Reversible binding of the anticancer drug KXO1 (tirbanibulin) to the colchicine-binding site of β-tubulin explains KXO1’s low clinical toxicity

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Running title: KXO1 reversibly binds to colchicine site of β-tubulin

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ABSTRACT

KXO1 (tirbanibulin or KX2-391) is a non-ATP–competitive inhibitor of SRC proto-oncogene non-receptor tyrosine kinase (SRC) and is being clinically investigated for the management of various cancers and actinic keratosis. Recently, KXO1 has also been shown to strongly inhibit tubulin. Interestingly, unlike conventional tubulin-targeting drugs, KXO1 has exhibited low toxicity in preclinical and clinical studies, but the reason for this remains elusive, as are the KXO1-binding site and other details of the interaction of KXO1 with tubulin. Here, cell-based experiments revealed that KXO1 induces tubulin depolymerization and G2/M phase cell cycle arrest at low nanomolar concentrations, similar to colchicine, used as a positive control. Results from biochemical experiments, including an N,N-ethylene-bis(iodoacetamide) competition assay, disclosed that KXO1 binds to the colchicine-binding site on β-tubulin, further confirmed by the crystal structure of the tubulin–KXO1 complex at 2.5 Å resolution. A high-quality electron density map of the crystallographic data enabled us to unambiguously determine the position and orientation of KXO1 in the colchicine-binding site, revealing the detailed interactions between KXO1 and tubulin. We also found that KXO1 binds reversibly to purified tubulin, induces a totally reversible cellular effect (G2/M cell cycle arrest), and possesses no cellular toxicity 5 days after drug washout, explaining KXO1’s low toxicity. In summary, we show that KXO1 binds to the colchicine-binding site of tubulin and resolved the crystal structure of the tubulin–KXO1 complex. Importantly, KXO1’s reversible binding to tubulin explains its clinically low toxicity, an insight that could guide further clinical applications of KXO1.

KXO1 (tirbanibulin or KX2-391), an agent with good oral bioavailability, has been shown to inhibit primary tumor growth and metastasis in many preclinical animal models of cancer (1-5). Since 2007, studies have demonstrated the effectiveness of KXO1 in various solid and liquid tumor types, as well as its low toxicity (5-8). In a phase I clinical trial in solid tumors, the maximum tolerated dose (MTD) in patients with advanced cancer was determined to be 40 mg for a twice daily (BID) dose with a continuous dosing schedule; even the highest dose administered (80 mg/dose BID) resulted in no evidence of drug accumulation after several days of administration (8). Another phase I study of KXO1 in elderly patients with advanced acute myeloid leukemia found an MTD of 120 mg/dose repeated once daily, and even a dose of 160 mg/daily for 12 days caused no obvious side effects (7). Most recently, in two phase III pivotal efficacy studies of actinic keratosis (AK) (ClinicalTrials.gov identifier: NCT03285490 and NCT03285477), KXO1 achieved its primary endpoint of 100% clearance of AK lesions in the face or scalp areas within 2 months of treatment. Notably, KXO1 showed dramatically less toxicity compared with the existing standard therapy. Mechanistic studies initially identified KXO1 as a non-ATP-competitive Src inhibitor (9). However, it also showed an effect on non-Src driving cells, suggesting that it may have other molecular targets (5). Photoaffinity labeling of KXO1 identified tubulin as another target of KXO1, and further study showed that KXO1 could inhibit tubulin polymerization in a low nanomolar range,
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suggesting that it is a strong tubulin inhibitor (5).

Tubulin is an abundant protein in all cell types and is involved in various physiological activities, including cell migration, protein transport, and mitosis. It is the target of many chemotherapeutic drugs in clinical cancer treatment, including paclitaxel, vinblastine, and eribulin (10). There are six established binding sites on αβ-tubulin heterodimers, including the well-known paclitaxel (11), vinblastine (12), and colchicine (13) sites, as well as the recently identified laulimalide (14), maytansine (15), and pironetin (16,17) sites. As tubulin inhibitors are non-selective cytotoxic compounds, the tubulin-binding agents in clinical use show good efficacy but exhibit non-negligible side effects, including chemotherapy-induced peripheral neuropathy, stomatitis, bone marrow suppression, severe weakness, and neurotoxicity (18,19). This inevitable toxicity limits the clinical applications of tubulin inhibitors.

KXO1 has exhibited a strong inhibitory activity toward tubulin together with extremely low toxicity in preclinical and clinical trials, in contrast to the characteristics of conventional toxic tubulin inhibitors. This deserves in-depth research, as insight into the underlying mechanism could be of great help in developing highly effective tubulin drugs with low toxicity. In addition, the binding site of KXO1 on tubulin and other details of the interaction are not well understood, hindering further investigation.

In this study, through observing the changes in microtubule morphology induced by KXO-1 and using an N,N-ethylene-bis(iodoacetamide) (EBI) competitive binding assay, we found that KXO1 directly binds to the colchicine site of β-tubulin. X-ray data of the KXO1-tubulin complex confirmed the binding of KXO1 at the colchicine site and clearly showed the details of the interaction. Reversibility experiments demonstrated that KXO1 binds to tubulin in a totally reversible manner and induces reversible cellular effects, providing a good explanation for the low toxicity of KXO1. Overall, our results suggest that reversible tubulin inhibitors that bind to the colchicine site of β-tubulin could be developed as low-toxicity drugs.

**Results**

**KXO1 inhibits tubulin polymerization and induces G2/M cell cycle arrest in a low nanomolar range**

We first examined the effects of KXO1 on the morphology of microtubules in HeLa cells. A tubulin polymerization agent (paclitaxel) and tubulin depolymerization agents (colchicine and vinblastine) were used as positive controls. As shown in Fig. 1A, microtubules in non-treated HeLa cells had a normal network structure (a tubular structure formed by assembly of αβ-tubulin heterodimers). Colchicine and vinblastine inhibit polymerization of tubulin, and paclitaxel promotes its excessive polymerization into abnormal microtubules. KXO1 at 30 nM inhibited tubulin polymerization in a colchicine- or vinblastine-like manner, confirming that KXO1 is a strong tubulin depolymerization agent. All tubulin inhibitors inhibit mitosis and block the cell cycle in the G2/M phase (20). We found that KXO1 induced G2/M phase cell cycle arrest in a concentration-dependent manner. As shown in Fig. 1B, KXO1 caused G2/M phase cell cycle arrest with 50% of maximal effect concentrations (EC50s) of 53.2±3.73 nM,
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A previous study using photoaffinity labeling of KXO1 showed that KXO1 directly bound to tubulin and inhibited tubulin polymerization in human peripheral blood mononuclear cells (5). However, the specific binding site and details of the interaction were not well clarified. Tryptophan fluorescence decrease (TFD) is commonly used to detect drugs binding to tubulin (21,22). Using a TFD assay, we showed that KXO1 bound to purified tubulin with a dissociation constant ($K_d$) of 1.26±0.27 μM, whereas the positive compound colchicine showed a $K_d$ value of 13.3±3.30 μM (Fig. 2A), suggesting that KXO1 probably binds more strongly to tubulin than does colchicine. In an in vitro tubulin polymerization assay, KXO1 inhibited tubulin polymerization in a concentration-dependent manner and performed a little better than colchicine (Fig. 2B). Next, we aimed to determine the binding site of KXO1 on tubulin. Tubulin polymerization can be inhibited by binding of inhibitors to either the colchicine or the vinblastine site; however, cells treated with high concentrations of vinblastine uniquely show formation of tubulin paracrystals (packing of unpolymerized tubulin in the cytoplasm) (12). High concentrations (2 μM) of inhibitors were incubated with HeLa cells for 2 h and tubulin was observed using immunofluorescence. As shown in Fig. 2C, vinblastine induced the formation of paracrystals in the cytoplasm, whereas colchicine and KXO1 did not, suggesting the possible binding of KXO1 to the colchicine site. For further confirmation, we carried out an EBI assay. One molecule of EBI bound covalently to cysteine 239 and cysteine 354 in the colchicine site of β-tubulin simultaneously to form an EBI-β-tubulin complex, which migrated faster than β-tubulin in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23). As shown in Fig. 2D, colchicine and KXO1 inhibited the formation of the EBI-β-tubulin complex, but vinblastine did not, thus confirming KXO1 binding to the colchicine site on tubulin.

X-ray crystal structure of the tubulin–KXO1 complex

Next, we determined the crystal structure of tubulin-bound KXO1 at 2.5 Å resolution by soaking KXO1 (Fig. 3A) in crystals of a protein complex composed of αβ-tubulin, stathmin-like protein RB3, and tubulin tyrosine ligase (T2R-TTL) (24). Details of the data collection and refinement statistics are summarized in Table 1. The resulting high-quality electron density map enabled us to determine unambiguously the position and orientation of the inhibitor KXO1 (Fig. 3B), thus revealing in detail the interaction between KXO1 and tubulin (Fig. 3C and 3D). Consistent with the tubulin polymerization and EBI data (Fig. 2C and 2D), KXO1 bound to the colchicine site (Fig. 3C and 3D). As could be seen in the crystal structure, upon binding to the colchicine site, KXO1 formed extensive hydrophobic interactions with various residues of β-tubulin, including βI4, βF169, βL242, βL248, βL252, βM259, βI318, βI376, and βK352. The amide and pyridine moieties of KXO1 formed hydrogen bonds with the side-chain of βE200 via a water molecule (Fig. 3D).

KXO1 is much longer than colchicine.
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Superimposition of the tubulin–KXO1 structure onto that of tubulin–colchicine showed that KXO1 occupied a much broader position upon binding to the colchicine site than did colchicine (Fig. 4). We have proposed a structure-based colchicine binding site inhibitor (CBSI) pharmacophore model consisting of three hydrophobic centers (I, II, and III) and two hydrogen bond centers (IV and V) (25). KXO1 occupied hydrophobic centers I and II and hydrophilic center IV of this model. Compared with all other reported CBSIs–tubulin structures, the morpholine group of KXO1 extended out of the colchicine pocket and approached the GTP molecule of α-tubulin.

KXO1 induced reversible G2/M cell cycle arrest and showed no toxicity to HeLa cells after wash out

KXO1 has exhibited good pharmacological activity together with low toxicity in preclinical and clinical studies. The reversibility of its binding to tubulin is an effective predictor of the toxicity of a microtubule inhibitor (19,26). Generally, irreversible tubulin inhibitors accumulate in cells over a long period and affect the functions of normal cells, resulting in toxicity (26). We used a method developed by Murray et al. (27) to determine the reversibility of KXO1. As shown in Fig. 5A, HeLa cells were treated with a wide range of concentrations of KXO1 (1–10,000 nM) at −12 h, and compounds were totally washed out at 0 h. Cell cycling was detected at 0 h and 10 h, and cell viability was detected at 5 days. Figure 5B shows cell cycle profiles of KXO1 and positive compound colchicine (an irreversible tubulin inhibitor (27,28)). A proportion of cells in the G2/M phase greater than 50% was considered to indicate significant mitotic block (SMB). At 0 h, both KXO1 and colchicine presented SMB at the concentration of 30 nM. However, 10 h after wash out, significant differences were observed regarding mitotic block; colchicine at 30 nM continued to induce SMB, whereas a very high concentration (10,000 nM) of KXO1 was required to sustain SMB at this time point. Detection of p-H3 (a marker of G2/M phase cell cycle arrest) by immunoblotting at 0 h and 12 h gave similar results (Fig. S1). All these results indicate that colchicine is an irreversible tubulin inhibitor but KXO1 is a totally reversible tubulin inhibitor. Figure 5C shows 5-day cell viability data for colchicine and KXO1. After 5 days of culture, both colchicine and KXO1 reduced cell viability at the same concentrations that were capable of inducing SMB at 10 h; colchicine inhibited cell growth at concentrations higher than 30 nM, whereas KXO1 only decreased cell viability at 10,000 nM. Overall, KXO1 exhibited no toxicity to cells after wash out at concentrations below 3000 nM, consistent with its observed low toxicity in clinical studies.

KXO1 binds reversibly to purified tubulin

We further investigated the mechanism underlying the difference in reversibility between colchicine and KXO1 inhibition by detecting the binding of compounds to purified tubulin, following our previously published method (29). Purified tubulin (25 μM) was incubated with 20 μM colchicine or 20 μM KXO1 for 1 h and then washed three times by ultrafiltration. Compounds in the filtrate and retentate were detected using high-performance liquid chromatography (HPLC). As shown in Fig. 6A, 20 μM colchicine and 20 μM KXO1 were detectable by HPLC in controls. After ultrafiltration, we
were able to detect almost the same amount of KXO1 in the filtrate as in the control group, whereas there was almost no detectable colchicine (Fig. 6B). By contrast, colchicine but not KXO1 was detected in the retentate (Fig. 6C). This suggests that KXO1 can be easily washed from tubulin, as almost all KXO1 was detached from tubulin after washing three times, implying that KXO1 binds to purified tubulin in a totally reversible manner.

Discussion

Tubulin inhibitors are a major class of chemotherapy drugs for the treatment of cancer; examples include docetaxel, eribulin, and cabazitaxel (30). Microtubule drugs are cytotoxic and do not show selectivity between normal cells and tumor cells, leading to non-negligible systemic side effects. Thus, there are severe restrictions on the use of tubulin inhibitors as chemotherapy drugs (20,31). KXO1 is a clinically effective and low-toxicity microtubule inhibitor (5). It has strong tubulin inhibitory activity and can significantly inhibit the polymerization of tubulin at low nanomolar levels. In contrast to conventional microtubule inhibitors, however, preclinical and clinical trials have confirmed that the toxicity of KXO1 is very low (5). In order to determine the mechanism of KXO1’s low toxicity, we systematically studied the interaction of KXO1 with tubulin.

We first confirmed that KXO1 is indeed a highly effective tubulin inhibitor. KXO1 began to induce G2/M phase arrest at 10 nM and caused SMB and inhibition of tubulin polymerization at 30 nM in HeLa cells, demonstrating that KXO1 inhibited tubulin activity to a similar extent to the positive compound colchicine. TFD assays showed that KXO1 bound to tubulin with a stronger affinity (as indicated by a lower $K_d$ value) than colchicine, and in vitro tubulin polymerization experiments confirmed that KXO1 was slightly more potent than colchicine in inducing tubulin depolymerization. Of note, the TFD assay used to determine the $K_d$ values could only approximate the relative binding affinity of compounds to tubulin and the results do not necessarily reflect the real binding affinity. We can only conclude that KXO1 showed greater affinity for tubulin than did colchicine.

The increase of turbidity in in vitro tubulin polymerization requires high concentrations of tubulin to form high-polymerized microtubules. The concentration of tubulin (30 μM) in the assay was high, but a lower concentration (5 μM) of inhibitors could break down high-polymerized microtubules to a low-polymerized form, thus decreasing the turbidity. By monitoring changes in microtubule morphology and performing EBI competitive binding experiments, we determined that KXO1 binds to the colchicine site on tubulin; this was further confirmed by the X-ray structure of a tubulin–KXO1 complex. The X-ray data also allowed us to accurately determine specific details of the interaction between KXO1 and the tubulin colchicine site, providing guidance for the subsequent optimization of KXO1 design.

The reversibility of the binding of a tubulin inhibitor to tubulin can generally predict its toxicity. In general, irreversible compounds have higher toxicity and reversible compounds are less toxic (26). Reversibility experiments showed that the irreversible inhibitor colchicine could maintain SMB at 30 nM after the drug had been washed out, whereas a much higher concentration of KXO1 (10,000 nM) was
required to maintain SMB. That is, at concentrations below 10,000 nM, cell viability was not affected after drug wash out in KXO1-treated cells, as confirmed by 5-day cell viability tests. We also found that colchicine bound irreversibly to purified tubulin, whereas KXO1 bound in a totally reversible manner (KXO1 could be easily eluted from purified tubulin). This reversible binding provides a plausible explanation for KXO1’s low toxicity.

Although we aimed to explain from a structural biology perspective why different compounds show different reversibility of binding to tubulin, we did not find significant differences when comparing the crystal structure of tubulin–KXO1 with that of tubulin–colchicine. It is worth noting that minor differences in the structures of compounds may lead to completely different reversibility of binding, as in the cases of colchicine (irreversible) and colcemid (reversible), eribulin (irreversible) and ER-076349 (partially reversible), and vinblastine (reversible) and vincristine (irreversible) (27,29). The differences in reversibility of these compounds are difficult to explain from a structural biology perspective. It is possible that the kinetics of binding to tubulin affect reversibility; however, this requires further validation.

In summary, in this study we showed that KXO1, a clinical phase III drug, binds to the colchicine site of tubulin, and resolved the crystal structure of a tubulin–KXO1 complex. Importantly, we found that the binding of KXO1 to tubulin was fully reversible, which could explain the clinically low toxicity of KXO1. Our results have the potential to guide further clinical applications of KXO1.

**Experimental procedures**

**Reagents**

Paclitaxel, colchicine, and vinblastine were obtained from Selleck. Propidium iodide (PI), EBI, β,γ-methylenedenosine 50-triphosphate disodium salt, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma. KXO1 was from MedChemExpress. All antibodies used in this study were obtained from Abcam: α-tubulin antibody (ab7291); β-tubulin antibody (ab179513); p-H3 antibody (ab47297); GAPDH antibody (ab181602). Purified tubulin was purchased from Cytoskeleton Inc. Other conventional agents were from Kelun Pharmaceutical or Sangon Biotech.

**Cell lines and cell culture**

Human cervical adenocarcinoma cell line HeLa, human hepatoma cell line HepG2, and human large-cell lung cancer H460 were sourced from American Type Culture Collection. Cells were cultured with Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum; 100 units/mL penicillin and 100 μg/mL streptomycin were also added to the medium to prevent bacterial contamination of cells. The culture environment was set to 5% CO2 at 37 °C. HeLa cells were authenticated by short tandem repeat testing by Coboier Biosciences Co., Ltd.

**Immunofluorescence**

HeLa cells cultured in a 24-well plate were incubated with compounds for the appropriate times, then washed with phosphate-buffered saline (PBS) for 2 min. The cells were then incubated with 50% methanol/50% acetone for 2 min for fixation and washed with PBS for another 2 min. Fixed cells were incubated with anti-α-tubulin antibody (1:200) at 4 °C for 12 h, then washed...
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with 1×PBST (PBS containing 0.1% Tween 20) for 20 min (5 min × 4) before incubation with a second antibody for 45 min at room temperature. After washing for another 20 min (5 min × 4), cells were stained with DAPI for 10 min before being washed and transferred to slides. Finally, cells were imaged using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell cycle analysis
Cells cultured in six-well plates were collected and washed with PBS before being fixed with 70% pre-cooled ethanol overnight at 4 °C. Cells were then washed three times with PBS again and stained with 50 μg/mL PI for 20 min. Cell cycling in these samples was analyzed using a flow cytometer (BD FACSCalibur).

Tryptophan-based binding assay
Purified tubulin (0.5 μM) in PEM buffer (80 mM PIPES, pH 6.9; 2 mM MgCl2; 0.5 mM EGTA; and 1 mM GTP) was incubated with different concentrations (0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μM) of KXO1 or colchicine for 30 min at 4 °C. Tryptophan fluorescence was detected with a Biotech Gen5 spectrophotometer at 295 nm (excitation) and 335 nm (emission). The GraphPad Prism software was used to determine dissociation constants by calculating fitting curves for the decrease in fluorescence.

In vitro tubulin polymerization assay
Purified tubulin (30 μM) in PEM buffer was incubated with different compounds at 4 °C for 1 min before transferred to a pre-warmed (37 °C) 384-well plate for immediate analysis with the Biotech Gen5 spectrophotometer. Optical density values at 340 nm were recorded at 1-min intervals for 18 min. The polymerization curve was obtained from the absorption values at consecutive time points.

EBI competition assay
The assay was carried out following the method published by Sébastien et al. (23). One molecule of EBI bound covalently to cysteine 239 and cysteine 354 in the colchicine site of β-tubulin simultaneously to form an EBI-β-tubulin complex, which migrated faster than β-tubulin in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Tubulin inhibitors which could inhibit the formation of EBI-β-tubulin complex were considered as CBSIs.

HeLa cells were cultured in six-well plates, then treated with or without different concentrations of KXO1, vinblastine, or colchicine for 2 h, before being treated with 100 μM EBI for another 2 h. Total protein was extracted with radio immunoprecipitation assay buffer and subjected to western blotting analysis of β-tubulin. GAPDH was used as a loading control. The western blotting procedure was as described previously (29).

Structural biology
Protein expression and purification
The T2R–TTL complex, which includes two tubulins, one stathmin-like domain of RB3 (RB3–SLD), and one tubulin tyrosine ligase (TTL), was prepared as described previously (25). Porcine brain tubulin was supplied at 10 mg/mL in PEM buffer and stored at 80 °C until use. RB3–SLD was overexpressed in Escherichia coli strain BL21 (DE3) and purified sequentially by anion-exchange chromatography (OFF, GE Healthcare) and size-exclusion chromatography (Superdex 75, GE-Healthcare). The purified RB3–SLD protein was concentrated to 10 mg/mL and stored at 80 °C until use. TTL was overexpressed in E. coli strain BL21 (DE3)
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and purified by nickel-affinity chromatography followed by size-exclusion chromatography (Superdex 200, GE-Healthcare). Purified TTL protein in Bis-Tris propane (pH 6.5), 200 mM NaCl, 2.5 mM MgCl₂, 5 mM β-mercaptoethanol, and 1% glycerol was concentrated to 20 mg/mL and stored at 80 °C until use. The T2R–TTL complex was formed by mixing tubulin, RB3–SLD, and TTL in a 2:1.3:1.2 molar ratio; 5 mM tyrosine, 10 mM DTT, and 1 mM β,γ-methyleneadenosine 50-triphosphate disodium salt were then added, and the complex was concentrated to 15 mg/mL at 4 °C.

Crystallization and crystal soaking

The T2R–TTL crystals were obtained by the sitting-drop vapor-diffusion method at 20 °C in a buffer consisting of 6% PEG4000, 8% glycerol, 0.1 M MES (pH 6.7), 30 mM CaCl₂, and 30 mM MgCl₂. The seeding method was used to obtain single crystals. Rod-like crystals appeared within 2 days and grew to maximum dimensions within 1 week. For crystal soaking, 0.1 μL of KXO1 (dissolved in dimethyl sulfoxide at a 10 mM concentration) was added to a 2 μL crystal-containing drop for 16 h at 20 °C.

Data collection and structure determination

The crystals were transferred into cryoprotectant (reservoir solution supplemented with 20% (v/v) glycerol) for a few seconds and then mounted in nylon loops and flash-cooled in liquid nitrogen. Diffraction data were collected on beamline BL19U1 at the Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, China). Data were processed using HKL3000 (32). The structures were determined by the molecular replacement method using the tubulin–tivantinib structure (Protein Data Bank (PDB) ID: 5CB4) as a search model. The refinement was performed using COOT (33) and PHENIX (34). The model quality was evaluated with MOLPROBITY (35). All figures were generated using PyMOL (http://www.pymol.org).

Mitotic block reversibility assay

This assay was carried out following the protocol of Murray et al. (27). As shown in Fig. 5A, HeLa cells were treated with increasing concentrations (0, 10, 30, 100, 300, 1,000, 3,000, 10,000 nM) of colchicine or KXO1 at the −12 h time point and cultured for 12 h. At 0 h, compounds were totally washed out and cells were incubated with fresh medium and further cultured for 5 days. At 0 h and 10 h, cell cycle analysis and p-H3 expression levels were detected by flow cytometry and western blotting, respectively. At the 5-day time point, cell viability was examined with a trypan blue staining assay, following a previously established method (36).

HPLC detection of compounds binding to tubulin

Purified tubulin (25 μM) in PEM buffer (100 μL) was incubated with 20 μM colchicine or 20 μM KXO1 for 60 min at room temperature, after which samples were transferred to ultrafiltration tubes (Millipore, 10 kDa) for centrifugation at 13,000 rpm for 15 min. Then 400 μL PEM buffer was added to the ultrafiltration tube to wash the tubulin with centrifugation at 13,000 rpm for 15 min again. This washing procedure was repeated three times. The filtrates were combined and dried under vacuum before being re-dissolved in 100 μM methanol to obtain the filtrate fraction. The retentate (tubulin) was heated to 80 °C to denature the tubulin protein and release the bound compounds. The denatured
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retentate was also dried under vacuum before being re-dissolved in 100 μM methanol to yield the retentate fraction. The filtrate fraction and retentate fraction were subjected to HPLC for detection of compounds. HPLC was also performed on 20 μM colchicine and 20 μM KXO1 in 100 μL methanol as controls. HPLC was performed using a Waters Alliance 2695 separation module (Empower Software) with a Waters 2996 photodiode array detector.

Statistical analysis
Data are shown as mean ± S.D. Statistical differences were determined using an unpaired, non-parametric student’s t-test; P-values are indicated in each graph or figure legend: *p<0.05; **p<0.01; ***p<0.001.
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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References

1. Anbalagan, M., Sheng, M., Fleischer, B., Zhang, Y., Gao, Y., Hoang, V. T., Matossian, M. D., Burks, H. E., Burow, M. E., and Collins-Burow, B. M. (2017) Dual Src Kinase/Pretubulin Inhibitor, KX-01, Sensitizes ERα-negative Breast Cancers to Tamoxifen Through ERα Re-expression. *Molecular Cancer Research*, molcanres 0297.2016

2. Kim, S., Min, A., Lee, K. H., Yang, Y., Kim, T. Y., Lim, J. M., Park, S. J., Nam, H. J., Kim, J. E., and Song, S. H. (2017) Antitumor Effect of KX-01 through Inhibiting Src Family Kinases and Mitosis. *Cancer Research & Treatment Official Journal of Korean Cancer Association* 49, 643-655

3. Anbalagan, M., Carrier, L., Glodowski, S., Hangauer, D., Shan, B., and Rowan, B. G. (2012) KX-01, a novel Src kinase inhibitor directed toward the peptide substrate site, synergizes with tamoxifen in estrogen receptor α positive breast cancer. *Breast Cancer Res Treat* 132, 391-409

4. Liu, T., Hu, W., Dalton, H. J., Choi, H. J., Huang, J., Kang, Y., Pradeep, S., Miyake, T., Song, J. H., and Wen, Y. (2013) Targeting Src and Tubulin in Mucinous Ovarian Carcinoma. *Clinical Cancer Research* 19, 6532-6543

5. Smolinski, M. P., Bu, Y., Clements, J., Gelman, I. H., Hegab, T., Cutler, D. L., Fang, J. W. S., Fetterly, G., Kwan, R., and Barnett, A. (2018) Discovery of Novel Dual Mechanism of Action Src Signaling and Tubulin Polymerization Inhibitors (KX2-391 and KX2-361). *Journal of Medicinal Chemistry*, acs.jmedchem.8b00164

6. Antonarakis, E. S., and Heat, E. I., Posadas E. M., Yu E. Y., Harrison M. R., Bruce J. Y., Cho S. Y., Wilding G. E., Fetterly G. J., Hangauer D. G., Kwan M. F., Oyster L. M., and Carducci M. A. (2013) A phase 2 study of KX2-391, an oral inhibitor of Src kinase and tubulin polymerization, in men with bone-metastatic castration-resistant prostate cancer. *Cancer Chemotherapy & Pharmacology* 71, 883-892

7. Van Herpen, C. M. L., Eskens, F. A. L. M., de Jonge, M., Desar, I., Hoofman, L., Bone, E. A., Timmer-Bonte, J. N. H., and Verweij, J. (2010) A Phase Ib dose-escalation study to evaluate safety and tolerability of the addition of the aminopeptidase inhibitor tosudostat (CHR-2797) to paclitaxel in patients with advanced solid tumours. *British Journal of Cancer* 103, 1362-1368

8. Naing, A., Cohen, R., Dy, G. K., Hong, D. S., Oyster, L., Hangauer, D. G., Kwan, R., Fetterly, G., Kurzrock, R., and Adjei A. A. (2013) A phase I trial of KX2-391, a novel non-ATP competitive substrate-pocket-directed SRC inhibitor, in patients with advanced malignancies. *Investigational New Drugs* 31, 967-973

9. Yahao B., Lingqiu G., Michael S., Taher H., Lyn D., David H., and Irwin G. (2008) KXO1 (KX2-391), a Src-family kinase inhibitor targeting the peptide-binding domain, suppresses oncogenic proliferation in vitro and in vivo. *Cancer Research*, 8, 4983-4983.

10. Kaur, R., Kaur, G., Gill, R. K., Soni, R., and Bariwal, J. (2014) Recent developments in tubulin polymerization inhibitors: An overview. *European Journal of Medicinal Chemistry* 87, 89-124

11. Ojedalopez, M. A., Needleman, D. J., Song, C., Ginsburg, A., Kohl, P. A., Li, Y., Miller, H. P., Wilson, L., Raviv, U., and Choi, M. C. (2015) Transformation of taxol-stabilized microtubules into inverted tubulin tubules triggered by a tubulin conformation switch. *Nature Materials* 108, 448a-448a

12. Gigant, B. t., Wang, C., Ravelli, R. B. G., Roussi, F., Steinmetz, M. O., Curmi, P. A., Sobel, A., and
Knossow, M. (2005) Structural basis for the regulation of tubulin by vinblastine. *Nature* **435**, 519–522

13. Ravelli, R. B. G., Gigant, B. t., Curmi, P. A., Jourdain, I., Lachkar, S., Sobel, A., and Knossow, M. (2004) Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. *Nature* **428**, 198–202

14. Prota, A. E., Bargsten, K., Northcote, P. T., Marsh, M., Altmann, K. H., Miller, J. H., Diaz, J. F., and Steinmetz, M. O. (2014) Structural Basis of Microtubule Stabilization by Laulimalide and Peloruside?A. *Angewandte Chemie* **53**, 1621–1625

15. Prota, A. E., Bargsten, K., Diaz, J. F., Marsh, M., Cuevas, C., Liniger, M., Neuhaus, C., Andreu, J. M., Altmann, K. H., and Steinmetz, M. O. (2014) A new tubulin-binding site and pharmacophore for microtubule-destabilizing anticancer drugs. *Proc Natl Acad Sci USA* **111**, 13817–13821

16. Yang, J., Wang, Y., Wang, T., Jiang, J., Bottig, C. H., Liu, H., Chen, Q., Yang, J., Naismith, J. H., and Zhu, X. (2016) Pironetin reacts covalently with cysteine–316 of α-tubulin to destabilize microtubule. *Nature Communications* **7**, 12103

17. Prota, A. E., Setter, J., Waight, A. B., Bargsten, K., Murga, J., Diaz, J. F., and Steinmetz, M. O. (2016) Pironetin Binds Covalently to αCys316 and Perturbs a Major Loop and Helix of α-Tubulin to Inhibit Microtubule Formation. *Journal of Molecular Biology* **428**, 2981–2988

18. Gutiérrez-Gutiérrez, G., Sereno, M., Miralles, A., Casado-Sáenz, E., and Gutiérrez-Rivas, E. (2010) Chemotherapy-induced peripheral neuropathy: Clinical features, diagnosis, prevention and treatment strategies. *Clinical & Translational Oncology* **12**, 81–91

19. Wei, Y., Tao, Y., Jianhong, Y., Taijin, W., Yamei, Y., Yuxi, W., Qiang, C., Peng, B., Dan, L., and Haoyu, Y. (2018) SKLB060 Reversibly Binds to Colchicine Site of Tubulin and Possesses Efficacy in Multidrug-Resistant Cell Lines. *Cellular Physiology & Biochemistry* **47**, 489–504

20. Bates, D., and Eastman, A. (2016) Microtubule destabilising agents: far more than just antimitotic anticancer drugs. *Br J Clin Pharmacol* **83**, 255–268

21. Sardar, P. S., Maity, S. S., Das, L., and Ghosh, S. (2007) Luminescence Studies of Perturbation of Tryptophan Residues of Tubulin in the Complexes of Tubulin with Colchicine and Colchicine Analogues. *Biochemistry* **46**, 14544–14556

22. Chinen, T., Liu, P., Shioda, S., Pagel, J., Cerikan, B., Lin, T.–c., Gruss, O., Hayashi, Y., Takeno, H., and Shima, T. (2015) The γ-tubulin-specific inhibitor gatatinat reveals temporal requirements of microtubule nucleation during the cell cycle. *Nature Communications* **6**, 8722

23. Fortin, S., Lacroix, J., C?té, M.–F., Moreau, E., Petitclerc, é., and C.–Gaudreault, R. (2010) Quick and Simple Detection Technique to Assess the Binding of Antimicrotubule Agents to the Colchicine-Binding Site. *Biological Procedures Online* **12**(2010–04–08) **12**, 113–117

24. Prota, A. E., Bargsten, K., Zurwerra, D., Field, J. J., Diaz, J. F., Altmann, K.–H., and Steinmetz, M. O. (2013) Molecular Mechanism of Action of Microtubule-Stabilizing Anticancer Agents. *Science* **339**, 587–590

25. Wang, Y., Zhang, H., Gigant, B. t., Yu, Y., Wu, Y., Chen, X., Lai, Q., Yang, Z., Chen, Q., and Yang, J. (2016) Structures of a diverse set of colchicine binding site inhibitors in complex with tubulin provide a rationale for drug discovery. *Fefs Journal* **283**, 102–111

26. Wozniak, K. M., Vornov, J. J., Wu, Y., Nomoto, K., and Slusher, B. S. (2016) Sustained Accumulation of Microtubule-Binding Chemotherapy Drugs in the Peripheral Nervous System: Correlations with Time Course and Neurotoxic Severity. *Cancer Research* **76**, 3332
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27. Towle, M. J., Salvato, K. A., Wels, B. F., Aalfs, K. K., Zheng, W., Seletsky, B. M., Zhu, X., Lewis, B. M., Kishi, Y., and Yu, M. J. (2011) Eribulin Induces Irreversible Mitotic Blockade: Implications of Cell-Based Pharmacodynamics for In vivo Efficacy under Intermittent Dosing Conditions. Cancer Research 71, 496-505

28. Banerjee, A. C., and Bhattacharyya, B., . (1979) Colcemid and colchicine binding to tubulin. Similarity and dissimilarity. Fems Letters 99, 333-336

29. Jianhong, Y., Wei, Y., Yamei, Y., Yuxi, W., Tao, Y., Linlin, X., Xue, Y., Caofeng, L., Zuowei, L., and Xiaoxin, C. (2018) The compound millepachine and its derivatives inhibit tubulin polymerization by irreversibly binding to the colchicine-binding site in β-tubulin. Journal of Biological Chemistry, jbc:RA117.001658

30. Van Vuuren, R. J., Visagie, M. H., Theron, A. E., and Joubert, A. M. (2015) Antimitotic drugs in the treatment of cancer. Cancer Chemotherapy & Pharmacology 76, 1101-1112

31. Visconti, R., and Grieco, D. (2017) Fighting tubulin-targeting anticancer drug toxicity and resistance. Endocrine Related Cancer 24, T107-T117

32. Minor, W., Czyborowski, M., Otwinowski, Z., and Chruszcz, M. (2010) HKL-3000: The integration of data reduction and structure solution - From diffraction images to an initial model in minutes. Acta Crystallographica 62, 859-866

33. Emsley P., and Cowtan K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallographica Section D: Biological Crystallography, 60, 2126-2132.

34. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., and Grossekunstleve, R. W. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr 66, 213-221

35. Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallographica 66, 12-21

36. Yang, J., Zhou, Y., Cheng, X., Fan, Y., He, S., Li, S., Ye, H., Xie, C., Wu, W., and Li, C. (2015) Isogambogenic acid induces apoptosis-independent autophagic cell death in human non-small-cell lung carcinoma cells. Scientific Reports 5, 7697

FOOTNOTES
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The abbreviations used are: MTD, maximum tolerance dose; AK, actinic keratosis; EBI, bis(iodoacetamide); SMB, significant mitotic block; HPLC, high-performance liquid chromatography; TFD, tryptophan fluorescence decrease; PI, propidium iodide; PBS, phosphate-buffered saline; PDB, Protein Data Bank.
**Figure 1.** KXO1 inhibited tubulin polymerization and induced G2/M phase cell cycle arrest in HeLa cells. (A) HeLa cells were treated with 100 nM paclitaxel (PTX), 100 nM vinblastine (Vin), 100 nM colchicine (Col), or 30 nM or 100 nM KXO1 for 16 h, and microtubule morphology was monitored by immunofluorescence. (B) HeLa, HepG2 and H460 cells were treated with 0, 12.5, 25, 50, or 100 nM KXO1 for 16 h and cell cycling was analyzed by PI staining. The lower graph showed the EC50 of KXO1’s G2/M phase arrest effect on the three cell lines. Data are shown as mean ±S.D. (n=3). Con: control.

**Figure 2.** KXO1 binds directly to the colchicine site of β-tubulin. (A) Tryptophan-based binding assay to detect the $K_d$ values of colchicine and KXO1 binding to tubulin. Compounds at different concentrations (0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μM) were incubated with tubulin for 30 min and then monitored at 295 nm (excitation) and 335 nm (emission) using a Biotech Gen5 spectrophotometer. Data are shown as mean ±S.D. (n=3). **p<0.01, in comparison with the Col group. (B) Different compounds at various concentrations were co-incubated with tubulin (3 mg/mL) at 37 °C. Absorbance at 340 nm was detected every 1 min for 18 min. (C) HeLa cells were treated with a high concentration (2 μM) of paclitaxel (PTX), vinblastine (Vin), colchicine (Col), or KXO1 for 4 h. Cell microtubule morphology was monitored by immunofluorescence. Red arrows represent paracrystals. (D) HeLa cells were incubated with or without the indicated concentrations of compounds for 2 h before being treated with 100 μM EBI for another 2 h. Total protein was lysed and subjected to western blotting analysis for β-tubulin. GAPDH was used as a loading control. The right graph shows the quantitative data from western blotting. Data are shown as mean ±S.D. (n=3). **p<0.01, ***p<0.001, in comparison with the EBI treated group. Con: control.

**Figure 3.** Crystal structure of tubulin complexed with KXO1. (A) Chemical formula of KXO1. (B) Electron densities of KXO1. The Fo-Fc omit map is colored gray and contoured at 3 σ. (C) Overall structure of tubulin–KXO1 complex. RB3–SLD is colored green, TTL is yellow, α-tubulin is black, β-tubulin is gray, GTP is red, GDP is orange, and KXO1 is cyan. GTP, GDP, and KXO1 are shown as spheres. (D) Interactions between tubulin and KXO1. KXO1 is shown as sticks. Residues that form interactions with KXO1 are shown as sticks and are labeled. Hydrogen bonds are drawn with yellow dashed lines.

**Figure 4.** Comparison of the binding modes between KXO1 and colchicine. The crystal structures of tubulin–colchicine (PDB ID: 4O2B, purple) and tubulin–KXO1 (cyan) are superimposed.

**Figure 5.** KXO1 induced reversible cellular effects. (A) Time schedule of mitotic block reversibility assay. (B) HeLa cell cycle histograms for colchicine (Col) and KXO1 in mitotic block reversibility assay. Rectangle: minimum drug concentrations inducing SMB at 0 h or maintaining SMB at 10 h. Data are representative of three independent experiments. (C) Five-day HeLa cell viability detection after transient exposure to KXO1 or Col in mitotic block reversibility assay. Results are presented as mean ±S.D. (n=3). *p<0.05, **p<0.01, in comparison with the control group.

**Figure 6.** Tubulin (25 μM) was incubated with 20 μM KXO1 or 20 μM colchicine (Col) for 60 min
before being separated by ultrafiltration. The filtrate fraction and retentate fraction were subjected to HPLC for detection of compounds. (A) 20 μM Col and 20 μM KXO1 in 100 μL methanol were also detected by HPLC as controls. (B) Compounds in filtrate fractions were detected by HPLC. (C) Compounds in retentate fractions were detected by HPLC.
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Table 1. Data collection and refinement statistics.

| Data collection | Tubulin–KXO1 |
|-----------------|--------------|
| Space group     | $P \overline{2} \overline{1} \overline{2} \overline{1}$ |
| Cell dimensions | $a$, $b$, $c$ (Å): 104.9, 157.1, 182.2 |
|                 | $\alpha$, $\beta$, $\gamma$ (°): 90.0, 90.0, 90.0 |
| Resolution (Å)  | 50.0–2.50 (2.54–2.50)* |
| $R_{\text{meas}}$ | 0.113 (0.794) |
| $I/\sigma I$     | 19.5 (2.0) |
| Completeness (%) | 100.0 (100.0) |
| Redundancy       | 13.4 (13.3) |

| Refinement       | |
| Resolution (Å)   | 49.2–2.48 |
| No. reflections  | 106,355 |
| $R_{\text{work}}$, $R_{\text{free}}$ | 0.216/0.263 |
| No. atoms        | |
| Protein          | 17,451 |
| Ligand/ion       | 286 |
| Water            | 222 |
| RMS deviations   | |
| Bond lengths (Å) | 0.008 |
| Bond angles (°)  | 0.991 |

*Highest-resolution shell is shown in parentheses.
A

Con Col Vin

PTX KXO1 (30 nM)

B

KXO1(nM)

0 12.5 25 50 100

HeLa

HepG2

H460

DNA Content

Concentrations Log[nM]

G2/M Phase Arrest Percent (not shown)

Fig. 1

EC50=39.8±3.73 nM

EC50=53.2±3.73 nM

EC50=74.6±9.45 nM
Fig. 2

A

Kd=13.30±3.30 μM
Kd=1.26±0.27 μM**

B

Tryptophan Fluorescence Decrease

Con
Col
KXO1

Concentrations (μM)

Time

OD(340nm)

0.0
0.1
0.2
0.3

C

Con
Col
Vin
KXO1

D

Relative Protein Level

Con
KXO1
Col
Vin

+100μM EBI

**

***
**Fig. 5**

**A**

12-h dose/response pretreatment

-12 h 0 h 10 h 5-d recovery

Cell cycle sample

**B**

| KXO1 | 0h | 10h |
|------|----|-----|
| 0 nM | ![image] | ![image] |
| 10 nM | ![image] | ![image] |
| 30 nM | ![image] | ![image] |
| 100 nM | ![image] | ![image] |
| 300 nM | ![image] | ![image] |
| 1,000 nM | ![image] | ![image] |
| 3,000 nM | ![image] | ![image] |
| 10,000 nM | ![image] | ![image] |

**C**

Concentrations (nM)

Cell viability at 5 days (%)

- Col
- KXO1

Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001
Fig. 6
Reversible binding of the anticancer drug KX01 (tirbanibulin) to the colchicine-binding site of β-tubulin explains KX01’s low clinical toxicity
Lu Niu, Jianhong Yang, Wei Yan, Yamei Yu, Yunhua Zheng, Haoyu Ye, Qiang Chen and Lijuan Chen

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