This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This Accepted Manuscript will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Abstract

A novel series of acetyl-β-D-thio-glycoside modified shikonin derivatives were designed and synthesized and investigated for inhibition of cell proliferation against MG63, MCF-7, B16-F10, HepG2, MDA-231, L02, VERO and MCF10A cell lines. The biological study showed that most single, di- and tri-substituted shikonin derivatives exhibited better anti-proliferative activities against the five cancer cell lines but lower cytotoxic activity against normal cells than shikonin itself. Notably, compared to shikonin, IIb displayed much stronger anti-proliferative effect among them. Furthermore, the inhibition of tubulin polymerization results indicated that IIb showed the most potent anti-tubulin activity (IC$_{50}$ = 4.67±0.433 µM), which was compared with shikonin (IC$_{50}$ = 16.8±0.625 µM) and colchicine (IC$_{50}$ = 3.83±0.424 µM). Docking simulation, confocal microscopy and western blot results further confirmed that IIb can cause cell arrest in G2/M phase and induce cell apoptosis via binding to the active site of tubulin and inhibiting tubulin polymerization.

Keywords:
acetyl-β-D-thio-glycoside; anti-cancer; anti-tubulin; shikonin derivatives

Introduction

Microtubules are key cytoskeletal filaments which are involved in various crucial cellular processes such as cell motility, cell division, shape maintenance and vesicle transport$^{1,2}$. Inhibiting tubulin polymerization or interfering with microtubule disassembly will interrupt the dynamic equilibrium, leading to cell cycle arrest or apoptosis induction$^3$. Due to its significant role in the cellular functions, microtubule has been a proven molecular target for cancer chemotherapeutic agents$^4,5,6$. Acharya et al.$^6$ reported that naphthazarin is a microtubules inhibitor in cell-free system and in A549 cells. Naphthazarin plays a role as microtubule depolymerizing agent which induces both apoptosis and autophagy in A549 lung cancer cells.

Shikonin and its derivatives are active naphthoquinone compounds that are isolated from the root of Chinese herbal medicine Lithospermum erythrorhizon$^7$, have been reported to possess numerous biological activities, such as anti-oxidant activities$^8$, anti-inflammatory$^9$, inhibiting adipogenesis$^{10}$, anti-HIV$^7$ and anti-cancers activities$^{11-13}$. However, as a potential anti-cancer drugs, it is poorly soluble and believed to exert strong cytotoxic effects on normal cells$^{14}$. Hence, a large number of researchers are dedicated to synthesizing and preparing some new and effective shikonin derivatives.
As is summarized in Fig. 1, based on the shikonin skeleton, compound B, C and G used methyl to substitute hydroxyl hydrogen atoms\textsuperscript{14-16}; compound E not only replaced the phenol hydroxyl with methyl, but also completely changed the structure of its side chain to give a better anti-cancer agent, 2-hydm-DMNQ-S317; compound F and H completely changed the side chain to give the novel shikonin derivatives\textsuperscript{18-20}; compound D linked the ester group or O-glycoside to obtained functional molecules\textsuperscript{15, 16, 21-24} and compound I was added to the fragments which contain amino or thiol groups derivatization of acrlyshikonin\textsuperscript{25, 26}. To sum up, by changing the chain line or replacing a number of structural elements based on the 5, 8-Dihydroxy-1, 4-naphthoquinone ring, a series of newly functional compounds can be obtained. However, most compounds among them did not resolve the toxicity against normal cells or solubility problems. Based on these, we hope to get more effective structures of shikonin derivatives.

After investigation, we found that glycoside drugs consisting of more glycoside present better solubility and play an important role in the treatment of diseases\textsuperscript{27-29}. Su et al.\textsuperscript{22} synthesized some glycoside shikonin derivatives which use O atom as linkages and confirmed that those compounds are good candidates of anti-tumor agents. In addition, He et al.\textsuperscript{28} synthesized some aminoglycosides of shikonin or alkalamin which improve their DNA-binding affinity. Zhao et al.\textsuperscript{30} reported that the introduction of a thioether functional group at the 1\textsuperscript{st}-position in the side chain of shikonin is associated with an increase in cytotoxicity. Therefore, we used different methods to obtain fifteen glycoside shikonin derivatives which used S atom as linkages. And the results indicated that all of the shikonin acetyl-\(\beta\)-D-thiglycosyl derivatives exhibited stronger if not the same cytotoxicity against cancer cell lines than shikonin itself and arouse considerable interest of us to further study the underlying mechanism.

### Results and discussion

The synthetic routes for the novel thio-glycosyl shikonin derivatives Ia-IIId is outlined in Scheme 1. These compounds were synthesized from \(\beta\), \(\beta\)-dimethylacrylshikonin and acetyl-\(\beta\)-D-thio-glycosides. The desired acetyl-\(\beta\)-D-thio-glycoside could be obtained by three steps (acytlayting, brominating and thionation reaction\textsuperscript{31-32}) from six kinds of glycosides. Acetyl-\(\beta\)-D-thio-glycoside and \(\beta\), \(\beta\)-dimethylacrylshikonin were dissolved in ethanol to furnish the corresponding shikonin derivatives. The reaction occurred at the side chain ester group, benzene ring and quinine ring of \(\beta\), \(\beta\)-dimethylacrylshikonin, respectively. All the target compounds were characterized by IR, \(^1\)H NMR, elemental analysis and mass spectrum, which were in full accordance with their depicted structures.

All synthesized shikonin derivatives Ia-IIId were evaluated for their anti-proliferative activities against five cancer cell lines, human osteosarcoma cell (MG63), human breast cancer cell (MCF-7), melanoma cell (B16-F10), human hepatoma cell (HepG2), human breast cancer cell (MDA-231) and three normal cell lines, normal human liver cells (L02), African green monkey kidney cell (VERO) and normal breast cells (MCF-10A). The results are summarized in Table 1. A number of acetyl-\(\beta\)-D-thio-glycoside substituted shikonin compounds which bearing the same dithiocarbamate (DTC) moiety showed remarkable effects on anti-proliferation.

Generally speaking, all of the synthetic compounds have significant anti-proliferative effects on MG63, MCF-7, B16-F10 and MDA-231 cancer cell lines except HepG2. However, IIb (IC\textsubscript{50} = 1.22±0.110 \(\mu\)M) not only showed good anti-proliferative activities against MG63, MCF-7, B16-F10 and MDA-231, but also have good inhibition effect on HepG2. For glycoside, it seems that the compounds with different glycosides exhibited slightly more potent activities in order of xylene (b) > glucose (a) > galactose (c), mannose (d) > maltose (e) > arabinose (e). Among the three compounds which contain xylene moieties (Ib, IIb, IIIb), IIb with two xyloses showed the best anti-proliferative activities; IIIb with three xyloses took second place and Ib with single xylene was the worse.

According to MTT assay results, IIb (IC\textsubscript{50} = 1.22±0.221 \(\mu\)M, 2.37±0.165 \(\mu\)M and 1.22±0.110 \(\mu\)M for MG63, MCF-7 and HepG2, respectively) showed the best anti-proliferative activities against MG63, MCF-7 and HepG2 cell lines. Meanwhile, IIIB (IC\textsubscript{50} = 3.75±0.273 \(\mu\)M) has the best anti-proliferative effect on B16-F10 cell line and Ie (IC\textsubscript{50} = 4.97±0.37 \(\mu\)M) is the best proliferation inhibitor against MDA-231 cell line. In addition, the IC\textsubscript{50} values of all the compounds against three normal cell lines, L02, VERO and MCF-10A, indicated that all of them have hardly any cytotoxicity against normal cells.

Then we performed the tubulin assembly assay to examine whether the compounds interact with tubulin and inhibit tubulin polymerization in vitro. As is shown in Table 1, Ia, IIb, and IIb showed strong inhibitory effect and their 50\% tubulin polymerization inhibition are 10.0±0.311 \(\mu\)M, 4.67±0.433 \(\mu\)M, and 7.57±0.742 \(\mu\)M, respectively. Obviously, IIb displayed the most potent anti-tubulin polymerization activity. These findings indicate a continuing impairment of cell division and proved IIb is a potent anti-tubulin agent.

For better understanding of the potency of IIb and guide further SAR studies, we examined the interaction of IIb with tubulin...
bound to the colchicine binding site of tubulin via four hydrogen bond with CYS 241 (distance = 2.19 Å), ASN 258 (distance = 2.07 Å), ASN 101 (distance = 2.35 Å) and LYS 254 (distance = 1.99 Å). And in the second binding mode, Ilb can also bind to the colchicine binding site of tubulin well via three hydrogen bond with LYS 254 (distance = 2.36 Å), ASN 258 (distance = 2.36 Å), ASN 101 (distance = 1.96 Å) and two π bonds with LYS 352 (distance = 6.72 Å and 6.44 Å). For Fig. 2(B) and 2(D), the 3D models of the interaction between Ilb and the colchicine binding site of tubulin was depicted. The molecular docking results argue that Ilb may be a potential anti-tubulin agent via binding to the colchicine binding site of tubulin and then inhibit tubulin polymerization.

To further determine the mechanism by which acetyl-β-D-thioglycoside modified shikonin derivatives induced cell death, we assessed cell cycle distribution of HepG2 cells by flow cytometry. Treatment of HepG2 cells with Ilb for different time could arrest cells at G2/M transition. This study further showed that treatment with Ilb led to an obvious G2/M arrest in concentration- and time-dependent manners in HepG2 cells as shown in Fig. 3. Treatment of HepG2 cells with varying doses of Ilb for 24 hours resulted in the increased accumulation of the cells in G2/M phase (Fig. 3(A)). Incubation of cells with 3 µM Ilb for 24 hours caused 17.39% enrichment of cells in G2/M phase compared with the control. When the drug concentration increases to 10 µM, 26.45% cells are arrest in G2/M phase. In a time-dependent experiment, maximum accumulation of cells in the G2/M phase was observed after treatment of cells with 3 µM Ilb for 48 hours (Fig. 3(B)).

We also treated HepG2 cells with varying concentrations of Ilb and analyzed cells for changes in apoptotic markers by flow cytometry in vitro. The results are shown in Fig. 4(A), after treated with increasing concentrations of Ilb, the percentage of apoptotic cell has a significant increase. Meanwhile, the time-dependent assay result which was shown in Fig. 4(B) indicates that cell apoptosis presents a time-dependent manner.

The preliminary results make us to speculate that Ilb causes HepG2 cells arrest at G2/M phase and thus induces cell apoptosis possibly by inhibiting the polymerization of tubulin.

To assess the effect of drug on cell microtubules, confocal microscopy analyses on HepG2 cells was carried out. The results were shown in Fig. 5. After treating cells with 3 µM Ilb for 12 hours and 24 hours, we found the morphology of cells changed obviously. In the control group, cells are round and the cytoskeleton is integrated. When treated with drug for 12 hours, cytoskeleton is affected significantly. Drug inhibited the formation of spindle, which lead to the chromosome cannot move toward the poles and finally formed multinuclear cells. After 24 hours of drug treating, we found that the entire cytoskeleton suffered serious damage; cell membranes and nucleus are deformed and eventually induce apoptosis. These results indicate that Ilb can inhibit the formation of microtubules during mitosis of HepG2 cells, and this is in line with the molecular docking simulation results.

In order to further investigate its’ effect on microtubule organization, we did an in vitro microtubule assembly assay. As shown in Fig. 6, HepG2 cells were treated with 3 µM Ilb, 1 µM paclitaxel, and 1 µM colchicine for 24 hours, respectively. With comparison, Ilb and colchicine caused inhibition of microtubule assembly. However, paclitaxel significantly induced promotion of tubulin polymerization. To sum up, our results demonstrate that Ilb induced mitotic arrest and inhibited the polymerization of microtubules in HepG2 cells.

**Experimental**

**General information and materials**

The eluates were monitored using TLC. Melting points (uncorrected) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). Infrared (IR) spectra were recorded on a NEXUS870 spectrometer (NICOLET Co. USA), using KBr pellet (solid). 1H NMR spectra were determined on Varian Mercury-300 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards, Chemical shifts are reported in ppm (d), Elemental analyses were performed on a CHN-O-Rapid instrument and were within 0.4% of the theoretical values. ESI mass spectra were obtained on a Mariner Bio-spectrometry Workstation (ESI-TOF) mass spectrometer. Reagents and solvents were commercially available. Solvents were dried and purified using standard techniques. Column chromatography was run on silica gel (200-300 meshes) from Qingdao Ocean Chemical Factory. Paclitaxel and colchicine were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO. MgCl₂, EGTA, NPM-40, PMSF, Aprotinin, amino caproic acid, benzamidine and Tris-HCl, pH 6.8 were purchased from Sangon (Shanghai, China).
China) and dissolved in PBS. β-tubulin antibody were purchased from Sino Biological Inc. (Beijing, China).

General procedure for Preparation of β, β-dimethylacrylshikonin

In 5 litre beaker, 1 Kg of the crushed herb Lithospermum erythrorhizon (100 meshes) was soaked in petroleum ether for two days. Then the residue was filtered and washed with petroleum ether twice; the organic layer was collected and the solvent was evaporated to dryness. After that, the residue was dissolved in 200 mL of petroleum ether and was dumped into the 500 mL beaker to be slowly crystallization. Until a large number of the red powder appeared at the bottom of the beaker, we filtrated and washed residua with petroleum ether (20 mL×2). Finally, the residua was dried in the air to given red power of β, β-dimethylacrylshikonin.

General procedure for the preparation thio-glycoside (Ia-II, Ila-IIId and IIla-IIId)

Adding β, β-dimethylacrylshikonin (1 mmol dissolved in 15 mL EtOH) to a solution of acetyl-β-D-thio-glycoside (1 mmol dissolved in 15 mL EtOH) at room temperature and the resultant mixture was stirred at room temperature for 30 minutes under a nitrogen atmosphere. Until no further changes in TLC, the reaction was stop. Removal of solvent and purification of the residue by column chromatography gave the product. Purification by silica gel chromatography (ethylacetate: petroleum ether V/V = 1:2) afforded the shikonin thio-glycoside. The disubstituted method is similar to the above one, while just changing thio-glycoside moles to 2 mmol and reaction system’s temperature to 40 °C, then the corresponding dissubstituted compounds can be obtained. The tri-substituted product can be obtained by changing thio-glycoside moles to 3 mmol, reaction system’s temperature to 60 °C and reaction time to 1 hour, then separated the target compounds by column chromatography solvent ratio (ethylacetate: petroleum ether V/V = 1:1).

2, 3, 4, 6-tetra-O-acetyl-1-thio-β-D-glucopyranosyl shikonin (Ia)

Red powder, yield 20%; mp: 97.5-99.7 °C; IR (KBr) 3436, 2927, 1752, 1610, 1570, 1455, 1369, 1225, 1053 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.61(s, 3H), 1.68(s, 3H), 2.02(s, 9H), 2.05(s, 3H), 2.59(s, 2H), 3.62-3.65 (m, 1H), 4.02(d, J = 11 Hz, 1H), 4.40(t, J = 7 Hz, 1H), 4.47(d, J = 10 Hz, 1H), 4.55(t, J = 7 Hz, 1H), 5.00-5.11 (m, 3H), 5.17-5.21 (m, 1H), 7.12(s, 1H), 7.22(s, 2H), 12.45 (s, 1H), 12.66(s, 1H); ESI-TOF, calec for C₃₀H₃₄O₁₃S ([M+Na⁺]⁺) 657.1720, found 657.1745. Anal. Calec for C₃₀H₃₄O₁₃S: C, 56.77; H, 5.40; O, 32.77; S, 5.05. Found: C, 69.31; H, 6.71; O, 23.94, S, 5.01.

2, 3, 4, 6-tetra-O-acetyl-1-thio-β-D-galactopyranosyl shikonin (Ib)

Red powder, yield 25%; mp: 86.3-88.7 °C; IR (KBr) 3444, 2925, 1755, 1608, 1572, 1435, 1372, 1223, 1053 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.60(s, 3H), 1.67(s, 3H), 2.04(s, 9H), 2.56-2.62(m, 2H), 3.61-3.64(m, 1H), 4.38-4.47(m, 1H), 4.57(d, J = 8 Hz, 1H), 4.84(t, J = 8 Hz, 1H), 4.87-4.91(m, 1H), 4.96(t, J = 8 Hz, 1H), 5.06(s, 1H), 5.09-5.12(m, 1H), 7.04(s, 1H), 7.22(s, 2H), 12.45(s, 1H), 12.62(s, 1H); ESI-TOF, calec for C₂₇H₃₉O₁₁S ([M+Na⁺]⁺) 585.1509, found 585.1573. Anal. Calec for C₂₇H₃₉O₁₁S: C, 57.64; H, 5.37; O, 31.28; S, 5.70. Found: C, 56.89; H, 6.01; O, 31.94; S, 5.75.

2, 3, 4, 6-tetra-O-acetyl-1-thio-β-D-mannopyranosyl shikonin (Ic)

Red powder, yield 18%; mp: 95.0-98.2 °C; IR (KBr) 3436, 2927, 1752, 1610, 1570, 1455, 1369, 1225, 1053 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.62(s, 3H), 1.67(s, 3H), 2.00(s, 9H), 2.01(s, 3H), 2.52(s, 2H), 3.60-3.62 (m, 1H), 4.05(d, J = 10.5 Hz, 1H), 4.37(t, J = 6.0 Hz, 1H), 4.47 (d, J = 9.5 Hz, 1H), 4.58(t, J = 7 Hz, 1H), 4.95-5.08 (m, 3H), 5.13-5.19 (m, 1H), 7.10(s, 1H), 7.18(s, 2H), 12.38(s, 1H), 12.63(s, 1H); ESI-TOF, calec for C₃₀H₃₄O₁₁S ([M+Na⁺]⁺) 657.1720, found 657.1764. Anal. Calec for C₃₀H₃₄O₁₁S: C, 56.77; H, 5.40; O, 32.77; S, 5.05. Found: C, 69.31; H, 6.71; O, 23.94, S, 5.01.

2, 3, 4, 6-tetra-O-acetyl-1-thio-β-D-mannopyranosyl shikonin (Id)

Red powder, yield 16%; mp: 96.3-99.6 °C; IR (KBr) 3447, 2969, 1753, 1614, 1565, 1437, 1372, 1225, 1053, 975 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.58(s, 3H), 1.65(s, 3H), 2.02(s, 3H), 2.03(s, 3H), 2.04 (s, 3H), 2.05(s, 3H), 2.55(s, 2H), 3.60-3.62 (m, 1H), 4.00(d, J = 10 Hz, 1H), 4.35(t, J = 7.5 Hz, 1H), 4.48(d, J = 10.5 Hz, 1H), 4.58(t, J = 7.5 Hz, 1H), 5.05-5.13 (m, 3H), 5.20-5.25 (m, 1H), 7.14(s, 1H), 7.25(s, 2H), 12.48 (s, 1H), 12.69(s, 1H); ESI-TOF, calec for C₃₀H₃₄O₁₁S ([M+Na⁺]⁺) 657.1720, found 657.1756. Anal. Calec for C₃₀H₃₄O₁₁S: C, 56.77; H, 5.40; O, 32.77; S, 5.05. Found: C, 69.31; H, 6.71; O, 23.94, S, 5.01.

2, 3, 4-tri-O-acetyl-1-thio-β-D-arabinopyranosyl shikonin(ie)

Red powder, yield 11%; mp: 85.7-87.5 °C; IR (KBr) 3446, 2926, 2857, 1751, 1610, 1572, 1454, 1435, 1372, 1221, 1054 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.55(s, 3H), 1.65(s, 3H), 2.03(s, 3H), 2.04(s, 3H), 2.05(s, 3H), 2.50-2.60(m, 2H), 3.60-3.61 (m, 1H), 4.40(d, J = 8.5 Hz, 1H), 4.57(d, J = 7.5
2. 3, 6-tri-O-acetyl-(2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl)-1-thio-β-D-glucopyranosyl shikonin (II)

Red powder, yield 19%; mp: 103.1-105.6 °C; IR (KBr) 3441, 2938, 1753, 1683, 1652, 1611, 1436, 1370, 1229, 1050 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.59(s, 3H), 1.66(d, J = 7 Hz, 3H), 1.99-2.05(m, 15H), 2.01(d, J = 6 Hz, 6H), 2.58(s, 2H), 3.53-3.61(m, 1H), 3.92(t, J = 8.5 Hz, 2H), 3.96-4.01(m, 1H), 4.05(t, J = 22 Hz, 1H), 4.16-4.26(m, 1H), 4.32-4.40(m, 1H), 4.50(t, J = 10.5 Hz, 1H), 4.69-4.76(m, 1H), 4.82-4.86(m, 1H), 4.90(t, J = 10 Hz, 1H), 5.04(t, J = 9.5 Hz, 1H), 5.23(t, J = 9 Hz, 1H), 5.32(t, J = 10.5 Hz, 1H), 5.37(s, 1H), 7.08(d, J = 35 Hz, 1H), 7.23(s, 2H), 12.46(s, 1H), 12.64(s, 1H); ESI-TOF, calcld for C₄₂H₇₀O₃₇S ([M+Na]⁺) 945.2565, found 945.2652. Anal. Calcld for C₄₂H₇₀O₃₇S: C, 53.61; H, 5.46; O, 35.14; S, 3.43. Found: C, 53.61; H, 5.46; O, 35.14; S, 3.43.

3, 4, 6-tetra-O-acetyl-1, 6′ or 7′-di-thio-β-D-galactopyranosyl disubstituted shikonin (IIC)

Red powder, yield 11%; mp: 115.0-118.9 °C; IR (KBr) 3437, 2918, 1738, 1602, 1558, 1362, 1218, 1039 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.61(s, 3H), 1.65(s, 3H), 1.95-2.26(m, 24H), 2.60-2.65(m, 2H), 3.98-4.22(m, 6H), 4.48(s, 1H), 4.81(s, 1H), 4.91-5.08(m, 1H), 5.12-5.19(m, 1H), 5.36-5.56(m, 4H), 6.22(s, 1H), 6.98(s, 1H), 12.24(s, 1H), 12.76(s, 1H); ESI-TOF, calcld for C₄₄H₇₂O₃₆S₂ ([M+Na]⁺) 1019.2392, found 1019.2415. Anal. Calcld for C₄₄H₇₂O₃₆S₂: C, 53.01; H, 5.26; O, 35.30; S, 6.43. Found: C, 52.54; H, 5.76; O, 35.87; S, 6.54.

3, 4, 6-tetra-O-acetyl-1, 6′ or 7′-di-thio-β-D-mannopyranosyl disubstituted shikonin (IID)

Red powder, yield 20%; mp: 116.5-119.1 °C; IR (KBr) 3435, 2936, 1755, 1683, 1528, 1475, 1325, 1230, 1063, 980 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.61(s, 6H), 1.96(s, 3H), 2.04(d, J = 18 Hz, 6H), 2.11(d, J = 9 Hz, 6H), 2.17(t, J = 8 Hz, 6H), 2.26(s, 3H), 2.66(s, 2H), 3.47-3.61(m, 1H), 3.92(d, J = 17.25 Hz, 1H), 4.03-4.27(m, 4H), 4.45-4.59(m, 1H), 4.94-5.05(m, 2H), 5.16(d, J = 8 Hz, 2H), 5.21(t, J = 10.5 Hz, 1H), 5.29(t, J = 5 Hz, 1H), 5.38(d, J = 10.3 Hz, 1H), 5.67(s, 1H), 6.96(s, 1H), 7.34(s, 1H), 12.15(s, 1H), 12.68(s, 1H); ESI-TOF, calcld for C₄₄H₇₄O₃₆S₂ ([M+Na]⁺) 1019.2392, found 1019.2415. Anal. Calcld for C₄₄H₇₄O₃₆S₂: C, 53.01; H, 5.26; O, 35.30; S, 6.43. Found: C, 52.54; H, 5.76; O, 35.87; S, 6.54.

3, 4, tri-O-acetyl-1, 6′ or 7′-di-thio-β-D-arabinopyranosyl disubstituted shikonin (IIE)

Red powder, yield 21%; mp: 108.9-109.6 °C; IR (KBr) 3439, 2913, 2848, 1746, 1607, 1537, 1389, 1368, 1211, 1048 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.61(s, 3H), 1.68(s, 3H), 2.04(d, J = 4.5 Hz, 3H), 2.08(d, J = 12.5 Hz, 6H), 2.14(d, J = 3 Hz, 9H), 2.20(t, J = 11.25 Hz, 3H), 2.54-2.73(m, 2H), 3.75(d, J = 12.5 Hz, 1H), 3.84(t, J = 15.25 Hz, 1H), 4.18-4.22(m, 1H), 4.38-4.45(m, 1H), 4.99-5.10(m, 2H), 5.17(t, J = 12 Hz, 1H), 5.21-5.25(m, 2H), 5.35(d, J = 12.5 Hz, 1H), 5.39(d, J = 9.5 Hz, 2H), 5.45-5.52(m, 2H), 6.14(d, J = 5 Hz, 1H), 7.12(s, 1H), 7.21(s, 1H), 12.20(s, 1H), 12.76(s, 1H); ESI-TOF, calcld for C₄₃H₄₄O₁₈S₂ ([M+Na]⁺) 875.1996, found 875.1996. Anal. Calcld for C₄₃H₄₄O₁₈S₂: C, 53.51; H, 5.20; O, 33.77; S, 7.52. Found: C, 52.54; H, 5.76; O, 33.87; S, 7.54.
2, 3, 6-tri-O-acetyl-(2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl)-1,6'- or 7'-di-thio-β-D-glucopyranosyl disubstituted shikonin (IIf)

Red powder, yield, 18%; mp: 125.6-127.1 °C; IR (KBr) 3448, 2927, 1749, 1675, 1628, 1427, 1355, 1224, 1045 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.55(s, 3H), 1.64(s, 3H), 1.98-2.21(m, 42H), 2.60(d, J = 6.5 Hz, 2H), 3.54-3.79 (m, 2H), 3.93-4.11(m, 8H), 4.14-4.29 (m, 4H), 4.36-4.44(m, 2H), 4.55-4.69(m, 2H), 4.83-4.91(m, 3H), 4.97-5.17(m, 5H), 5.29-5.46(m, 6H), 7.00(s, 1H), 7.36(s, 1H), 12.17(s, 1H), 12.69(s, 1H); ESI-TOF, calcd for C₉₆H₇₇O₃₇S₇ ([M+Na]⁺) 1595.4082, found 1595.4123. Anal. Calcd for C₉₆H₇₇O₃₇S₇: C, 51.91; H, 5.38; O, 38.64; S, 4.08. Found: C, 50.87; H, 6.02; O, 38.87; S, 4.54.

2, 3, 4, 6-tetra-O-acetyl-1, 3, 6’ or 7’-tetra-thio-β-D-glucopyranosyl trisubstituted shikonin (IIia)

Red powder, yield, 26%; mp: 178.7-180.6 °C; IR (KBr) 3559, 2932, 2917, 1789, 1667, 1635, 1425, 1217, 1038, 927 cm⁻¹; ¹H NMR(300 MHz, CDCl₃) δ: 1.59(s, 3H), 1.66(s, 3H), 1.98-2.07(m, 30H), 2.13(s, 6H), 2.61(s, 2H), 3.59-3.62(m, 4H), 3.76-3.98 (m, 3H), 4.11-4.41(m, 5H), 5.03-5.10(m, 3H), 5.28-5.43(m, 5H), 5.56-5.81(m, 7H), 7.37(s, 1H), 12.58(s, 1H), 13.23(s, 1H); ESI-TOF, calcd for C₃₅H₇₂O₃₇S₇ ([M+Na]⁺) 1381.3063, found 1381.3198. Anal. Calcd for C₃₅H₇₂O₃₇S₇: C, 51.25; H, 5.19; O, 36.49; S, 7.54. Found: C, 50.69; H, 5.76; O, 36.87; S, 7.54.

2, 3, 4- tri-O-acetyl-1, 3, 6’ or 7’-tetra-thio-β-D-xylopyranosyl trisubstituted shikonin (IIb)

Red powder, yield, 28%; mp: 148.7-150.5 °C; IR (KBr) 3537, 2984, 2938, 1746, 1628, 1408, 1013, 910 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.58(s, 3H), 1.65(s, 3H), 2.04-2.09(m, 27H), 2.18(s, 2H), 2.58-2.67(m, 1H), 3.27-3.37(m, 2H), 3.73-3.83(m, 3H), 3.97-4.29(m, 2H), 4.44-4.50(m, 3H), 4.56-4.67(m, 1H), 4.83-5.03(m, 3H), 5.09-5.29(m, 2H), 5.48-5.86(m, 3H), 7.34(s, 1H), 12.59(s, 1H), 13.16(s, 1H); ESI-TOF, calcd for C₄₉H₇₉O₂₇S₇ ([M+] 1165.2429, found 1165.3647. Anal. Calcd for C₄₉H₇₉O₂₇S₇: C, 51.48; H, 5.11; O, 34.99; S, 8.41. Found: C, 50.69; H, 5.56; O, 35.27; S, 8.54.

2, 3, 4, 6-tetra-O-acetyl-1, 3, 6’ or 7’-tetra-thio-β-D-mannopyranosyl trisubstituted shikonin (IIId)

Red powder, yield, 19%; mp: 174.6-176.8 °C; IR (KBr) 3528, 2937, 2910, 1737, 1618, 1604, 1423, 1227, 1022, 929 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ: 1.54(s, 3H), 1.62(s, 3H), 1.91-2.08(m, 30H), 2.13(s, 6H), 2.58(s, 2H), 3.59-3.76(m, 3H), 3.98-4.09(m, 4H), 4.11-4.26(m, 3H), 4.41-5.03(m, 3H), 5.17-5.30(m, 6H), 5.48-5.85(s, 4H), 7.37(s, 1H), 12.58(s, 1H), 13.23(s, 1H); ESI-TOF, calcd for C₃₉H₇₉O₃₇S₇ ([M+Na]⁺) 1381.3063, found 1381.3276. Anal. Calcd for C₃₉H₇₉O₃₇S₇: C, 51.25; H, 5.19; O, 36.49; S, 7.08. Found: C, 50.69; H, 5.76; O, 36.87; S, 7.54.

Cell lines and culture conditions

The cell lines used in this study were following: MG63, MCF-7, B16-F10, HepG2, MDA-231, L02, VERO and MCF-10A were obtained from State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University. MG63, MCF-7 and B16-F10 cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine; L02, VERO and MCF-10A cell lines were maintained in DMEM (High Glucose) Mixture; MDA-231 cell line was maintained in L15 medium and all of the cell lines were supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂.

Anti-proliferation assay

The anti-proliferative activity of the prepared compounds against five cancer cell lines, MG63, MCF-7, B16-F10, HepG2 and MDA-231 and three normal cell lines, L02, VERO and MCF-10A were evaluated as described elsewhere with some modifications. Target tumor cell lines were grown to log phase in DMEM medium supplemented with 10% fetal bovine serum. After diluting to 2×10⁴ cells mL⁻¹ with the complete medium, 100 µL of the obtained cell suspension was added to each well of 96-well culture plates and then allowed to adhere for 12 hours at 37 °C, 5% CO₂ atmosphere. Tested samples at pre-set concentrations (0.1 µM, 1 µM, 10 µM, 100 µM) were added to 96 wells with shikonin as positive reference. After 24 hours exposure period, 20 µL of PBS containing 2.5 mg mL⁻¹ of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well. Plates were then incubated for further 4 hours, and then were centrifuged (1500 rpm at 4 °C for 10 minutes) to remove supernatant. 150 µL of DMSO was added to each well for coloration. The plates were shaken vigorously to ensure complete solubilization for 10 minutes at room temperature. The absorbance was measured and recorded on an ELISA reader (ELx800, BioTek, USA) at a test wavelength of 570 nm. In all experiments three replicate wells were used for each drug concentration. Each assay was carried out at least three times. The results are summarized in Table1.

Effects on tubulin polymerization

To evaluate the effect of the compounds on tubulin assembly in vitro, varying concentrations of IIb were pre-incubated with tubulin (10 µM) in glutamate buffer at 30 °C and then cooled to
0 °C. After addition of GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed-up to 30 °C and the assembly of tubulin was observed turbid metrically. The IC₅₀ was defined as the compound concentration that inhibited the extent of assembly by 50% after 20 minutes incubation.

Cell-Cycle Distribution by Flow Cytometry

HepG2 cells were plated in 6-well plates (5.0×10³ cells/well) and incubated at 37 °C for 24 hours. Exponentially growing cells were then incubated with the IIb at 3 µM and 10 µM. And in the time-dependent assays, exponentially growing cells were incubated with 3 µM IIb at 37 °C for 12 hours, 24 hours and 48 hours. Untreated cells (control) or cells treated with the compounds solvent (DMSO) were included. DMSO was used at the highest concentration used in the experiments. After then, cells were centrifuged and fixed in 70% ethanol at 4 °C for at least 12 hours and subsequently resuspended in PBS containing 0.1 mg mL⁻¹ RNase A and 5 µg mL⁻¹ propidium iodide (PI). Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry using FACScan cytometer. The percentage of cells in the G1, S and G2/M phases of the cell cycle and the percentage of cells in the sub-G1 peak were determined using the Flowjo 7.6.1 software after cell debris exclusion.

Flow Cytometric Analysis of Apoptosis

For Annexin V/PI assays, HepG2 cells were stained with Annexin V-FITC and PI and then monitored for apoptosis by flow cytometry. Briefly, 5×10⁴ cells were seeded in 6-well plates for 24 hours and then were treated with IIb (0-3 µM) for 0-48 hours. Then cells were collected and washed twice with PBS and stained with 5 μL of Annexin V-FITC and 2.5 μL of PI (5 μg mL⁻¹) in 1× binding buffer (10mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl₂) for 30 minutes at room temperature in the dark. Apoptotic cells were quantified using a FACScan cytometer. The graphical user interface AutoDock Tool Kit (ADT 1.4.6) was employed to set up the docking interface Auto-Dock Tool Kit (ADT 1.4.6)³⁴. The graphical user interface ADT was employed to set up the enzymes: all hydrogens were added, Gasteiger charges were calculated and nonpolar hydrogens were merged to carbon atoms. For macromolecules, generated pdbqt files were saved.

0.1 mg mL⁻¹ RNase A and 5 µg mL⁻¹ propidium iodide (PI). Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry using FACScan cytometer. The percentage of cells in the G1, S and G2/M phases of the cell cycle and the percentage of cells in the sub-G1 peak were determined using the Flowjo 7.6.1 software after cell debris exclusion.

In vitro microtubule assembly assay

We used an established method to measure soluble (depolymerized) and assembled (polymerized) tubulin³³. HepG2 cells (5×10³/flask) were seeded into the 75-T flask. Cells were exposed to paclitaxel (1 µM), colchicine (1 µM), and IIb (3 µM) for 24 hours. After treatment, cells were collected and washed twice with PBS then lysed at 37 °C for 5 minutes with 50 µL of hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 2 mM PMSF, 200 units ml⁻¹ aprotinin, 5 mM amino caproic acid, 1 mM benzamidine, and 20 mM Tris-HCl, pH 6.8). The cell lysates were centrifuged at 13,000 rpm for 10 minutes at 25 °C. The supernatants containing soluble (cytosolic) tubulin were separated from the pellets containing polymerized (cytoskeletal) tubulin. The pellets were resuspended in 100 µL of hypotonic buffer, sonicated on ice, mixed with 5×sample buffer, and heated for 5 minutes at 100 °C. Equal amounts of the two fractions were partitioned by SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with β-tubulin monoclonal antibody and secondary HRP-conjugated antibody. The blots were developed by using an ECL kit and Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY). All results are from three independent experiments.

Docking simulations

Molecular docking of IIb into the 3D X-ray structure of tubulin (PDB code: 1SA0) was carried out using the AutoDock software (version 4.0) as implemented through the graphic user interface Auto-Dock Tool Kit (ADT 1.4.6)³⁴. The graphical user interface ADT was employed to set up the enzymes: all hydrogens were added, Gasteiger charges were calculated and nonpolar hydrogens were merged to carbon atoms. For macromolecules, generated pdbqt files were saved.
The 3D structures of ligand molecules were built, optimized (PM3) level, and saved in Mol2 format with the aid of the molecular modeling program SPARTAN (Wavefunction Inc.). These partial charges of Mol2 files were further modified by using the ADT package (version 1.4.6) so that the charges of the nonpolar hydrogens atoms assigned to the atom to which the hydrogen was attached. The resulting files were saved as pdbqt files.

Auto-Dock software (version 4.0) was employed for all docking calculations. The AUTODOCKTOOLS program was used to generate the docking input files. In all docking a grid box size of 42×45×43 points in x, y, and z directions was built, and a population of 50 randomly placed individuals, a maximum number of 2.5×10^6 energy evaluations, and a maximum number of 2.7×10^4 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were chosen. Results differing by less than 0.5 Å in positional root-mean-square deviation (RMSD) were clustered together and the results of the most favorable free energy of binding were selected as the resultant complex structures.

### Abbreviations

IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectrum; TLC, thin layer chromatography; TMS, trimethylsilane; DMSO, dimethyl sulfoxide; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMSF, phenylmethane sulfonyl fluoride; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; BSA, bovine serum albumin.

### Conclusion

In our present work, a series of novel anti-tubulin polymerization agents (Ia-IIId) containing shikonin skeleton and acetyl-β-D-thio-glycoside moieties were synthesized and their biological activities were also evaluated. Among them, 11b with two xylose moieties exhibits potent anti-proliferating effect against HepG2 cell line (IC_{50}=1.22±0.110 µM), being comparable with shikonin (IC_{50}=2.73±0.286 µM) and showed lower cytotoxicity against normal cells. The docking simulation and flow cytometry results demonstrated that 11b can bind to the colchicine binding site of tubulin and cause HepG2 cells arrest in G2/M phase then induce cell apoptosis. Confocal microscopy assay and western blot results further confirmed that 11b can really inhibit tubulin polymerization. These findings prompt us to consider it as a potent anti-cancer agent.

### Acknowledgements

The authors are grateful to the National Natural Science Foundation of China (NSFC) (Nos.31071082, 31170275, 31171161), the Program for Changjiang Scholars and Innovative Research Team in University (IRT1020), the Project of New Century Excellent Talents in University (NECT-11-0234) and the Natural Science Foundations of the Jiangsu (BK2011414).

### Notes and references


**Figure and table captions:**

**Fig. 1** Chemical structures of compound A-I.

**Fig. 2** Molecular docking analysis of IBb, showing proposed binding modes at the colchicine binding pocket β-tubulin (PDB code: 1SA0). Hydrogen bonds are displayed as lime dashed lines. (A) and (C) Interaction of IBb with the amino acid residues of colchicine binding site (carbon atom, gray; oxygen atom, red; hydrogen atom, white; sulphur atom, yellow). (B) and (D) Binding pose of IBb in the protein surface of tubulin (carbon atom, green; oxygen atom, red; hydrogen atom, white; sulphur atom, yellow and nitrogen atom; light blue).

**Fig. 3** Effect of IBb on the cell cycle distribution of HepG2 cells. (A) Cells treated with 0, 3 and 10 µM IBb for 24 hours were collected and processed for analysis. (B) Cells treated with 3 µM IBb for different time was collected and analyzed.

**Fig. 4** AnnexinV/PI dual-immuno- fluorescence staining after treatment with different concentrations of IBb for different time revealed significantly increased number of apoptotic and necrotic cells (measured with Annexin V+/PI+ cells). (A) Cells treated with 0, 0.3, 1 and 3 µM IBb for 24 hours were collected and processed for analysis. (B) Cells treated with 3 µM IBb for different time (0 h, 12 h, 24 h and 48 h) was collected and analyzed. The percentage of early apoptotic cells in the lower right quadrant (annexin V-FITC positive/PI negative cells), as well as late apoptotic cells located in the upper right quadrant (annexin V-FITC positive/PI positive cells).

**Fig. 5** Effect of IBb (3 µM) on interphase microtubules of HepG2 cells. Microtubules tagged with rhodamine (red) and nuclei tagged with DAPI (blue) were observed under a confocal microscope.

**Fig. 6** IBb affected microtubule assembly in vitro. After 24 hours treatment with IBb (3 µM), paclitaxel (1 µM) and colchicine (1 µM), cytosolic (S, soluble) and cytoskeletal (P, polymerized tubulin) tubulin fractions were separated and immunoblotted with antibody against β-tubulin.

**Scheme 1** Regents and conditions: a) Etanol, nitrogen, room temperature, 30 minutes; b) Etanol, nitrogen, room temperature to 0 °C, 30 minutes; c) Etanol, nitrogen, room temperature to 0 °C, 30 minutes; d) Etanol, nitrogen, room temperature to 0 °C, 1 hour.
Table 1 Inhibition of tubulin polymerization and cell proliferation against MG63, MCF-7, B16-F10, HepG2, MDA-231, L02, VERO and MCF-10A cells by Ia-IIId
Fig. 1 Chemical structures of compound A-I.
183x132mm (300 x 300 DPI)
Fig. 2 Molecular docking analysis of IIB, showing proposed binding modes at the colchicine binding pocket β-tubulin (PDB code: 1SA0). Hydrogen bonds are displayed as lime dashed lines. (A) and (C) Interaction of IIB with the amino acid residues of colchicine binding site (carbon atom, gray; oxygen atom, red; hydrogen atom, white; sulphur atom, yellow). (B) and (D) Binding pose of IIB in the protein surface of tubulin (carbon atom, green; oxygen atom, red; hydrogen atom, white; sulphur atom, yellow and nitrogen atom; light blue).

101x51mm (300 x 300 DPI)
Fig. 3 Effect of IIb on the cell cycle distribution of HepG2 cells. (A) Cells treated with 0, 3 and 10 µM IIb for 24 hours were collected and processed for analysis. (B) Cells treated with 3 µM IIb for different time was collected and analyzed.
Fig. 4 AnnexinV/PI dual-immuno-fluorescence staining after treatment with different concentrations of IIb for different time revealed significantly increased number of apoptotic and necrotic cells (measured with Annexin V+/PI+ cells). (A) Cells treated with 0, 0.3, 1 and 3 µM IIb for 24 hours were collected and processed for analysis. (B) Cells treated with 3 µM IIb for different time (0 h, 12 h, 24 h and 48 h) was collected and analyzed. The percentage of early apoptotic cells in the lower right quadrant (annexin V-FITC positive/PI negative cells), as well as late apoptotic cells located in the upper right quadrant (annexin V-FITC positive/PI positive cells).
Fig. 5 Effect of IIb (3 µM) on interphase microtubules of HepG2 cells. Microtubules tagged with rhodamine (red) and nuclei tagged with DAPI (blue) were observed under a confocal microscope.
Fig. 6 IIb affected microtubule assembly in vitro. After 24 hours treatment with IIb (3 µM), paclitaxel (1 µM) and colchicine (1 µM), cytosolic (S, soluble) and cytoskeletal (P, polymerized tubulin) tubulin fractions were separated and immunoblotted with antibody against β-tubulin.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IIb 3 µM</th>
<th>Paclitaxel 1 µM</th>
<th>Colchicine 1 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>1</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>0.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

149x63mm (300 x 300 DPI)
Scheme 1 Regents and conditions: a) Etanol, nitrogen, room temperature, 30 minutes; b) Etanol, nitrogen, room temperature to 0 °C, 30 minutes; c) Etanol, nitrogen, room temperature to 60 °C, 1 hour.

137x159mm (300 x 300 DPI)
Table 1 Inhibition of tubulin polymerization and cell proliferation against MG63, MCF-7, B16-F10, HepG2, MDA-231, L02, VERO and MCF-10A cells by Ia–IIId

<table>
<thead>
<tr>
<th></th>
<th>MG63 IC50 (µM)</th>
<th>MCF-7 IC50 (µM)</th>
<th>B16-F10 IC50 (µM)</th>
<th>HepG2 IC50 (µM)</th>
<th>MDA-231 IC50 (µM)</th>
<th>L02 IC50 (µM)</th>
<th>VERO IC50 (µM)</th>
<th>MCF-10A IC50 (µM)</th>
<th>Tubulin IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>4.37±0.231</td>
<td>4.75±0.255</td>
<td>7.07±0.363</td>
<td>85.9±2.73</td>
<td>62.3±1.56</td>
<td>134±4.68</td>
<td>83.3±2.04</td>
<td>121±4.96</td>
<td>10.0±0.311</td>
</tr>
<tr>
<td>IIa</td>
<td>2.61±0.165</td>
<td>6.30±0.344</td>
<td>3.98±0.276</td>
<td>99.7±3.44</td>
<td>49.8±1.25</td>
<td>122±4.05</td>
<td>87.9±2.16</td>
<td>90.5±3.56</td>
<td>18.5±1.47</td>
</tr>
<tr>
<td>IIIa</td>
<td>3.99±0.372</td>
<td>7.84±1.23</td>
<td>4.62±0.698</td>
<td>91.5±3.02</td>
<td>96.7±2.99</td>
<td>107±3.98</td>
<td>93.1±2.98</td>
<td>120±4.21</td>
<td>14.0±1.69</td>
</tr>
<tr>
<td>Ib</td>
<td>9.16±0.640</td>
<td>29.7±1.66</td>
<td>14.1±0.786</td>
<td>98.3±3.46</td>
<td>137±4.49</td>
<td>125±3.48</td>
<td>84.8±2.56</td>
<td>132±4.68</td>
<td>41.2±1.79</td>
</tr>
<tr>
<td>IIb</td>
<td>1.22±0.221</td>
<td>2.37±0.165</td>
<td>4.53±0.247</td>
<td>1.22±0.110</td>
<td>10.6±0.97</td>
<td>132±4.71</td>
<td>82.5±2.00</td>
<td>78.1±2.27</td>
<td>4.67±0.433</td>
</tr>
<tr>
<td>IIIb</td>
<td>1.38±0.178</td>
<td>3.13±0.342</td>
<td>3.75±0.273</td>
<td>89.1±2.98</td>
<td>47.9±1.04</td>
<td>146±4.89</td>
<td>99.7±3.27</td>
<td>70.1±2.08</td>
<td>7.57±0.742</td>
</tr>
<tr>
<td>Ic</td>
<td>4.67±0.127</td>
<td>14.5±0.862</td>
<td>6.88±0.293</td>
<td>98.9±3.06</td>
<td>4.97±0.37</td>
<td>88.2±2.37</td>
<td>78.5±1.77</td>
<td>87.2±2.28</td>
<td>32.8±1.98</td>
</tr>
<tr>
<td>IIc</td>
<td>3.58±0.63</td>
<td>15.3±1.03</td>
<td>6.26±0.970</td>
<td>82.4±2.06</td>
<td>93.2±3.01</td>
<td>73.8±2.21</td>
<td>99.7±3.05</td>
<td>94.5±2.26</td>
<td>28.1±2.01</td>
</tr>
<tr>
<td>Id</td>
<td>3.20±0.212</td>
<td>6.67±0.423</td>
<td>9.24±0.524</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18.0±1.02</td>
</tr>
<tr>
<td>IIId</td>
<td>2.71±0.0924</td>
<td>12.2±1.01</td>
<td>11.6±1.29</td>
<td>76.0±1.99</td>
<td>94.6±2.99</td>
<td>89.1±2.79</td>
<td>96.3±2.27</td>
<td>42.5±1.88</td>
<td>40.8±2.98</td>
</tr>
<tr>
<td>IIIId</td>
<td>4.47±0.148</td>
<td>7.97±0.991</td>
<td>5.93±0.571</td>
<td>97.9±3.08</td>
<td>76.5±2.96</td>
<td>95.3±3.44</td>
<td>85.5±2.21</td>
<td>92.3±2.69</td>
<td>17.3±2.23</td>
</tr>
<tr>
<td>Ie</td>
<td>15.58±1.84</td>
<td>24.3±1.913</td>
<td>14.2±0.553</td>
<td>94.6±2.97</td>
<td>81.8±2.34</td>
<td>95.2±3.35</td>
<td>74.2±1.88</td>
<td>95.3±3.07</td>
<td>53.8±2.35</td>
</tr>
<tr>
<td>IIe</td>
<td>11.06±0.913</td>
<td>10.28±0.873</td>
<td>11.4±0.980</td>
<td>97.2±2.11</td>
<td>78.5±2.01</td>
<td>90.9±3.10</td>
<td>77.6±2.03</td>
<td>113±4.42</td>
<td>26.4±1.09</td>
</tr>
<tr>
<td>If</td>
<td>3.34±0.115</td>
<td>11.2±0.830</td>
<td>9.72±0.475</td>
<td>95.1±2.84</td>
<td>67.3±1.94</td>
<td>81.2±2.55</td>
<td>71.6±1.99</td>
<td>91.5±2.99</td>
<td>31.1±1.88</td>
</tr>
<tr>
<td>IIIf</td>
<td>2.63±0.267</td>
<td>10.0±0.871</td>
<td>6.79±0.856</td>
<td>78.3±2.00</td>
<td>69.8±1.88</td>
<td>126±4.85</td>
<td>30.5±1.45</td>
<td>58.8±1.57</td>
<td>13.2±1.04</td>
</tr>
<tr>
<td>Shiko</td>
<td>7.36±0.374</td>
<td>23.6±1.21</td>
<td>17.9±0.895</td>
<td>2.73±0.286</td>
<td>108±4.89</td>
<td>83.8±2.07</td>
<td>6.76±0.684</td>
<td>93.4±3.37</td>
<td>16.8±0.625</td>
</tr>
<tr>
<td>-nin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.83±0.424</td>
</tr>
<tr>
<td>Colch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.83±0.424</td>
</tr>
<tr>
<td>-icine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Inhibition of the growth of tumor cell lines.

b Inhibition of tubulin polymerization.

- Not determined.