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SHORT REPORT

An orally antitumor chalcone hybrid inhibited HepG2 cells growth and migration as the tubulin binding agent

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Summary
Liver cancer is a kind of high mortality cancer due to the difficulty of early diagnosis. It is necessary to develop the anticancer agents to treat liver cancer. Here, a novel chalcone derivative was synthesized and evaluated for anticancer activity in vitro against liver cancer cell lines (HepG2, SNU-423, SMMC7221, and SNU-398). The chalcone hybrid **9** displayed the antiproliferative effect against HepG2, SNU-423, SMMC7221 and SNU-398 cells with IC_{50} values of 0.9 μM, 2.7 μM, 6.2 μM and 4.6 μM, respectively. Cellular mechanisms showed that derivative **9** could obviously inhibit HepG2 cells growth and colony formation in a concentration-dependent manner. Analogue **9** inhibited the migration by regulating the expression levels of migration-releated markers and transcription factors (Snail and Slug). Tubulin polymerization inhibition assay illustrated that chalcone hybrid **9** might be a potent tubulin polymerization inhibitor. Importantly, compound **9** displayed the antitumor activity against liver cancer HepG2 cells in vivo with the low toxicity toward mice. Therefore, compound **9** as a novel tubulin polymerization inhibitor deserves further investigation to treat liver cancer.

Keywords Chalcone · Liver cancer · Cell growth · Migration · Tubulin

Introduction

Liver cancer is highly fatal, and death rates in the United States are increasing faster than for any other cancer [1–3]. Recently, there have been progresses to help patients with hepatocellular carcinoma by the liver resection or chemical drugs [4]. However, patients usually present with underlying advanced liver disorders and the successful treatment of liver cancer remains a challenge [5]. Therefore, it is very necessary to discovery novel antiproliferative agents to treat liver cancer.

Chalcone bearing an α,β-unsaturated carbonyl moiety has been used as the antitumor agent against a variety of cancer cell lines [6–10]. Chalcone 1 (Fig. 1) could activate intracellular reactive oxygen species levels and activate programmed death via the caspase-dependent intrinsic mitochondrial pathway against HepG2 cells [11]. Natural chalcone 2 arrested cell cycle at G2/M phase against HER2-overexpressing breast cancer cells by inhibiting phosphorylation of Cdc2 and Cdc25C [12]. Chalcone 3 inhibited cell growth and induced apoptosis against MCF-7/ADR cells in a dose-dependent manner [13]. Chalcone 4 as a phosphate salt inhibited tumor growth in xenograft models in vivo without apparent toxicity [14].

Molecular hybridization is a rational design strategy to obtain new ligands based on the recognition of pharmacophoric subunits in the molecular structure of two or three known bioactive derivatives [15, 16]. Based on the anticancer activity of chalcones, we designed and synthesized a novel chalcone-1,2,3-triazole hybrid 9 by the molecular hybridization strategy. In this work, we tested its antiproliferative activity against liver cancer cells and explored its anticancer mechanisms.

Materials and methods

General procedure for the synthesis of chalcone-1,2,3-triazole derivative 9

Chalcones **7** (5 mmol), 3-azidoprop-1-ene (5 mmol), propargyl bromide (6 mmol), $CuSO_4.5H_2O$ (1 mmol) and sodium

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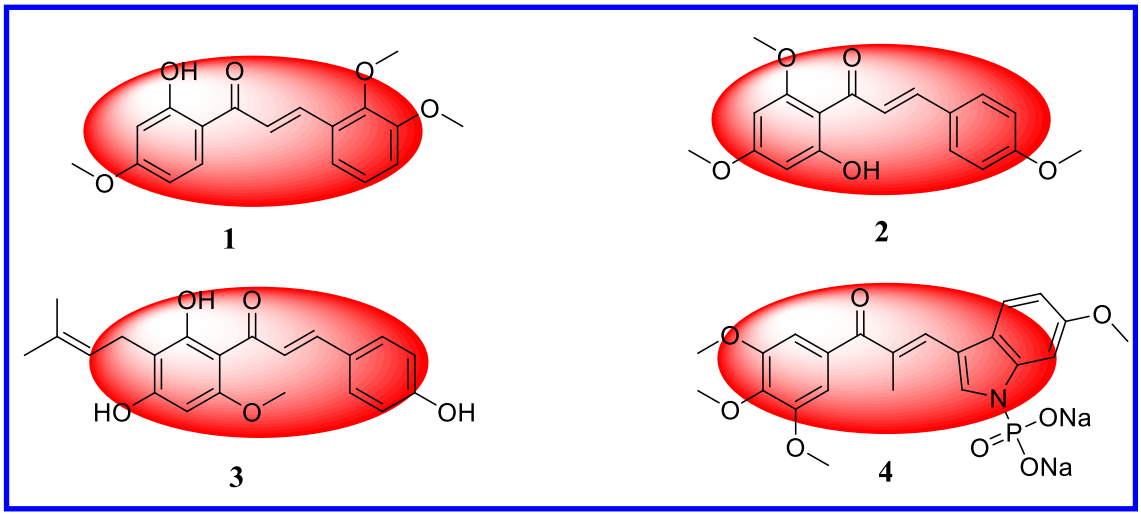


Fig. 1 Chemical structures of anticancer chalcones

ascorbate (0.5 mmol) were dissolved in THF/H_2O (15 mL/15 mL) and stirred for 10 h at room temperature. The crude product was filtered and purified with column chromatography on silica gel (EtOAc/hexane = 8/1). The yield to get compound **9** is 86%.

(E)-1-(4-((1-allyl-1*H*-1,2,3-triazol-4-yl)methoxy) phenyl)-3-(2,4-dichlorophenyl)prop-2-en-1-one (9)

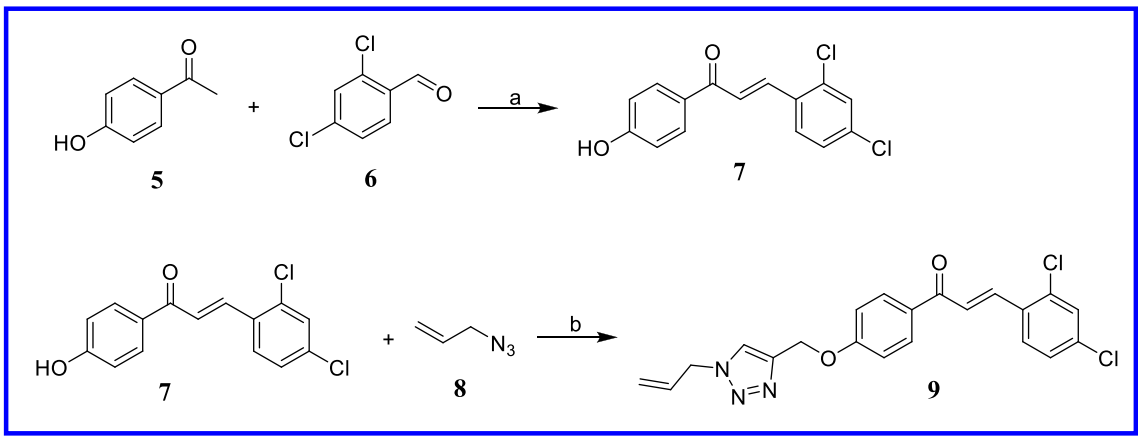
White solid, yield: 86%; m.p.:175–177 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 15.7 Hz, 1H), 8.03 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 8.5 Hz, 1H), 7.65 (s, 1H), 7.48(d, J = 15.7 Hz, 1H)7.47(d, J = 6.8 Hz, 1H), 7.30 (dd, J = 8.5, 2.0 Hz, 1H), 7.09 (d, J = 8.8 Hz, 2H), 6.03 (s, 1H), 5.37 (m, 2H), 5.31(s, 1H), 5.01 (d, J = 6.2 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.26, 162.17, 143.66, 138.66, 136.29, 135.99, 132.01, 131.17, 130.98, 130.12, 128.49, 127.53, 124.85, 122.66, 120.57, 114.73, 77.23, 62.17, 52.89. HRMS (ESI) calcd. For $C_{21}H_{18}C_{l2}N_3O_2$ $[M+H]^+$: 414.0776, found: 414.0779.

MTT assay

Liver cancer cell lines (HepG2, SNU-423, SMMC7221, and SNU-398) were from shanghai research science limited company. Cells were cultured with RPMI 1640 medium in an atmosphere containing 5% CO_2. We added 10,000 cells in each well of 24 wells plate. All wells were added 100uL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) solution to culture 1 h. The next steps were processed according to the reported reference [17].

Colony formation assay

1000 HepG2 cells were selected and added in each well of 6 wells plate. The next day, derivative **9** at different



Scheme 1 Reagents and conditions: (a) NaOH, EtOH, reflux. (b) Propargyl bromide, $CuSO_4.5H_2O$, sodium ascorbate, THF:H_2O (1:1), r.t

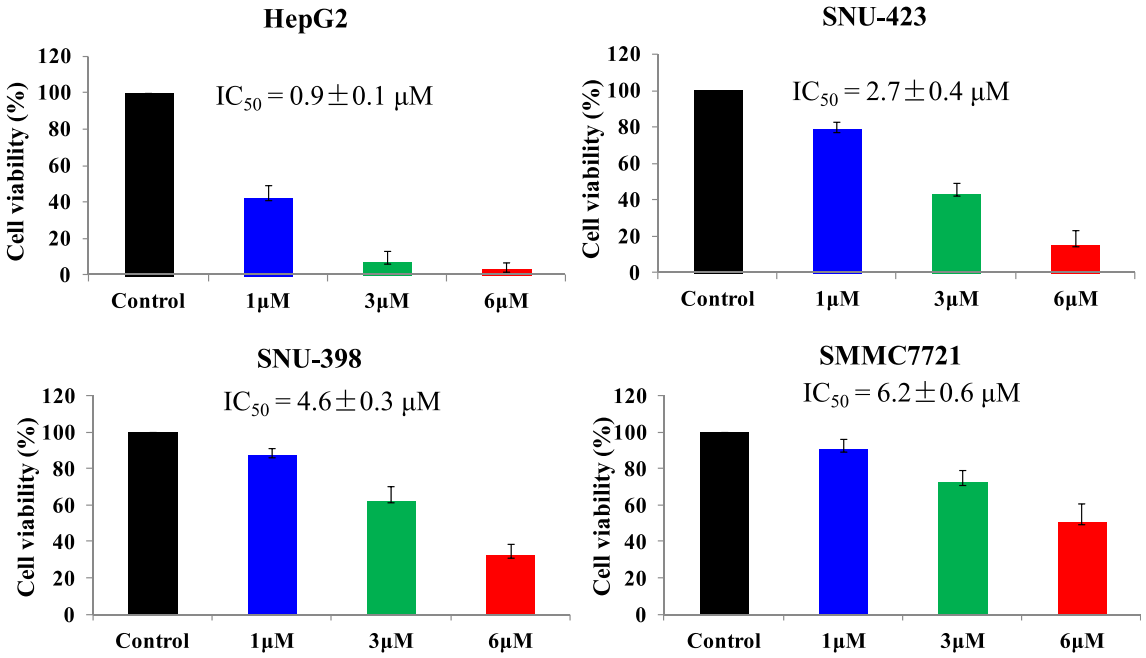


Fig. 2 The cell viability and IC_{50} values of compound **9** for 72 h

concentrations (0 μM, 0.1 μM and 0.5 μM) was added and cells were cultured for 1 week. Then, all cells were washed by PBS solution and stained by crystal violet solution for 1 h. The system was washed by water and took photos. The detailed method was referenced the report [18].

Migration assay

Migration assay was assessed by the 24-well cell culture plate (BD Falcon, NJ). The top chambers were seeded with 2×10^4 HepG2 cells in 400 μL serum-free 1640 medium containing derivative 9. The bottom chambers were filled with 700 μL complete medium. After 24 h incubation, top chambers were washed with PBS. The bottom chambers were fixed with 4% paraformaldehyde and stained with 0.4% crystal violet for 1 h. Then the chambers were washed with water, and the membrane was left to dry. The detailed method was referenced the report [19].

Animals study in vivo

Animals were treated according to protocols established by the ethics committee of zhengzhou university and the in vivo experiments were carried out in accordance with the approved guidelines. Mice were subcutaneously implanted with HepG2 cells (1×10^7 cells per mouse) on the right flank of nude mice. The mice were randomly divided into corresponding saline and **9** (70 mg/kg) treatment groups ($n = 5$ mice for each group). The treatment group received intragastric administration of **9** per day for a period of 21 days.

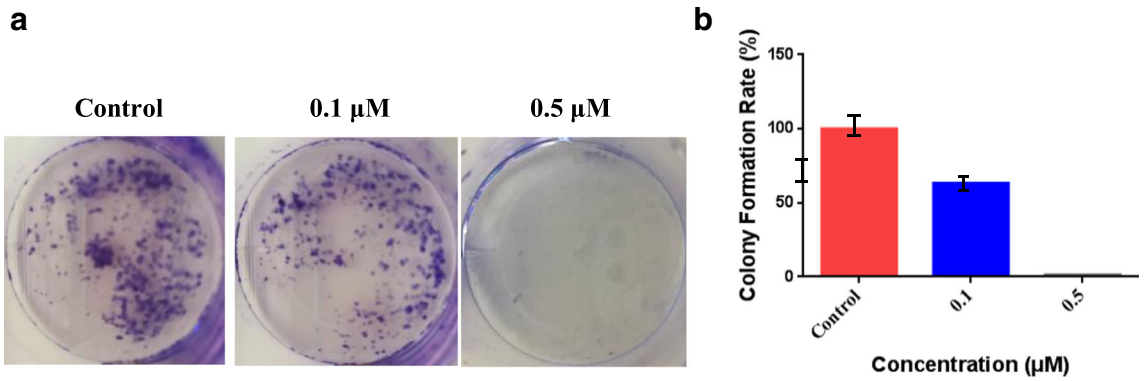


Fig. 3 Colony formation (**a**) Representative images of HepG2 cells colonies after the treatment of derivative **9** for a week (**b**) Colony formation rate

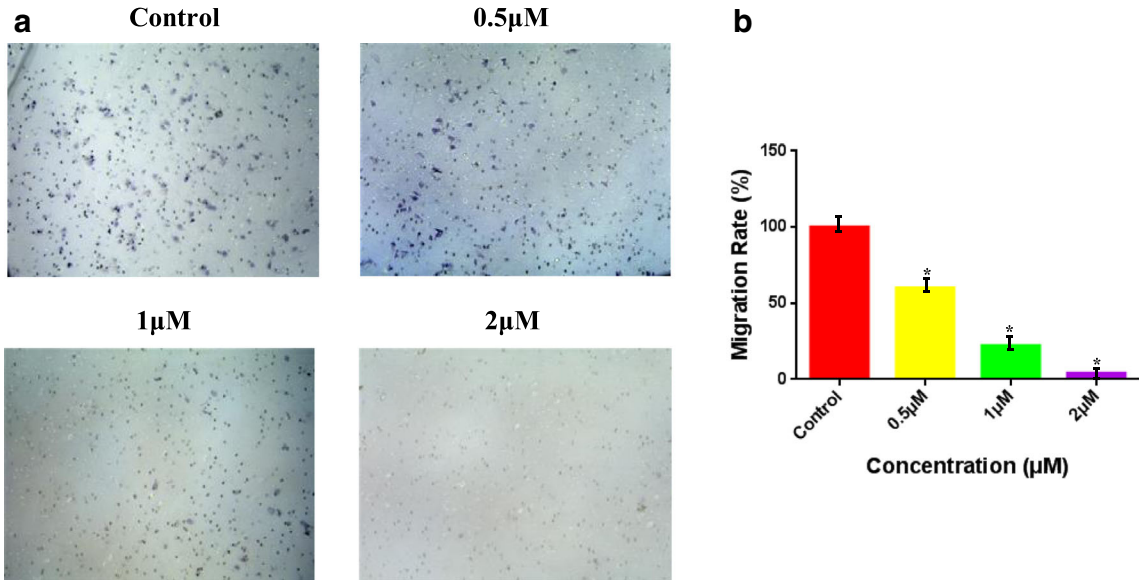


Fig. 4 Derivative **9** inhibited the migration against HepG2 cells (**a**) Representative images of HepG2 cells migration (**b**) migration rate. *: $p < 0.05$ verse control

Results

Synthesis

The synthesis of chalcone-1,2,3-triazole hybrid 9 in this work was shown in Scheme 1. Acetophenone 5 was reacted with the aromatic aldehyde 6 to obtain the chalcone intermediate 7 via the claisen-schmidt condensation reaction based on reported references [20–22]. 1,2,3-Triazole fragment is widely applied in organic chemistry and medicinal chemistry because of its anticancer activity [23–25]. The 1,2,3-triazoles might increase the antitumour activity of chemical compounds [26, 27]. Based on these findings, chalcone-1,2,3-triazole analogue 9 was designed and synthesized. Chalocne intermediate 7 was

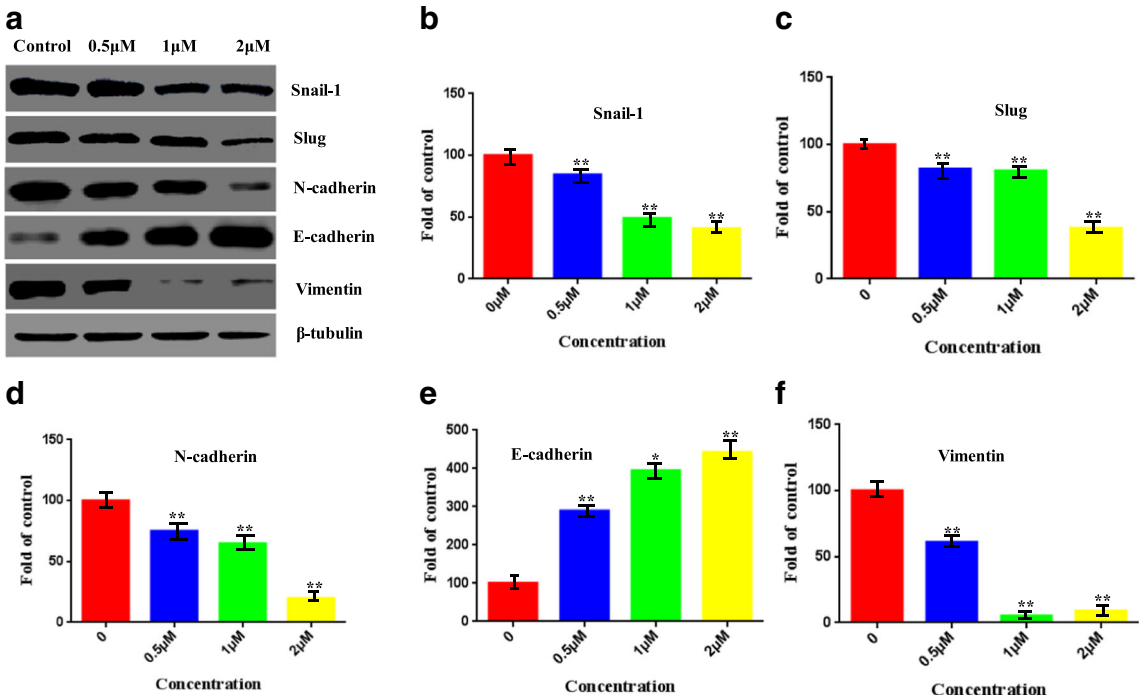


Fig. 5 Expression changes of migration releated biomarkers and transcription factors (**a**) Analogue **9** regulated the expression levels of migration-releated markers and transcription factors in HepG2 cells (**b**-**f**) Statistical analysis of protein expression levels. *: $p < 0.05$ verse control, **: $p < 0.01$ verse control

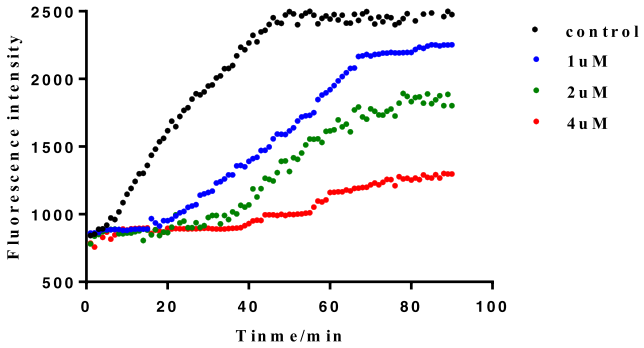


Fig. 6 Tubulin polymerization activity of derivative **9**

reacted with 3-azidoprop-1-ene 8 to form (*E*)-1-(4-((1-allyl-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-3-(2,4-dichlorophenyl)prop-2-en-1-one 9 in the yield of 86%.

Antiproliferative activity of analogue 9

MTT assay was performed to investigate the antiproliferative activity of chalcone derivatives against liver cancer cells for 72 h [28]. The antiproliferative activity results of chalcone-1,2,3-triazole hybrid 9 against liver cancer cells (HepG2, SNU-423, SMMC7221, and SNU-398) were listed in Fig. 2. In this work, 5-fluorouracil (5-Fu) was used as the control drug. The IC_{50} values of 5-fluorouracil against HepG2, SNU-423, SMMC7221 and SNU-398 cells were 9.2 μM, 10.3 μM, 15.1 μM, and 17.9 μM, respectively. From MTT assay, chalcone 7 showed the weak inhibitory activity with IC_{50} values >20 μM against all four cancer cell lines. However, chalcone-1,2,3-triazole hybrid 9 displayed the potently antiproliferative activity with IC_{50} values from 0.9 μM to 6.2 μM against all four cancer cell lines. These resuts illustrated that the 1,2,3-triazole unit might be very important for antiproliferative activity.

Analogue 9a inhibited HepG2 cells growth

Based on the best antiproliferative activity result of chalcone-1,2,3-triazole derivative 9 against HepG2 cells, HepG2 cell line was selected to do anticancer mechanisms in vitro. Colony formation was used to investigate whether derivative 9 could inhibit HepG2 cells growth and proliferation [29]. The colony formation results were shown in Fig. 3. Compared with the control group, HepG2 cells with the treatment of derivative 9 at 0.1 μM and 0.5 μM exhibited fewer colonies obviously. From the colony formation results, chalcone-1,2,3-triazole derivative 9 could significantly inhibit HepG2 cells growth in a concentration-dependent manner.

Analogue 9 inhibited HepG2 cells migration

The effect of migration about chalcone-1,2,3-triazole derivative 9 was explored by the reported method [30]. HepG2 cells were treated with derivative 9 at different concentrations (0 μM, 0.5 μM, 1 μM and 2 μM) for 24 h and were stained by hematoxylin. The migration rates at 0.5 μM, 1 μM and 2 μM were about 62%, 21% and 4%, respectively. All migration results in Fig. 4 indicated that chalcone-1,2,3-triazole derivative 9 could inhibit HepG2 cells migration by a concentration-dependent manner.

Analogue 9 regulated the expression levels of migration-releated markers and transcription factors

Epithelial-mesenchymal transition (EMT) as a biologic process displayed a pivotal role during the embryonic development and carcinoma progression [31]. Due to the migration inhibitory effect of derivative 9 against HepG2 cells, we examined the expression level of the typical proteins of epithelial-mesenchymal transition. As shown in Fig. 5, chalcone-1,2,3-triazole derivative 9 could upregulate the expression level of E-cadherin. Meanwhile, this compound decreased the mesenchymal cells' biomarkers, N-Cadherin and Vimentin. The expression levels of upstream transcription factors, Snail and Slug were both decreased. These results indicated that chalcone-1,2,3-triazole derivative 9 could inhibit liver cancer cells migration by regulating EMT related biomarkers (E-cadherin, N-Cadherin and Vimentin) and inhibiting the expression of upstream transcription factors (Snail and Slug).

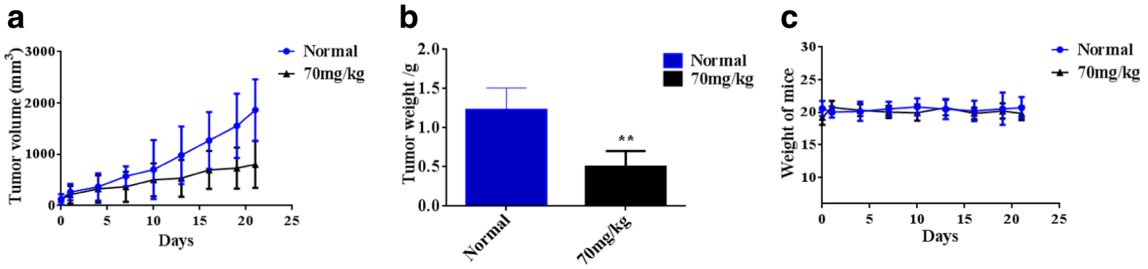


Fig. 7 The in vivo antitumor activity of chalcone-1,2,3-triazole derivative **9** (**a**) Tumor volumes of mice (**b**) Tumor weight from each group (**c**) Body weights of mice. $^{**}P < 0.01$ verse control

Chalcone analogue 9 inhibited tubulin polymerization

Microtubules as an important framework supporting cellular morphology in interphase are the major components of the mitotic spindle which allows the controlled segregation of the chromosomes during mitosis [32]. Recently, a series of phenstatin/isocombretastatin-chalcone conjugates were synthesized and screened as potent tubulin polymerization inhibitors [33]. These phenstatin/isocombretastatin-chalcones possess the structural similarity with our target compounds. Based on these findings, the tubulin activity of chalcone hybrid **9** was detected using the tubulin polymerization inhibition assay [34]. The IC_{50} value of chalcone hybrid **9** was 2.34 μM against tubulin polymerization. All these results indicated that derivative **9** was a novel tubulin polymerization inhibitor (Fig. 6).

Analogue 9 inhibited HepG2 cells growth in vivo

To evaluate the potential anticancer effects of chalcone-1,2,3-triazole derivative **9** in vivo, a HepG2 xenograft model was established in nude mice by subcutaneously injecting HepG2 cells. Tumor bearing mice were then randomly assigned to two groups (control, 70 mg/kg **9**) with 5 mice per group. The growth rate of HepG2 xenograft tumors from mice which were treated with chalcone-1,2,3-triazole derivative **9** was lower than those from the control group (Fig. 7). The average tumor weights of control and 70 mg/kg **9** were 1.311 ± 0.216 g and 0.524 ± 0.172 g (inhibitory rate: 60.03%), respectively. Importantly, there is no significant difference in mean body weights between control and treated group. All these results demonstrated the potent antitumor activity of chalcone-1,2,3-triazole derivative **9** against liver cancer HepG2 cells in vivo and low toxicity toward mice.

Discussion

A novel chalcone-1,2,3-triazole derivative **9** displayed the potent inhibitory effect against HepG2 cells with an IC_{50} value of 0.9 μM. Analogue **9** obviously inhibited HepG2 cells growth and migration in a concentration-dependent manner. In addition, compound **9** was a novel tubulin polymerization inhibitor. Importantly, derivative **9** effectively inhibited the liver cancer cells growth in vivo with no signs of adverse side effects. In summary, derivative **9** deserves further investigation as a lead compound to treat liver cancer.

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Author's contribution Wang Yan., Chen Xiangyu., Li Ya., and Wang Yu. performed the design work and experiments. Wang Yan., and Xu Feng. witten the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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