Rosthorin A inhibits non-small cell lung cancer cell growth and metastasis through repressing epithelial-mesenchymal transition via downregulating Slug

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Lung cancer always ranks first in the number of cancer deaths every year, accounting for 18.4% of total cancer deaths in 2018. Metastasis is the main cause of death in lung cancer patients. The identification of bioactive components of traditional Chinese medicine is very important for the development of novel reagents against non-small cell lung cancer (NSCLC). Rosthorin A has originated from *Rabdosia rosthornii (Diels) Hara* which excerpts from 'Chinese materia medica', and is known to have 'clear heat phlegm' properties in the folk. Little is known about the biological functions and mechanisms of Rosthorin A in cancer cells at present. The role of EMT in metastasis of a tumor cell is self-evident. Slug is an important EMT inducer, which is related to the development of lung cancer. Cell growth, clone assay, cell migration, cell invasion, and protein expression, and NSCLC transplanted tumor growth were performed in A549, H1299, and H1975 cells. Rosthorin A significantly inhibited the growth of NSCLC cells, it could prolong the survival of nude mice. Rosthorin A inhibited the migration and invasion of A549, H1299, and H1975 cells. Rosthorin A up-regulated E-cadherin expression level and down-regulated the expression of β-catenin, N-cadherin, vimentin, Slug, and Twist. Rosthorin A could promote the expression of E-cadherin and inhibit the development of EMT by downregulating Slug, to inhibit the development and metastasis of NSCLC cells. In summary, Rosthorin A could be used as a promising candidate for the treatment of NSCLC patients with recurrence and metastasis.

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Introduction

Lung cancer which has attracted researchers' attention for many years is still a serious public health problem in the world. In the past decade, about 1.6 million people have been diagnosed with lung cancer every year, and its incidence is still increasing [1]. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers [2]. In recent years, researchers have been made great progress in the diagnosis and treatment of lung cancer. However, lung cancer always ranks first in the number of cancer deaths every year, accounting for 18.4% of total cancer deaths in 2018 [3], and most of them are related to the metastasis of lung cancer. At the time of diagnosis, more than 60% of lung cancer patients have advanced stage or metastasis [4]. Metastasis is the main cause of the death of lung cancer patients. Therefore, many researchers turned to study lung cancer metastasis. Metastasis is thought to be associated with the expression of embryonic transcription factors Slug and Twist, which promote the invasion and metastasis of tumors-epithelial-mesenchymal transition (EMT) [5]. EMT is a process in which epithelial cells with polarity are transformed into stromal cells with migration ability, and the invasion and migration ability is acquired at the same time. EMT can promote metastasis in the progression of many types of cancer, including NSCLC [6]. EMT provides the diffusion of tumor cells with self-renewal characteristics and enhanced proliferation ability, which makes these tumor cells spread to a distant place and increase the chance of metastasis [7]. Therefore, the study of EMT is expected to reduce the recurrence and metastasis of lung cancer and improve the quality of life of lung cancer patients.

Systemic chemotherapy, radiotherapy, or combined targeted therapy are the main treatment methods for locally advanced or metastatic NSCLC. However, high toxicity is a common problem. Many reports show that natural drugs

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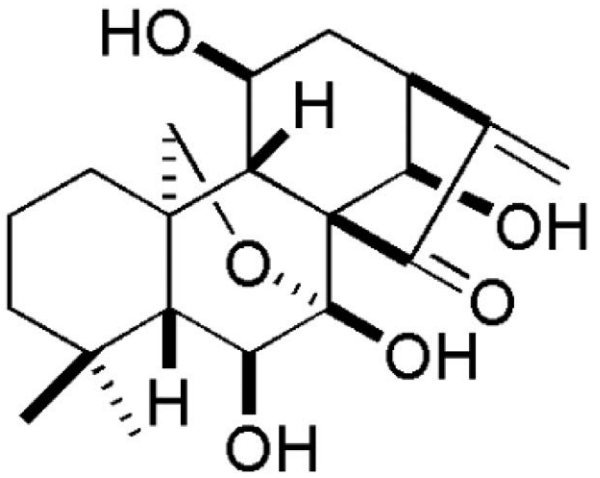
can reduce the side effects of NSCLC patients and improve the effectiveness of chemotherapy [8–10]. Therefore, we screened the monomer composition Rosthorin A that has a direct inhibitory effect in non-small cell lung cancer cells by the ATP method. Rosthorin A has originated from *Rabdosia rosthornii (Diels) Hara*. *Rabdosia rosthornii (Diels) Hara* excerpts from 'Chinese materia medica', and produced in the Mount Emei area in the Sichuan province of China. The role of the grass of *Rabdosia rosthornii (Diels) Hara* is 'clear heat phlegm' and 'swelling and blood stasis' in the folk. The application of traditional Chinese medicine and natural medicine has its unique advantage in lung cancer treatment. Compared with surgery, radiation, and chemotherapy, the treatment of traditional Chinese medicine in lung cancer attaches great importance to the vital qi of the body, to eliminate pathogenic without harming the vital qi. According to the theory of traditional Chinese medicine, the formation of lung cancer is closely connected with 'phlegm condensed', and the original plant of Rosthorin A is from 'Chinese materia medica', have phlegm effect. Therefore, we choose Rosthorin A for studying the anti-lung cancer effect.

Materials and methods

Experiment reagents

Rosthorin A (Fig. 1) was purchased from BioBioPha Co., Ltd (Yunnan, China). The monomer was dissolved in dimethylsulfoxide (DMSO). The dissolved liquid was stored at –20°C. RPMI1640 and Dulbecco's modified eagle medium (DMEM) for culture were purchased from Gibco (Grand Island, New York, USA). Thiazoles [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; methylthiazolyldiphenyl-tetrazolium bromide (MTT)] were purchased from Sigma (St. Louis, Missouri, USA). Matrigel was purchased from BD Biosciences (San Jose, California, USA). Antibodies against Slug and Twist were purchased from Abcam, E-cadherin, N-cadherin, β-catenin, Vimentin, and actin, as well as secondary antibodies, were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Western blotting substrate kit was supplied from Merck Millipore (Billerica, Massachusetts, USA).

Fig. 1



Chemical structure of Rosthorin A.

Cell culture

Human NSCLC cell lines, A549, H1299, and H1975 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. A549 cells were cultured with DMEM medium and H1299 and H1975 cells were grown in RPMI 1640 medium. Both DMEM medium and RPMI1640 medium were supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Grand Island, New York, USA), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation assay

To assess the effect of Rosthorin A on the survival and proliferation of NSCLC cells, MTT colorimetric analysis was performed. A549, H1299, and H1975 cells in the logarithmic phase were respectively seeded into a 96-well plate, at a density of 6000 cells/well and maintained at 37°C in a humidified 5% CO_2 atmosphere for 16 h. Then the Rosthorin A at the indicated final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM were added, respectively. The total volume was 200 μl. At 24 h, MTT was added to a final concentration of 0.5 mg/ml in the 96-well plate. Then the plate was kept at 37°C for 4 h. After removing the supernatants, 150 μl of DMSO was supplemented into each well, and then the plate was maintained at 37°C for 15 minutes. Absorbance at a wavelength of 570 nm was determined by using the Synergy 2 Multi-Mode Microplate Reader (BioTek Instrument, Int., Winooski, Vermont, USA). The cell inhibition was calculated as follows: inhibition rate (%) = [(A of the negative control group – A of the test group)/A of the negative control group] × 100%. Independent experiments were performed at least three times.

Clone assay

To clarify the effect of Rosthorin A on the tumorigenesis ability of NSCLC cells, plate clone assay was applied. A549, H1299, and H1975 cells (500 for each type) in the logarithmic phase were seeded in a six-well plate for 16 h. Then Rosthorin A at a density of 0, 0.25, 0.5, and 1 μM was added to NSCLC cells, respectively. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Twelve days later, the colonies were fixed by 4% paraformaldehyde for 15 minutes, stained with crystal violet for 15 minutes and then imaged and counted under a phase-contrast microscope.

Tumor mouse xenograft assay

All animal procedures, including tumor transplantation, tumor volume monitoring, and killing, were approved

by the Institutional Animal Care and Use Committee at Medical College of Shanghai University of Traditional Chinese Medicine. For the subcutaneous xenograft model, Six- to 8-week-old male Balb/c nude mice, weighing 18–20 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China) and fed in SPF environmental condition. H1975 cells (5×10^6) were suspended in a medium at 100 µl and then subcutaneously injected into the right axilla of Balb/c nude mice. When tumor volume reached approximately 100 mm^3, Rosthorin A groups were given an intraperitoneal injection of Rosthorin A (10 mg/kg). The blank control group was administered intraperitoneally injected with the same volume PBS (phosphate buffer saline). The volume of the tumor and body weight was measured every 3 days. The formula for volume was as follows: $V = (\pi/8) \times a \times b^2$. Where 'a' represents the maximum diameter of the tumor, while 'b' reflects the shorter diameter vertical to 'a'. The Balb/c mouse with the tumor over 2000 mm^3 was put to death by CO_2 suffocation.

Cell migration assay

To determine the intervention of Rosthorin A on the metastasis of NSCLC cells, a cell scratch test was used. A549, H1299, and H1975 cells in the logarithmic phase were seeded in a six-well plate, at a density of 6×10^5. Sixteen hours later, cells were scratched with spearhead. Unattached cells were removed with PBS. Simultaneously, Rosthorin A solutions at a density of 0, 0.25, 0.5, and 1 µM were added to A549, H1299, and H1975 cells. Photographs were taken by microscopes at 24 h after treatment, respectively, and the crawling distance of cells was compared from three independent experiments.

Invasion assay

To investigate the effect of Rosthorin A on NSCLC cell invasion, cell invasive ability was measured using 24-well Matrigel Transwell chambers (BD Biosciences) with 12.0 µm pore polycarbonate membrane. A549, H1299, and H1975 cells treated with 0, 0.25, 0.5, and 1 µM of Rosthorin A for 24 h, and then seeded onto the top compartment of the chamber with low serum medium (0.5% FBS in DMEM for A549 or in RPMI 1640 for H1299 and H1975). Medium with 20% FBS as a chemoattractant was added to the lower chamber. After 24 h incubation, non-invaded cells were carefully removed with a cotton swab. The invaded cells were stained and quantified under a phase-contrast microscope (×10). The data are presented with the average number of cells in 10 randomly selected fields from three independent experiments.

Western blotting for determining apoptotic protein

A549, H1299, and H1975 cells in the logarithmic phase were seeded in the six-well plate. When cells occupied 80% of the dish, Rosthorin A was used for intervention. Rosthorin A at a density of 0, 1, 2, and 4 µM was added to A549, H1299, and H1975 cells, respectively. Twenty-four hours later, 100 µl RIPA was added to the dishes, and cells were fully soaked. After centrifugation for 30 minutes, supernatants were collected for protein measurement. After being washed with 95°C water for 10 minutes, the proteins were separated with gel and transferred to a membrane. The membranes were incubated overnight at 4°C with the primary antibodies and then further incubated with secondary antibodies and finally visualized.

Statistical analysis

Statistical software GraphPad Prism 5 was used for analysis. All data were presented as mean ± SEM. Differences were judged to be statistically significant at $P < 0.05$.

Results

Inhibition of Rosthorin A on the proliferation of non-small cell lung cancer cells

Rosthorin A at different densities (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 µM) was applied to treat A549, H1299, and H1975 cells for 24 h. Results showed that the inhibition rate of Rosthorin A at the density of 1 µM was between 6 and 13% (Fig. 2a). With the increase of the density, the inhibition was improved. *P*-value was lower than 0.01 when the inhibition was over 20% with the corresponding density at 24 h (Fig. 2a). These data suggested that Rosthorin A may be a potential inhibitor for the proliferation of NSCLC cells.

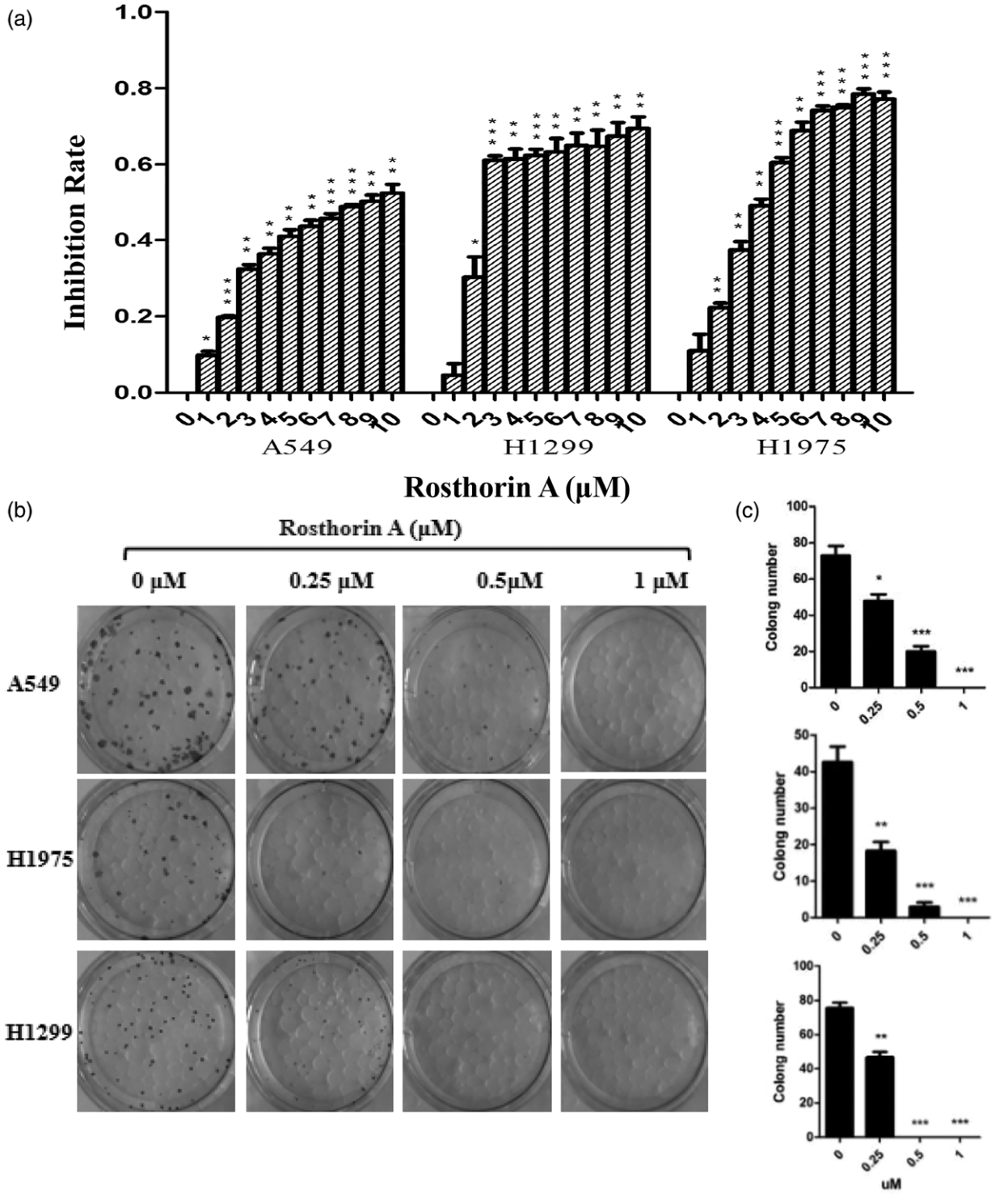
Inhibition of Rosthorin A on the tumor formation of non-small cell lung cancer cells

A further study on the effect of Rosthorin A on the tumor formation of NSCLC cells was performed with plate clone assay. It was found that treated with Rosthorin A at different densities (0, 0.25, 0.5, and 1 µM), the clones of A549, H1299, and H1975 cells declined with the increase of the density (Fig. 2b). When treating A549, H1299, and H1975 cells with Rosthorin A at the density of 0.25 µM, there were fewer clones ($P < 0.05$). The Rosthorin A at the density of both 0.5 and 1 µM can entirely inhibit the formation of NSCLC cells ($P < 0.001$) (Fig. 2c). The results suggested that Rosthorin A can greatly inhibit the tumor formation of NSCLC cells.

Inhibition of Rosthorin A on the growth of non-small cell lung cancer cells *in vivo*

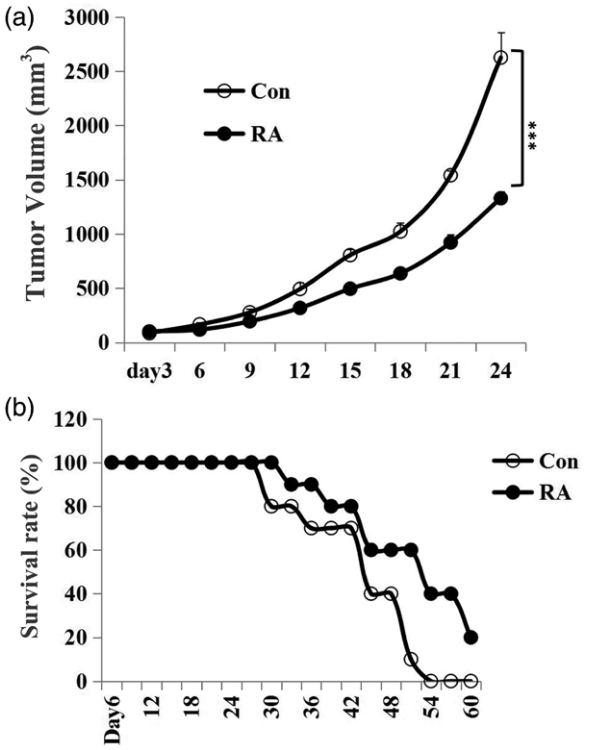
Since Rosthorin A can inhibit the proliferation and the tumor formation of NSCLC cells *in vitro*, it was wondered whether Rosthorin A can inhibit the tumor growth of NSCLC cells *in vivo*. To confirm this, Balb/c nude mice were selected for the study. H1975 cells were injected into the right axilla of Balb/c nude mice. Based on the findings, compared with the control groups, Rosthorin A can distinctively inhibit the growth of NSCLC cells (Fig. 3a). It is suggested that Rosthorin A can markedly prolong the lifetime of mice with NSCLC (Fig. 3b).

Fig. 2



Effect of Rosthorin A on the proliferation of NSCLC cells. (a) A549, H1299, and H1975 cells viability were analyzed by the MTT assay. (b) Effect of Rosthotin A on colony formation of A549, H1299, and H1975 cells. (c) The number of colony formation. Data are presented as mean ± SEM from three individual experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group. NSCLC, non-small cell lung cancer.

Fig. 3



Rosthorin A (RA) inhibits tumor growth and prolongs the survival of tumor-bearing mice. (a) Tumor volume (n = 6). (b) Survival on different days (n = 10). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group.

The intervention of Rosthorin A on the invasion and migration of non-small cell lung cancer cells

We also examined the effects of Rosthorin A on NSCLC cell invasion and migration. According to the proliferation results, Rosthorin A lower than 1 μM did not significantly inhibit cell proliferation in A549, H1299, and H1975 cells after 24 h treatment (Fig. 2a); as a result, 0.25, 0.5, and 1 μM of Rosthorin A were used to investigate its effects on NSCLC cell invasion and migration. Results showed that Rosthorin A treatment decreased the invasive capacity of NSCLC cells (Fig. 4c). Moreover, Rosthorin A treatment inhibited NSCLC cell migration (Fig. 4a). NSCLC cell invasion and migration were effectively inhibited in A549, H1299, and H1975 cells compared to the untreated cells, respectively (both $P < 0.05$, Fig. 4b and d).

Western blotting for determining proliferation and invasion proteins

Our data confirm that Rosthorin A inhibited tumor proliferation and invasion. Next determined whether Rosthorin A regulated the expression of β-catenin, E-cadherin, N-cadherin, Vimentin, Slug, and Twist. The results showed that Rosthorin A could up-regulate the expression of E-cadherin and down-regulate the expression of Slug and Twist. Besides, Rosthorin A could down-regulate the level of β-catenin. For metastatic proteins, Rosthorin A could significantly down-regulate the expression of N-cadherin and Vimentin in a dose-dependent manner in three types of NSCLC cells (Fig. 5a and b). These findings indicated that Rosthorin A could significantly inhibit the growth and metastasis of NSCLC cells.

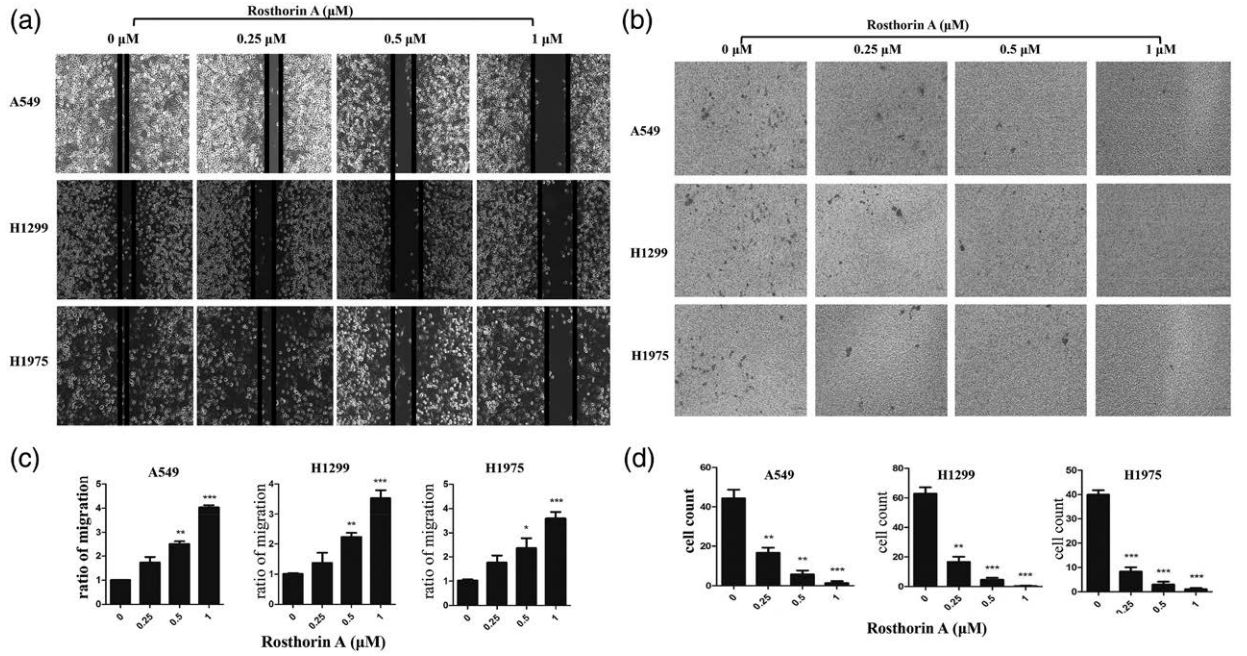
Discussion

In this study, Rosthorin A can significantly inhibit the growth of NSCLC cells *in vitro*, and *in vivo*, it can prolong the survival of nude mice. Scratch and Transwell experiments showed that Rosthorin A significantly inhibited the migration and invasion of A549, H1299, and H1975 cells. EMT is related to many signal pathways *in vivo*, especially the Wnt/β-catenin pathway. The regulation of β-catenin stability is the core mechanism of Wnt/β-catenin signal transduction pathway. Wnt signaling pathway is closely related to lung adenocarcinoma [11–13]. β-catenin is the most important channel transfer molecule in the Wnt signaling pathway. Our study found that Rosthorin A can significantly down-regulated β-catenin expression.

E-cadherin is one of the markers of epithelial morphogenesis. The cells without E-cadherin are loose tumor cells, which are easy to escape from the primary tumor, so tumor cells cannot maintain the integrity of tissue through the formation of adhesion. The decreased expression of E-cadherin is one of the main characteristics of EMT activation. EMT is considered to be the main driving force of tumor from initiation to metastasis [14]. EMT is a highly conserved cellular procedure and plays a key role in promoting tumor invasion and metastasis [15]. In EMT, polarized epithelial cells lose their epithelial characteristics, intercellular adhesion complex, and cytoskeleton specific structure, and change into mobile mesenchymal type cells, which have the ability of invasion and metastasis [14,16]. The expression of E-cadherin was down-regulated in the later stage of epithelial tumor progression [17]. This study found that Rosthorin A can significantly up-regulate the expression of E-cadherin, which indicates that Rosthorin A can inhibit the development of EMT in NSCLC cells.

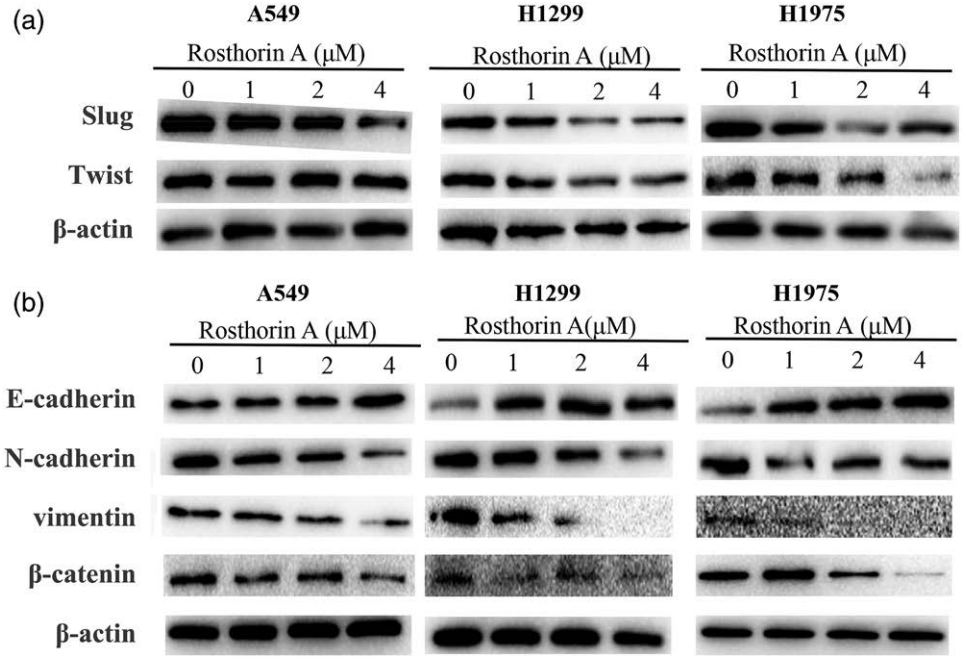
Slug and Twist have been found to induce EMT as transcription factors of E-cadherin. The Twist is the cause of EMT and is associated with poor survival [17,18]. When Twist expression is significantly inhibited, the metastatic proficiency of cells may be impaired. Previous studies have shown that the overexpression of Twist reduces overall survival and recurrence-free survival in lung cancer patients [19,20]. Slug is a transcriptional factor of EMT with zinc finger structures, which plays a crucial role in the process of EMT, migration, invasion, metastasis, and sustain of stem cell-like characteristics of cancer cells, and it is related to the development of lung cancer [5]. As one of the key factors in the EMT process, Slug

Fig. 4



Effect of Rosthorin A on NSCLC cells migratory and invasion ability. A549, H1299, and H1975 cells were treated with Rosthorin A (0, 0.25, 0.5, and 1 μM) for 24 h. (a) Scratch wound healing assays were performed. (b) The ratio of migration. (c) Invaded NSCLC cells were imaged. (d) The number of invaded cells was counted. Data are presented as mean ± SEM from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. NSCLC, non-small cell lung cancer.

Fig. 5



Rosthorin A-induced differential protein expressions were analyzed. (a) Rosthorin A down-regulated Slug and Twist expressions at protein levels in A549, H1299, and H1975 cells. (b) Rosthorin A up-regulated E-cadherin and downregulated N-cadherin, Vimentin, and β-catenin expressions at protein levels in A549, H1299, and H1975 cells.

has been detected in many malignant tumors, and the high expression of Slug has been proved to be related to the clinical results of malignant tumors (such as lung cancer [21] and colorectal cancer [22]). When Slug overexpression can eliminate E-cadherin-mediated intercellular adhesion and promote tumor cell invasion in lung cancer [23,24]. In this study, Rosthorin A down-regulated the expression of Slug in A549, H1299, and H1975 cells, but Rosthorin A only down-regulated the expression of Twist in H1299 and H1975 cells, and had little effect on the expression of Twist in A549 cells. Also, Rosthorin A can down-regulate the expression of transfer related proteins N-cadherin, and Vimentin. Therefore, we can conclude that Rosthorin A can promote the expression of E-cadherin and inhibit the development of EMT by down-regulating Slug, to inhibit the development and metastasis of NSCLC cells.

Taken together, we, for the first time, showed that Rosthorin A could significantly down-regulate the Slug expression at the protein levels in a dose-dependent manner, leading to the increase of E-cadherin expression, the inhibition of EMT and the subsequent inhibition of NSCLC cell growth and metastasis. These results demonstratively showed that Rosthorin A had an effective inhibitory effect on NSCLC growth and metastasis, and Rosthorin A could be used as a promising candidate for the treatment of NSCLC patients with recurrence and metastasis. This study focused on the target of anti-lung cancer of Rosthorin A *in vitro*, but there was no in-depth study in animals. Our later research will focus on the establishment of the lung cancer metastasis model and the intervention of Rosthorin A *in vivo*.

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L.L.N., Z.J.L., and X.L.S. performed the experiments. C.Y., J.N.S., M.A., S.S.L., Y.X.L., F.X., and Y.T.Z. participated in the experiments. L.Y.Q. conceived the study, participated in its design. J.A.L. wrote the manuscript. All authors read and approved the final manuscript.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflicts of interest

There are no conflicts of interest.

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