Original Paper

Magnolol Inhibits the Growth of Non-Small Cell Lung Cancer via Inhibiting Microtubule Polymerization

Jia Shen^b Hailin Ma^c Tiancheng Zhang^{d,e} Hui Liu^f Linghua Yu^g Guosheng Li^a
Huishuang Li^a Meichun Hu^a

^aResearch Center of Basic Medical Sciences, School of Basic Medical Sciences, Hubei University of Science and Technology, Xianning, ^bInstitute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, ^cCollege of Biotechnology, Guilin Medical University, Guilin, ^dInstitute of Reproduction & Development, Fudan University, ^eChina National Population and Family Planning Key Laboratory of Contraceptive Drugs and Devices, Shanghai Institute of Planned Parenthood Research (SIPPR), Shanghai, ^fInstitute for Clean Energy & Advanced Materials, Southwest University, Chongqing, ^gCenter for Gastroenterology and Hepatology, The First Affiliated Hospital of Jiaxing College, Jiaxing, China

Key Words

Magnolol • Microtubule polymerization • NSCLC • Apoptosis • Autophagy

Abstract

Background: The tubulin/microtubule system, which is an integral component of the cytoskeleton, plays an essential role in mitosis. Targeting mitotic progression by disturbing microtubule dynamics is a rational strategy for cancer treatment. **Methods:** Microtubule polymerization assay was performed to examine the effect of Magnolol (a novel natural phenolic compound isolated from *Magnolia obovata*) on cellular microtubule polymerization in human non-small cell lung cancer (NSCLC) cells. Cell cycle analysis, mitotic index assay, cell proliferation assay, colony formation assay, western blotting analysis of cell cycle regulators, Annexin V-FITC/PI staining, and live/dead viability staining were carried out to investigate the Magnolol's inhibitory effect on proliferation and viability of NSCLC cells *in vitro*. Xenograft model of human A549 NSCLC tumor was used to determine the Magnolol's efficacy *in vivo*. **Results:** Magnolol treatment effectively inhibited cell proliferation and colony formation of NSCLC cells. Further study proved that Magnolol induced the mitotic phase arrest and inhibited G2/M progression in a dose-dependent manner, which were mechanistically associated with expression alteration of a series of cell cycle regulators. Furthermore, Magnolol treatment disrupted the cellular microtubule organization *via* inhibiting the polymerization of microtubule. We also found treatment with NSCLC cells with Magnolol resulted in apoptosis activation through a p53-independent pathway, and autophagy induction *via* down-regulation of the Akt/mTOR pathway. Finally, Magnolol treatment significantly suppressed the NSCLC

J. Shen, H. Ma and T. Zhang contributed equally to this work.

Dr. Meichun Hu

Research Center of Basic Medical Sciences, School of Basic Medical Sciences, Hubei University of Science and Technology, Xianning 437100 (China)
E-Mail humeichun.530@163.com

tumor growth in mouse xenograft model *in vivo*. **Conclusion:** These findings identify Magnolol as a promising candidate with anti-microtubule polymerization activity for NSCLC treatment.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

In eukaryotic cells, microtubule is the main component of the cellular cytoskeleton, plays important roles in cell shape maintenance, intracellular transport, cell motility, meiosis, and mitosis [1, 2]. Specifically, microtubule is required for the process of mitosis in orchestrating the separation and segregation of chromosomes, which suggests that microtubule is a rational target for the development of anticancer drugs [3]. Microtubule-targeted drugs disrupt the dynamics of microtubule and induce mitosis arrest of tumor cells. These drugs are commonly classified into two major groups. The first group drugs inhibit microtubule polymerization, such as colchicine and vinorelbine, while the second group drugs stimulate microtubule depolymerization, such as paclitaxel and docetaxel [4].

Lung cancer is the most common malignant tumor and the leading cause of cancer-related death in the world [5, 6]. More than 1.5 million new cases of lung cancer are diagnosed every year, approximately 80% of which are non-small cell lung cancer (NSCLC) [7-9]. The morbidity is rapidly increasing mainly due to environmental pollution and unhealthy lifestyles (smoking, occupational exposure, diet, et al). Therefore, there is an urgent need to identify biomarkers and drug targets for early diagnosis and treatment for NSCLC [10]. Currently, chemotherapy plays a crucial role in comprehensive NSCLC therapy. Cisplatin-based drugs are the most widely used chemotherapy medicine for NSCLC [11, 12]. Additionally, some chemo-drugs targeting at microtubule including docetaxel, cabazitaxel, epothilones and vinorelbine have been used for NSCLC treatment [13]. These agents have demonstrated overall survival benefit clinically [3]. Nevertheless, drug resistance often appears after prolonged treatment [14-16]. Therefore, development of novel generation of anti-microtubule candidates is a promising and urgent task for NSCLC treatment.

Magnolia officinalis belongs to Magnoliaceae family and distributes over China, Japan, and South Korea. Magnolia officinalis is used as a folk remedy for gastrointestinal disorders, cough, acute pain, anxiety, and allergic diseases. Magnolol (5,5'-diallyl-2,2'-dihydroxybiphenyl, Fig. 1A, MW: 308.3495), a hydroxylated biphenyl compound isolated from the root and stem bark of Magnolia officinalis, was shown to have muscle relaxant, anti-oxidative, anti-atherosclerosis, anti-inflammatory, and anti-microbial effects [17-22]. Recent studies have shown that Magnolol exhibits anti-cancer properties by inhibiting proliferation, inducing differentiation and apoptosis, suppressing angiogenesis, countering metastasis, and reversing multidrug resistance [23-28]. However, the function and mechanism underlying the antitumor activity of Magnolol against lung cancer remain unknown. In this study, we investigated the efficacy of Magnolol against human NSCLC cells both *in vitro* and *in vivo*. Our results indicated that Magnolol induced G2/M phase cell cycle arrest through inhibiting microtubule polymerization. Further study demonstrated that Magnolol treatment led to inhibition of proliferation, apoptosis activation and autophagy induction of NSCLC cells. Importantly, Magnolol suppressed xenograft of A549 tumor growth *in vivo*. All these results suggest that Magnolol is a promising candidate for NSCLC treatment.

Materials and Methods

Cell Lines, animals and reagents

Human NSCLC cell lines PGCL3, SK-MES-1, NCI-H460, A549, NCI-1299 and immortalized human normal bronchial epithelium cell line 16HBE were purchased from ATCC [29]. A549 cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific). PGCL3, SK-MES-1, NCI-H460, NCI-1299 and 16HBE cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific). Both mediums were supplemented with 10% fetal bovine serum (Wisent). All cells were incubated at 37°C with 5% humidified

Fig. 1. (A) Chemical structure and molecular weight of Magnolol. (B) Cell viability assay was performed to investigate Magnolol's efficacy on several NSCLC cell lines and normal bronchial epithelium 16HBE cells. (C) Magnolol inhibited colony formation of NSCLC cells. After treatment with Magnolol for a week, cells were fixed and stained with crystal violet and the numbers of cell colonies were counted ($n=3$; *** $P<0.001$ versus the control group).

CO_2 . Mice were obtained from National Rodent Laboratory Animal Resources, Shanghai Branch of China. Cdc 2, p-Cdc 2, Cdc 25c, Cyclin B1 and actin antibodies were purchased from Cell Signaling Technology, and phospho-Ser/Thr-Pro MPM-2 antibody were obtained from Millipore. Magnolol with a purity of up to 98% was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Magnolol was dissolved in DMSO (Sigma) at a stock solution of 100 mM and stored at -20°C .

Cell cycle distribution analysis

Cell cycle analysis was performed according to previous report [30]. Cells were treated with four different doses (0, 5, 10, 20 μM) of Magnolol for 24 hrs. After washed with PBS and digested with trypsin, adherent and floating cells were collected, washed once with PBS, and then fixed in cold 70% ethanol overnight in 4°C . After ethanol fixation, cells were washed in PBS once and suspended in PBS with 200 $\mu\text{g}/\text{mL}$ RNAase and 50 $\mu\text{g}/\text{mL}$ propidium iodide (PI) in the dark for 30 minutes. Then cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Immunofluorescence staining

Immunofluorescence staining assay was performed as previously described [31] with minor modification. Briefly, Then cells treated were incubated with 4% paraformaldehyde for 20 min, washed with PBS, and treated with 0.5% Triton-X 100, washed with PBS. After blocking in 0.5% BSA, cells were incubated with primary antibody overnight at 4°C before further incubation with secondary antibody at 37°C in the dark. Images were taken by confocal microscopy (Leica).

Mitotic index assay

Cells were seeded on gelatin-coated coverslips. After fixed with 4% paraformaldehyde for 20 min at room temperature, cells were permeabilized with 0.2% Triton-X 100 in PBS for 5 min. The nuclei were stained with DAPI. After washed with PBS, cells were visualized and photographed with Leica microscopy. Five random fields were counted and analyzed. Mitotic index was calculated by dividing the total number of examined cells by the number of cells in mitosis.

Cell proliferation assay (Sulforhodamine B assay)

Cell proliferation was assessed by Sulforhodamine B (SRB) assay. Briefly, 4000 cells per well were seeded in 96-well plates. After 24 hrs, cells were exposed to five different dose (0, 1, 5, 10, 20 μ M) of Magnolol for 72 hrs. Cells were fixed with 10% trichloroacetic acid for 1 hr at 4 °C, washed five times with flowing water, and then air-dried. Cells were stained with 50 μ L 0.4% (w/v) SRB for 20 min at room temperature, washed five times with 1% acetic acid, and then air-dried. 100 μ L 10 mM Tris was added per well, and absorbance was measured at 515 nm.

Colony formation assay

Cells were seeded in 6-well dishes. Then cells were allowed to attach overnight and then exposed to different doses of Magnolol for a week. After fixed with 4% paraformaldehyde for 20 min room temperature, cells were stained with 0.2% crystal violet. The number of cell colonies was calculated and analyzed as the ratio of the number of treated samples to untreated samples. Triplicate wells were set up for each dose.

Western blotting

Cells were exposed to drug treatment for indicated time and lysed in RIPA buffer as detailed in previous report [32]. Lysate was mixed with sample loading buffer and heated at 100°C for 10 min. After separated by SDS-polyacrylamide gel electrophoresis (PAGE), extracted protein were transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in PBS and 0.1% Tween-20 and then incubated with specific primary antibodies overnight at 4°C. Then membranes were exposed to secondary antibodies for 2 hrs at room temperature.

Apoptosis analysis

Apoptosis analysis was carried out by flow cytometry (FACSCalibur; BD Biosciences). Cells were treated with different doses of Magnolol for 48 hrs or with 10 μ M Magnolol for different times. The procedure was performed as previous report [8]. Cells were washed with PBS, then harvested by 0.25% trypsin and washed with PBS again. After that, 5 μ L Annexin V fluorescein isothiocyanate and 5 μ L propidium iodide were added and the mixture was kept in the dark for 15 min at room temperature, then 400 μ L of binding buffer was added and analyzed immediately with flow cytometry (FACSCalibur; BD Biosciences).

Live/dead viability staining assay

Viability assay was performed using the live/dead viability/cytotoxicity kit (Molecular Probes). This kit contains calcein-AM to stain the living cells (green) and ethidium homodimer-1 (EthD-1) to stain the dead cells (red). Briefly, cells were exposed to Magnolol treatment for 48 hrs, then 2 μ M calcein-AM and 4 μ M EthD-1 working solution was added. After incubated at room temperature for 30 min, the green living cells and the red dead cells were visualized by fluorescence microscopy and photographed. Cells from three random areas per sample were counted for statistical analysis.

In vitro microtubule polymerization assay

In vitro microtubule polymerization assay was performed according to previous reports with modification [33, 34]. A549 and NCI-1299 cells were seeded on 6-well plates. Then cells were exposed to treatment of Magnolol, 50 nM colchicine and 50 nM paclitaxel for 24 hrs, respectively. The cells were washed with PBS for three times before adding lysis buffer containing 20 mM Tris-HCL (pH6.8), 1 mM MgCl₂, 2 mM EGTA and Protease inhibitor and phosphatase inhibitor and 0.5% Nonidet. Supernatants were collected after centrifugation at 14000 rpm for 10 min. Supernatant and the pellet were dissolved in SDS-PAGE sampling loading buffer at 95°C for 10 min. Supernatant containing soluble tubulin and the pellet (the polymerized tubulin lysates) were subjected to 10% SDS-PAGE before Western blotting.

Xenograft model of human A549 NSCLC tumor

A549 cells (2×10⁶) were implanted subcutaneously on the right side of the dorsal area of 4-week-old male nude mice. 10 days later, mice were divided into two groups (n=8) randomly as reported [35]. Magnolol (25 mg/kg) was injected intraperitoneally every other day. Control group was treated with DMSO. Twenty days later, mice were sacrificed, tumors were removed and images were taken. The growth rate of the tumor xenograft was evaluated by determining the tumor volume using digital caliper everyday. Tumor

growth rate was measured as the following equation, $\text{volume} = \text{length} \times \text{width}^2 \times 0.52$. Mice were continually observed and were treated consecutively for 20 days with the measurement of body weight until they were sacrificed.

Statistical analysis

All values are given as means $\pm$ standard error of the mean (s.e.m.) or standard deviation (s.d.) as indicated. Comparisons between two groups were made either by Student's t-test or Mann-Whitney U-Test; for more than two groups' one way analysis of variance (ANOVA) with Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's multiple comparison test was performed. Tumor growth was analyzed with two-way analysis of variance and Bonferroni multiple comparison testing. For the Kaplan-Meier analysis a log-rank test was performed. $P < 0.05$ was accepted as statistically significant. For all graphs: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Magnolol inhibits proliferation and colony formation of NSCLC cells

Firstly we performed SRB assay to examine the anti-proliferative effect of Magnolol against NSCLC cells (PGCL3, SK-MES-1, NCI-H460, A549, NCI-1299 cells) and human normal bronchial epithelium cells (16HBE cells). As shown in Fig.1B, Magnolol inhibited the proliferation of PGCL3, SK-MES-1, NCI-H460, A549 and NCI-1299 cells, while it showed slightly inhibitory effect on the 16HBE cells. Notably, the half maximal inhibitory concentrations (IC_{50}) of Magnolol at 72hrs was $\sim 5 \mu\text{M}$ on both NCI-1299 and A549 NSCLC cells, but the toxicity on 16HBE cells was negligible. Next, we sought to investigate the anti-proliferative effect of Magnolol on lung cancer cells by using colony formation assay. In

Fig. 2. (A) Magnolol induced cell cycle arrest on NSCLC cells. A549 and NCI-H1299 cells were analyzed by flow cytometry after treatment with Magnolol for 24 hrs. (B) Western blotting analysis of cellular G2/M phase regulators under Magnolol treatment. Cells were lysed and the protein levels of Cdc2, p-Cdc2, Cdc25c, Cyclin B1 and β -actin were detected with specific antibodies. (C) Western blotting analysis of several key cyclins, the cyclin dependent kinases and cyclin dependent kinase inhibitor proteins under Magnolol treatment.

Fig. 3. (A, B) Magnolol treatment led to accumulation of mitotic phase of NSCLC cells. A549 cells were exposed to different doses of Magnolol for 24 hrs. The mitotic index was represented as percentage of mitosis phase cells ($n=3$; one-way ANOVA statistic test was used). (C) Magnolol treatment resulted in induction of MPM-2. A549 cells were treated with different doses of Magnolol for 24 hrs and MPM-2 protein was detected by western blotting.

accordance with the result of SRB assay, as shown in Fig.1C, Magnolol treatment with low concentration (5 μM) led to significant inhibition on A549 and H1299 cells colony formation compared to the controls. Taken together, these results demonstrate that Magnolol not only has strong efficacy on the proliferation of NSCLC cells but also possesses a great selectivity against NSCLC cells over the normal bronchial cells.

Magnolol induces cell cycle arrest of NSCLC cells

It is commonly known that cell proliferation defect is mainly attributed to cell cycle alteration. Subsequently, the effect of Magnolol on cell cycle progression of NSCLC cells was assessed. After 24 hrs' treatment, the flow cytometry analysis revealed that Magnolol treatment caused dramatic accumulation of cell populations at the G2/M phase on both A549 and NCI-H1299 cells in a dose-dependent manner. Accordingly, cell populations in S and G1 phases decreased under Magnolol treatment (Fig. 2A). To elucidate the molecular mechanisms through which Magnolol induced the G2/M phase arrests, the levels of the G2/M phase regulatory proteins were evaluated by western blotting. As expected, the data showed that treatment of A549 and NCI-1299 cells with Magnolol for 24 hrs resulted in a dose-dependent downregulation of phosphorylated Cdc2, total Cdc25c, and an accumulation of Cyclin B1 (Fig. 2B). Nevertheless, the protein level of total Cdc2 was not affected by the treatment of Magnolol. In addition, we examined the other key cell cycle regulators under the treatment. As shown in Fig. 2C, the reduction of Cyclin A2, Cyclin D1, Cdk1 and Cdk4 was observed when the cells treated with Magnolol, but Cdk2, p21 and p27 increased as compared with untreated control. Taken together, these findings indicate that the effect of Magnolol on the G2/M phase cell cycle arrest is associated with accumulation of Cyclin B1/Cdk2/p21/p27 and suppression of phosphorylated Cdc2/Cdc25c/Cyclin A2/Cyclin D1/Cdk1/Cdk4 in the NSCLC cells.

Next, the mitosis index assay [36] that stains the cells with DAPI was performed to investigate whether Magnolol treatment could affect cell mitosis. As shown in Fig. 3A and B, with treatment of an increasing doses of Magnolol, more A549 cells were accumulated at mitotic (M) phase as indicated by condensed nuclei DAPI staining. In addition, NCI-H1299

Fig. 4. (A) Magnolol reduced polymerized tubulin in A549 and NCI-1299 cells. Cells were treated with different doses of Magnolol or 50 nM Colchicine (Col, served as positive control) or 50 nM Paclitaxel (Pal, served as negative control) for 24 hrs. Polymerized tubulin (P) and soluble tubulin (S) were detected by western blotting with tubulin antibody. (B, C) Magnolol disrupted microtubule organization in interphase and mitotic phase of A549 cells. Cells were fixed and micro-tubulin was stained with α -tubulin antibody (green) and the nuclear DNA was stained by DAPI (blue). Images of interphase cells (B) and mitotic phase cells (C) were taken by confocal microscopy.

cells showed the similar M phase cells accumulation responding to Magnolol treatment (data not shown). Furthermore, we found a marked increase in mitosis-specific MPM-2 expression by western blotting in Magnolol-treated cells (Fig. 3C), further suggesting Magnolol treatment could arrest cells at M phase.

Magnolol disrupts microtubule organization in NSCLC cells

Microtubules are composed of α -tubulin and β -tubulin heterodimers and play critical roles in regulating cell cycle and cell proliferation [4]. Conventionally, α -tubulin and β -tubulin heterodimers exist in two forms, soluble monomer and polymerized tubulin heterodimers. Our above results showed that the inhibitory effect of Magnolol on NSCLC cell growth was associated with cell mitotic arrest, which might be related to microtubular dynamics. Hence, the *in vitro* microtubule polymerization assay was performed to investigate the effect of Magnolol on microtubule polymerization in A549 and NCI-H1299 cells. Herein, 50 nM treatment of Colchicine or Paclitaxel was used as positive control or negative control, respectively. As shown in Fig. 4A, Magnolol treatment resulted in polymerized tubulin decrease in a dose-dependent manner. Immunofluorescent staining of the cellular microtubule at interphase and mitotic phase was performed to further examine how Magnolol affected microtubule polymerization. As shown in Fig. 4B, in the absence of Magnolol treatment, A549 cells that were at interphase exhibited the normal microtubule organization and arrangement. In contrast, the Magnolol-treated cells presented disorganized microtubule distribution and more diffused staining pattern throughout the cytoplasm, which was

Fig. 5. (A, B) Magnolol treatment led to apoptosis activation in A549 cells. Cells were treated with different doses of Magnolol for 48 hrs (A) or with 10 μ M Magnolol for 24 hrs, 48 hrs, and 72 hrs (B). Then the percentage of apoptosis cells was analyzed with flow cytometry. (C) Western blotting analysis of apoptosis and autophagy markers.

Fig. 6. (A) Live-dead staining assay for NSCLC cells under Magnolol treatment. Cells were treated with different doses of Magnolol for 48 hrs and stained with live-dead kit. Representative images showed cells under treatment of 10 μ M Magnolol. The red staining represented dead cells while the green staining showed live cells. Quantitative data was showed in (B). One-way ANOVA statistic test was used.

similar to those Colchicines-treated cells. Mechanistically, Colchicines inhibits microtubule polymerization by binding to tubulin directly. In Fig. 4C, mitotic A549 cells without Magnolol treatment showed intact spindle assembly, while the Magnolol-treated cells showed disrupted spindle structure and irregular organization. These observations suggest that Magnolol depolymerizes microtubules in NSCLC cells.

Magnolol provokes NSCLC cell apoptosis and autophagy

It is well documented that there is a crosstalk between anti-microtubule activity with apoptotic induction [37, 38]. Hence we sought to explore the effect of Magnolol on NSCLC cell apoptosis. As shown in Fig. 5A and B, the Annexin V-FITC/PI staining and flow

Fig. 7. (A) Representative photo for tumor burden from A549 tumor-bearing mice. Mice were intraperitoneally treated with Magnolol (25 mg/kg) or DMSO (served as control) for 20 days. (B) Tumor volume was measured every day during Magnolol administration (n=8; one-way ANOVA statistic test was used). (C) Mice body weight was measured during Magnolol treatment (n=8; one-way ANOVA statistic test was used).

cytometric assay revealed that Magnolol treatment induced apoptosis of A549 cells in both concentration-dependent and time-dependent manners. Western blotting analysis further indicated that Magnolol treatment up-regulated the protein levels of several apoptotic markers such as Cleaved-caspase 9, cleaved-PARP and Bax. Instead, anti-apoptotic Bcl-2 protein level was downregulated in the Magnolol-treated cells compared to the control (Fig. 5C). The similar result was obtained when we treated NCI-H1299 cells with Magnolol (data not shown). P53 gene is null in NCI-H1299 cells, suggesting that the apoptosis induction in NSCLC cells upon Magnolol treatment was not dependent on p53 status. Interestingly, we also found treatment of A549 cells with Magnolol led to activation of ATG5, ATG12 and LC3B-II/-I ratio, suggesting cellular autophagy induction. The mTOR kinase serves as a signal hub for autophagy induction and the activation of mTOR by Akt suppresses autophagy. As shown in Fig. 5C, both p-Akt (S473) and mTOR levels decreased in Magnolol-treated cells compared to the control, indicating Magnolol induced autophagy *via* downregulation of the Akt/mTOR pathway. Since both apoptosis activation and autophagy induction could provoke cell death, we hypothesized Magnolol treatment would lead to cell death of NSCLC cells. Following Magnolol treatment, the A549 and NCI-H1299 NSCLC cells were co-stained with Calcein AM and propidium iodide (PI) to differentiate live (green) and dead (red) cells, respectively. As revealed in Fig. 6A and B, in the case of control cells, no acute cell death or loss of cell viability was observed. In contrast, the percentage of dead cells increased under Magnolol treatment for 48 hrs in A549 and NCI-H1299 NSCLC cells. These data indicate that Magnolol provokes apoptosis and autophagy which could lead to cell death in NSCLC cells.

Magnolol inhibits the growth of A549 tumor xenograft

To further evaluate the tumor-suppressing effect of Magnolol *in vivo*, a model for tumorigenicity of A549 NSCLC cells in nude mice was established. A549 cells (2×10^6) were implanted subcutaneously into 4-week-old male nude mice. The effect of Magnolol (25mg/kg) on tumor xenograft was examined as indicated in Fig. 7A. As anticipated, Magnolol treatment significantly reduced the tumor sizes compared to the control. Specifically, the average tumor weight of control group was 312.54 ± 64.13 mg, whereas the tumor weight in Magnolol-treated group was 176.32 ± 10.25 mg. For the average tumor size, the control group showed 2510.62 ± 800.91 mm³, whereas Magnolol-treated group was 1190.85 ± 296.91 mm³ (Fig. 7B). Notably, Magnolol treatment at the given concentration had little effect on the body weight of the mice (Fig. 7C). Therefore, in animal model, Magnolol also exhibited strong tumor suppression efficacy and low toxicity as it did in *in vitro* cell culture system.

Discussion

Natural sources provide beneficial pharmacotherapy for people worldwide. In this study, we reported for the first time that the effect of Magnolol, a hydroxylated biphenyl compound isolated from the root and stem bark of *M. officinalis*, against NSCLC cells both *in vitro* and *in vivo*. Several previous studies have documented the anti-cancer activity of Magnolol [23-28]. For example, Magnolol-induced apoptosis of HCT-116 colon cancer cells was associated with the AMP-activated protein kinase signaling pathway. In PC-3 prostate carcinoma cells, Magnolol reduced MMP-2/-9 and cyclooxygenase-2 at the mRNA level. In this current work, we demonstrated that Magnolol significantly inhibited NSCLC cell proliferation, growth, and colony formation, as well as suppressed tumor growth in a xenograft model of human lung cancer, suggesting its potential in lung cancer control. Our work further proved that Magnolol might exert its anti-tumor efficacy through the disruption of cellular polymerization of microtubule.

Lung cancer especially NSCLC causes more deaths than any other types of cancer in both men and women worldwide. In the past few years, great strides have been made in the discovery of effective and new therapies for NSCLC [39, 40]. However, most NSCLC cases ultimately relapsed and progressed to intractable late-stage metastatic disease. The successful use of docetaxel and cabazitaxel in NSCLC chemotherapy suggested that microtubule-targeting agents might be a feasible strategy. Drugs that disrupt microtubule dynamics have been used in the clinic as anti-cancer drugs for over twenty years. These drugs bind to tubulin and thus cause an increase or decrease in the interphase microtubule mass at high concentrations [3]. However, drug resistance and concomitant side effects hinder the clinical use for effective cancer therapy [41]. Therefore, it is necessary to develop new agents that overcome these hurdles.

In our study, we showed that cellular polymerized tubulin decreased significantly under the Magnolol exposure in microtubule polymerization assay (Fig. 4A). It was similar when we treated the cells with Colchicine, a known chemical inhibitor for microtubule polymerization, suggesting Magnolol is a potential microtubule-targeting agent. Further Immunofluorescence staining for tubulin in cells treated with Magnolol revealed disorganized microtubule distribution at interphase and disrupted spindle at mitotic phase (Fig. 4B and C). In accordance with the fact that microtubule-targeting agents arrest the cell cycle at G2/M phase, our results showed that Magnolol induced a G2/M blockade, as indicated by flow cytometry analysis (Fig. 2A). We further investigated the in-depth molecular mechanism of cell cycle arrest induced by Magnolol treatment. Previous reports have shown that Cyclin B and Cdc2 kinase regulate the start of M phase. The activation of Cdc2 kinase is dependent on accumulation of Cyclin B1 and dephosphorylation of Cdc2 [42]. We observed an obvious dose-dependent decrease of phosphorylated Cdc2 and total Cdc25c, while an accumulation of Cyclin B1 protein under Magnolol treatment, suggesting cell cycle was blocked at mitophase. Another evidence is that we found MPM-2, a protein marker for mitotic phase [43], increased upon Magnolol treatment in a dose-dependent manner.

Cell cycle arrest at mitotic phase might be an upstream event leading to apoptosis. Under Magnolol treatment, we indeed found activation of cellular apoptosis by using Annexin V-FITC/PI staining (Fig. 5A and B). Western blotting analysis confirmed the change of apoptotic protein markers (Cleaved-caspase 9, Cleaved-PARP, Bax, Bcl-2). Meanwhile, we also detected the strong induction of autophagy in cells treated with Magnolol. The Akt-mTOR signaling pathway is critical for controlling autophagy induction. We found that Magnolol treatment resulted in down-regulation of both p-Akt and mTOR levels but activation of autophagy marker LC3B-II. Calcein AM is a nonfluorescent dye that could be hydrolyzed by intracellular esterases to a green fluorescent calcein dye in live cells. Propidium iodide (PI), a dead cell staining, is generally excluded from viable cells. It binds to the DNA of dead cells, generating red fluorescence. Our live-dead staining assay suggested that cell death increased upon Magnolol treatment (Fig. 6). Since apoptosis and autophagy are two important pathways led to cell death, we speculate that induction of cell cycle arrest

and apoptosis, along with promoted activation of autophagy might mechanistically underlie the efficacy of Magnolol on NSCLC cells. Furthermore, we used mouse xenograft model to confirm the Magnolol's efficacy on NSCLC cells *in vivo*.

In addition to Magnolol's remarkable anticancer efficacy shown both *in vitro* and *in vivo*, another advantage of Magnolol is its acceptable toxicity. Current commercial microtubule targeting agents are widely used in the clinic but side effects are significant due to drug toxicity. Although Magnolol also induces cell cycle arrest in 16HBE cells, an immortalized human normal bronchial epithelium cell line, the effect was negligible compared to the effect on the cancer cells. Besides, in subcutaneous xenograft tumor model, *in vivo* treatment of Magnolol at the effective concentration had little effect on the body weight of the mice (Fig. 7C).

Although Magnolol had the inhibitory effect on microtubule polymerization, we still did not know the specific molecular mechanism. It is not clear how Magnolol exerted its inhibitive function on microtubule polymerization. Does Magnolol bind to microtubule directly or through binding to other indirect molecules? In addition, although there was a report to test some symbolic parameters of pharmacokinetics and pharmacodynamics (PDPK) on Magnolol *in vivo* [44], there is still an urgent need to determine more specific PDPK characteristics before its clinical utilization in NSCLC treatment.

In conclusion, our data demonstrate Magnolol is efficacious in suppressing the growth of NSCLC cells both *in vitro* and *in vivo* via perturbing the microtubule polymerization. These findings suggest Magnolol may foster novel therapeutic application for NSCLC treatment.

Acknowledgements

This work was supported by Hubei Provincial Natural Science Foundation of China (No. 2016CFB210) and the Startup Scientific Research Foundation for Doctors of Hubei University of Science and Technology (No. BK1506).

Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Jordan MA, Wilson L: Microtubules as a target for anticancer drugs. *Nature Reviews Cancer* 2004;4:253-265.
- 2 Wilson L, Jordan MA: Microtubule Dynamics - Taking Aim at a Moving Target. *Chemistry & Biology* 1995;2:569-573.
- 3 Risinger AL, Giles FJ, Mooberry SL: Microtubule dynamics as a target in oncology. *Cancer Treatment Reviews* 2009;35:255-261.
- 4 Desbene S, Giorgi-Renault S: Drugs that inhibit tubulin polymerization: the particular case of podophyllotoxin and analogues. *Current medicinal chemistry Anti-cancer agents* 2002;2:71-90.
- 5 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: Global Cancer Statistics, 2012. *Ca-a Cancer Journal for Clinicians* 2015;65:87-108.
- 6 Song GS, Shen J, Jiang FR, Hu RG, Li WY, An L, Zou RJ, Chen ZG, Qin ZY, Hu JQ: Hydrophilic Molybdenum Oxide Nanomaterials with Controlled Morphology and Strong Plasmonic Absorption for Photothermal Ablation of Cancer Cells. *Acs Appl Mater Inter* 2014;6:3915-3922.
- 7 Shen J, Song GS, An M, Li XQ, Wu N, Ruan KC, Hu JQ, Hu RG: The use of hollow mesoporous silica nanospheres to encapsulate bortezomib and improve efficacy for non-small cell lung cancer therapy. *Biomaterials* 2014;35:316-326.

- 8 Li C, Hu JQ, Li WY, Song GS, Shen J: Combined bortezomib-based chemotherapy and p53 gene therapy using hollow mesoporous silica nanospheres for p53 mutant non-small cell lung cancer treatment. *Biomaterials Science* 2017;5:77-88.
- 9 Shen J, Sheng X, Chang Z, Wu Q, Xie D, Wang F, Hu R: The heme-p53 interaction: Linking iron metabolism to p53 signaling and tumorigenesis. *Mol Cell Oncol* 2016;3:e965642.
- 10 Passiglia F, Bronte G, Castiglia M, Listi A, Calo V, Toia F, Cicero G, Fanale D, Rizzo S, Bazan V, Russo A: Prognostic and predictive biomarkers for targeted therapy in NSCLC: for whom the bell tolls? *Expert Opin Biol Ther* 2015;15:1553-1566.
- 11 He CS, Liu YC, Xu ZP, Dai PC, Chen XW, Jin DH: Astragaloside IV Enhances Cisplatin Chemosensitivity in Non-Small Cell Lung Cancer Cells Through Inhibition of B7-H3. *Cellular Physiology and Biochemistry* 2016;40:1221-1229.
- 12 Hu W, Jin P, Liu W: Periostin Contributes to Cisplatin Resistance in Human Non-Small Cell Lung Cancer A549 Cells via Activation of Stat3 and Akt and Upregulation of Survivin. *Cellular Physiology and Biochemistry* 2016;38:1199-1208.
- 13 Vrignaud P, Semiond D, Lejeune P, Bouchard H, Calvet L, Combeau C, Riou JF, Commercon A, Lavelle F, Bissery MC: Preclinical antitumor activity of cabazitaxel, a semisynthetic taxane active in taxane-resistant tumors. *Clin Cancer Res* 2013;19:2973-2983.
- 14 Zhao J, Zhuo M, Wang Z, Duan J, Wang Y, Wang S, An T, Wu M, Wang J: A phase I study of nimotuzumab plus docetaxel in chemotherapy-refractory/resistant patients with advanced non-small-cell lung cancer. *Chin J Cancer Res* 2016;28:12-18.
- 15 Shen W, Pang H, Liu J, Zhou J, Zhang F, Liu L, Ma N, Zhang N, Zhang H: Epithelial-mesenchymal transition contributes to docetaxel resistance in human non-small cell lung cancer. *Oncol Res* 2014;22:47-55.
- 16 Chang A: Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. *Lung Cancer* 2011;71:3-10.
- 17 Maruyama Y, Kuribara H, Morita M, Yuzurihara M, Weintraub ST: Identification of magnolol and honokiol as anxiolytic agents in extracts of Saiboku-to, an Oriental herbal medicine. *Journal of Natural Products* 1998;61:135-138.
- 18 Wang JP, Hsu MF, Raung SL, Chen CC, Kuo JS, Teng CM: Anti-inflammatory and analgesic effects of magnolol. *Naunyn Schmiedeberg's Arch Pharmacol* 1992;346:707-712.
- 19 Seo JU, Kim MH, Kim HM, Jeong HJ: Anticancer potential of magnolol for lung cancer treatment. *Arch Pharm Res* 2011;34:625-633.
- 20 Tse AK-W, Wan C-K, Zhu G-Y, Shen X-L, Cheung H-Y, Yang M, Fong W-F: Magnolol suppresses NF-kappaB activation and NF-kappaB regulated gene expression through inhibition of IkappaB kinase activation. *Molecular immunology* 2007;44:2647-2658.
- 21 Ho KY, Tsai CC, Chen CP, Huang JS, Lin CC: Antimicrobial activity of honokiol and magnolol isolated from *Magnolia officinalis*. *Phytotherapy Research* 2001;15:139-141.
- 22 Fong WF, Tse AK, Poon KH, Wang C: Magnolol and honokiol enhance HL-60 human leukemia cell differentiation induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Int J Biochem Cell Biol* 2005;37:427-441.
- 23 Hwang ES, Park KK: Magnolol Suppresses Metastasis via Inhibition of Invasion, Migration, and Matrix Metalloproteinase-2/-9 Activities in PC-3 Human Prostate Carcinoma Cells. *Bioscience Biotechnology and Biochemistry* 2010;74:961-967.
- 24 Hwang ES, Park KK: Magnolol suppresses metastasis via inhibition of invasion, migration, and matrix metalloproteinase-2/-9 activities in PC-3 human prostate carcinoma cells. *Biosci Biotechnol Biochem* 2010;74:961-967.
- 25 McKeown BT, Hurta RA: Magnolol affects expression of IGF-1 and associated binding proteins in human prostate cancer cells in vitro. *Anticancer Res* 2014;34:6333-6338.
- 26 Kang YJ, Park HJ, Chung HJ, Min HY, Park EJ, Lee MA, Shin Y, Lee SK: Wnt/beta-catenin signaling mediates the antitumor activity of magnolol in colorectal cancer cells. *Mol Pharmacol* 2012;82:168-177.
- 27 Li ML, Zhang F, Wang XA, Wu XS, Zhang BT, Zhang N, Wu WG, Wang Z, Weng H, Liu SB, Gao GF, Mu JS, Shu YJ, Bao RF, Cao Y, Lu JH, Gu J, Zhu J, Liu YB: Magnolol inhibits growth of gallbladder cancer cells through the p53 pathway. *Cancer Science* 2015;106:1341-1350.

- 28 Liu Y, Cao W, Zhang B, Liu YQ, Wang ZY, Wu YP, Yu XJ, Zhang XD, Ming PH, Zhou GB, Huang L: The natural compound magnolol inhibits invasion and exhibits potential in human breast cancer therapy. *Sci Rep* 2013;3:3098.
- 29 Grundner-Culemann K, Dybowski JN, Klammer M, Tebbe A, Schaab C, Daub H: Comparative proteome analysis across non-small cell lung cancer cell lines. *Journal of Proteomics* 2016;130:1-10.
- 30 Shen J, Sheng XP, Chang ZN, Wu Q, Wang S, Xuan ZL, Li D, Wu YL, Shang YJ, Kong XT, Yu L, Li L, Ruan KC, Hu HY, Huang Y, Hui LJ, Xie D, Wang FD, Hu RG: Iron Metabolism Regulates p53 Signaling through Direct Heme-p53 Interaction and Modulation of p53 Localization, Stability, and Function. *Cell Reports* 2014;7:180-193.
- 31 Shen J, Zhang S, Li Y, Zhang W, Chen J, Zhang M, Wang T, Jiang L, Zou X, Wong J, Li X, Cui Y, Wang C: p14(ARF) inhibits the functions of adenovirus E1A oncoprotein. *Biochem J* 2011;434:275-285.
- 32 Jin R, Shen J, Zhang T, Liu Q, Liao C, Ma H, Li S, Yu Z: The highly expressed COL4A1 genes contributes to the proliferation and migration of the Invasive ductal carcinomas. *Oncotarget* 2017; DOI: 10.18632/oncotarget.17345.
- 33 Zhao HB, Quan HT, Xie CY, Xu YP, Xie FC, Hu YH, Lou LG: YHHU0895, a novel synthetic small-molecule microtubule-destabilizing agent, effectively overcomes P-glycoprotein-mediated tumor multidrug resistance. *Cancer Letters* 2012;314:54-62.
- 34 Kuo CC, Hsieh HP, Pan WY, Chen CP, Lion JP, Lee SJ, Chang YL, Chen LT, Chen CT, Chang JY: BPR0L075, a novel synthetic indole compound with antimitotic activity in human cancer cells, exerts effective antitumoral activity in vivo. *Cancer Research* 2004;64:4621-4628.
- 35 Wang SG, Chen Y, Li X, Gao W, Zhang LL, Liu J, Zheng YY, Chen HR, Shi JL: Injectable 2D MoS₂-Integrated Drug Delivering Implant for Highly Efficient NIR-Triggered Synergistic Tumor Hyperthermia. *Adv Mater* 2015;27:7117-+.
- 36 Chiang NJ, Lin CI, Liou JP, Kuo CC, Chang CY, Chen LT, Chang JY: A Novel Synthetic Microtubule Inhibitor, MPT0B214 Exhibits Antitumor Activity in Human Tumor Cells through Mitochondria-Dependent Intrinsic Pathway. *Plos One* 2013;8
- 37 Huang YT, Huang DM, Guh JH, Chen IL, Tzeng CC, Teng CM: CIL-102 interacts with microtubule polymerization and causes mitotic arrest following apoptosis in the human prostate cancer PC-3 cell line. *J Biol Chem* 2005;280:2771-2779.
- 38 Yang JS, Chung JG, Hour MJ, Wang SF, Lin KL: MJ-29 interacts with microtubule polymerization and induces mitotic arrest following apoptosis in human leukemia U937 cell line. *Faseb Journal* 2007;21:A794-A794.
- 39 Chen Y, Li C, Pan Y, Han S, Feng B, Gao Y, Chen J, Zhang K, Wang R, Chen L: The Emerging Role and Promise of Long Noncoding RNAs in Lung Cancer Treatment. *Cell Physiol Biochem* 2016;38:2194-2206.
- 40 Zhang L, Wang S, Che X, Li X: Vitamin D and lung cancer risk: a comprehensive review and meta-analysis. *Cell Physiol Biochem* 2015;36:299-305.
- 41 Kavallaris M: Microtubules and resistance to tubulin-binding agents. *Nature Reviews Cancer* 2010;10:194-204.
- 42 Abrieu A, Doree M, Fisher D: The interplay between cyclin-B-Cdc2 kinase (MPF) and MAP kinase during maturation of oocytes. *Journal of Cell Science* 2001;114:257-267.
- 43 Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD, Pietenpol JA: Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and taxol-induced growth arrest. *Journal of Biological Chemistry* 1998;273:30777-30784.
- 44 Kim SB, Kang HE, Cho HJ, Kim YS, Chung SJ, Yoon IS, Kim DD: Metabolic interactions of magnolol with cytochrome P450 enzymes: uncompetitive inhibition of CYP1A and competitive inhibition of CYP2C. *Drug Development and Industrial Pharmacy* 2016;42:263-269.