Mere15, a novel polypeptide from Meretrix meretrix, inhibits proliferation and metastasis of human non-small cell lung cancer cells through regulating the PI3K/Akt/mTOR signaling pathway

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Mere15, an anticancer polypeptide with a molecular weight of 15 kDa, is extracted from the marine species *Meretrix meretrix*. A previous study in our laboratory has confirmed that Mere15 displays a potent antitumor activity. However, the underlying mechanism of Mere15 still remains unclear. The effect of Mere15 on the growth of a variety of tumor cells was measured by the CCK-8 assay. Hoechst33342/PI double staining and flow cytometry assays were used to detect the apoptosis status of cancer cells. Western blotting was used to detect the expression of apoptosis-related proteins, migration and invasion-related protein, and the changes in the PI3K/Akt/mTOR signaling pathway-related proteins. Treatment with Mere15 inhibited cancer cell growth significantly. Scratch wound-healing assay, as well as Transwell experiments, revealed that the polypeptide was able to inhibit the invasion and migration of NSCLC cells significantly. Western blotting analysis confirmed that treatment with Mere15 inhibited the phosphorylation of PI3K, Akt, and mTOR significantly. The effects of Mere15 were also evaluated in the presence of an activator or inhibitor of the PI3K/Akt/mTOR pathway. Downregulated expression of MMP-2, MMP-9, and Snail, and increased expression of E-cadherin were also found in cells treated with Mere15. *In vivo* study revealed that Mere15 inhibited tumor growth significantly in xenograft nude mice bearing NCI-H460 cancer cells. The study provides evidence that Mere15 has the potential to be developed as a novel antimetastatic agent for the treatment of NSCLC patients. The work also provides further evidence that targeting PI3K/Akt/mTOR pathway is an important strategy for overcoming cancer metastasis.

Key words: Mere15, anticancer, migration, invasion, PI3K/Akt/mTOR signaling

Lung cancer is the second most common malignancy and the leading cause of cancer death among both men and women worldwide [1]. The majority of patients with lung cancer are diagnosed at the advanced stages, resulting in a 5-year survival rate below 15% [2]. The poor prognosis makes lung cancer the leading cause of cancer-associated mortality, with the estimate of more than 1.8 million new cases and 1.6 million mortalities per year worldwide [3]. It is well established that most of the patients with lung cancer present with a locally metastatic condition at diagnosis due to its high metastatic potential. Therefore, developing novel anticancer agents with anti-metastatic activity is promising for the treatment of patients with lung cancer.

PI3K/Akt/mTOR signaling pathway plays a key role in cancer development, proliferation, and metastasis [4, 5]. Activation of PI3K can catalyze the phosphorylation of phosphatidylinositol-3,4,5-triphosphate (PIP) 2 to PIP3, and PIP3 is able to bind with Akt to form the PIP3/Akt complex. The activated Akt is capable of catalyzing the phosphorylation of the checkpoint kinase1 (ChK1), murine double minute (MDM2), BclxL/Bcl-2 associated death promoter (BAD), nuclear factor-kappa B (NF-κB), and glycogen synthase kinase (GSK) 23 [6]. The complex of mTOR and raptor can also catalyze the phosphorylation of Akt. Therefore, activation of the PI3K/Akt/mTOR signaling can enhance the proliferation and mobility of cells [7–9]. It is also well

established that activation of the PI3K/Akt/mTOR signaling could increase the expression of matrix metalloproteinases (MMP)-2 and MMP-9 of cancer cells, and thus enhance their ability of metastasis [10, 11].

Mere15, a polypeptide with a molecular weight of 15 kDa was isolated and extracted from *M. meretrix* [12]. Previous studies from our laboratory revealed that Mere15 was able to inhibit the proliferation of several cancer cells and lead to apoptosis and the G0/G1 cell cycle arrest in human lung cancer cells [12, 13]. Further study showed that Mere15 could inhibit the adhesion, migration, and invasion of human lung cancer cells [14]. However, the underlying mechanism of the polypeptide on cancer cell metastasis has not been well addressed. In the present study, we studied the anticancer effect of Mere15 in both *in vitro* and *in vivo* and the role of the PI3K/Akt/mTOR signaling pathway in proliferation, migration, and invasion of human lung cancer cells.

Materials and methods

Chemical reagents. Mere15 was isolated, extracted, and purified from *Meretrix meretrix* L. as described previously [13]. Rabbit anti-human PI3K (4249S), Akt (4691S), mTOR (2983S), p-PI3K (17366S), p-Akt (4060S), and p-mTOR (5536S) monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The monoclonal antibodies of E-cadherin (ab40772), Snail (ab216347), MMP-2 (ab92536), MMP-9 (ab76003), Caspase-3 (9664S), and Caspase-9 (ab2324) were purchased from Abcam (Cambridge, MA, USA). CCK-8, dimethyl sulfoxide (DMSO), PARP, and Actin monoclonal antibodies were purchased from Beyotime (Shanghai, China).

Cell culture. Human non-small cell lung cancer (NSCLC) cells including NCI-H460, PC-9, and A549, human liver cancer cells Bel-7402, human colon cancer cells Caco2, human pancreatic cancer cells Panc-28, and human normal umbilical vein endothelium cells (HUVEC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in modified RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific Inc., New York, NY, USA) at 37°C with 5% CO_2.

Cell viability assay. CCK-8 assay was performed to evaluate the anti-proliferative effects of Mere15 on cancer cells. Briefly, cells ($5–8×10^3$) were seeded in 96-well culture plates. After incubation for 24 h, the cells were treated with various concentrations of Mere15 (0, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μM) for 12, 24, and 48 h respectively. Then, 10 μl of CCK-8 solution was added to each well and the cells were incubated for an additional 30 min. The CCK-8-formazan product was estimated by measuring the absorbance at 450 nm with a microplate reader (Biotek, Winooski, VT, USA). All experiments were performed at least 3 times in triplicates, and the percent of inhibitory rate was calculated by the following formula; inhibitory rate (%) = [(Ac–As)/(Ac–Ab)]

× 100% (As: absorbance of experimental wells; Ac: absorbance of control wells; Ab: absorbance of blank wells).

Determination of apoptosis in cancer cells. Hoechst33342/ PI double staining was performed to detect apoptotic cancer cells. Briefly, NSCLC NCI-H460 and PC-9 cells were seeded in 6-well plates at a density of $2×10^5$ cells/well and cultured for 24 h and then treated with various concentrations of Mere15 (0, 4, 8, and 16 μM for NCI-H460 cells and 0, 2, 4, and 8 μM for PC-9 cells). After incubation for another 48 h, the cells were washed with PBS twice and stained with Hoechst33342 and propidium iodide (PI) using the dual staining kit (Solarbio, Beijing, China) in the dark for 30 min at room temperature. Apoptotic cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometry analysis was also performed to detect the apoptosis of cancer cells using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Briefly, NCI-H460 and PC-9 cells ($2 × 10^5$) were seeded in 6-well culture plates and incubated for 24 h at 37°C. The same concentrations of Mere15 as in the abovementioned Hoechst33342/PI double staining study were added into the plates and incubated for an additional 48 h. Cells were harvested and resuspended in 1× binding buffer, and then Annexin V-FITC (5 μl) and PI (5 μl) were added to the wells and incubated in the dark for 15 min at room temperature. Apoptotic cells were examined by flow cytometry (BD Biosciences, San Jose, CA, USA). All experiments were performed at least 3 times in triplicates.

Determination of cancer cell metastasis. A scratch wound-healing assay was performed to determine the migration of NSCLC NCI-H460 and PC-9 cells. Briefly, cells were seeded in a 6-well plate. After grown to 90–100% confluence, wounds were made by scraping the monolayer of cells with a 10 μl tip and extensively washed with PBS to remove the detached cells and debris. Various concentrations of Mere15 (0, 2, 4, and 8 μM for NCI-H460 cells and 0, 1, 2, and 4 μM for PC-9 cells) were added into the wells and cultured for 24 h. Then, the cells were photographed under an inverted microscope to observe the wound repair, and scratch-healing rate (%) = (initial scratch width – scratch width of specified time)/initial scratch width × 100%.

Transwell assay was also carried out to determine the migration and invasion of NCI-H460 and PC-9 cells. Briefly, the cells ($5–10×10^4$) were plated to the upper chamber of the Transwell culture plates (Corning, NY, USA) and Mere15 with the same concentrations as in scratch wound-healing assay was added to the wells. The lower chamber was filled with 500 μl 1640 medium containing 10% FBS. After incubation for 24 h, the medium in the upper chamber was removed, and the non-migrated cells on the upper surface of the membrane were scrubbed gently with a cotton-tipped swab. The migratory cells on the lower surface of the membrane were fixed with 4% paraformaldehyde (Solarbio, Beijing, China), stained with 1% crystal violet (Beyotime, Haimen, Jiangsu, China), and the number of the migrated

cells was manually counted with an inverted microscope in three randomly selected fields.

For the Transwell invasion assay, the upper wells of the Transwell were coated with 100 µl (250 µg/ml) Matrigel (Corning, NY, USA). The effect of Mere15 on cells invasion was measured by Transwell assay as described above as the cell migration assay. Cells penetrated to the lower chamber were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The stained invasive cells were photographed under an inverted light microscope (OLYMPUS company, Shinjuku-ku, Tokyo, Japan) and quantified by manual counting in three randomly selected fields. All experiments were performed for at least 3 times in triplicates.

Western blotting analysis. NSCLC NCI-H460 and PC-9 cells (2×10^5) were seeded in six-well plates. After incubated for 24 h, the cells were left untreated or treated with 0.1% DMSO and various concentrations of Mere15 (0, 4, 8, and 16 µM for NCI-H460 cells and 0, 2, 4, and 8 µM for PC-9 cells) for 24 or 48 h, respectively. The cells were collected by centrifugation at 1500× g for 5 min and lysed in ice-cold RIPA lysate buffer. The protein concentrations were determined by the BCA method. Whole-cell lysates (30 µg protein) were resolved on 10% SDS-PAGE and then electroblotted onto polyvinylidene fluoride (PVDF) membranes using the Bio-Rad electrophoresis system (Bio-Rad, Hercules, CA, USA). The membranes were incubated in blocking solution (1× TBS, 0.1% Tween-20, and 5% non-fat dry milk powder) for 1 h at room temperature, and subsequently incubated with the corresponding primary antibodies (1:1000) overnight at 4°C. After three 10 min washes with 1× TBST (1× TBS, and 0.1% Tween-20), the membranes were incubated with secondary antibodies (1:1000) for 1 h at room temperature. Actin was used as a loading control. Chemiluminescence substrate solution (Millipore, Burlington, MA, USA) was used for visualization of the protein signals on the membrane after being washed with 1× TBST three times. All experiments were performed three times in triplicate.

Analysis of the antitumor activity *in vivo*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Southwest Medical University (Luzhou, Sichuan, China; Permit No. 201903-227) and strictly followed the guidelines for the investigation of experimental pain in conscious animals for improving animals' welfare to minimize animals' suffering [15]. Female BALB/c nude mice (Tengxin Biotechnology Co, Chongqing, China) aged 4–5 weeks (body weights of 16–18 g) were housed in constant laboratory conditions of a 12 h light/dark cycle and fed with water and food *ad libitum*. All of the mice were inoculated subcutaneously into the right axilla with NCI-H460 cells (3×10^6) in 100 µl PBS. After the tumors were developed more than 100 mm³, the mice were randomly assigned into 6 groups with 8 mice for each group; A) normal saline control, B) DMSO control, C) cyclophosphamide (CTX, 40 mg/kg), D) Mere15 5 mg/kg, E) Mere15 10 mg/kg, and F) Mere15 20 mg/kg. Normal saline, 10% DMSO, and Mere15 were administered by intraperitoneal injection (i.p.) once a day for 14 days (daily x 14) and CTX by i.p. 3 times a week for 2 weeks. The body weight and tumor volume were measured every other day. The tumor-bearing mice were sacrificed and weighed on day 15 (24 hours after the last treatments), then the tumors were removed and photographed. Tumor inhibitory rate was calculated two weeks after treatments using the following formula: Tumor inhibitory rate (%) = [(average tumor volume of control group – average tumor volume of treatment group)/average tumor volume of control group] × 100%.

Statistical analysis. Statistical analyses were performed using the software Excel 2007 and GraphPad 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as mean ± standard deviation (SD). The results of western blotting were quantified by ImageJ (National Institutes of Health, Bethesda, MD, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine statistical significances. Statistical significance was accepted for *$p<0.05$ (marked as *) and higher significance levels of $p<0.01$ and $p<0.001$ were marked as ** and ***, respectively.

Results

Mere15 inhibited cancer cell growth. The CCK-8 assay was performed to evaluate the anti-proliferative effect of Mere15 against several cancer cell lines. The results showed that Mere15 exhibited a significant growth-inhibition against various cancer cells in a dose-dependent manner (Figure 1A). The values of IC_{50} of Mere15 were 3.65±0.17, 5.93±2.83, 6.37±0.12, 6.39±0.19, 9.17±0.23, 10.38±1.20, and 15.84±1.07 µM for NCI-H460, PC-9, Bel-7402, Panc-28, A549, Caco2, and HUVEC, respectively. The data indicate that NCI-H460 and PC-9 cells were the most sensitive cells to Mere15 treatment among all the tested cancer cell lines. We then studied the time-dependent effect of Mere15 by treating Mere15 at 8.0 µM for 12, 24, and 48 h respectively, against NCI-H460 and PC-9 cells and the results showed that the inhibitory effect of Mere15 on NCI-H460 and PC-9 cells was time-dependent (Figure 1B). Therefore, we selected NCI-H460 and PC-9 cells for subsequent studies of apoptosis, migration, and invasion.

Mere15 induced cellular apoptosis in NCI-H460 and PC-9 cells. The pro-apoptotic effects of Mere15 on NCI-H460 and PC-9 cells were studied by Hoechst33342/PI double staining. The results showed that the effect of Mere15 on induction of apoptosis was concentration-dependent with the increase of Mere15 concentrations, more apoptotic cells were observed in both NCI-H460 (Figure 2A) and PC-9 cells (Figure 2B). Flow cytometry analysis was performed to quantitatively analyze the pro-apoptotic effect of Mere15 in NCI-H460 and PC-9 cells. As shown in Figures 2C and 2E, a dose-dependent increase of total apoptotic cells was observed in cells treated with Mere15; the percentages of total apoptotic
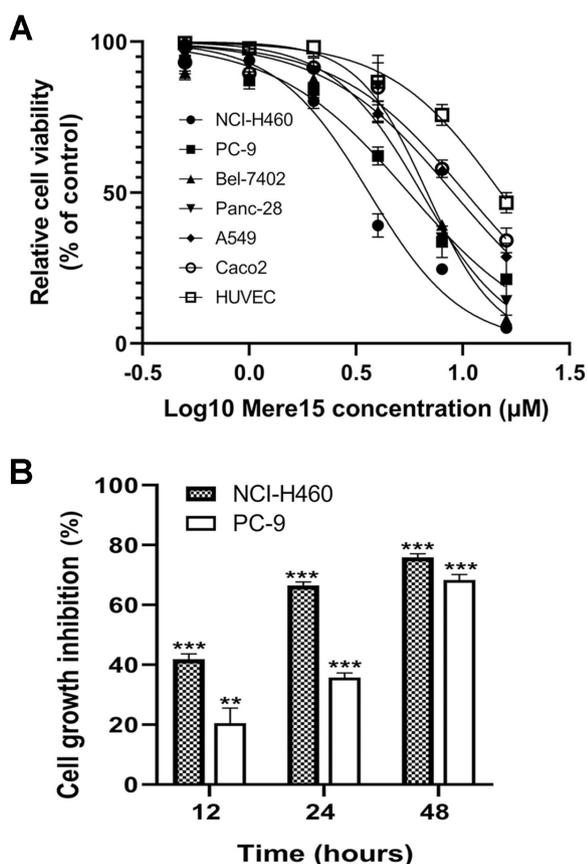
Figure 1. Mere15 inhibited cancer cell growth. Cancer cells were treated with various concentrations of Mere15 for 48 h against NCI-H460, PC-9, Bel-7402, Panc-28, A549, and Caco2 cancer cells and HUVEC (A) or treated with 8.0 μM of Mere15 for 12, 24, and 48 h, respectively; against NCI-H460 and PC-9 cells (B). CCK-8 assays were performed to determine the effect of Mere15 on growth inhibition of cancer cells as described in the Materials and Methods section. The data are representative of at least three independent experiments (n>3) run in triplicate and expressed as mean ± SD. *p<0.05, **p<0.01, and ***p<0.001 vs. control by one-way ANOVA followed by Tukey's multiple comparison test.

was performed to study the effect of Mere15 on cancer cells migration. As shown in Figures 3A and 3C, Mere15 inhibited the cell migration significantly in a dose-dependent manner in NCI-H460 cells; the scratch-healing rate was 37.08%, 28.46%, and 13.42% after treatment with Mere15 at the concentrations of 1.0, 2.0, and 4.0 μM, respectively. A similar result was also found in PC-9 cells treated with Mere15 at the concentrations of 2.0, 4.0, and 8.0 μM (Figures 3B, 3D).

A Transwell experiment was also performed to determine the effect of Mere15 on the migration and invasion of cancer cells. The result showed that treatment of NCI-H460 and PC-9 cells with Mere15 significantly inhibited cell mobility. The numbers of migrated cells were 145±34, 111±15, 85±10, and 35±13; while the numbers of invaded cells were 272±22, 230±10, 192±13, and 108±5 in NCI-H460 cells treated with Mere15 at the concentrations of 0, 1.0, 2.0, and 4.0 μM, respectively (Figures 3E, 3G). Similar results were also found in PC-9 cells treated with Mere15 at the concentrations of 0, 2.0, 4.0, and 8.0 μM (Figures 3F, 3H). These results demonstrated that Mere15 was able to inhibit the migration and invasion of cancer cells.

We then studied the underlying mechanism of Mere15 for inhibiting cancer cell proliferation and metastasis. It has been well documented that activation of the PI3K/Akt/mTOR signaling plays a critical role in the proliferation and metastasis of cancer cells. Western blotting analysis showed that treatment with Mere15 inactivated the PI3K signaling significantly; the phosphorylation of PI3K, Akt, and mTOR was significantly inhibited in both NCI-H460 and PC-9 cancer cells (Figure 4A). Additionally, Mere15 also downregulated the expression of MMP-2, MMP-9, and Snail while upregulated the expression of E-cadherin in both NCI-H460 and PC-9 cells (Figure 4B).

In order to further verify the role of the PI3K/Akt/mTOR signaling on the Mere15-induced anti-metastasis effect, we studied the effect of Mere15 on the expression of related proteins in the presence of activator LY294002 or inhibitor 1,3-dicaffeoylquinin acid of the PI3K/Akt/mTOR pathway. The results showed that the expression of p-PI3K, p-Akt, and p-mTOR was partially reversed after being treated with the combination of Mere15 and LY294002 compared to Mere15 alone in NCI-H460 cells (Figure 4Ca). In contrast, the expression of p-PI3K, p-Akt, and p-mTOR was increased significantly after NCI-H460 cells were treated with the combination of Mere15 and 1,3-dicaffeoylquinin acid LY294002 compared to Mere15 alone (Figure 4Cb). These results suggested that Mere15 inhibited the metastasis of cancer cells via regulating the PI3K/Akt/mTOR signaling pathway.

Mere15 inhibited the tumor growth of NCI-H460 xenografts *in vivo*. A cancer model of tumor xenograft bearing in nude mice was developed to evaluate the antitumor effect of Mere15 *in vivo*. The results showed that i.p. administration of Mere15 inhibited the tumor growth significantly (Figure 5A); the inhibitory rates of tumor growth were 25.3, 45.2, and 61.2% after the mice were treated with Mere15 at the

cells were 37.30, 55.82, 78.35% in the NCI-H460 cells treated with Mere15 at 4.0, 8.0, and 16.0 μM, respectively. Similar results were also found in PC-9 cells, and the percentages of total apoptotic cells were 24.03, 37.01, and 65.44% treated with 2.0, 4.0, and 8.0 μM of Mere15, respectively (Figures 2D, 2F). These results suggest that Mere15 induces cell death perhaps via the apoptotic pathway. Western blotting analysis was performed to investigate the underlying mechanism associated with Mere15-induced cancer cell apoptosis. The results indicated that the expressions of anti-apoptosis proteins, caspase-3, caspase-9, and PARP were decreased but the expression of cleaved PARP, cleaved caspase-3, and cleaved PARP was significantly increased in NCI-H460 and PC-9 cells treated with Mere15 (Figures 2H, 2G).

Mere15 inhibited the migration and invasion in NCI-H460 and PC-9 cells. A scratch wound-healing assay
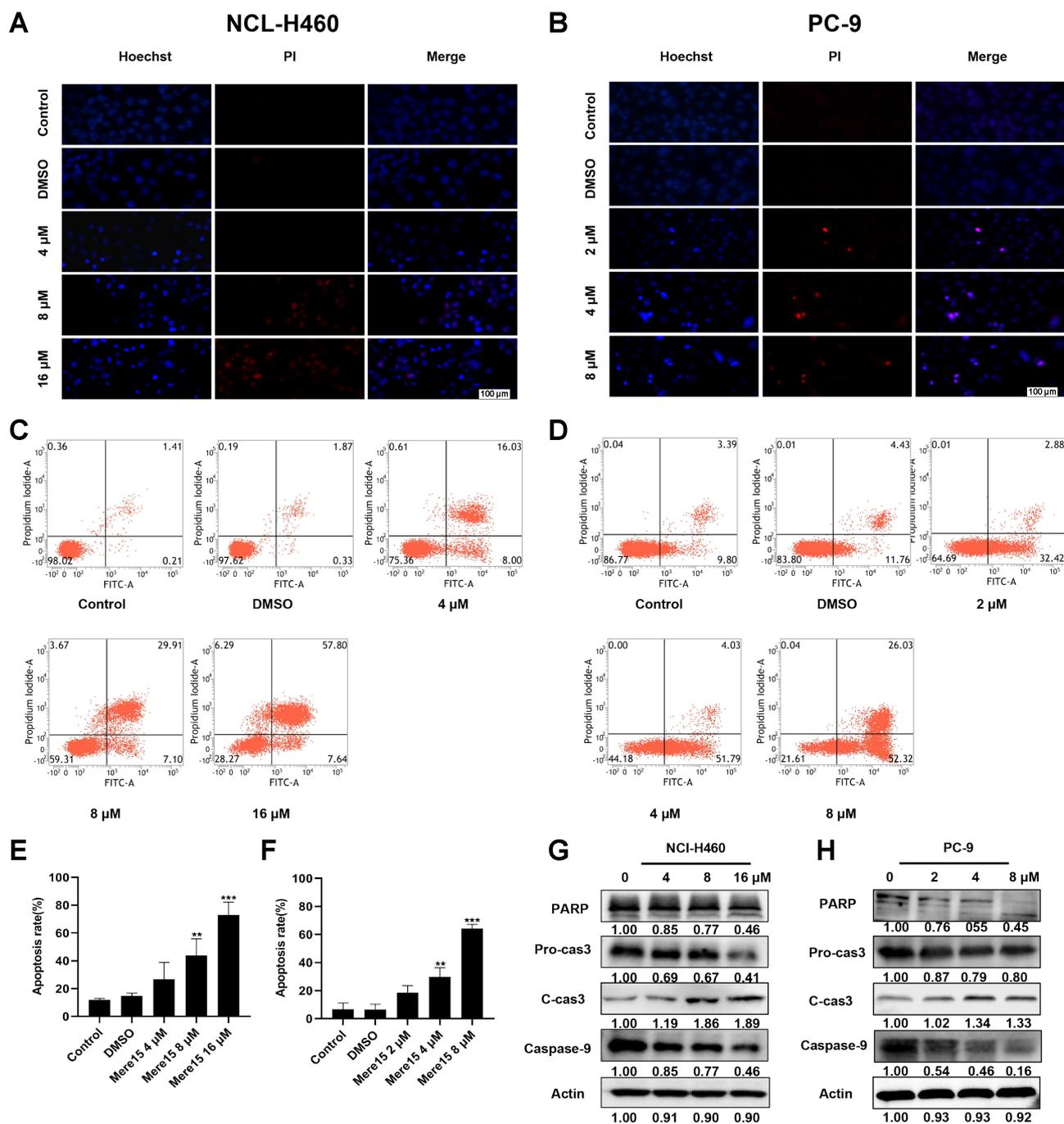
Figure 2. Mere15 induced apoptosis in both human non-small lung cancer (NSCLC) NCI-H460 and PC-9 cells. Effect of Mere15 on the apoptosis detected by Hoechst33342/PI double stains and observed under a fluorescence microscope in NCI-H460 (A) and PC-9 cells (B) scale bars: 100 μm; Flow cytometry was performed to quantitatively analyses the effect of Mere15 on the apoptosis of NCI-H460 (C, E) and PC-9 cells (D, F). Western blotting analysis was carried out to determine the expression of apoptotic related proteins in NCI-H460 (G) and PC-9 cells (H). The cells were treated with various concentrations of Mere15 (0, 4, 8, and 16 μM for NCI-H460 cells and 0, 2, 4, and 8 μM for PC-9 cells). The data are representative of at least three independent experiments (n > 3) run in triplicate and expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control by one-way ANOVA followed by Tukey's multiple comparison test.

doses of 5.0, 10.0, and 20.0 mg/kg, respectively (Figure 5B), similar therapeutic efficacy as the positive control CTX (61.8% inhibitory rates), a commonly used anticancer drug clinically. In addition, there was no significant bodyweight change in Mere15 treated mice, but CTX induced a significant decrease in the body weight of mice compared to that

of the negative (normal saline-treated) and solvent-treated control mice (Figure 5C). The results demonstrated that Mere15 displays a potent antitumor activity *in vivo* with low toxicity to the hosts.

Discussion

In the present study, we studied the antitumor and anti-metastatic effects of Mere15 on human NSCLC NCI-H460 and PC-9 cells, and the results showed that Mere15 was able to inhibit the growth of NSCLC cells via inducing cellular apoptosis. Mere15 can also inhibit migration and invasion of

NSCLC cells via inactivating the PI3K/Akt/mTOR pathway. *In vivo* study demonstrated that i.p. administration of Mere15 at 5–20 mg/kg significantly inhibited tumor growth of NCI-H460 xenografts bearing in nude mice. Our study provides evidence that Mere15 has the potential to be developed as a novel anticancer agent for the treatment of patients with lung cancer.

Metastasis is the main reason for the low survival rate of NSCLC patients and targeting the MMPs is one of the important therapeutic strategies for overcoming the metastatic ability of cancer cells [16–18]. MMPs play important role in cell migration and invasion. In addition to facilitating
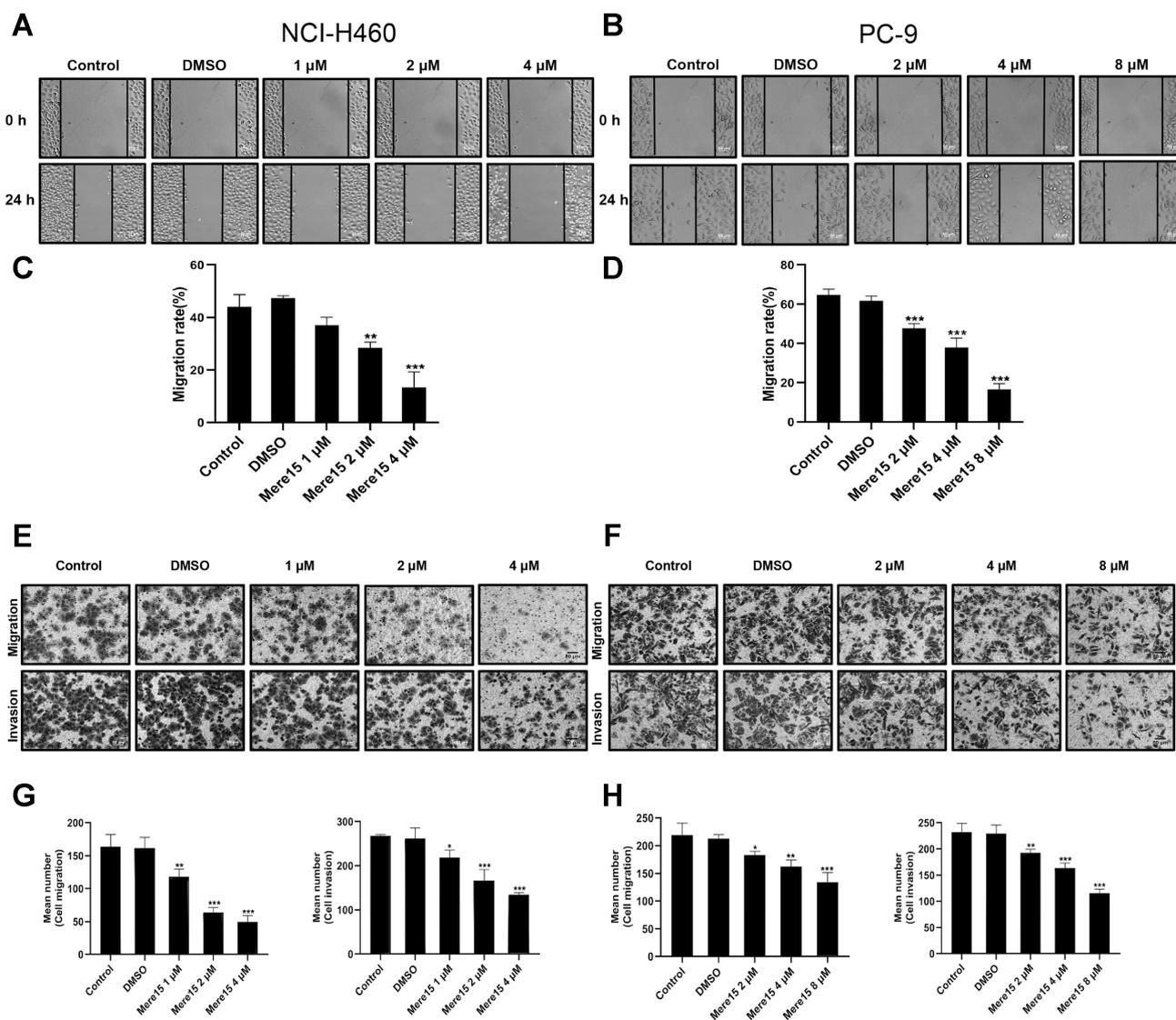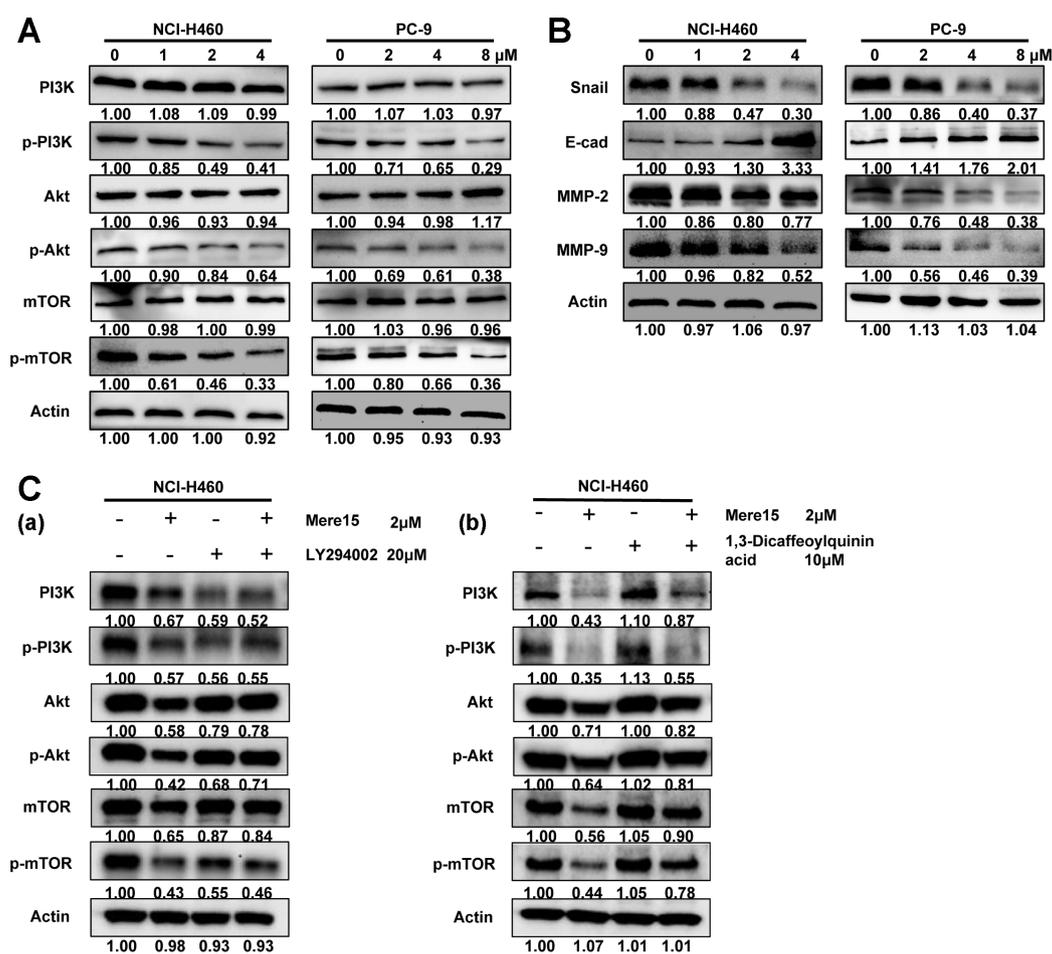

Figure 3. Mere15 inhibited migration and invasion of human NSCLC NCI-H460 and PC-9 cells. The cells were treated with various concentrations of Mere15 (0, 1, 2, and 4 μM for NCI-H460 cells and 0, 2, 4, and 8 μM for PC-9 cells), and the migration of NCI-H460 cells (A, C) and PC-9 cells (B, D) was determined by the scratch wound-healing assay (scale bars: 50 μm). Transwell assay was also performed to determine the migration and invasion of NCI-H460 cells (E, G) and PC-9 cells (F, H) treated with Mere15. The data are representative of at least three independent experiments (n > 3) run in triplicate and expressed as mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. control by one-way ANOVA followed by Tukey's multiple comparison test.

tumor invasion, these enzymes may generate a diverse array of bioactive cleaved peptides. These products can modulate cancer-cell proliferation, survival, tumor angiogenesis, and migration. It has been well established that activation of the PI3K/Akt/mTOR signaling pathway is able to increase the expression of MMP-2 and MMP-9, and thus increase the ability of metastasis of cancer cells [10, 11]. Sinulariolide was found to suppress cell migration and invasion by inhibiting MMP-2/-9 in a human bladder cancer cell [19]. Dryofragin, a phloroglucinol derivative extracted from *Dryopteris fragrans* (L.) Schott, could inhibit the migration and invasion of human osteosarcoma U2OS cells by reducing the expression of MMP-2 and MMP-9 through the PI3K/Akt signaling pathways [20]. In the present study, we confirmed that Mere15 was able to inhibit the migration and invasion of human NSCLC NCI-H460 and PC-9 cells via inactivating the PI3K/Akt/mTOR pathway. Treatment

of NSCLC cells with Mere15 resulted in the inhibitory effect of migration and invasion via downregulating MMP-2/-9 in both NCI-H460 and PC-9 cells. Our study provides more evidence that targeting the PI3K/Akt/mTOR signaling is an effective approach for overcoming tumor metastasis.

Over the last decades, a lot of marine polypeptides have been found from marine organisms with potent anticancer activity and some of them have been applied clinically for the treatment of human cancer. Dolastatin 10, a linear pentapeptide containing several unique amino acid subunits, is derived from the marine mollusk *Dolabella auricularia* [21]. The compound is considered the most potent member of a large class of related peptides with anticancer activity [22, 23]. A dolastatin derivative, auristatin E, linked to a CD30 monoclonal antibody developed as a novel antibody drug conjugate (ADC), called Adcetris, has been approved for the treatment of Hodgkin's lymphoma in 65 countries [24–26].

Figure 4. Mere15 inhibited the activation of the PI3K/Akt/mTOR pathway in human NSCLC NCI-H460 and PC-9 cells. The cells were treated with various concentrations of Mere15 (0, 1, 2, and 4 μM for NCI-H460 cells and 0, 2, 4, and 8 μM for PC-9 cells) for 24 h. The expression of PI3K, p-PI3K, Akt, p-Akt, mTOR, and p-mTOR (A) and snail, E-cad, MMP-2, and PPM-9 (B) in both NCI-H460 and PC-9 cells; and the expression of PI3K, p-PI3K, Akt, p-Akt, mTOR, and p-mTOR in NCI-H460 cells treated with Mere15 (2 μM) and LY294002 (20 μM) or 1,3-dicaffeoylquinin acid (10 μM) alone or in combination (C). The protein expression was detected by western blotting analysis and actin was used as a loading control.
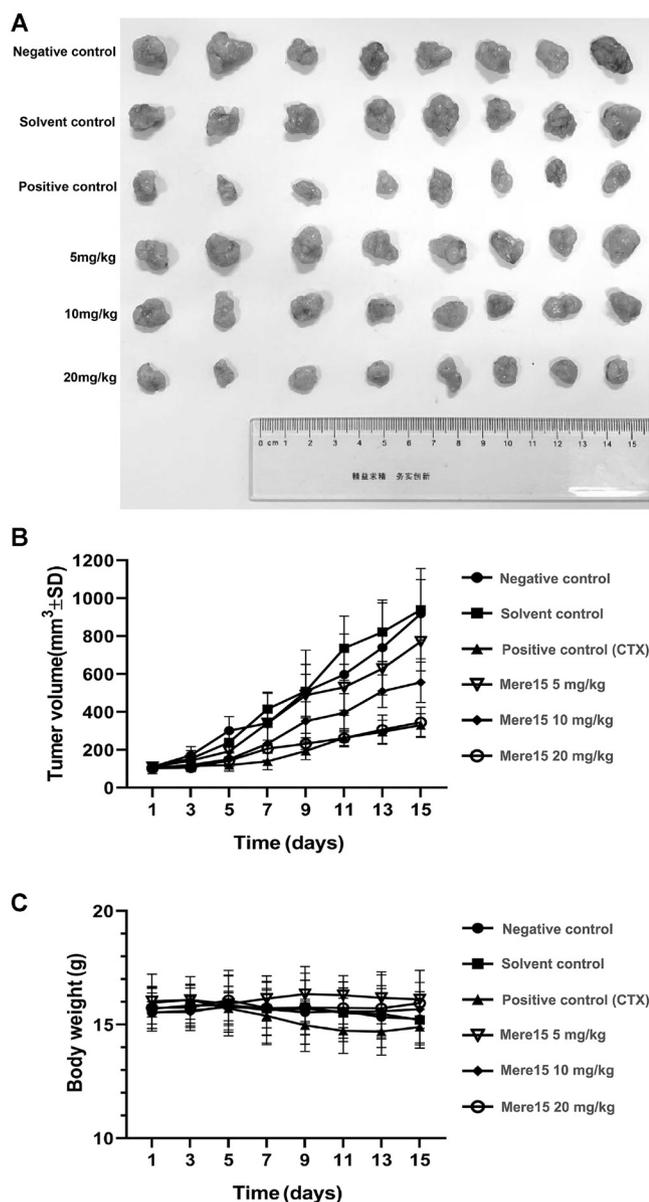
Figure 5. Mere15 inhibited the tumor growth of NCI-H460 xenografts bearing in nude mice. BALB/c nude mice were inoculated with human NSCLC NCI-H460 cells (3×10^6) in 100 μl PBS. After the growth of tumors reached ~100 mm³, the mice were administrated by intraperitoneal injection (i.p.) with normal saline (negative control), 10% DMSO (solvent control), cyclophosphamide (CTX) at 40 mg/kg (positive control), and Mere15 at 5, 10, and 20 mg/kg, respectively; CTX was given 3 times a week for 2 weeks while other groups by once a day for 14 days (daily × 14). A) photographs of tumors, the tumors were removed and photographed on day 15 (24 h after last treatments); B) Tumor volumes curve; and C) body weight curve of mice during treatment. Tumor volumes and body weights were measured and weighed every other day. There were 8 mice for each experimental group and expressed as mean ± SD.

Iturin A, a marine Bacillus microbe-derived lipopeptide was found to inhibit cancer cell growth via the PI3K/Akt/mTOR pathway, and the peptide also exhibit an anti-metastasis effect against several cancer cells [27]. Our present study

confirmed that Mere15 displayed a potent anticancer effect both *in vitro* and *in vivo*, suggesting that the polypeptide has the potential to be developed as a novel anticancer agent. However, the low amount of polypeptide in the species of *Meretrix meretrix* limits its application as an anticancer drug. Studies are ongoing in our laboratory to develop a biotechnique approach to prepare a large amount of the polypeptide for its application clinically.

In conclusion, our study demonstrated that Mere15 could induce cellular apoptosis and inhibit cell proliferation and metastasis through the PI3K/Akt/mTOR signaling pathway in human NSCLC NCI-H460 and PC-9 cells. Furthermore, Mere15 significantly inhibited tumor growth of NCI-H460 xenografts bearing in nude mice *in vivo*. Therefore, the polypeptide is promising for developing as a novel anticancer agent in the treatment of patients with NSCLC.

References

[1] KNIGHT SB, CROSBIE PA, BALATA H, CHUDZIAK J et al. Progress and prospects of early detection in lung cancer. Open Biol 2017; 7: 170070. https://doi.org/10.1098/rsob.170070

[2] TORRE LA, SIEGEL RL, JEMAL A. Lung cancer statistics. Adv Exp Med Biol. 2015; 893: 1–19. https://doi.org/10.1007/978-3-319-24223-1_1

[3] KEITH RL, MILLER YE. Lung cancer chemoprevention: current status and future prospects. Nat Rev Clin Oncol 2013; 10: 334–343. https://doi.org/10.1038/nrclinonc.2013.64

[4] POLIVKA J JR, JANKU F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther 2014; 142: 164–175. https://doi.org/10.1016/j.pharmthera.2013.12.004

[5] PORTA C, PAGLINO C, MOSCA A. Targeting PI3K/Akt/mTOR signaling in cancer. Front Oncol 2014; 4: 64. https://doi.org/10.3389/fonc.2014.00064

[6] JIN Y, FENG SJ, QIU S, SHAO N, ZHENG JH. LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. Eur Rev Med Pharmacol Sci 2017; 21: 3176–3184.

[7] SATHE A, CHALAUD G, OPPOLZER I, WONG KY, VON BUSCH M et al. Parallel PI3K, AKT and mTOR inhibition is required to control feedback loops that limit tumor therapy. PLoS One 2018; 13: e0190854. https://doi.org/10.1371/journal.pone.0190854

[8] LEE H, KIM JS, KIM E. Fucoidan from seaweed Fucus vesiculosus inhibits migration and invasion of human lung cancer cell via PI3K-Akt-mTOR pathways. PLoS One 2012; 7: e50624. https://doi.org/10.1371/journal.pone.0050624

[9] XU Z, LIU D, FAN C, LUAN L, ZHANG X et al. DIXDC1 increases the invasion and migration ability of non-small-cell lung cancer cells via the PI3K-AKT/AP-1 pathway. Mol Carcinogen. 2014; 53: 917–925. https://doi.org/10.1002/mc.22059

[10] BROUXHON SM, KYRKANIDES S, TENG X, ATHAR M, GHAZIZADEH S et al. Soluble E-cadherin: a critical oncogene modulating receptor tyrosine kinases, MAPK and PI3K/Akt/mTOR signaling. Oncogene 2014; 33: 225–235. https://doi.org/10.1038/onc.2012.563

[11] YANG N, HUI L, WANG Y, YANG H, JIANG X. SOX2 promotes the migration and invasion of laryngeal cancer cells by induction of MMP-2 via the PI3K/Akt/mTOR pathway. Oncol Rep 2014; 31: 2651–2659. https://doi.org/10.3892/or.2014.3120

[12] WANG C, LIU M, CHENG L, WEI J, WU N et al. A novel polypeptide from Meretrix meretrix Linnaeus inhibits the growth of human lung adenocarcinoma. Exp Biol Med 2012; 237: 442–450. https://doi.org/10.1258/ebm.2012.011337

[13] LIU M, ZHAO X, JIN Z, XIAO L, LIU H et al. Induction of apoptosis, G0/G1 phase arrest and microtubule disassembly in K562 leukemia cells by Mere15, a novel polypeptide from Meretrix meretrix Linnaeus. Mar Drugs 2012; 10: 2596–2607. https://doi.org/10.3390/md10112596

[14] WANG H, WEI JT, WU N, LIU M, WANG C et al. Mere15, a novel polypeptide from Meretrix meretrix, inhibits adhesion, migration and invasion of human lung cancer A549 cells via down-regulating MMPs. Pharm Biol 2013; 51: 145–151. https://doi.org/10.3109/13880209.2012.715169

[15] ZIMMERMANN M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16: 109–110. https://doi.org/10.1016/0304-3959(83)90201-4

[16] BLACK RC, KHURSHID H. NSCLC: An update of driver mutations, their role in pathogenesis and clinical significance. R I Med J (2013) 2015; 98: 25–28.

[17] CRONIN KA, LAKE AJ, SCOTT S, SHERMAN RL, NOONE AM et al. Annual report to the nation on the status of cancer, part I: national cancer statistics. Cancer 2018; 124: 2785–2800. https://doi.org/10.1002/cncr.31551

[18] ETTINGER DS, WOOD DE, AGGARWAL C, AISNER DL, AKERLEY W et al. NCCN guidelines insights: non–small cell lung cancer, version 1.2020. J Natl Compr Canc Netw 2019; 17: 1464–1472. https://doi.org/10.6004/jnccn.2019.0059

[19] WU YJ, NEOH CA, TSAO CY, SU JH, LI HH. Sinulariolide suppresses human hepatocellular carcinoma cell migration and invasion by inhibiting matrix metalloproteinase-2/-9 through MAPKs and PI3K/Akt signaling pathways. Int J Mol Sci 2015; 16: 16469–16482. https://doi.org/10.3390/ijms160716469

[20] SU Y, WAN D, SONG W. Dryofragin inhibits the migration and invasion of human osteosarcoma U2OS cells by suppressing MMP-2/9 and elevating TIMP-1/2 through PI3K/AKT and p38 MAPK signaling pathways. Anti-Cancer Drug 2016; 27: 660–668. https://doi.org/10.1097/CAD.0000000000000381

[21] BAI R, PETTIT GR, HAMEL E. Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. Biochem Pharmacol 1990; 39: 1941–1949. https://doi.org/10.1016/0006-2952(90)90613-p

[22] BAI R, ROACH MC, JAYARAM SK, BARKOCZY J, PETTIT GR et al. Differential effects of active isomers, segments, and analogs of dolastatin 10 on ligand interactions with tubulin. Correlation with cytotoxicity. Biochem Pharmacol 1993; 45: 1503–1515. https://doi.org/10.1016/0006-2952(93)90051-w

[23] FENNELL BJ, CAROLAN S, PETTIT GR, BELL A. Effects of the antimitotic natural product dolastatin 10, and related peptides, on the human malarial parasite Plasmodium falciparum. J Antimicrob Chemother 2003; 51: 833–841. https://doi.org/10.1093/jac/dkg151

[24] GRAVANIS I, TZOGANI K, VAN HENNIK P, DE GRAEFF P, SCHMITT P et al. The European medicines agency review of brentuximab Vedotin (Adcetris) for the treatment of adult patients with relapsed or refractory CD30+ hodgkin lymphoma or systemic anaplastic large cell lymphoma: summary of the scientific assessment of the committee. Oncologist 2015; 21: 102–109. https://doi.org/10.1634/theoncologist.2015-0276

[25] MEI M, THOMAS S, CHEN R. Management of relapsed or refractory hodgkin lymphoma with second-generation antibody–drug conjugates: focus on brentuximab vedotin. BioDrugs 2014; 28: 245–251. https://doi.org/10.1007/s40259-013-0077-7

[26] SASSOON I, BLANC V. Antibody-drug conjugate (ADC) clinical pipeline: a review. Methods Mol Biol 2013; 1045: 1–27. https://doi.org/10.1007/978-1-62703-541-5_1

[27] DEY G, BHARTI R, DAS AK, SEN R, MANDAL M. Re-sensitization of Akt induced docetaxel resistance in breast cancer by 'Iturin A' a lipopeptide molecule from marine bacteria Bacillus megaterium. Sci Rep 2017; 7: 17324. https://doi.org/10.1038/s41598-017-17652-z