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Design, Synthesis, and Biological Evaluation of Chalcone-Containing Shikonin Derivatives as Inhibitors of Tubulin Polymerization

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The biological importance of microtubules in mitosis makes them an interesting target for the development of anticancer agents. In this study, a series of novel chalcone-containing shikonin derivatives was designed, synthesized, and evaluated for biological activities. Among them, derivative PMMB-259 [(*R*)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl (*E*)-2-(4-(3-oxo-3-(3-(trifluoromethoxy)phenyl)prop-1-en-1-yl)phenoxy)acetate] was identified as a potent inhibitor of tubulin polymerization. Further investigation confirmed that PMMB-259 can induce MCF-7 cell apoptosis, reduce the mitochondrial transmembrane potential, and arrest the cell cycle at the G_2/M phase. Moreover, the morphological variation of treated cells was visualized by confocal microscopy. The results, along with docking simulations, further indicated that PMMB-259 can bind well to tubulin at the colchicine site. Overall, these studies may provide a new molecular scaffold for the further development of antitumor agents that target tubulin.

Introduction

Mitosis is an elaborate and essential biological activity in somatic proliferating cells, resulting in the division of duplicated sets of chromosomes into two genetically identical daughter cells.[1] In the whole process of cell mitosis, the dynamic equilibrium between microtubules and its heterodimers plays an extremely important role.[2] When perturbed, the polymerization/depolymerization dynamics lead to cell-cycle arrest and apoptosis.[3] Microtubule polymerization dynamics also affect the cell's capacity to maintain or change shape and undergo critical processes such as intracellular transport, cell signaling, and secretion.[4] Considering the significant role of microtubules in actively proliferating and newly divided cells, it is not surprising that microtubules are among the most important molecular targets for the development of anticancer agents.[5,6] To date, various classes of anti-tubulin agents have been explored and pre-clinically or clinically examined, with some already being used in cancer therapy.[7,8] However, the search for new tubulin inhibitors with safer and more effective profiles remains an important ongoing task.

Natural products are currently gaining significant status in the drug design field. Strategies based on modifying natural products have benefited from the innately complex scaffolds of natural products, which are rarely covered in commercial synthetic compound libraries.[9] In fact, the most prominent tubulin inhibitors, paclitaxel and colchicine, were originally extracted from plants.[10,11] However, with natural products, there remains considerable space for optimization in terms of oral bioavailability, safety, efficacy, and other pharmacological properties.[12,13] As the main efficacious component isolated from the root of the Chinese herbal medicine *Lithospermum erythrorhizon*, shikonin has been used in Europe and Asia since ancient times.[14] It is attracting considerable attention in the field of natural product chemistry due to its manifold pharmacological properties such as wound healing, antioxidant, antibacterial, anti-inflammatory, and antitumor.[15–19] Unfortunately, shikonin also exerts nonselective cytotoxic effects on normal cells, which hinders its further development. Structural modifications aimed at improving target selectivity help solve this dilemma. Our previous study also indicated that the toxicity of shikonin against non-cancer cells can be decreased by modifying the side chain hydroxy group.[20–22] Moreover, in our previous research, various types of shikonin derivatives were designed and found to have notable anti-tubulin activity with lower cytotoxicity.[23] Given these previous research results, in this study we attempted to design a type of shikonin derivative that has improved tumor targeting. We designed and synthesized a series of chalcone-containing shikonin derivatives. Biological evaluations indicated that some of these compounds can act as potent inhibitors of tubulin polymerization. In addition, mo-

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lecular docking simulations were performed in an attempt to rationalize the biological results.

Results and Discussion

Chemistry

The routes to synthesize the novel chalcone-containing shikonin derivatives PMMB-248–PMMB-265 are outlined in Scheme 1. All of these compounds are reported for the first time (Table 1), and gave satisfactory analytical and spectroscopic data. ^{1}H NMR and ESI-MS spectra are in full accordance with the assigned structures, which are presented in the Supporting Information (SI).

In vitro antiproliferative activity

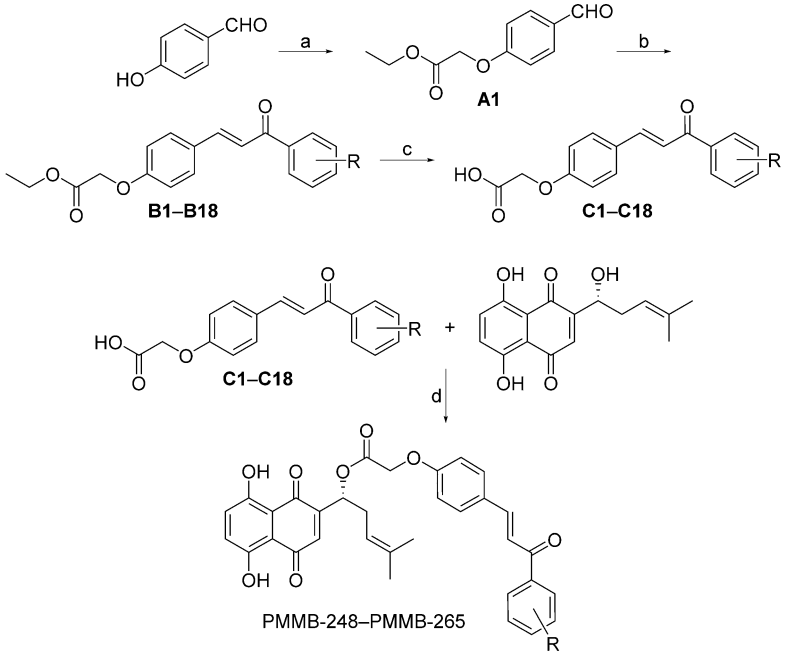
Compounds PMMB-248–PMMB-265 were evaluated for their antiproliferative activities by MTT assay against three cancer cell lines: HeLa (human cervical cancer), MCF-7 (human breast cancer), and A549 (human lung adenocarcinoma epithelial), as well as two non-cancer cell lines: 293T (human kidney epithelial) and L02 (human hepatic). The IC_{50} values were calculated and are listed in Table 2. The data for intermediate compounds **B1**–**B18** were also collected and are provided in SI Table S1 for comparison. We found that all compounds show moderate to excellent antiproliferative effects on MCF-7 and A549 cancer cell lines, but not HeLa. Especially against the MCF-7 cell line, PMMB-259 showed the best antiproliferative activity, with a lower IC_{50} value (2.36 ± 0.32 μM) than that of shikonin (7.61 ± 0.16 μM). Subsequently, MTT assays against the two non-cancer cell lines, 293T and L02, were performed to test the cytotoxicity of the obtained compounds. As shown in Table 2, none of the compounds have any clear cytotoxic effects on the two non-cancer cell lines, hinting at considerable selectivity.

Table 1. The CDOCKER interaction energy (ΔG_b) between target compounds and 1SA0 and in vitro inhibition of tubulin polymerization.

Compound	R	ΔG_b [−kcal mol^{-1}]	Tubulin IC_{50} [μM]
PMMB-248	H	57.2987	16.14 ± 0.65
PMMB-249	4-OCH_3	55.9599	17.29 ± 0.51
PMMB-250	3,4,5-3OCH_3	63.6994	6.26 ± 0.29
PMMB-251	4-CF_3	60.4691	15.36 ± 0.46
PMMB-252	2,3,4-3Cl	58.2318	18.35 ± 0.36
PMMB-253	2-CF_3	60.0841	13.65 ± 0.22
PMMB-254	2-OCH_3	66.6288	3.85 ± 0.39
PMMB-255	3-CF_3	60.6049	16.99 ± 0.25
PMMB-256	4-CH_2CH_3	63.2935	10.23 ± 0.46
PMMB-257	2-F	52.9641	28.35 ± 0.27
PMMB-258	3,5-2CF_3	64.3526	4.63 ± 0.11
PMMB-259	3-OCF_3	64.6074	2.98 ± 0.53
PMMB-260	3-OCH_3	53.5111	33.67 ± 0.45
PMMB-261	2-CH_3	55.3211	24.14 ± 0.57
PMMB-262	2-Cl	57.4367	20.15 ± 0.66
PMMB-263	2-Br	54.2322	23.56 ± 0.71
PMMB-264	3-CH_3	51.1432	19.68 ± 0.57
PMMB-265	4-CH_3	53.0311	21.39 ± 0.66
shikonin			15.20 ± 0.25
colchicine			3.10 ± 0.35

Inhibition of tubulin polymerization

To determine whether the antiproliferative activity of compounds PMMB-248–PMMB-265 result from their interaction with tubulin, we evaluated their capacity to inhibit tubulin polymerization in vitro. The results are listed in Table 1, indicating that the IC_{50} values for inhibiting tubulin polymerization



Scheme 1. Reagents and conditions: a) ethyl bromoacetate, K_2CO_3, acetone, 60 °C, 8 h; b) acetophenone, NaOH, 0 °C, 2 h; c) NaOH, EtOH, 90 °C, 6 h; d) DCC, DMAP, CH_2Cl_2, 0 °C, 1 h.

Table 2. Inhibition of proliferation of HeLa, MCF-7, A549, L02 and 293T cells by PMMB (248–265).

Compound	IC_{50} [μM]				
	HeLa	MCF-7	A549	L02	293T
PMMB-248	11.70 ± 0.01	8.42 ± 0.21	12.49 ± 0.10	> 100	> 100
PMMB-249	14.82 ± 0.08	9.68 ± 0.05	17.68 ± 0.27	> 100	> 100
PMMB-250	8.91 ± 0.07	5.22 ± 0.19	8.10 ± 0.17	> 100	> 100
PMMB-251	11.59 ± 0.13	8.32 ± 0.03	11.36 ± 0.22	> 100	> 100
PMMB-252	13.9 ± 0.12	8.91 ± 0.04	13.66 ± 0.32	> 100	> 100
PMMB-253	8.56 ± 0.05	7.54 ± 0.05	8.67 ± 0.13	> 100	> 100
PMMB-254	5.29 ± 0.04	4.58 ± 0.43	6.61 ± 0.13	> 100	> 100
PMMB-255	11.11 ± 0.42	8.14 ± 0.15	9.66 ± 0.05	> 100	> 100
PMMB-256	9.13 ± 0.17	6.71 ± 0.15	8.17 ± 0.03	> 100	> 100
PMMB-257	17.17 ± 0.03	13.20 ± 0.07	10.33 ± 0.09	> 100	> 100
PMMB-258	10.41 ± 0.09	4.82 ± 0.13	7.27 ± 0.32	> 100	> 100
PMMB-259	4.53 ± 0.07	2.36 ± 0.32	5.84 ± 0.18	> 100	> 100
PMMB-260	14.82 ± 0.08	15.47 ± 0.18	21.21 ± 0.07	> 100	> 100
PMMB-261	17.39 ± 0.21	12.55 ± 0.11	20.66 ± 0.01	> 100	> 100
PMMB-262	14.57 ± 0.23	9.18 ± 0.25	16.71 ± 0.44	> 100	> 100
PMMB-263	17.37 ± 0.01	11.58 ± 0.26	6.81 ± 0.14	> 100	> 100
PMMB-264	16.17 ± 0.23	9.48 ± 0.02	9.51 ± 0.18	> 100	> 100
PMMB-265	14.17 ± 0.11	10.48 ± 0.22	12.51 ± 0.47	> 100	> 100
shikonin	6.44 ± 0.04	7.61 ± 0.16	11.68 ± 0.01	10.52 ± 0.02	9.27 ± 0.01
colchicine	0.15 ± 0.02	0.22 ± 0.06	0.47 ± 0.06	2.3 ± 0.01	1.03 ± 0.03

show a similar trend with the respective IC_{50} values from the cell antiproliferation assay. Among them, PMMB-259 showed the most potent activity of anti-tubulin polymerization, with an IC_{50} value of 2.98 ± 0.53 μM. We therefore conclude that most of the synthesized compounds can inhibit tubulin assembly and that the antiproliferative effects might be produced in part by their interaction with tubulin.

PMMB-259 causes apoptosis in MCF-7 cells in a dose- and time-dependent manner

We next performed an Annexin V–FITC/PI double staining assay to validate the apoptosis-stimulating effects of PMMB-259 on MCF-7 cells. We treated MCF-7 cells with varying concentrations (0, 2, 4, and 8 μM) of PMMB-259 for 24 h, and analyzed cells for changes in apoptotic markers with a flow cytometer. As shown in Figure 1, the results indicate that increasing concentrations of PMMB-259 yield an increased apoptotic rate in MCF-7 cells. Meanwhile, we treated MCF-7 cells with PMMB-259 at 4 μM for 0, 12, 24, and 36 h, and found that the percentage of apoptotic cells is markedly elevated in a time-dependent manner. In conclusion, it is clear from the aforementioned results that PMMB-259 can induce apoptosis in MCF-7 cells in a dose- and time-dependent manner.

PMMB-259 reduces mitochondrial transmembrane potential in MCF-7 cells

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was next monitored by JC-1 staining. Figure 2 shows representative JC-1 fluorescence in both FL-1 and FL-2 channels. Red fluorescence represents the mitochondrial aggregate form of JC-1, with high $\Delta\Psi_m$ in normal cells. Green fluorescence represents the monomeric form of JC-1, with low $\Delta\Psi_m$ in apoptotic cells. Cells treated with PMMB-259 exhibited increased green fluorescence and decreased red fluorescence, suggesting the depolarization of mitochondria and reduced $\Delta\Psi_m$. The ratio of cells with high membrane potential exposed to PMMB-259 at 4 and 8 μM decreased from 86.6% to 78.9% and 53.3% after 4 h treatment. Moreover, the ratio of cells with high membrane potential decreased from 81.8% to 78.1% and 66.9% after cells exposed to PMMB-259 at 4 μM for 4 and 8 h treatment.

PMMB-259 induces cell-cycle arrest in MCF-7 cells in a dose- and time-dependent manner

We further assessed the effect of PMMB-259 on the cell cycle to ascertain whether MCF-7 cells are blocked in mitosis. MCF-7 cells were treated with PMMB-259 at increasing concentrations (0, 2, 4, and 8 μM) for 24 h and then incubated with propidium iodide (PI) to analyze their genomic DNA content in flow cytometry assays. As illustrated in Figure 3, PMMB-259 induced a gradual accumulation of cells in the G_2/M phase in a dose-dependent manner, and 43.76% of cells were arrested in G_2/M phase upon exposure to 8 μM PMMB-259 for 24 h. We then treated MCF-7 cells with 4 μM PMMB-259 for 0, 12, 24, and 36 h. The results show that PMMB-259 could block the cell cycle in G_2/M phase in a time-dependent manner, and the maximum accumulation of cells in G_2/M phase was observed after treatment with PMMB-259 at 4 μM for 36 h. Furthermore, we performed western blotting on PMMB-259 with cycle-related proteins to confirm its G_2/M arrest effect. As research has revealed that cyclin B1 and CDK1 play crucial roles in regulating the transition from G_2 to M phases, their expression was examined in the test. The western blot results show that PMMB-259 can increase the expression of cyclin B1 and CDK1 in a dose-dependent manner, which are in accordance with the above results.

PMMB-259 inhibits tubulin polymerization and disrupts the microtubule system

To further investigate the effect of PMMB-259 on the cytoskeletal network of tubulin, MCF-7 cells were immunostained and analyzed by confocal microscopy. As illustrated in Figure 4, the microtubule network of control cells displayed a normal arrangement and organization, which is slim and fibrous. Treatment with paclitaxel at 1 μM dramatically enhanced microtubule polymerization, with an increase in the density of cellular microtubules and formation of long thick microtubule bundles. In contrast, the cell membrane microtubules of cells treated with 1 μM colchicine showed depolymerization and solubility. With reference to these two groups and an untreated control group, we found that a significant decrease in the density of microtubules and the radial organization of microtubules was perturbed after treatment with PMMB-259 at 2 μM. We therefore conclude that PMMB-259 can inhibit tubulin polymeri-

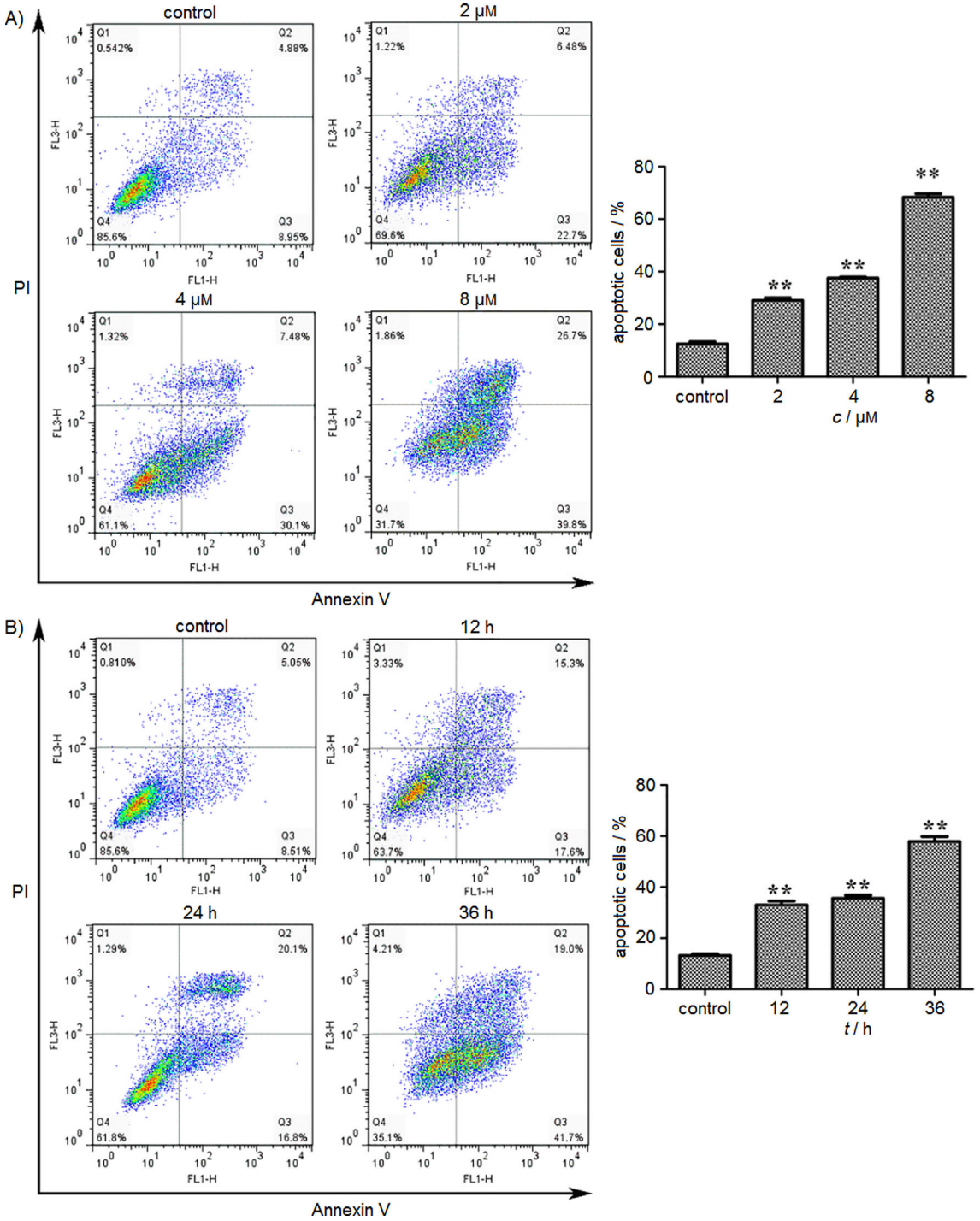


Figure 1. Apoptosis study of PMMB-259 tested on MCF-7 cells. A) Cells treated with various concentrations (0, 2, 4, and 8 μM) of PMMB-259 for 24 h were collected and analyzed. B) Cells treated with 4 μM PMMB-259 at different time points (0, 12, 24, and 36 h) were collected and analyzed. The percentage of early apoptotic cells is shown in the lower right quadrant (Annexin V–FITC-positive/PI-negative cells), and late apoptotic cells are located in the upper right quadrant (Annexin V–FITC-positive/PI-positive cells). Images are representative of three independent experiments. Data are the mean ± SD of three independent experiments; $*p < 0.05$, $**p < 0.01$.

zation just like colchicine, and these results further confirm that the observed effects are due to its interaction with tubulin, resulting in a prolonged mitotic arrest that ultimately leads to cell death.

Molecular docking

To gain a better understanding into the binding mode of the synthesized compounds, we proceeded to examine the interaction of all compounds with the crystal structure of tubulin (PDB ID: 1SA0) using the Discovery Studio 3.5 software pack-

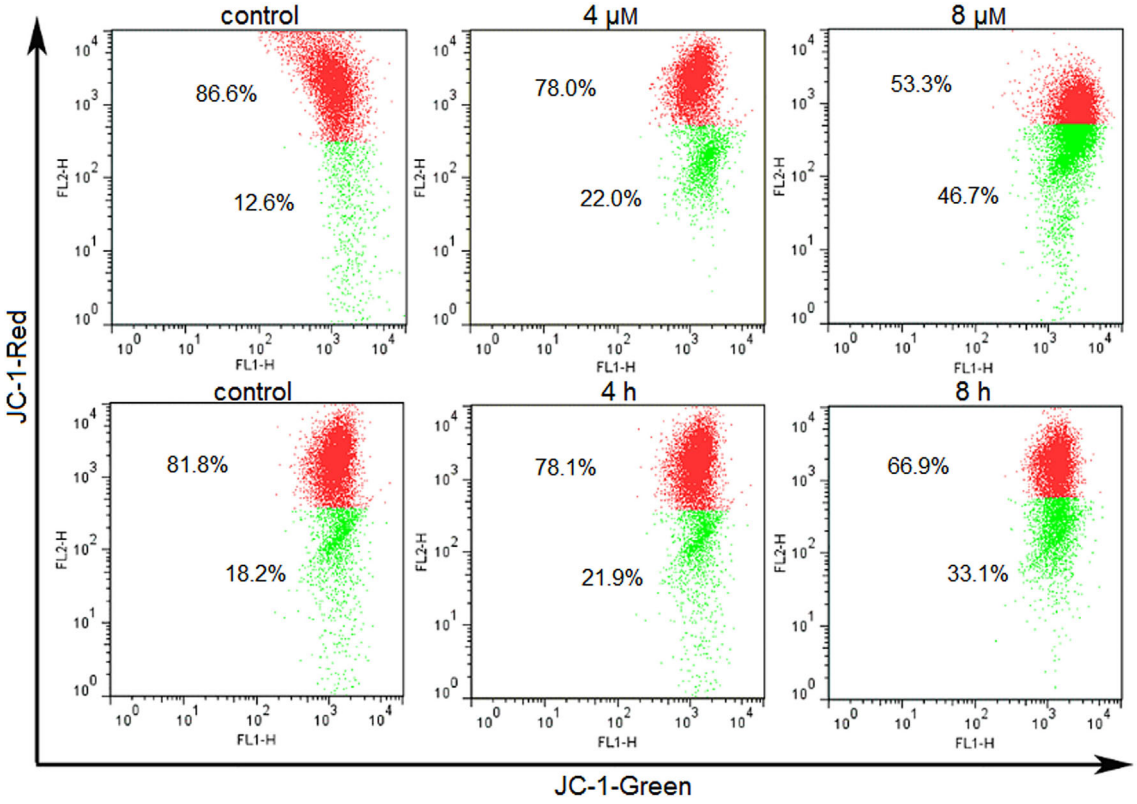


Figure 2. Mitochondrial transmembrane potential was analyzed in MCF-7 cells by JC-1 staining.

age (Accelrys, Co. Ltd). Molecular docking was performed by inserting the compounds into the colchicine binding site of tubulin, and the general workflow is described in the Experimental Section below. The CDOCKER_INTERACTION_ENERGY values of molecular docking are listed in Table 1. It is clear that PMMB-259 shows the best interaction energy of all the synthesized compounds, with a calculated interaction energy value of -64.6074 kcal mol^{-1}. The model of PMMB-259 docked with tubulin is depicted in Figure 5. In the binding model, PMMB-259 is nicely bound to the colchicine binding site of tubulin by three hydrogen bonds with Ser178, Tyr202, and Lys254. These molecular docking results, along with the biological assay data, suggest that PMMB-259 is a potential inhibitor of tubulin polymerization.

Conclusions

In conclusion, a series of novel chalcone-containing shikonin derivatives, PMMB-248–PMMB-265, were designed, synthesized, and evaluated as potential inhibitors of tubulin polymerization. Most of them showed potent antiproliferative activity, and PMMB-259 displayed the most potent activity against tubulin polymerization (IC_{50}: 2.98 ± 0.53 μM) and MCF-7 cell proliferation (IC_{50}: 2.36 ± 0.32 μM). Furthermore, mechanism of action studies proved that PMMB-259 can induce apoptosis in MCF-7 cells, reduce the mitochondrial membrane potential, cause an accumulation of cells in the G_2/M phase of the cell cycle, and effect severe disruption of the microtubule system in a manner similar to that of the positive control compound, colchicine. According to the analysis of the binding model of PMMB-259 with tubulin, three hydrogen bonds were observed with residues in the colchicine binding site, which may contribute to its anti-tubulin polymerization activity. Our findings indicate that PMMB-259 could be a lead molecule for further research into tubulin polymerization inhibitors.

Experimental Section

Materials and measurements: All chemicals (reagent grade) used were purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Paclitaxel and colchicine were purchased from Sigma–Aldrich (St. Louis, MO, USA). All ^{1}H NMR spectra were recorded on a Bruker DPX 500 model spectrometer in $CDCl_3$, and chemical shifts (δ) are reported as parts per million (ppm). ESI-MS spectra were recorded a Mariner System 5304 mass spectrometer. Melting points were determined on an XT4 MP apparatus (Taike Corp., Beijing, China). Thin-layer chromatography (TLC) was performed on silica gel plates (silica gel 60 GF_{254}) and visualized by UV light (λ 254 nm). Column chromatography was performed using silica gel (200–300 mesh) eluting with ethyl acetate and petroleum ether (bp: 30–60 °C). The Annexin V–FITC Apoptosis Detection Kit (A211-01/02) was purchased from Vazyme Biotech Co. Ltd. (Nanjing, China); the JC-1 Mitochondrial Membrane Potential Assay Kit (C2006) was purchased from Beyotime Biotech (Shanghai, China); the Cell-Cycle Assay Kit (A411-01/02) was purchased from Vazyme Biotech Co. Ltd. (Nanjing, China).

General procedure for the synthesis of compounds PMMB-248–PMMB-265: A mixture of 4-hydroxybenzaldehyde (10 mmol) and anhydrous potassium carbonate (15 mmol) were dissolved in ace-

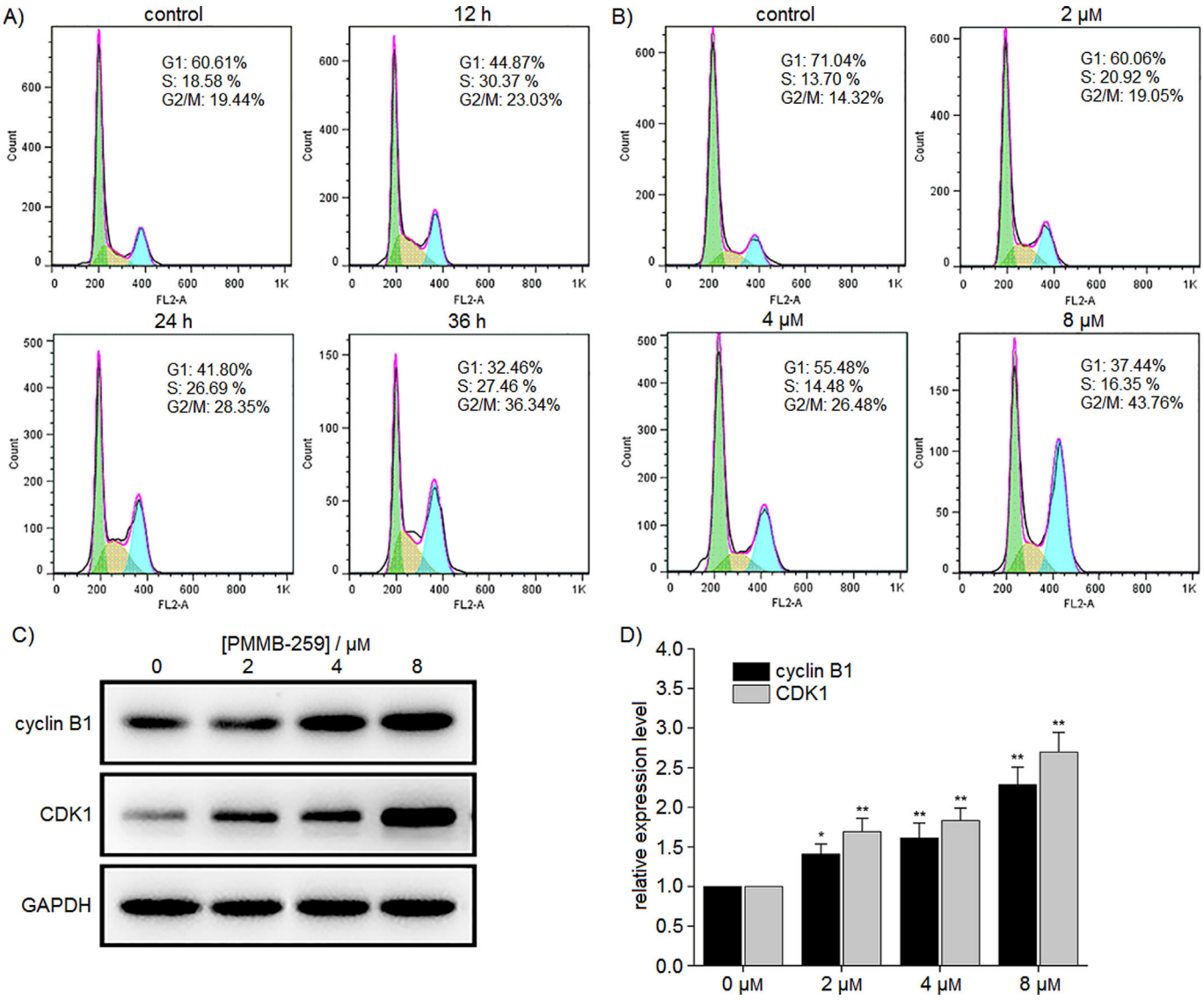


Figure 3. Effect of PMMB-259 on the cell-cycle distribution of MCF-7 cells. A) Cells treated with 4 μM PMMB-259 at different time points (0, 12, 24, and 36 h). B) Cells treated with various concentrations (0, 2, 4, and 8 μM) of PMMB-259 for 24 h were collected and disposed for detection. C) The expression of cyclin B1 and CDK1 were detected by western blot analysis. D) Relative expression levels were analyzed by ImageJ software.

tone, and ethyl bromoacetate (12 mmol) was thereafter added dropwise. The reaction mixture was held at reflux for 8 h, and the precipitate was filtered before vacuum distillation. The solvent was then concentrated, water (150 mL) was added, and the solution was adjusted to pH 5.0 with dilute HCl. Product extraction was carried out with ethyl acetate, dried over Na_2SO_4, and the organic layer was then concentrated in vacuo to afford compound **A1**.

A mixture of **A1** (5 mmol), substituted acetophenones (6 mmol) and sodium hydroxide (10 mmol) in ethanol (40 mL) was stirred at 0 °C for 2 h. After completion of the reaction, the precipitate was filtered and washed with ethanol three times to furnish compounds **B1**–**B18**. Without further purification, a mixture of **B1**–**B18** (1 mmol), sodium hydroxide (1.5 mmol) in ethanol (20 mL) and water (10 mL) was stirred at 90 °C for 6 h. The solution was cooled, and the solution was adjusted to pH 3.0 by diluted HCl. The precipitated solid was collected by filtration and recrystallized form ethanol to afford pure **C1**–**C18**.

Shikonin, **C1**–**C18**, 4-dimethyaminopyridine (DMAP), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were dissolved in dichloromethane. The reaction was stirred on an ice bath for 1 h and monitored by TLC. After completion of the reaction, the products PMMB-248–PMMB-265 were obtained and purified by column chromatography.

Cell culture: Human cervical cancer (HeLa), human breast cancer (MCF-7), human lung adenocarcinoma epithelial (A549), human hepatic (L02) and human kidney epithelial (293T) cell lines were purchased from Nanjing Keygen Technology (Nanjing, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone; high glucose) with L-glutamine supplemented with 10% fetal bovine serum (FBS, BI), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (Hyclone), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Antiproliferation assays: The antiproliferative activities of the prepared compounds against HeLa, MCF-7, A549, L02, and 293T cell lines were evaluated using a standard 3-(4,5-dimethylthiazol-2-yl)-

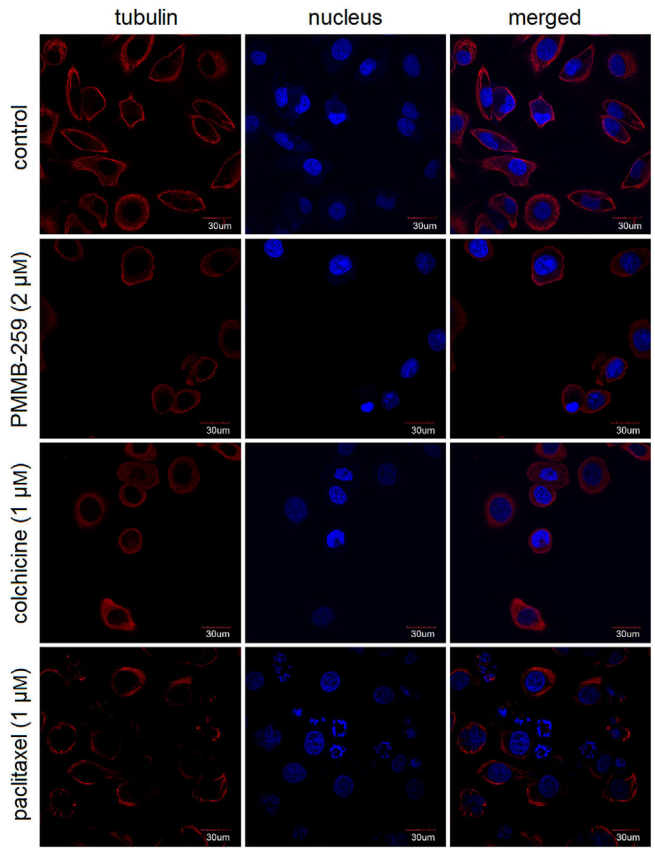


Figure 4. Effect of PMMB-259 on the tubulin network of MCF-7 cells. Microtubules labelled with rhodamine (red) and nuclei tagged with DAPI (blue) were observed by confocal microscopy.

2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay with some modification. Cell lines were grown to log phase in DMEM supplemented with 10% FBS. Cell suspensions were prepared, and 100 μL per well were dispensed into 96-well plates, giving 10^4 cells per well. The subsequent incubation was carried out at 37 °C under 5% CO_2 atmosphere for 24 h to allow the cells to reattach. Stock solutions of the synthesized compounds and positive control reference drugs (20 mM) in DMSO were prepared. Cells were then treated with the target compounds at 0, 1, 10, and 100 μM in the presence of 10% FBS for 24 h. Cell viability was then assessed by conventional MTT reduction assay, carried out strictly according to the manufacturer's instructions (Sigma). The absorbance (OD_{570}) was read on an ELISA reader (Tecan, Austria). In all experiments, three replicate wells were used for each drug concentration. Each assay was carried out at least three times.

Tubulin polymerization assays: Tubulin was purified from bovine brain as described previously. To characterize the effect of the compounds on tubulin assembly in vitro, compounds (ranging from 0.1 to 50 μM) were incubated with bovine brain tubulin (10 μM) in glutamate (0.8 M, pH 6.6 with HCl) at 30 °C and then cooled to 0 °C. After GTP (0.4 mM) was added, the mixtures were transferred to cuvettes at 0 °C in a recording spectrophotometer and warmed up to 30 °C. Finally, the assembly of tubulin was observed turbidimetrically at λ 350 nm. The IC_{50} value is defined as the compound concentration required to inhibit the extent of assembly by 50% after 20 min incubation.

Apoptosis assays: Approximately 10^5 cells per well were plated in a 24-well plate and allowed to adhere. The medium was then replaced with fresh culture medium containing PMMB-259. Untreated wells received an equivalent volume of ethanol (<0.1%). After treatment, they were trypsinized, washed in phosphate-buffered saline (PBS) and centrifuged at 2000 rpm (300×*g*) for 5 min. The pellet was then resuspended in 500 μL staining solution (containing 5 μL Annexin V–FITC and 5 μL PI in binding buffer), mixed gently and incubated for 15 min at room temperature in the dark. The samples were then analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Mitochondrial membrane potential evaluations: Mitochondrial transmembrane potential ($\Delta\Psi_m$) was detected using a JC-1 mitochondrial membrane potential assay kit (Beyotime Biotech), following the manufacturer's protocol. After treatment, the cells were incubated at 37 °C for 20 min with 5 μg mL^{-1} JC-1 (5,5,6,6-tetrachloro-1,1,3,3- tetraethylbenzimidazolylcarbocyanine iodide), then washed twice with PBS and placed in fresh medium without serum. The samples were analyzed using a FACSCalibur cytometer (Becton Dickinson) equipped with a 488 nm argon laser.

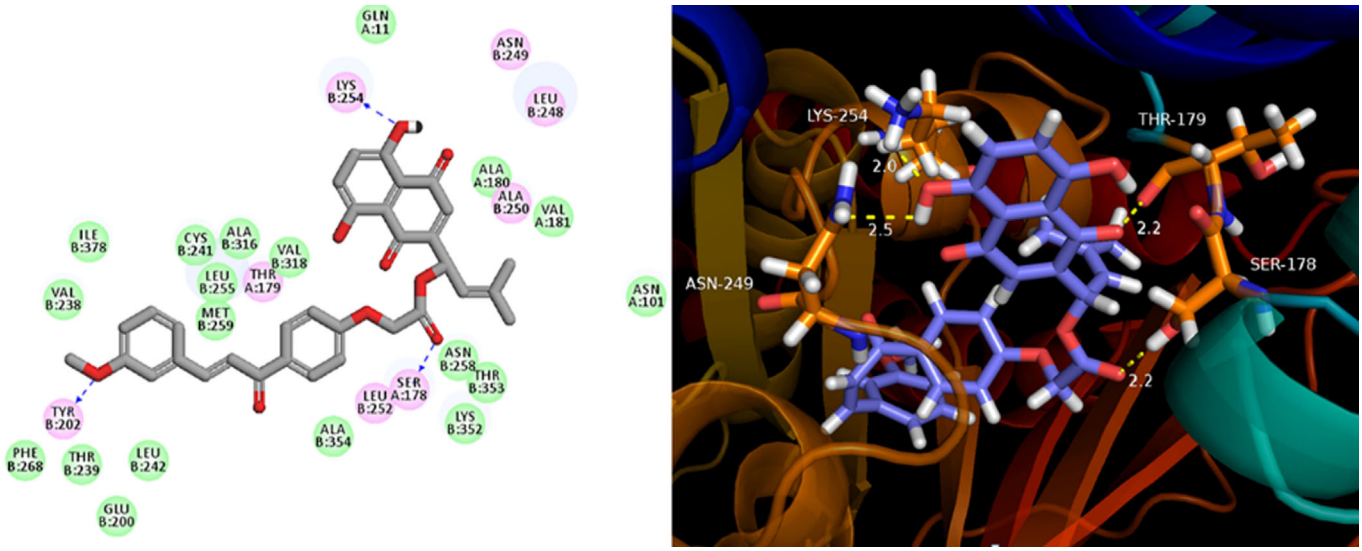


Figure 5. Molecular docking 2D and 3D modeling of PMMB-259 with the colchicine binding site of tubulin.

Cell-cycle analysis: Cells were plated in six-well plates (10^6 cells per well) and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated with PMMB-259. Cells were centrifuged at 2000 rpm (300×*g*) at 4 °C for 5 min after treatment, and then fixed in 70% ethanol at 4 °C for at least 12 h and subsequently re-suspended in PBS containing 0.1 mg mL^{-1} RNase A and 5 mg mL^{-1} PI. The cellular DNA content, for cell-cycle distribution analysis was measured by flow cytometry, plotting at least 10000 events per sample. The percentage of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle were determined using Flowjo 7.6.1 software.

Confocal microscopy: MCF-7 cells were grown on coverslips to 70% confluence and incubated with 2 μM PMMB-259, 1 μM colchicine, or 1 μM paclitaxel for 24 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 for a further 10 min. Cells were blocked with 3% BSA for 1 h, and then washed once with PBS, and treated with anti-tubulin antibody (1:200, Cytoskeleton, Inc.) in 3% BSA at 4 °C. Then 200 μL Cy3-labeled goat anti-mouse IgG (H + L) (1:500, Cytoskeleton, Inc.) was added to each coverslip, which were previously washed with 0.5% Triton X-100 (incubation for 5 min), in 5% BSA and incubated at room temperature followed by DAPI (5 μg mL^{-1}). Cells were then observed under an Olympus confocal microscope, and data were analyzed using FV-10-ASW 1.7 viewer software.

Docking simulations: Molecular docking of the compounds into the X-ray crystal structure of tubulin (PDB ID: 1SA0) was carried out using Discovery Studio software (version 3.5) as implemented through the graphical user interface DS-CDOCKER protocol. The three-dimensional structures of the aforementioned compounds were constructed using Chem.3D ultra 14.0 software [Chemical Structure Drawing Standard; Cambridge Soft Corp., USA (2010)], and then energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of 0.10. The crystal structures of the protein complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound water molecules and ligands were eliminated from the protein. Molecular docking was performed by inserting molecules into the binding pocket of 1SA0 based on the binding mode. The types of interactions of the docked protein with the ligand-based pharmacophore model were analyzed after the end of molecular docking.

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Keywords: antitumor agents · docking studies · shikonin · tubulin polymerization

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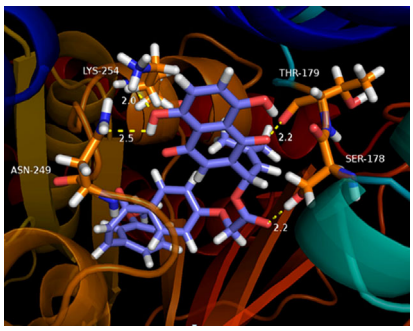
Combined effect: A series of chalcone-containing shikonin derivatives were designed, synthesized, and evaluated as potential inhibitors of tubulin polymerization. Most showed potent antiproliferative activity, and studies into the mechanism of action revealed induction of apoptosis, reduction of mitochondrial membrane potential, accumulation of cells in the G_2/M phase, and severe disruption of the microtubule system to an extent similar to that of the reference compound colchicine.



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Design, Synthesis, and Biological Evaluation of Chalcone-Containing Shikonin Derivatives as Inhibitors of Tubulin Polymerization