

Eupalinilide B as a novel anti-cancer agent that inhibits proliferation and epithelial–mesenchymal transition in laryngeal cancer cells

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Abstract

Objective: To investigate the anti-cancer effects and potential mechanisms of eupalinilide B in laryngeal cancer cells.

Methods: Laryngeal cancer cell lines were selected to study the anti-tumor effects of eupalinilide B *in vitro* and *in vivo*. Lysine-specific demethylase 1 (LSD1) activity was assessed *in vitro* and dialysis experiments were performed to identify the anti-tumor target of the drug.

Results: Eupalinilide B concentration-dependently inhibited the proliferation of laryngeal cancer cells, exhibiting potent inhibitory activity against TU686 ($IC_{50} = 6.73 \mu M$), TU212 ($IC_{50} = 1.03 \mu M$), M4e ($IC_{50} = 3.12 \mu M$), AMC-HN-8 ($IC_{50} = 2.13 \mu M$), Hep-2 ($IC_{50} = 9.07 \mu M$), and LCC cells ($IC_{50} = 4.20 \mu M$). Subsequent target verification experiments demonstrated that eupalinilide B selectively and reversibly inhibited LSD1. Furthermore, eupalinilide B, as a natural product, suppressed epithelial–mesenchymal transition in TU212 cells. An *in vivo* experiment further indicated that eupalinilide B could significantly reduce the growth of tumors in TU212 xenograft mouse models.

Conclusions: Eupalinilide B might be a novel LSD1 inhibitor for treating laryngeal cancer.

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Keywords

Eupalinilide B, laryngeal cancer, proliferation, epithelial–mesenchymal transition, xenograft, lysine-specific demethylase 1, monoamine oxidase

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Introduction

Laryngeal cancer is a common head and neck cancer and a serious threat to human health.^{1,2} Although some progress has been made in the treatment of laryngeal cancer, improving the survival and quality of life of patients remains a challenge.³ Chemotherapy has become increasingly important because of its protective effects on laryngeal function.⁴

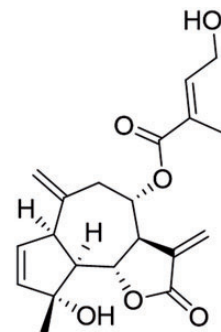
Lysine-specific demethylase 1 (LSD1) is a highly conserved flavin adenine dinucleotide-dependent oxidase with an amine oxidase domain.⁵ LSD1 inhibition can induce tumor cell apoptosis and block tumor cell migration and proliferation.⁶ In addition, LSD1 is overexpressed in many malignant tumors, including stomach, laryngeal, cervical, and prostate cancers.⁷ Because of its important biological roles, LSD1 has been a promising anti-cancer target for identifying new anti-tumor drugs.⁸

Natural compounds, representing an important source of new cancer therapies, have potent anti-cancer properties and few side effects.⁹ Paclitaxel, podophylotoxin, ginsenoside, and colchicine have been widely used to treat cancer.¹⁰ *Eupatorium lindleyanum* is a perennial herbaceous plant that has been used to treat cough and tracheitis because of its anti-microbial and anti-inflammatory activities.¹¹ Recently, eupalinilide B (Figure 1), a novel sesquiterpene lactone isolated from *E. lindleyanum*, displayed potential anti-proliferative activity against P388 and A549 tumor cell lines.¹² However, the anti-cancer effects and mechanisms of eupalinilide B against laryngeal cancer cells remain unknown. In this study, we investigated the anti-cancer activity of eupalinilide B in laryngeal cancer. Importantly, its inhibitory effects on LSD1 in laryngeal cancer cells were also explored.



Eupatorium lindleyanum

Isolation



Eupalinilide B

Figure 1. *Eupatorium lindleyanum* and the chemical structure of eupalinilide B.

Materials and methods

Drug and reagents

Eupalinilide B was purchased from Innochem (Beijing, China), and its purity was greater than 98%. Eupalinilide B was dissolved in dimethyl sulfoxide (Innochem) as a stock solution. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Innochem) solution (5 mg/mL) was created for subsequent experiments.

Cell culture and MTT assay

TU686, TU212, M4e, AMC-HN-8, Hep-2, and LCC laryngeal cancer cells (Procell Life Science & Technology Co., Ltd., Wuhan, China) were cultured in DMEM (Procell Life Science & Technology Co., Ltd.) or RPMI-1640 medium (Hyclone, Merck KGaA). Cancer cells were seeded in 96-well microplates (Corning, Corning, NY, USA) for 24 hours. Then, eupalinilide B was added, and plates were incubated for 48 or 72 hours. MTT solution (Innochem) was added, followed by incubation for 4 hours. OD at 570 nm was measured using a microplate reader (PerkinElmer, Waltham, MA, USA).

LSD1 activity and monoamine oxidase (MAO)-A/B activity

cDNA encoding LSD1 (Hyclone, Merck KGaA) was used to prepare pET28b-LSD1 plasmids, which were incubated with eupalinilide B. The inhibition rate of LSD1 was measured using a Varioskan LUX microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Commercial kits were used to evaluate the inhibitory effects of eupalinilide B on MAO-A and MAO-B activity (Hyclone, Merck KGaA).

Dialysis assay

Eupalinilide B and LSD1 were incubated and dialyzed at 4°C for 24 hours. Then,

the relative activity of LSD1 was measured to investigate the binding mode of eupalinilide B. GSK-LSD1 (Selleck Chemicals, Houston, TX, USA) was also selected as a LSD1 inhibitor to perform the dialysis experiment. Finally, the each tube was measured with a spectrophotometer (PerkinElmer).

Western blotting analysis

Cancer cells were cultured in six-well plates (Hyclone, Merck KGaA). Cells were incubated for 24 hours with eupalinilide B and then washed with PBS and lysis buffer (Hyclone, Merck KGaA). The protein extract was denatured and run on 10% SDS-PAGE gels (Procell Life Science & Technology). Primary antibodies against H3K9me1 and H3K9me2 (1:500 to 1:1500, Procell Life Science & Technology) were used for western blotting. Then, the membranes were incubated with secondary antibodies (Procell Life Science & Technology). The duration of incubation with the primary antibodies was 12 hours, whereas that for secondary antibodies was 1 hour. We performed the western blotting experiments three times.

Wound healing assay

TU212 cells were cultured in a six-well plate for 1 day. A pipette tip (Innochem) was used to scratch the surface of each well. Finally, TU212 cells were treated with eupalinilide B at different concentrations (0, 1, or 2 μM) for 48 hours, and images were taken using a camera (Procell Life Science & Technology) at ×400 magnification.

Migration assay

TU212 cells were cultured in a 24-well plate containing a Transwell membrane (Hyclone, Merck KGaA). Then, 20% fetal bovine serum (Hyclone, Merck KGaA) was added into the lower chamber separately.

TU212 cells in the upper chamber were treated with eupalinilide B at different concentrations for 48 hours. Then, TU212 cells in the lower chamber were washed and fixed with methanol. Finally, cells were stained with hematoxylin for 30 minutes (Hyclone, Merck KGaA).

Animal experiments and hematoxylin-eosin staining

Animals were treated according to the protocols established by the First Affiliated Hospital of Harbin Medical University. The reporting of this study conforms to ARRIVE 2.0 guidelines. The *in vivo* experiments were also conducted in accordance with the guidelines of the ethics committee of the First Affiliated Hospital of Harbin Medical University. The care of the animals followed the 'Guide for the Care and Use of Laboratory Animals, 8th Edition'. We have made efforts to minimize the number of animals used and to decrease their suffering.

Animal experiments were performed according to the guidelines from the ethics committee of the First Affiliated Hospital of Harbin Medical University (approval number: 2020-JLL03; approval date: January 23, 2020). BALB/c mice were obtained from Servicebio (Wuhan, China). Xenograft models were established by implanting mice with 1×10^6 TU212 cells. Then, mice were separated ($n = 5/\text{group}$) into vehicle (PBS) and treatment groups (10 and 50 mg/kg). The animals were treated for 21 days by intragastric administration. Finally, all mice were euthanized prior to assessments of tumor growth.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad, La Jolla, CA, USA). A *t*-test or analysis of variance was used to compare the measurement

data. $P < 0.05$ was considered statistically significant.

Results

Eupalinilide B shows the potentially anti-proliferative activity

Eupalinilide B was evaluated for its potential anti-proliferative activity by the MTT assay in nine cancer cell lines, including HepG2 and SMCC-7721 human liver cancer cells, BFTC-905 and RT-112 human bladder cancer cells, HL-60 human leukemia cells, MGC803 human gastric cancer cells, KYSE-70 human esophageal cancer cells, TU686 human laryngeal cancer cells, and A549 human lung cancer cells. 5-Fluorouracil was selected as a reference drug to perform the anti-proliferative assay in cancer cell lines (BFTC-905 and A549). The IC_{50} values of 5-fluorouracil in BFTC-905 and A549 cells were 30.27 and 9.74 μM , respectively. As reported in Figure 2, eupalinilide B displayed potent anti-proliferative activity against BFTC-905, MGC803, HL-60, TU686, and A549 cells (all $P < 0.05$). In particular, eupalinilide B displayed the strongest anti-proliferative effects against TU686 laryngeal cancer cells.

Eupalinilide B potently inhibits proliferation against laryngeal cancer cells

Because eupalinilide B displayed strong inhibitory activity in TU686 cells among the examined cancer cell lines, different laryngeal cancer cell lines (TU686, TU212, M4e, AMC-HN-8, Hep-2, and LCC) were chosen to investigate its anti-proliferative effects. As presented in Figure 3, the viability of TU212 cells was obviously decreased. Eupalinilide B potently inhibited proliferation with IC_{50} values of 6.73, 1.03, 3.12, 2.13, 9.07, and 4.20 μM in TU686, TU212, M4e, AMC-HN-8, Hep-2, and LCC cells

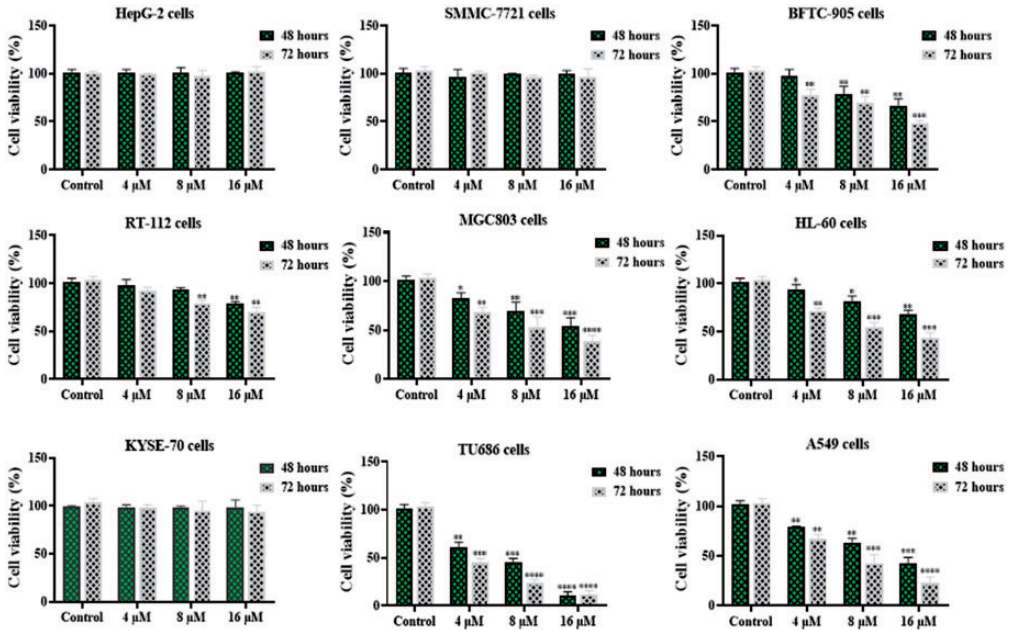


Figure 2. Anti-proliferative activity of eupalinilide B. Cell lines were treated with eupalinilide B (0, 4, 8, or 16 μM) for 48 or 72 hours. Significant differences were identified between the treatment and control groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

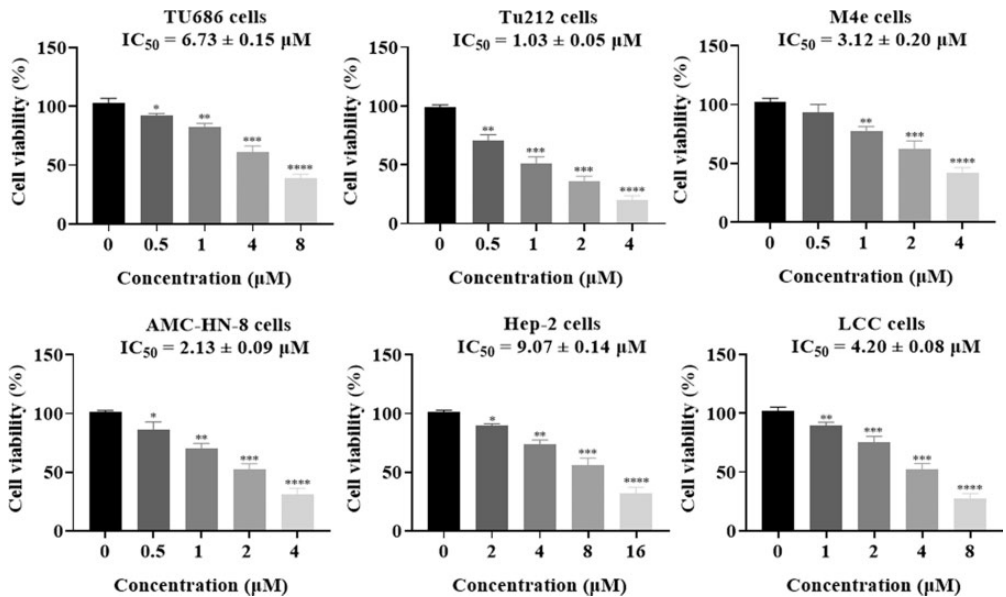


Figure 3. Six laryngeal cancer cell lines were treated with eupalinilide B at different concentrations for 48 hours. The data are presented as the mean \pm SEM. Significant differences were identified between the treatment and control groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

(all $P < 0.05$). These findings indicated that eupalinilide B inhibited the proliferation of laryngeal cancer cell lines in a concentration-dependent manner.

Inhibition of eupalinilide B on LSD1 activity

LSD1 belongs to the flavin adenine dinucleotide-dependent amine oxidase family, and it is similar to other flavin-dependent amine oxidases, including MAOs.¹³ Therefore, eupalinilide B was assessed for selectivity between LSD1 and MAO-A/B. From the results in Figure 4, eupalinilide B at 1000 nM inhibited LSD1, MAO-A, and MAO-B activity with inhibitory rates of 78%, 15%, and 16.7%, respectively. The results indicated that eupalinilide B was a selective LSD1 inhibitor. Then, a dialysis experiment was conducted to reveal the potential binding mode of eupalinilide B to LSD1.¹⁴ Based on the results, eupalinilide B might inhibit LSD1 in a reversible manner. As previously reported, H3K9me1 and H3K9me2 are substrates of LSD1.¹⁵ To further investigate the LSD1-inhibitory effects of eupalinilide B at the cellular level, western blotting was employed to examine whether eupalinilide B could affect the expression of LSD1 substrates. In the experiment, eupalinilide B increased the expression of H3K9me1 and H3K9me2 in a concentration-dependent manner in TU212 cells.

Eupalinilide B inhibits epithelial–mesenchymal transition (EMT) in laryngeal cancer cells

EMT describes a common pathophysiological process that plays critical roles in wound healing, fibrosis, and tumor development.¹⁶ Because LSD1 contributes to cell proliferation and EMT, we investigated the effects of eupalinilide B on EMT in laryngeal cancer cells. From the

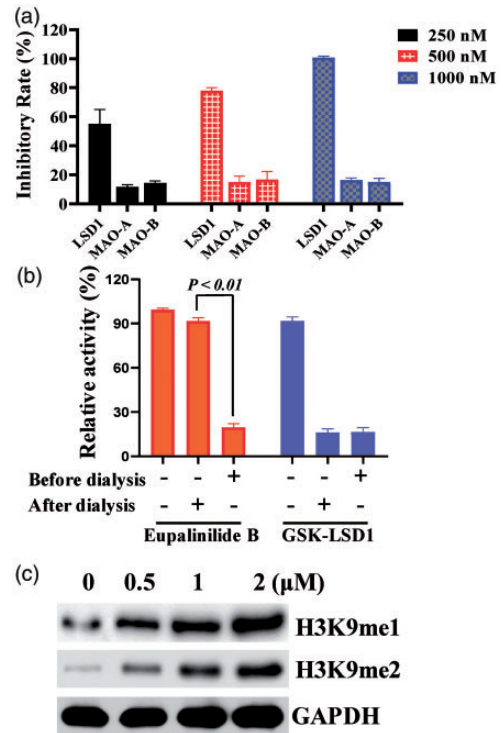


Figure 4. Inhibitory effect of eupalinilide B on LSD1 activity. (a) Inhibitory effects of eupalinilide B on LSD1 and MAO-A/B. (b) Reversibility of the inhibition of LSD1 by eupalinilide B in dialysis experiments. (c) Western blotting of H3K9me1 and H3K9me2 following treatment with eupalinilide B for 24 hours. LSD1, lysine-specific demethylase 1; MAO, monoamine oxidase.

results in Figure 5, eupalinilide B suppressed wound healing ($P < 0.05$). The migration assay demonstrated that eupalinilide B hindered the migration of TU212 cells in a concentration-dependent manner ($P < 0.05$). E-cadherin and N-cadherin, as the related proteins of EMT, were also investigated. Eupalinilide B increased the expression of E-cadherin and downregulated N-cadherin (both $P < 0.05$). According to these results, eupalinilide B could inhibit EMT in laryngeal cancer cells.

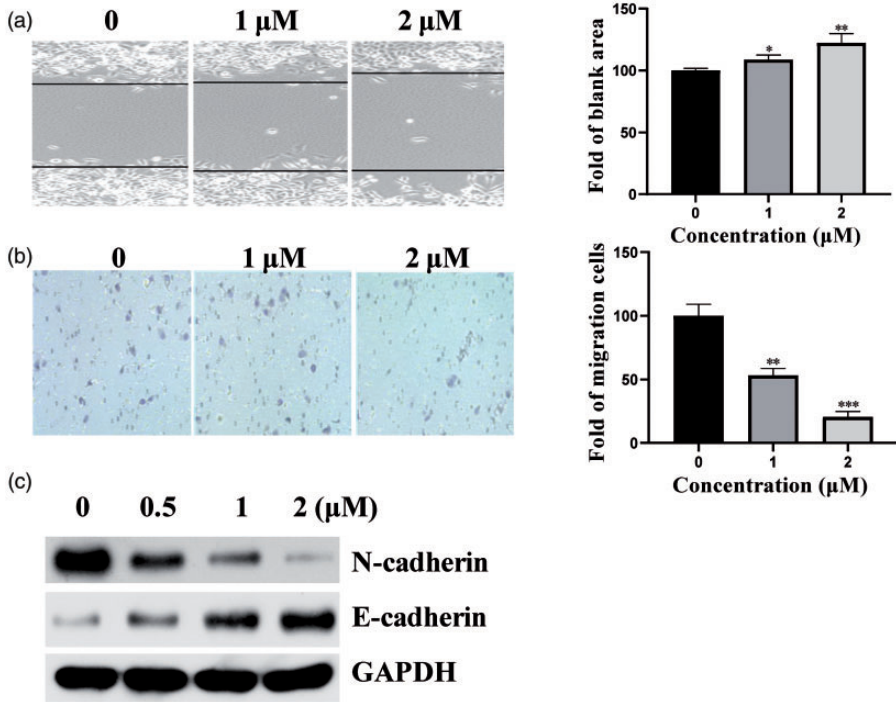


Figure 5. Eupalinilide B inhibited epithelial–mesenchymal transition. (a) TU212 cells were treated with eupalinilide B for 48 hours to perform wound-healing assays. (b) TU212 cells were treated with eupalinilide B for 48 hours to perform migration assays. (c) Western blotting of N-cadherin and E-cadherin following treatment with eupalinilide B for 24 hours. The data are presented as the mean \pm SEM. Significant differences were identified between the treatment and control groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In vivo anti-tumor study

The anti-cancer effects of eupalinilide B was further investigated in xenograft models established with TU212 cells. As presented in Figure 6, eupalinilide B significantly suppressed tumor growth ($P < 0.01$). Furthermore, the tumor volume was smaller in eupalinilide-treated mice than in vehicle-treated mice ($P < 0.01$). During the entire treatment period, no obvious changes of weight were observed in the treatment groups. In addition, hematoxylin–eosin staining results also suggested that eupalinilide B caused no obvious cytotoxicity in the major organs (kidneys, liver, heart, lungs, and spleen). These results demonstrated that eupalinilide B exhibits excellent anti-tumor activity against laryngeal cancer.

Discussion

LSD1 plays crucial roles in cancer and affects many cellular processes.¹⁷ Recent studies revealed that LSD1 was highly expressed in breast, prostate, gastric, and laryngeal cancers.¹⁸ LSD1 inhibition has been an effective strategy for treating cancer.¹⁹ In addition, many LSD1 inhibitors have been discovered from natural products.²⁰ Eupalinilide B, a novel natural product isolated from *E. lindleyanum*, has attracted the attention and interest of scientists. In this work, eupalinilide B was characterized as a novel LSD1 inhibitor for the first time by enzyme activity experiments and cellular experiments. Eupalinilide B at 1000 nM inhibited LSD1, MAO-A, and MAO-B with rates of 78%, 15%, and

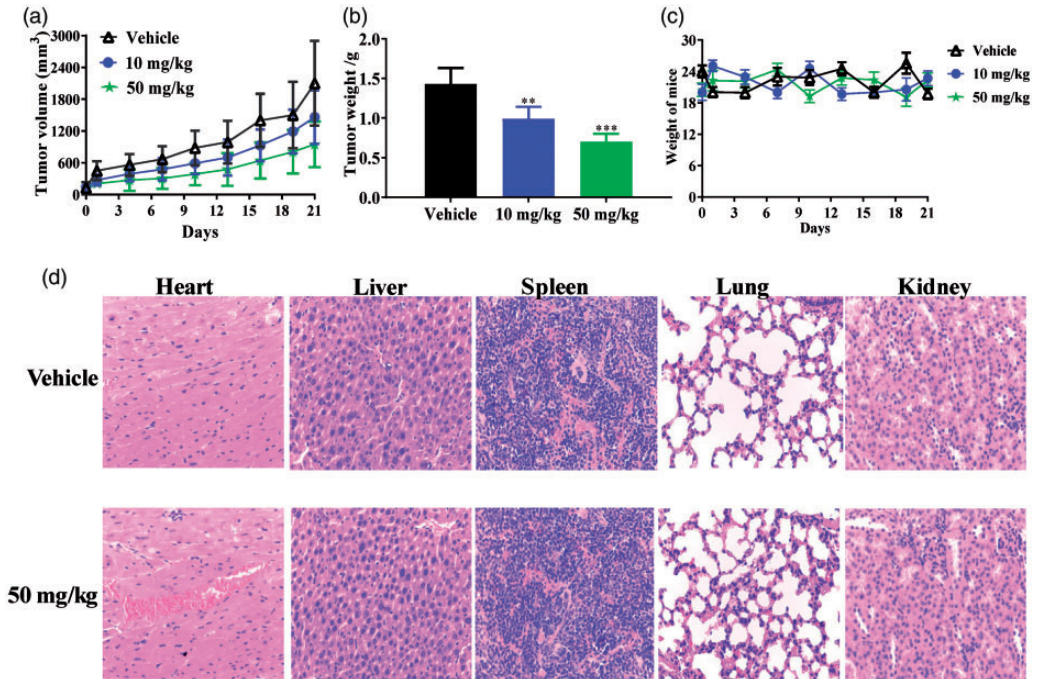


Figure 6. Anti-cancer effects of eupalinilide B. (a) Tumor volume over 21 days. (b) Tumor weight. (c) Weight of mice over 21 days. (d) Hematoxylin–eosin staining of major organs. Significant differences were identified between the treatment and control groups. ** $P < 0.01$, *** $P < 0.001$.

16.7%, respectively, indicating that the agent was a selective LSD1 inhibitor.

Laryngeal cancer is the most common cancer in the larynx and the second most common cancer of the respiratory system after lung cancer.²¹ It is necessary to develop novel chemical drugs to treat laryngeal cancer. To date, the anti-tumor effects of eupalinilide B against laryngeal cancer remained unknown. We demonstrated for the first time that the natural sesquiterpene lactone eupalinilide B is a novel anti-tumor agent against laryngeal cancer.

Based on the proliferation experiments, eupalinilide B potently inhibited laryngeal cancer cell proliferation with IC_{50} values of 6.73, 1.03, 3.12, 2.13, 9.07, and 4.20 μ M in TU686, TU212, M4e, AMC-HN-8, Hep-2, and LCC cells, respectively. In addition, eupalinilide B suppressed migration and

wound healing in TU212 cells. The agent suppressed N-cadherin expression and increased the expression of E-cadherin, revealing that eupalinilide B blocked EMT in TU212 cells. Meanwhile, eupalinilide B remarkably inhibited tumor growth with low global toxicity.^{22,23}

Conclusion

Eupalinilide B, a natural product isolated from *E. lindleyanum*, potently inhibited the proliferation of laryngeal cancer cells *in vivo* and *in vitro* without obvious cytotoxicity. In this work, eupalinilide B was identified as a novel selective and reversible LSD1 inhibitor. The agent concentration-dependently increased the expression of H3K9me1 and H3K9me2 in TU212 laryngeal cancer cells. Collectively, eupalinilide B might be

a promising candidate for developing anti-cancer drugs against laryngeal cancer.

Authors' contributions

Linlin Jiang, Lei Zhang, and Xinran Zhang performed the MTT assay and animal experiments. Linlin Jiang and Lei Zhang analyzed the experimental data. Linlin Jiang, Lei Zhang, and Xinran Zhang approved the final manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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