Oligomannururate sulfate, a novel antimitotic agent, exerts anti-cancer activity by binding to tubulin on novel site

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Key words: JG3, tubulin, antimicrotubule agent, G2/M, antineoplastic agent

Abbreviations: SPR, surface plasmon resonance; FITC, fluorescein-5-isothiocyanate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); BSA, bovine serum albumin; DTT, dithiothreitol; IAA, iodoacetamide; FCM, flow cytometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography-tandem mass spectrometry; bis-ANS, bis-8-anilinonaphthalene-1-sulfonate; RTV, relative tumor volume

A number of structurally diverse compounds have been reported to attack microtubules by targeting tubulin, and thereby induce mitotic arrest and cell death. The well characterized antimitotic drugs such as colchicines, vinca alkaloids, and taxanes have been used to treat various kinds of human cancers in clinic.10-12-15 Taxanes stabilize microtubules by blocking their disassembly.16,17 In contrast, colchicines and vinca alkaloids are microtubule-destabilizing agents that block microtubule assembly and induce the formation of tubulin spiral oligomers.9,16,19 All the microtubule inhibitors lead to inhibition of chromosome segregation in mitosis, resulting in inhibition of cell division, proliferation, and survival, despite that different inhibitors abolish different processes of microtubule dynamics.10,12-15

Since the well known small molecule antimicrotubule agents exhibit severe drawbacks especially high cytotoxicity and undesired side-effects in vivo,9,20 They may also increase the likelihood

Introduction

Microtubules are highly dynamic cytoskeletal fibers and integral components of mitotic spindles that are composed of α- and β-tubulin.1-2 Microtubules play important roles in a variety of fundamental functions, including chemotaxis, membrane and intracellular scaffolding, transport, secretory processes, maintenance of cell shape, cell adhesion and movement, mitosis and cell division.3-5 Microtubules are at their highest dynamic instability during spindle formation and separation of chromosomes,6,7 and disruption of microtubules results in formation of abnormal mitotic spindles and cell cycle arrest at G2/M phase, and finally leads cells to apoptotic death because of their crucial roles in the regulation of the mitotic apparatus. All these make tubulin a potential target for development of novel antineoplastic agents, especially for solid tumors.8-11

Tubulin-binding agents have received considerable interest as potential anti-cancer drugs. These compounds interfere with tubulin dynamics and microtubule organization and thereby induce cell growth arrest at G2/M phase, and eventually lead to apoptotic cell death. Herein, we report that sulfated oligosaccharide JG3 was effective at inhibiting viability of cancer cells, and suppressing tumor growth in vivo. Our studies showed that JG3 significantly arrested cell cycle at G2/M phase and led cancer cells to apoptotic death. Consistent with this, tubulin was identified as a binding protein of JG3 using affinity chromatography and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The interaction between JG3 and tubulin was characterized by surface plasmon resonance (SPR) analysis. Furthermore, it was demonstrated both at cellular level and in cell free system that JG3 potently prevented tubulin polymerization, thereby demolished microtubule organization in cancer cells. The underlying mechanism lies in its binding to tubulin on a unique site distinct from other conventional binding sites. All these suggest that JG3 is a novel antimitotic reagent, and this might shed new light on the understanding of anti-cancer activities and mechanisms of novel antimitotic reagents, and help develop new antimitotic agents in cancer therapy.
of development of resistance.\textsuperscript{21-23} So there comes an urgent requirement to develop new antimicrotubule agents with novel scaffolds. In the present study, oligomannuronic sulfate JG3, a newly semi-synthesized, structurally novel oligosaccharide derived from marine oligomannurinate blocks (Fig. 1A), was found to be a potent antitumor agent both in vitro and in vivo via binding to tubulin on a novel site. All these help provide proof of concept that JG3 in particular and, oligosaccharides possible found to be a potent antitumor agent both in vitro and in vivo of development of resistance.\textsuperscript{21-23}

**Results**

JG3 inhibits cancer cell proliferation and reduces cell viability in vitro by arresting cells at G\textsubscript{2}/M phase. We first tested the effect of JG3 on viability of numerous kinds of cancer cell lines in vitro. We found that JG3 was effective at inhibiting proliferation and reducing viability of multiple cancer cell lines from different tissues and organs (K562, HO-8910, BEL-7402, OVCAR-3, P388, A549, ts-FT-210, SMMC-7721, etc.). JG3 strongly suppressed the proliferation and viability of leukemia cells (K562, P388), ovarian cancer cells (HO-8910, OVCAR-3), and hepatocellular carcinoma cells (BEL-7402) with a lowest IC\textsubscript{50} value of 12.52 \textmu M on K562 cells (Fig. 1B).

We then characterize the anti-cancer activity and mechanism of JG3. As shown in Figure 1C, JG3 concentration-dependently reduced the viability of HO-8910 and K562 cells (p < 0.01), and exposure of cells to 20 \textmu M JG3 for 72 h resulted in an inhibition of 63.53 and 64.01\% respectively compared to control. The effect of JG3 on cell cycle progression and apoptosis of cancer cells was examined by flow cytometry. JG3 treatment resulted in a time-dependent accumulation of cancer cells at G\textsubscript{2}/M phase with concomitant loss from G\textsubscript{0}/G\textsubscript{1} phase (Fig. 1D). After treatment with 20 \textmu M JG3 for 48 h, the amount of HO-8910 and K562 cells in G\textsubscript{2}/M phase was increased from 4.23-71.1%, and from 7.87-69.15\%, respectively. In addition, the percentage of characteristic hypodiploid DNA content peak (sub G\textsubscript{0}), indicated as apoptotic cells was increased from 0.23-15.22\%, and from 0.15-19.35\%, respectively (Fig. 1E and F). These data suggest JG3 inhibits cancer cell proliferation by dramatically arresting cell cycle at G\textsubscript{2}/M phase, and leads cancer cells to apoptotic death.

**JG3 binds to tubulin in cancer cells.** We next want to identify and characterize the engagement of JG3 with proteins in cancer cells. For this, we first examined the binding of JG3 to cancer cells using JG3-fluorescein-5-isothiocyanate (FITC). Flow cytometry analysis showed that the cells in JG3-FITC group showed much stronger fluorescence compared with those in control group (Fig. 2A, p < 0.01) demonstrating the binding of JG3 to cancer cells. We next used JG3 affinity chromatography in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to purify and identify the JG3 binding proteins in cancer cells. After solubilization, the protein preparation from HO-8910 cells was applied to a JG3-Sepharose affinity column and the proteins were eluted with a linear gradient of NaCl (0.15-2 M) in Tris buffer (Fig. 2B). Numerous proteins were eluted at NaCl concentrations between 0.15 and 1 M. Between 1 and 2 M of NaCl, a single symmetric peak comprising relatively fewer proteins was obtained (Fig. 2B and C). These proteins exhibited relatively strong affinity for JG3, and they were subsequently analyzed by LC-MS/MS. The analysis showed that JG3 could bind to tubulin in cancer cells. Table 1 lists all peptides identified by MS/MS that match the sequences of the two subunits of tubulin. The MS/MS spectrums of the mass peak at m/z 2410.67 of alpha subunit, and the peak at m/z 1871.19 of beta subunit were shown in Figure 2D and E as examples. These results provide substantial support for the direct binding of JG3 to tubulin in cancer cells.

The JG3 affinity chromatography and subsequent LC-MS/MS analysis provided convincing evidence that JG3 binds to tubulin. We next used surface plasmon resonance (SPR) assay to characterize the binding profiles between JG3 and tubulin. As indicated in Figure 2F, JG3 and tubulin exhibited a dramatic interaction, and the binding response increased with increases in tubulin concentrations.

**JG3 inhibits tubulin polymerization and demolishes microtubules in cancer cells.** The suppression of cell proliferation by JG3 through arresting cell cycle at G\textsubscript{2}/M phase, together with the binding of JG3 to tubulin, led us to examine the effect of JG3 on tubulin dynamics. As shown in Figure 3A, in cell free system, tubulin polymerization was remarkably inhibited by JG3 in a concentration-dependent manner with an IC\textsubscript{50} of 8.58 \textmu M. The inhibition increased from 18.26 to 73.44\% with concentration increased from 2.5 to 40 \textmu M (Fig. 3B). We further examined the effect of JG3 on cellular microtubule networks. As shown in Figure 3C, JG3 efficiently disrupted microtubule network, and it triggered a diffuse microtubule network in cells by inhibition of microtubule assembly. Similar results were also obtained using colchicine and vinblastine, two well known microtubule depolymerizers. Figure 3D showed the percentage of cells with deminished microtubule network after treatment with the agents. These data revealed that JG3 could bind to tubulin and interfere with polymerization of microtubules in cancer cells, which provides mechanical explanation to arrest of cell cycle and inhibition of cell proliferation induced by JG3.

**The binding site of JG3 on tubulin is distinct from the well-recognized ones.** JG3 binds to tubulin and inhibits tubulin...
Figure 1. (see opposite page)
Figure 2. JG3 binds to tubulin in cancer cells. (A) HO-8910 and K562 cells were incubated with 10 µM JG3-FITC for 12 h at 37°C, and cells were harvested and analyzed with FCM (1, control; 2, JG3-FITC). (B) The elution curve for quantification of JG3-bound proteins from HO-8910 cells using JG3 affinity chromatography. (C) The cytosolic proteins purified by the JG3-sepharose affinity column were separated by SDS-PAGE and stained with 0.2% (w/v) Coomassie brilliant blue R-250. Lane 1 and 2 present fraction 1 and 2 in (B). (D) MS/MS spectrum of the mass peak at m/z 2410.67 of alpha subunit of tubulin. (E) MS/MS spectrum of the mass peak at m/z 1871.19 of beta subunit of tubulin. (F) Tubulin (0.313–2.5 µM) in PIPES buffer was passed over the JG3 sensor chip surface and the changes in mass were measured, and the sensorgrams were recorded in real time and analyzed after subtracting the control. The sensorgram line of interaction between 0.313 µM tubulin and JG3 sensor chip was shown as an example. (G) The maximum response values of different concentrations of JG3. The data shown is a representative of three independent experiments with similar results, and the data points represent mean of triplicate measurements with error bars corresponding to standard deviation.
polymerization. We next try to figure out whether JG3 shares the same binding sites on tubulin with colchicine or vinblastine, whose binding sites are two typical and well-characterized binding sites for microtubule depolymerizing agents.14,24 For that, we first compared the changes of tubulin conformation induced by JG3, colchicine or vinblastine binding. As shown in Figure 4A, JG3 enhanced the intrinsic tryptophan fluorescence of tubulin in a concentration-dependent manner. In contrast, colchicine and vinblastine reduced the intrinsic tryptophan fluorescence of tubulin, suggesting that JG3 binding triggered different conformational changes compared to colchicine and vinblastine. The extreme environmental sensitivity of bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) makes it a useful probe for examining the conformational state of the tubulin dimers. Consistently, JG3 binding increased tubulin-bis-ANS fluorescence dose-dependently, while colchicine and vinblastine slightly reduced the fluorescence intensity (Fig. 4B). The sulfhydryl groups in tubulin locate in the regions important for tubulin polymerization, so the changes in chemical reactivity of these residues directly reflect polymerization-related conformational changes. Figure 4C showed that JG3 increased the reactivity of tubulin sulfhydryl groups with DTNB. In contrast, binding of colchicine or vinblastine significantly decreased the reaction between tubulin and DTNB. These data further confirmed the JG3 binding to tubulin, through a different way and triggered different conformational changes compared to colchicine and vinblastine.

We next performed a competitive inhibition assay. It was shown that both colchicine and vinblastine (10–2,560 μM) failed to inhibit the binding of tubulin to immobilized JG3. In contrast, free JG3 dramatically inhibited the interaction between tubulin and immobilized JG3 (Fig. 4D), which further demonstrated that the binding site of JG3 on tubulin is distinct from that of colchicine or vinblastine.

**JG3 inhibits in vivo tumor growth.** Based on the significant inhibition of cancer cell proliferation and viability by JG3 in vitro, we next examined the antitumor activity of JG3 in vivo. We first examined the effect of s.c. administration of JG3 on hepatocellular carcinoma BEL-7402 growth in nude mice. As indicated in Table 2 and Figure 5A and B, administration of 40 mg/kg JG3 exhibited a suppression on tumor growth in vivo with an inhibition of 63.85% after 21-day treatment compared with control, and administration of 80 mg/kg JG3 significantly attenuated tumor growth (p < 0.05) with a T/C value of 47.4% and an inhibition of 70.7%. Similarly, JG3 significantly inhibited S180 tumor growth in vivo. Administration of JG3 at dosage of 25, 50 and 100 mg/kg resulted in inhibition of 18.9%, 40.2% (p < 0.05) and 56.8% (p < 0.01) respectively after 10-day treatment (Fig. 5D). The tumor weight in JG3-treated groups was 1.1, 0.8 and 0.57 g respectively compared to 1.3 g in control group (Fig. 5C). At the same time, administration of JG3 was well tolerated by healthy mice without any signs of overt toxicity or loss of weight (Table 2).

**Table 1. Identification of JG3/tubulin binding by LC-MS/MS (A, α-tubulin; B, β-tubulin)**

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>MH⁺</th>
<th>Charge</th>
<th>Xcorr</th>
<th>Delta Cn</th>
<th>Ions</th>
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<td></td>
<td></td>
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<td>2</td>
<td>5.0962</td>
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Discussions

Microtubules are important cytoskeletal component involved in numerous cellular functions, especially cell mitosis.3,5 Microtubules are in a highly dynamic process of polymerization and depolymerization in cells undergoing replication and division.6,7 The discovery that the cytotoxic activity of various compounds is through
interference with the mitotic spindle apparatus has attracted much attention within the past two decades, and microtubule has become an attractive pharmacologic target for anticancer drug development. Microtubule inhibitors interfere with the dynamics of tubulin polymerization and/or depolymerization, resulting in inhibition of chromosome segregation in mitosis and inhibition of cell division and induction of apoptotic cell death. 

Although the current antimicrotubule agents are widely used in cancer therapy, they usually have some severe drawbacks: (1) dose-limiting toxicity such as neutropenia, peripheral neuropathy and vehicle (Cremaphor EL)-related hypersensitivity reactions; (2) development of drug resistance and tumor metastases in brain and (3) poor selectivity and high toxicity especially neurotoxicity.

So there has been great interest in identifying novel antimicrotubule agents with new mode of action and improved pharmacology profiles especially with reduced toxicity.
Microtubule depolymerizing agents have been reported to bind one of them. A series of biochemistry data as well as competitive inhibition result showed that JG3 binds tubulin on a unique site different from both colchicine and vinblastine binding sites. The dramatic structural differences between JG3 and other well characterized small antimitotic molecules provide substantial structural basis for this.

One direction for development of novel antineoplastic agents focuses on biochemical abnormalities that differentiate malignant tumors from most normal tissues. JG3 was effective at inhibiting cancer cell viability in vitro and suppressing tumor growth in vivo. In contrast, JG3 exhibited poor cytotoxicity to normal cells including B and T lymphocytes, NK cells and macrophages, exhibiting a good selectivity towards cancer cells, which may be attributed to its specific binding and entrance to cancer cells and also the high mitosis in cancer cells.

The oligosaccharides bear unique backbone totally different from that of small molecules that have never been challenged in this setting. JG3, a novel form of sulfated oligosaccharide isolated from brown alga is structurally characterized as a 1,4-linked β-D-mannururate of pyranohexuronic acid residues bearing an average of 1.5 sulfates at 2-hydroxyl and partial 3-hydroxyl groups and 6-carboxyl groups per sugar residue, with an additional C1 carboxyl group at the reducing end. JG3 is able to interact with tubulin, as is evident from the JG3-based affinity chromatography and LC-MS/MS analysis, together with SPR analysis. As a consequence, JG3 inhibits tubulin polymerization and disrupts intracellular microtubule network in cancer cells. These findings gave us a better understanding of the anti-cancer mechanism of JG3. The colchicine and vinblastine binding sites are two kinds of important conventional binding sites on tubulin, and a vast number of microtubule depolymerizing agents have been reported to bind one of them. A series of biochemistry data as well as competitive inhibition result showed that JG3 binds tubulin on a unique site different from both colchicine and vinblastine binding sites. The dramatic structural differences between JG3 and other well characterized small antimitotic molecules provide substantial structural basis for this.

One direction for development of novel antineoplastic agents focuses on biochemical abnormalities that differentiate malignant tumors from most normal tissues. JG3 was effective at inhibiting cancer cell viability in vitro and suppressing tumor growth in vivo. In contrast, JG3 exhibited poor cytotoxicity to normal cells including B and T lymphocytes, NK cells and macrophages, exhibiting a good selectivity towards cancer cells, which may be attributed to its specific binding and entrance to cancer cells and also the high mitosis in cancer cells.
which provides an opportunity for JG3 to synergize with other antimitotic drugs in cancer therapy.

**Materials and Methods**

**Drugs and reagents.** JG3, JG3-sepharose and JG3-FITC were provided by Marine Drug and Food Institute, Ocean University of China, China. Tubulin was purchased from Cytoskeleton (Denver, CO). Mouse anti-human tubulin monoclonal antibody (IgG) was purchased from Lab Vision (Saint Louis, MI). Cy3-conjugated goat anti-mouse IgG, aprotinin, pepstatin A, leupeptin, DTNB, bis-ANS, PMSF, bovine serum albumin (BSA), dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO). RNase was obtained from Hyclone (Logan, UT) and Trypsin was from Roche (Mannheim, Germany).

**Table 2. Inhibition of JG3 on BEL-7402 tumor growth in vivo**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Routes</th>
<th>Number</th>
<th>Weight (g)</th>
<th>TV (mm³, mean ± SD)</th>
<th>RTV (x ± SD)</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-</td>
<td>s.c.</td>
<td>d₀</td>
<td>10</td>
<td>10</td>
<td>d₂₁</td>
<td>18.8</td>
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<td>JG3</td>
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<td>s.c.</td>
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<td>5</td>
<td>19.8</td>
<td>21.7</td>
<td>91 ± 52</td>
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<td>JG3</td>
<td>80 mg/kg</td>
<td>s.c.</td>
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<td>5</td>
<td>19.0</td>
<td>21.7</td>
<td>91 ± 52</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with control group.

Figure 5. JG3 inhibits in vivo tumor growth. (A) Human hepatocellular carcinoma BEL-7402 xenograft was established and the mice were received JG3 at indicated doses by s.c. administration everyday for 3 weeks. The size of tumors were measured individually twice per week with micropipettes. The individual relative tumor volume was shown. (B) The inhibition of JG3 on BEL-7402 growth in vivo. (C) S₁₈₀ tumor cells were implanted and the mice were received JG3 at indicated doses by s.c. administration everyday for 10 days. Then mice were sacrificed and the tumor growth was quantified by weighting the tumor tissues. (D) The inhibition of JG3 on S₁₈₀ tumor growth in vivo. The data points represent mean of quintuplicate or ten measurements with error bars corresponding to standard deviation.
Cells and cell culture. Cancer cell lines of BEL-7402, HO-8910, OVCAR-3, K562, P388, A549, ts-FT210 were purchased from the American Type Culture Collection (Rockville, MD, USA). P388 was cultured in DMEM medium, and the other cells were cultured in RPMI 1640 medium. All media were supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/l streptomycin and 2 mM glutamine. All the cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Cell viability assay. Cancer cells were incubated with different concentrations (1.25, 2.5, 5, 10, 20, 40, 80 µM) of JG3 for 72 h, and MITT solution was added at 20 h and incubated for additional 4 h. Then culture supernatant was removed and 200 µl DMSO was added per well to dissolve the formazan crystals. Colorimetric determination was made at 570 nm using microplate reader. The inhibition rates were calculated as: inhibition (%)= [(1 - (JG3 treated group/control group)) x 100%].

Flow cytometry analysis. HO-8910 and K562 cells were seeded in 6-well plates (5 x 10^5 cells/well) and treated with JG3 (20 µM) for 12, 24 or 48 h. Cells were harvested, washed with PBS, and stained with a mixture of RNase (1 g/l) and PI (5 mg/l) in 1 g/l sodium citrate containing 0.5% TritonX-100 (v/v) in dark for 30 min. Then cells were collected for cell cycle analysis using flow cytometry (FCM) (Becton and Dickinson, USA). The percentage of hypodiploidy and cell cycle distribution were analyzed with CellQuest and ModFIT LT software (Becton and Dickinson).

Confocal microscope analysis. To evaluate the influence of JG3 on microtubule organization, HO-8910 cells were seeded on FN-coated coverslips, and incubated with JG3 (5, 10 or 20 µM) or colchicine (0.5 µM) or vinblastine (0.5 µM) for 24 h. Then cells were washed, harvested three times with PBS, and analyzed using FCM with a 488-nm laser excitation and a 513-nm emission filter. Data were analyzed with CellQuest software.

Confocal microscopy. To detect the binding of JG3 to cancer cells, HO-8910 and K562 cells were seeded and incubated with 10 µM JG3-FITC for 12 h, then cells were harvested, washed three times with PBS, and analyzed using FCM with a 488-nm laser excitation and a 513-nm emission filter. Data were analyzed with CellQuest software.

LC-MS/MS analysis. The purified proteins were dissolved in 200 µl of 6 M hydrochloric carbamidine (pH 8.3), reduced with 1 M dithiothreitol, and alkylated with 1 M iodoacetamide. 100 mM NH4HCO3 was added and ultrafiltrated at 12,000 rpm for 2 h at 4°C. Samples were digested with trypsin for 20 h at 37°C. After another ultrafiltration step, samples were separated using a reverse-phase (C18) capillary column (0.15 mm x 120 mm, Thermo Hypersil-Keystone, USA) and analyzed by electrospray ionization MS/MS. The mass spectrometer took one full-scan with mass range 400–2,000 m/z, followed by three MS/MS spectra of the three most intense peaks. MS/MS spectra was analyzed using SEQUEST (Thermo Finnigan, USA) against the International Protein Index database.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The cytosolic proteins eluted from the column was separated by 12% SDS-PAGE. The gels were stained with 0.2% Coomassie brilliant blue R-250 (w/v) in methanol, acetic acid and water (5:1:5, v/v), and destaining in methanol, acetic acid and water (5:7:88, v/v).

Biochemical assays. Intrinsinc tryptophan fluorescence of tubulin. Tubulin (2 µM) was incubated with JG3 (5, 10 or 20 µM), or colchicine (20 µM) or vinblastine (20 µM) for 30 min at 37°C. The fluorescent intensity was analyzed using a Spectrofluorometer (Jasco, Japan) with 295 nm as the excitation wavelength to specify excite the tubulin tryptophan residues, and the emission were eluted with a linear gradient of 0.15–2 M NaCl in Tris-HCl (pH 7.4) containing 0.1% TritonX-100 (v/v). The elution was monitored at OD 280 nm, protein content was estimated with 0.01% Coomassie brilliant blue G-250 (w/v) in ethanol, phosphoric acid and water (1:2:20, v/v), and the absorbance was measured at 395 nm with a microplate reader. The fractions were pooled and dialyzed with redistilled water, then concentrated and lyophilized.
was examined at 310–370 nm. Control value was subtracted from all measurements.

Probe conformational state of tubulin using bis-ANS. Tubulin (2 µM) was incubated with JG3 (5, 10 or 20 µM), or colchicines (20 µM) or vinblastine (20 µM) for 30 min at 37°C. Then 15 µM bis-ANS was added, incubated for 15 min, and the fluorescent intensity was measured using a Spectrofluorometer (excitation at 400 nm, emission at 430–590 nm). Control value was subtracted from all measurements.

Probe conformational state of tubulin using DTNB. Tubulin (5 µM) was incubated with JG3 (5, 10 or 20 µM), or colchicines (20 µM) or vinblastine (20 µM) for 30 min at 37°C. Then 300 mM DTNB was added, and the amount of modified sulfhydryl groups was examined at 412 nm using a microplate reader. Control value was subtracted from all measurements.

In vivo tumor growth assay. Human hepatocellular carcinoma BEL-7402 xenograft was established by inoculating 5 x 10^6 cells subcutaneously in nude mice. The experiment began when the xenograft had three passages in nude mice. Under a sterilization condition, the well grown tumors were cut into 1 mm³ fragments and the fragments were transplanted subcutaneously into the right flank of nude mice by trocar. When tumors reached a volume of 100 mm³, the mice were randomized to control and treated groups, and received vehicle or JG3 (40 and 80 mg/kg) by s.c. administration everyday for 3 weeks. The size of tumors were measured individually twice per week with microcalipers. Tumor volume (V) was calculated as follow: \[ V = \frac{\text{length} \times \text{width}^2}{2} \]. The individual relative tumor volume (RTV) was calculated as follow: \[ \text{RTV} = \frac{V}{V_0} \], where \( V \) is the volume of everyday measurement and \( V_0 \) is the volume of initial measurement. Therapeutic effect of compound was expressed in terms of T/C % and the calculation formula is: \( T/C(\%) = \text{mean RTV of the treated group/mean RTV of the control group} \times 100\% \).

The sterilly harvested Sarcoma 180 (S180) tumor cells were diluted to 1 x 10^3 cells/ml with sterilized physiological saline, and implanted subcutaneously into the right flank of Swiss albino mice (0.2 ml per mouse). The mice were randomly divided into control group and three JG3 treatment groups (10 mice per group) 24 h after transplantation of cancer cells. The mice were received vehicle or JG3 (25, 50 100 mg/kg body weight) by s.c. administration everyday for 10 days. Then mice were sacrificed, and the tumor growth was quantified by weighting the tumor tissues. The inhibition of JG3 on tumor growth was calculated as follow: \( \text{inhibition}(\%) = [1 - (\text{tumor weight of JG3 treated group/tumor weight of control group})] \times 100\% \).

Statistics. Student t-test and analysis of variance (ANOVA) were performed using Statview. p < 0.05 was considered significant and p < 0.01 as highly significant. Five parallel samples were prepared in each group and all experiments were replicated at least three times.

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References