possibly via perturbing microtubule dynamics. In fact, such an effect has been demonstrated for griseofulvin, another tubulin binder that can prevent extra centrosomes from clustering (Rebacz et al., 2007; Raab et al., 2012; Ronnest et al., 2012). Interestingly, griseofulvin enhances Sas-4 (in human CPAP)-dependent PCM protein binding and recruitment (Gopalakrishnan et al., 2012). From this aspect, it appears that griseofulvin could also function through centrosome activation mechanism by elevating PCM recruitment.

The complete mechanisms of action of CCB02 remain unknown. Given the inherent nature of small molecules such as off-target effects and cross-reactivity, it is plausible that CCB02 can compete with microtubule-binding proteins including kinesins such as HSET which has been shown to promote clustering of extra centrosomes (Kwon et al., 2008; Fielding et al., 2011). We could not exclude these additional possibilities that warrant for future experiments. However, based on our current data, CCB02’s action is likely to be selective for extra centrosomes-containing cells. Various experimental data support this notion. First, half-maximal inhibition value (IC50) of CCB02 in various cancer cells ranges from 0.86 to 2.9 μM. Interestingly, PC9 cells which display a relatively weak inhibitory response (IC50 = 2.9 μM) harbor only a mild centrosome amplification. In contrast, cell lines with a high degree of centrosome amplification display strong inhibitory responses (Fig EV2A and B). Thus, the higher inhibitory response observed with these cells suggests that CCB02 is more potent in cancer cells with a high incidence of centrosome amplification.

EGFR activating mutations are some of the most common oncogenic driver mutations found in NSCLC patients, which reduces the overall survival rate of cancer patients. Although patients respond to initial EGFR-TKI treatment, subsequent development of secondary resistance leads to treatment failure. Recent clinical and preclinical evidence suggests that combined approaches using multiple kinase inhibitors or a combination of kinases and small-molecule inhibitors of cell proliferation and cell migration can overcome secondary resistance (Brugger & Thomas, 2012). Interestingly, in contrast to erlotinib, we found CCB02 is able to perturb proliferation of EGFR-mutant NSCLC (Fig 3B and Fig EV2C). Indeed, these cells exhibited an extreme increase in centrosome numbers. These findings suggest that developing a combination therapeutic strategy that uses centrosome-activating agents could be beneficial in drug-resistant cancers.

In conclusion, our work identifies a vulnerability of cancer cells to extra centrosomal activation providing a conceptually new strategy to specifically prevent cancer cell proliferation. Recent studies have linked extra centrosomes to tumor aggressiveness identifying differential functions of extra centrosomes in cancer cells (Ganem et al., 2009; Godinho et al., 2014; Wong et al., 2015). Our work provides mechanistic evidence that activating these extra centrosomes via enhanced PCM recruitment and microtubule nucleation may be a broadly useful tool to target cancers that exhibit extra centrosomes. The CPAP-tubulin inhibitor CCB02 may not only serve as a useful tool to study centrosome functions in cells, but also a starting point for developing combinatorial treatment strategies, specifically when extra centrosomes indirectly contribute to a “bypass track” by which therapy-resistant cancers develop.

Materials and Methods

Screening instruments

We used a HTS platform with an integrated instrumentation for plate and liquid handling. The screening was performed using aSciclon G3 Liquid Handler from PerkinElmer (Waltham, MA, USA) with a Mitsubishi robotic arm (Mitsubishi Electric, RV-3S11) and a Flexdrop dispenser (PerkinElmer, Waltham, MA, USA). The AlphaScreen assay was performed in white 384-well Optiplates™ (PerkinElmer, 6007299). AlphaScreen signal was detected on the EnVision® Multilabel Reader (PerkinElmer, Waltham, MA, USA).

Compound library

The small-molecule diversity set used in the CPAP-tubulin HTS campaign was composed of compounds acquired from three providers, namely ChemDiv, Inc. (10,000 compounds), Enamine, Ltd. (10,000 compounds), and ChemBridge, Corp. (5,000 compounds). The purity of the compounds was > 90% as reported by the providers of the compounds. More detailed information on compound selection criteria is provided in Schorpp et al (2013).

AlphaScreen reagents

The AlphaScreen™ detection system (PerkinElmer, USA) used in this study consists of streptavidin donor beads and nickel chelate acceptor beads (AlphaScreen Histidine, Nickel Chelate Detection Kit, product #6760619C).

Assay development and screening of the CPAP-tubulin AlphaScreen protein–protein interaction assay

Biotinylated tubulin (Tebu-Bio, product number: 02T333P-B) was captured by the streptavidin donor beads. His-tagged PN2-3 domain (aa 319–389) of CPAP that has tubulin-binding region has been purified and bound with NiNTA Acceptor beads (Hsu et al., 2008; Zheng et al., 2016). All proteins and beads were diluted in assay buffer containing 1× PBS (pH 7.4), 0.5% bovine serum albumin (BSA) and 0.01% Tween-20. Prior to performing the screening with 25,000 small molecules, the PPI assay was adapted to automation using a liquid handler and a compound transfer station (see instruments). The CPAP-tubulin HTS campaign was performed in white 384-well Optiplates™ as follows: (i) dispensation of 30 µl of 2× concentrated (20 nM, 10 nM final) biotinylated tubulin into white 384-well plates using a robotic liquid handler (ii) transfer of 0.6 µl of compounds in DMSO (1 mM stock) or DMSO alone into each well using a compound transfer station with a nanoliter head yielding a final assay concentration of each compound of 10 µM and 1% v/v DMSO; for IC50 determination.
compounds were diluted in 100% DMSO (20 concentrations, 0.2 nM–100 μM final) (iii) dispensation of 10 μl of 6x concentrated (75 nM, 12.5 nM final) His-CPAP or His-CPAP\textsuperscript{F375A} (CPAP\textsuperscript{AT}). CPAP\textsuperscript{AT} cannot interact with tubulin and was therefore included as a negative control to all assay plates; (iv) incubation of the plates for 1 h at room temperature; (v) addition of 10 μl of streptavidin donor and nickel chelate acceptor beads (30, 5 μg/ml final) followed by a further incubation for 1 h at room temperature in the dark; (vi) reading of the assay plates using laser excitation at 680 nm, with emission detected at 520–620 nm in an EnVision 2102 Multilabel Reader (PerkinElmer, USA). Subsequently, AlphaScreen and His-tag frequent hitters (FH) were identified and could bioinformatically be excluded to create a final hit list (Schorpp et al, 2013). The quality and robustness of the assay, represented as Z', were calculated.

**Kinase screening assay**

Kinase screening assay was performed commercially using KINOMEScan\textsuperscript{TM} screening platform from DiscoverX. CCB02 was used at a concentration of 5,000 nM in duplicates. Kinase assay was performed as previously described (Fabian et al, 2005).

**Cell culture**

Unless and otherwise stated, all cancer cell lines were originally purchased from American Type Culture Collection (ATCC) and the German Resource Centre for Biological Material (DSMZ). Cell lines were cultivated at 37°C, 5% CO\textsubscript{2}, and 80–90% humidity with DMEM (#11965-062, Gibco) or RPMI 1640 (#61870010, Gibco) containing 10% FBS (#P30-19375, PAN Biotech), MEM (minimum non-essential amino acids, #11140-035, Gibco), 100 units/ml penicillin (#15140-122, Gibco), and 100 units/ml streptomycin (#15140-122, Gibco). All handling steps with the cell lines were done under a laminar flow. When the cells reached a density of about 90% the adherent cells were passaged beginning with removing the medium and a washing step with PBS (#18912014, Gibco). To detach the cells from the bottom of the 75 cm\textsuperscript{2}/150 cm\textsuperscript{2} flask 0.5–2 ml of trypsin/EDTA (#25200056, Gibco) was added for 5 min at 37°C. The cells were then re-suspended in at least 7 ml fresh medium and seeded at the desired density. Suspension cell lines were passaged by suitable dilution of the cell suspension. Cells were tested for mycoplasma using MycoAlert mycoplasma detection kit (#LT07-418, Lonza).

**Depletion of CPAP by siRNA**

For depleting CPAP in cells, siRNA against 5'-CCAAACACCUU CAUUCAUU-3' (Dharmacon siRNA, D-010209-02) CPAP was generated as previously described (Tang et al, 2009; Zhao et al, 2010; Zheng et al, 2014). Cells were treated with scramble and CPAP siRNA for 48 h. The depletion of CPAP was confirmed by Western blot and immunofluorescence.

**Double thymidine block of HeLa cells**

Double thymidine block of HeLa cells was performed as previously described (Harper, 2005). Briefly, cells were treated with 2 mM thymidine for 18 h. After this first thymidine block, cells were washed and released for 9 h. Second thymidine (2 mM) block was done for 15 h. Finally, cells released from the block and collected for G1/S stage of cell cycle.

**Cell viability assay**

Cell lines were plated as triplicates into sterile 96-well plates at 5,000 cells/well densities for adherent cells. Every well was filled with 100 μl cell-containing medium. To determine the cell number, improved Neubauer cell-counting chamber (Marienfeld, Germany) was used. After 24 h of incubation of the plates at 37°C, CCB02 was added at increasing dosages, ranging from 0.1 to 15 μM together with a separate DMSO control. After 72–96 h, cell proliferation was performed colorimetrically by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay, using 20 μl of CellTiter Aqueous One (#G3582, Promega) Solution reagent to each well. After 1–3 h of incubation at 37°C in a humidified, 5% CO\textsubscript{2} atmosphere, the absorbance at 490 nm was recorded using a multimode plate reader (Mithras, Berthold Technologies, Germany) and the raw data were obtained. The IC\textsubscript{50} (concentration needed to prevent cell proliferation by 50%) value was calculated using GraphPad Prism by plotting the percentage of cell survival as a function of drug concentration.

Three independent experiments were performed per cell line.

**3D organotypic culture system**

Spheroid model was adapted from previously described methods (Friedrich et al, 2009). In short, 100 μl of culture medium containing 5,000 cells was added to an ultra-low-attachment 96-well plate. The cells were supplemented with 2.5% of Matrigel (#356230, BD Biosciences) for compact spheroid formation (Ivascu & Kubbies, 2007). After 48–96 h, spheroids were transferred to a Labtek multi-well plate (#154534, Thermo Scientific) or Ibidi μ-Slide chamber plates (#80426 and #80286, Ibidi) containing polymerized Matrigel: collagen-1 mixture in each well. For spheroid treatment, the supernatant medium on top of Matrigel: collagen-1 mixture was replaced with vehicle or diluted drugs supplemented in a standard medium.

**Wound-healing assay**

Wound-healing assay was performed as previously described (Wang et al, 2012; Jonkman et al, 2014). In brief, MDA-MB-231 cells were grown as monolayer of cells to confluence in 2-well ibidi chamber plate (#80286), and at experimental time point 0 h, a scratch was made in each well using a pipette tip (p10). The cells were washed twice with PBS before their subsequent incubation with culture medium in the presence of vehicle or CCB02 (1 μM). The cells were pretreated with CCB02 for 12 h before the scratch. In order to monitor cell migration in the scratched area, live cell imaging was performed using Leica DMI6000B widefield microscope with 10×/0.22. During live cell imaging, cells were maintained at 37°C with humidified CO\textsubscript{2} (3–5%) using an enclosed temperature and CO\textsubscript{2} controller. Images were collected at 0, 10, 24, and 48 h. All the captured images from each experiment were processed using Fiji/ImageJ.
2D and 3D indirect immunofluorescence microscopy

For 2D immunofluorescence, cells were grown in glass coverslips (18 mm, No. 1.5H, #0117580, Marienfeld) in multi-well plates. Cancer cells were treated with CCB02 in a concentration range of 1–2 μM depending on the cell type. After vehicle or CCB02 treatment, cells were washed with PBS and fixed with ice-cold methanol at −20°C for 10 min or 4% PFA. Fixed cells were blocked with 0.5% fish gelatin in PBS (wash buffer) for 10–30 min at room temperature or at 4°C overnight and then incubated with primary antibodies (centrosomal markers, as in figure legends) for 60 min at room temperature or at 4°C overnight. After incubation, cells were washed with wash buffer for three times and incubated with species-specific secondary antibodies (#A11001, Alexa Fluor 488 anti-mouse, #A11037, Alexa Fluor 488 anti-rabbit, Alexa Fluor 594 anti-rabbit, #A11005 Alexa Fluor 594 anti-mouse; Molecular Probes, Invitrogen) at 1:1,000 dilution for 30 min. 3D spheroids were washed with wash buffer for 30 min for three times. DAPI (1:1,000 in wash buffer) was used to stain DNA for 30 min. 3D spheroids were imaged using 10×/0.4 plan Apo air objective on Olympus FV1000 laser scanning confocal microscope. Images were processed using Fiji/ImageJ and Adobe Photoshop.

Lentiviral production and transduction of target cells

Constitutive overexpression of GFP-tagged CPAP-WT and CPAPΔT lentiviral vectors was prepared using pSinEF2. The cloned vectors were packed into lentivirus using second-generation packaging plasmids (pPMD, addgene #12259 and pPAX, addgene #12260). Briefly, GFP-tagged CPAP vectors and packaging plasmids were transfected into HEK293TS cells using calcium chloride. After 16 h, medium was changed and the virus was collected after 48 h. The freshly collected virus was used to transduce target cells in 1:1 ratio for 24–48 h. For live cell imaging, pcDNA CPAP-Myc and CPAP-ΔT versions were introduced transiently into MCF10A cells using TransIT-X2 dynamic transfection reagent. These plasmids were kindly provided Dr. Tang TK (Tang et al., 2009).

Immunoprecipitations and Western blotting

Immunopurification of cytoplasmic CPAP complex was done as previously described (Gopalakrishnan et al., 2012). In brief, as previously described, cell extracts were prepared by lysing the cells using BRB80 buffer. The cell extracts were centrifuged 100,000 g for at least 60 min, and the high-speed lysate (the supernatant) was used for further purifications. Protein G beads (#17061801, GE healthcare) were coated with anti-CPAP antibody overnight at 4°C. The antibody-coated beads and vehicle- or CCB02-treated extracts were mixed and incubated at 4°C for 4 h. Then, it was washed with extract buffer containing 0.1% Triton X-100, and then twice with cell extract buffer. For eluting the complexes, beads were boiled with 2× Laemmli buffer. Protein lysates were subjected to SDS–PAGE on 8 or 10% polyacrylamide gel, transferred onto nitrocellulose membranes, which were incubated with indicated primary antibodies, washed, and probed with HRP-conjugated secondary antibodies (#G21040, goat mouse IgG (H + L) and #G21234, goat rabbit IgG (H + L)). The band intensities were quantified from two independent experiments (n = 3, technical replicates) using Fiji. Pull-down experiments were performed using GST-tagged PN2-3 domain of CPAP. In Brief, GST-PN2-3 was expressed in BL21 and we purified the protein using GST beads. Once the GST-PN2-3 bound to GST beads, we performed a pull-down experiment using cellular extracts or using purified tubulin in the presence of different concentrations of CCB02. Porcine/bovine tubulin was purified as previously described (Zheng et al., 2016). Similarly, CCB02-biotin pull-down assay was performed using cellular extracts. Before this, the CCB02-biotin was bound to streptavidin sepharose resins (#2-1201-002, IBA, Germany) and washed several times with wash buffer (#2-1003-100, IBA, Germany) before cellular extracts were loaded.

Mass spectrometric analysis

The pull-down proteins were on-bead digested and processed for mass spectrometric analysis. Briefly, the beads were boiled in 10 μl of RapiGest™ at 95°C for 10 min followed by reduction of disulfide bridges by incubating with 5 mM TCEP for 30 min at RT, 750 rpm. The free cysteines were alkylated by adding 10 mM of chloroacetylamine and incubating the vials for 30 min, 750 rpm, RT. Finally, the proteins were digested using 1 μg of trypsin (Promega) at 37°C, 750 rpm overnight. The protease activity was quenched by adding...
20 μl of 10% formic acid (Sigma). The eluted peptides were desalted using C18 STAGE-Tips and dried in SpeedVac. For LC-MS analysis, each sample was reconstituted in 20 μl of 5% ACN and 0.1% formic acid and 5 μl was injected into the mass spectrometer. LC separation was carried on an Agilent 1100 nano-flow LC system (Agilent Technologies). Buffer A was 0.1% formic acid in water. Buffer B was 95% acetonitrile, 0.1% formic acid in water. Injected peptides were loaded on an in-house packed C18 trap column (1.5 cm, 360 μm outer diameter, 150 μm inner diameter, Reprosil-Pur 120 Å, 5 μm, C18-AQ, Dr. Maisch) at a flow rate 10 μl/min and washed for 5 min with Buffer A. Peptide separation was done on an analytical C18 capillary column (15 cm, 360 μm outer diameter, 75 μm inner diameter, Reprosil-Pur 120 Å, 5 μm, C18-AQ, Dr. Maisch) at a flow rate of 300 nl/min with a gradient from 5–38% of Buffer B for 90 min. Eluting peptides were analyzed on a LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Electron) in positive ion mode. The instrument was operated in a data-dependent acquisition mode where the 30 most intense ions in the MS scan (m/z range from 350 to 1,600, resolution set to 60,000 at m/z 400) were selected for fragmentation by HCD mode. Automatic gain control target was set at 106 and 105 for MS1 and MS2, respectively. Sequenced precursors were put on an exclusion list for 30 s. The lock mass option (m/z 445.1200 was used for internal recalibration (Olsen et al, 2005).

The acquired RAW data were analyzed using MaxQuant software (Cox & Mann, 2008) version 1.5.2.8 based on Andromeda search engine (Cox et al, 2011). Each sample was given a unique name. Trypsin protease was selected, and LFQ option was highlighted. The human UniProt database (downloaded in December 2016; containing 20,129 reviewed entries) was used for identifying proteins. The protein and the peptide FDR was set to 0.01. The identified protein-Group.txt file was processed and analyzed using Perseus (Hubner et al, 2010; Tyanova et al, 2016) version 1.5.5.3. Briefly, the logarithm of LFQ intensities of CCB02/lysate was analyzed by Student’s t-test (n = 3). For volcano plot, the obtained P-values and logarithm of CCB02/lysate were plotted with S0 = 0.1, FDR = 0.05. The significant hits were categorized into various functional categories manually.

Centrosome fractionation
Discontinuous sucrose gradient (35–70%) was used to isolate centrosomes as described previously (Moritz et al, 1995; Gopalakrishnan et al, 2011, 2012). In brief, cells were treated with vehicle or CCB02 and lysed using BRB80 buffer containing 100 mM KCl. Lysed extracts were first centrifuged at 1,500 g for 20 min. The resulting supernatant was then layered on top of the discontinuous sucrose gradient of 35–70% prepared manually. After centrifugation at 243,000 g for 2 h at 4°C, fractions were collected and resolved using 8–10% SDS–PAGE. Proteins were transferred to nitrocellulose membrane and incubated with primary antibodies overnight at 4°C after blocking. Following this, membranes were incubated with species-specific secondary antibody at room temperature for 1 h and developed using chemiluminescence (Thermo Scientific).

Long-term time-lapse imaging
Cells expressing inducible CPAPWT and CPAPF375A as monolayer cells (MCF10A with Plk4 overexpression) or spheroids (MDA-MB-231) were grown in Labtek multi-well plate (#154534, Thermo Scientific) or Ibidi µ Slide chamber plates (#80426 and #80286, Ibidi) for 2D and 3D imaging. The microtubules (MTs) were stained with live SiR-Tubulin (Spirochrome AG). Images were captured using Leica DMi6000B widefield microscope with 10×/0.22 or 20×/0.40 Objective. The microscope is equipped with Leica DFC365 FX camera, a high-precision Pecon motorized stage, and Leica Adaptive focus control. During live cell imaging, cells were maintained at 37°C with humidified CO2 (3–5%) using an enclosed temperature and CO2 controller. All the captured images from each experiment were processed using Fiji/ImageJ.

NMR spectroscopy
All NMR spectra were recorded on an 800-MHz Bruker spectrometer equipped with a TCI cryoprobe, in buffer containing 1.5 mM phosphate, 1.5 mM calcium, and sodium, 5% DMSO at pH 7 at 298 K. For NOESY spectra, the H2O buffer was exchanged to D2O buffer in the concentrator at 4°C. The NOESY spectra were collected with mixing times of 40, 70, 100, and 150 ms, processed with NMRPipe (Delaglio et al, 1995) and analyzed using CCPNmr analysis (Vranken et al, 2005).

Modeling by docking
CCB02.3 structure was built in Maestro 2012 version 9.3.5 (Schrödinger, LLC) and imported into UCSF Chimera after minimization (Pettersen et al, 2004). The docking poses of compound CCB02.3 with tubulin were generated using AutoDock Vina tool in UCSF Chimera (Trott & Olson, 2010). Docking was performed using grid box as the peptide-binding interface on tubulin. Ten docking poses of CCB02.3 were generated, out of which eight accessed Phe385 binding pocket while two were docked into Phe375 pocket.

Isothermal titration calorimetry
Calorimetric experiments were conducted at 15°C with a MicroCal PEAQ-ITC instrument (Malvern). Tubulin samples were dialyzed against the 1×BRB80 buffer (80 mM PIPES-K, 1 mM MgCl2, 1 mM EGTA, pH 6.8, 0.5% DMSO) prior to titration. And the lyophilized compound CCB02 was solubilized using the dialysis buffer. For all ITC titration curves, 25 μM tubulin and 173 μM compound CCB02 were used. Protein concentration was determined by absorbance spectroscopy at 280 nm. Compound was quantified by weighing on a large scale. For fitting the ITC titration curve, acquired calorimetric titration data were analyzed using MicroCal PEAQ-ITC Analysis Software using the One Set of Binding Sites fitting model.

Microtubule regrowth assay
The microtubule (MT) regrowth assay was performed as previously described (Sankaran et al, 2005; Choi et al, 2010). For microtubule regrowth assay, MDA-MB-231 and MCF10A (+Dox, extra centrosomes) cells were pretreated with vehicle or CCB02 for 72 h and then treated with nocodazole for 16 h. After this, cells were placed in ice with cold medium for 90 min to completely depolymerize the microtubules. MT regrowth was induced by replacing the cold medium with prewarmed medium at 37°C. At the indicated time
points after the medium replacement, the cells were subsequently fixed with 4% PFA and stained for anti-α-tubulin and anti-γ-tubulin.

**Microtubule end-tracking assay**

Microtubule polymerization assay was conducted following the previously reported method (Bieling et al., 2010). For preparation, the reaction chambers, microscope slides, and biotin-coated coverslips were assembled using double-sided tape. After blocking reaction chambers with 1% pluronic F127 and 0.5 mg/ml κ-casein for 5 min, 50 μg/ml streptavidin was flowed in and incubate for 5 min. Microtubule seeds assembled from 50 μM tubulin mixed with 10% rhodamine-labeled tubulin and 10% biotin-labeled tubulin under 1 mM GMPCPP were then specifically attached to the functionalized surface by previously bound streptavidin. After washing the chamber with 1×BRB80 buffer, tubulin polymerizing was initiated by flowing in 1, 2 or 5 μM of CCB02 and Taxol, 20 nM GFP-tagged EB1, 15 μM tubulin, and 1.5 μM rhodamine-labeled tubulin, which were diluted in oxygen scavenger system (50 mM glucose, 400 μg/ml glucose-oxidase, 200 μg/ml catalase, and 4 mM DTT) supplied tubulin polymerization buffer (80 mM PIPES-K, 150 mM KCl, 1 mM EGTA, 5 mM GTP, 4 mM MgCl₂, pH 6.8). After immediately sealing the reaction chamber with candle wax, images were collected every 3 s using a TIRF (total internal reflection fluorescence) microscope (Nikon Eclipse Ti). ImageJ software (http://rsbweb.nih.gov/ij/) was used for kymograph presentation and image analysis.

Live EB3 imaging was performed using EB3-EGFP constructs (kindly provided by Dr. Anna Akhmanova). Briefly, cells were grown in Ibidi μ-Slide chamber plates (#80426 and #80286, Ibidi) and transiently transfected with EB3-EGFP plasmids. After 24 h of transfection, cells were treated with DMSO vehicle or CCB02 (1, 2 and 5 μM) and EB3 dynamics was observed using laser scanning confocal microscope (Leica SP8, Leica Germany). The microtubules (MTs) were stained with live SiR-Tubulin (Spirochrome AG). The images were recorded every 2 s. During live cell imaging, cells were maintained at 37°C with humidified CO₂ (3–5%) using an enclosed temperature and CO₂ controller. ImageJ software and MTrackJ plugin (http://rsbweb.nih.gov/ij/) was used for tracking and measuring EB3 and MT dynamics.

**Animals**

For mouse xenograft experiments, NMRI-nu (RjOrl-NMRI-Foxn1nu/Foxn1nu) female mice of 4–6 weeks old were used for experiments. All experiments and protocols were performed in accordance with the guidelines of the responsible national authority and approved by the local Governmental Committee for Animal Experimentation (license: 84-02.04.2015.A541).

**Breast cancer xenograft model**

Proof-of-principle xenograft model: For each xenograft (n = 4), 5 × 10⁶ MDA-MB-231 tumor cells expressing CPAP-WT and CPAPAT (a mutant version of CPAP that does not bind to tubulin) suspended in PBS were injected subcutaneously into the flank of male nude mice. Tumor size was monitored every second day by measuring perpendicular diameters. Tumor volumes were calculated by determination of the largest diameter and its perpendicular according to the following equation: tumor volume = a × (b²/2), where a represents the largest diameter and b represents the perpendicular diameter. The experimenter was not blinded.

**Lung cancer xenograft model**

Lung cancer (H197557[^8]) xenograft model: Immunodeficient NMRI-nu (RjOrl-NMRI-Foxn1nu/Foxn1nu) female mice from Harlan, the Netherlands, were delivered at the age of 4–6 weeks and were used for experiments after at least 1 week of quarantine. H1975 cells were cultivated at 37°C, 5% CO₂, and 80–90% humidity with DMEM (#61965-026, Gibco) containing 10% FBS (#P30-19375, PAN Biotech), MEM (minimum non-essential amino acids, #11140-035, Gibco), 100 units/ml penicillin (#15140-122, Gibco), and 100 units/ml streptomycin (#15140-122, Gibco). H1975 cells (5 × 10⁶ in 200 μl) were injected into 14 NMRI-nu female mice. Animals and tumor implants were monitored daily until solid tumor growth was detectable in a sufficient number of animals. At randomization, the volume of growing tumors was determined. Animals fulfilling the randomization criteria (i.e., bearing tumors of 80–100 mm³) were then distributed into experimental groups (n = 8 for vehicle control and n = 8 for CCB02 treatment), aiming at comparable median and mean group tumor volumes of approximately 100–120 mm³. CCB02 was administered (30 mg/kg of weight, daily) by oral gavage to mice. Animals were routinely monitored at least twice daily. Animals were weighed thrice a week or daily if body weight losses in excess of 15% were recorded. Tumor volumes were determined by two-dimensional measurement with a caliper on the day of randomization and then twice weekly according to the formula: (a × b²) × 0.5 where a represents the largest and b the perpendicular tumor diameter of the tumor representing an idealized ellipsoid. Institutional guidelines were strictly followed while performing the animal experiments. All experiments are approved by the local authorities and are conducted according to the guidelines of the German Animal Welfare Act (Tierschutzgesetz). The experimenter was not blinded.

**Statistical analysis**

The statistical analyses were performed using GraphPad Prism version 5-7. Statistical information including test results (P-value), definition of center values as mean, and definition of error bars as standard error mean is indicated in the text or the figure legends. Different compound treatments per cell line were done in triplicate (N = 3) as independent experiments. IC₅₀ values were calculated (N = 3–4 replicates per cell line) using dose–response curve. Western blot band intensities were calculated using Fiji. The intensity values from area under the curve (AUC) were used to calculate Western blot IC₅₀ values. Dose–response inhibition model was used to calculate IC₅₀ value. Unpaired Student’s t-test or ordinary one-way or two-way ANOVA was used to determine statistical significance of graphs (P < 0.01 vs. control was considered as significance). Error bars represent standard error mean.

**Chemical synthesis of CCB02**

Unless otherwise noted, all reagents were obtained from commercial sources and used without further purification. Technical grade
solvents used for aqueous workup were distilled prior to use. Dry tetrahydrofuran and methanol were purchased from Acros. Reverse-phase flash chromatography (TLC) was performed on a Grace Reveleris® Prep Purification System using C18 40 μM cartridges. Analytical thin-layer chromatography (TLC) was performed on silica (silica gel 60 F 254)-coated plates. Compounds were detected by ultraviolet (UV) irradiation at 254 or 366 nm. HPLC-UV/MS analysis was performed on a Waters X-Bridge C18 (4.6 × 30 mm, 3.5 μm) column using a Dionex UltiMate 3000 HPLC system coupled with a Thermo Finnigan LCQ ultraflext mass spectrometer (gradient: 0–95% of acetonitrile + 0.1% formic acid v/v in water + 0.1% formic acid v/v over 5-min period, then hold 95% of acetonitrile + 0.1% formic acid v/v in water + 0.1% formic acid v/v for 1 min; flow rate: 1.1 ml/min; UV detection at 214 and 280 nm). Infrared (IR) spectra were recorded on a JASCO IR-4100 (ATR). High-resolution mass spectrometry (HRMS) measurements were performed on a Thermo Finnigan LTQ FT apparatus using an electrospray ionization (ESI) detector. NMR spectra were recorded at 303 K on a Bruker Avance III HD 400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) relative to residual d5-DMSO (δH = 2.50 ppm) and d6-DMSO (δC = 39.52 ppm). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), td (triplet of doublets), ddd (doublet of doublets of doublets), m (multiplet), or bs (broad signal). The coupling constants (J) are reported in Hertz (Hz). 3-Chlorobenzo[b][1,6]naphthyridine-4-carbonitrile was synthesized as described in Ivanov et al (2002), Ivanov et al (2004) and Yalysheva et al (1986).

CCB02 (3-Methoxybenzo[b][1,6]naphthyridine-4-carbonitrile) was prepared from Chlorobenzo[b][1,6]naphthyridine-4-carbonitrile (Appendix Fig S5) according to the following procedure: A suspension of 3-Chlorobenzo[b][1,6]naphthyridine-4-carbonitrile (400 mg, 1.67 mmol) in a mixture of absolute methanol (30 ml) and dry tetrahydrofuran (20 ml) was stirred at reflux, and 0.5 M solution of sodium methoxide in methanol (4.00 ml, 2.00 mmol, 1.2 eq) was added dropwise over 1 h. The resulting brown solution was kept at reflux for further 30 min, cooled, quenched with saturated aqueous solution of ammonium chloride (2 ml), and concentrated in vacuo. The residue was partitioned between water (10 ml) and methylene chloride (50 ml). The layers were separated, and the aqueous phase was extracted with methylene chloride (2 × 15 ml). The combined organic extract was washed with saturated aqueous ammonium chloride, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The title compound (210 mg, 0.89 mmol, 54%) as a yellow solid was obtained as an orange solid (121 mg, 0.25 mmol, 82%).

Thin-layer chromatography (pentane: ethyl acetate, 2:1 v/v): Rf = 0.33; 1H NMR (400 MHz, d6-DMSO) δ 9.71 (s, 1H, H-1), 9.48 (s, 1H, H-10), 8.26 (d, J = 8.4 Hz, 1H, H-9), 8.15 (d, J = 8.8 Hz, 1H, H-6), 8.04 (ddd, J = 8.6, 6.6, 1.5 Hz, 1H, H-7), 7.70 (td, J = 6.8, 3.3 Hz, 1H, H-8), 4.23 (s, 3H, OCH3); 13C NMR (101 MHz, d6-DMSO) δ 166.6 (C-3), 160.4 (C-1), 152.6 (C-9a), 150.0 (C-10a), 141.5 (C-10), 135.2 (C-7), 130.5 (C-9), 128.8 (C-6), 126.8 (C-8), 126.3 (C-4a), 118.3 (C-5a), 115.2 (CN), 86.5 (C-4), 56.1 (OCH3); IR (ATR) vmax (cm–1) 3044, 3019, 2958, 2894, 2847, 2224, 1605, 1557, 1512, 1466, 1411, 1331, 1285, 1181, 1140, 1107, 1041, 969, 800, 741, 613; ESI-H R M S (m/z): [M + H]+ calcd. For C14H10N3O,

236.08184; found, 236.08194; LCMS (m/z): [M + H]+ 236, retention time 3.23 min.

Chemical synthesis of CCB02-Biotin and CCB02-2.5-Biotin

Under an argon atmosphere, 125 mg biotin (0.5 mmol, 1 eq.) was dissolved in 5 ml dry MeCN and the resulting reaction mixture was stirred to 0°C before 139 mg HOBr (1.0 mmol, 3 eq.) and 197 mg EDC-HCl (1 mmol, 3 eq.) was added. After 15 min, the free amine (90 mg, 0.34 mmol, 1 eq.) in 1 ml dry MeCN was added slowly. The resulting orange-red reaction mixture was stirred for 20 h at room temperature, before 25 μl H2O was added. The reaction was directly purified by chromatography (CHCl3/Methanol = 1:0 → 1:1) to obtain the desired compound as an orange solid (121 mg, 0.25 mmol, 82%).

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Author contributions

JG and AM conceived the concept and project. AM performed most of the syntheses, and uses of CCB02 and its biophysical experiments. MD, TK, AM, and H-GS did chemical synthesis of CCB02. SM and HU did mass spectrometry. IM, MO, KG, RU, CY, and RA and RB performed mouse xenograft experiments. JS, AR, AW, and AAH involved in the cell and biochemical experiments. JG and AM wrote the manuscript. JG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest. The University of Cologne has filed a European patent application related to the structures, syntheses, and uses of CCB02 and chemically related CCB02 inhibitors. Requests for CCB02 should be directed to JG (jay.gopalakrishnan@hhu.de).

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