A Novel Myricetin Derivative with Anti-cancer Properties Induces Cell Cycle Arrest and Apoptosis in A549 Cells

Hangtian Zhou, Lingling Xu, Yan Shi, Shihui Gu, Nan Wu, Fei Liu, Yinjiu Huang, Wei Xue, Xiaojing Wang, and Fuliang Chen*

*Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, Molecular Diagnosis Center, the Department of Pulmonary and Critical Care Medicine, First Affiliated Hospital, Bengbu Medical College, No. 287 Changhuai Road, Bengbu, Anhui Province 233000, China; †Department of Hand Surgery, Huashan Hospital, Fudan University, Shanghai, China; ‡Department of Basic Medical College, Bengbu Medical College, Bengbu, Anhui Province 233030, China; ‡Anhui Key Laboratory of Infection and Immunity of Bengbu Medical College, Bengbu, Anhui Province 233030, China; and ‡Research and Development Center for Fine Chemicals, Guizhou University, Huaxi District, Guiyang 550025, China.

Received July 6, 2022; accepted October 4, 2022

Lung cancer is the leading cause of cancer-related deaths worldwide, synthesizing and screening of novel anti-cancer drugs provides an alternative therapeutic strategy for renewal of the chemotherapy regimens against lung cancer. To this end, several compounds were synthesized based on the modification of the original myricetin, and their anti-tumor activity against the human non-small cell lung cancer (NSCLC) A549 cells were measured. Among the myricetin derivatives, S4-10 has displayed the highest antitumor efficacy in dose-dependent manner. The proliferation of A549 cells were significantly attenuated by given 6 μM of S4-10 both in vitro and in vivo. Further, the treatment of S4-10 also results in the inhibition of cell migration and invasiveness and the induction of cell apoptosis and G2 cycle arrest of A549 cells. Moreover, we found that S4-10 inhibits the progression of A549 cells through the sterol biosynthetic-cell apoptosis axis. These findings shed the light of developing S4-10 as a promising treatment agent for NSCLC.

Key words myricetin, non-small cell lung cancer, S4-10, chemotherapy

INTRODUCTION

Globally, lung cancer-associated morbidity and mortality rank first among malignant tumors with a five-year survival rate of only 18%.1 Projections for 2020 suggest over 1.8 million new lung cancer cases and 607000 cancer deaths are occurred only in the United States.2 About 80–85% of lung cancer cases are belong to non-small cell lung cancers (NSCLC) and the majority of patients were suffered with local or distant metastasis when diagnosed.3–5 As such, more effective drugs are needed to improve the therapeutic outcomes of this fatal disease.

Currently, the targeted therapy and immunotherapy has pushed the treatment of NSCLC towards a new era of precise, individualized treatment.6 Nevertheless, both the targeted therapy and immunotherapy are unavailable for patients in most cases due to the drug resistance, drug inaccessible for most genetic mutations, and tumor microenvironment.7 Chemotherapeutic drugs are powerful for killing tumor cells, while the adverse reactions are also strong that limit its clinical application. Antibody drug conjugates (ADCs) are targeted medicines that deliver chemotherapy agents to tumor cells and thus redefine the conventional chemotherapy.8,9 Developing novel chemotherapeutic agents and even its ADCs are critical and necessary subjects that can make complementary to current therapeutic regimens against NSCLC.

Myricetin is a natural flavonoid compound with multiple biological functions and promising development prospects.10–13 Previous studies have demonstrated myricetin has anti-tumor activity against multiple human cancers both in vitro and in vivo.14–19 In lung cancer, myricetin inhibits the invasion and migration of A549 cells by blocking the extracellular signal-regulated kinase (ERK) pathways.20 It is also a powerful radiosensitizer that enhances the radiotherapeutic-suppression of NSCLC cell lines.21 Moreover, studies have reported that myricetin has an inhibitory impact on the liver cancer cells and conversely harbors a protective effect on the normal liver cells,15,22 suggesting the selective activity of myricetin against tumor cells.

It is noteworthy that through the modification of original myricetin can develop myricetin derivatives with superior activity on tumor inhibition.23 Here, we synthesized novel myricetin derivatives and examined S4-10 can inhibit the cell proliferation, migration, and invasiveness and also induce cell apoptosis and cell cycle arrest of A549 cells. Further, we made the first-ever analysis that myricetin suppresses NSCLC cells via the lipid metabolism-cell apoptosis axis.

MATERIALS AND METHODS

Derivatization of Myricetin Myricetin derivatives were synthesized via a six step reaction: (a) Protect the group of myricitrin with methyl iodide, (b) Expose its three hydroxyl groups, (c) Bridge with 1,4-dibromobutane, (d) Replace the terminal bromine with 1-Boc-4-methylaminopiperidine, (e) Remove Boc and (f) React with sulfonyl chloride to obtain compounds S4-1 and S4-10, and react with carbon disulfide and benzyl chloride to obtain compounds S2-1, S2-2, S2-3, which

© 2023 The Pharmaceutical Society of Japan
belong to piperidine-1-dithiocarboxylic acid compounds. S4-1 and S4-10 were derivatives of myricetin and their chemical names are 5,7-dimethoxy-3-(4-(methyl(1-tosylpiperidin-4-yl)-amino)piperidine-1-carbodithioate, respectively. All the agents were cultured in a Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

### 3-(4,5-Dimethylthiazol-2-yl)-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (S4-10) and Cell Counting Kit-8 (CCK-8) Assays

**For MTT Assay**

A549 cells were seeded into 96-well plates at a seeding density of 4 × 10³ cells/well and were further cultured for 12 h that followed by the treatment of S2-L, S2-2, S2-3, S4-1 and S4-10 at 10 µM final concentration, respectively (n = 6). Brightfield images were captured at 24 and 48 h post treatment, respectively. Then, the cells were harvested for endpoint detection by treating with 10 µL MTT (5 mg/mL) and were incubated for another 4 h. The medium was then replaced with 100 µL of 100% DMSO and then the optical density (OD value at 450 nm) was measured using a microplate reader.

**For CCK-8 Assay**

The CCK-8 assay was performed to detect the cell viability. A total of 4 × 10³ numbers of A549 cells were seeded into 96-well plates and cultured for another 12 h. The cells were then treated with S4-10 at final concentration of 1, 3, 5, 6, 8, and 10 µM for 24, 48, and 72 h (n = 6), respectively. Then, 10 µL of CCK-8 solution was added into each well and the cells were further incubated for 1 h at 37°C. The spectrophotometric absorbance at 450 nm was immediately determined using a scanner.

**Ki67 Staining**

The immunofluorescence staining was performed to detect the expression of the cell proliferative marker Ki67 by. A549 cells were cultured in 24-well plates and were treated with S4-10 for 15 h. Then, the cells were fixed with 4% paraformaldehyde and were subsequently blocked with 10% FBS. Cells were incubated with Ki67 antibody overnight at 4°C, followed by incubation with an Alexa Fluor 568-conjugated secondary antibodies for 2 h at room temperature (r.t.). The proliferative index was calculated using the formula: cell number of Ki67^+ /cell number of DAPI^+.

### Wound Healing Assay

Wound healing assay was used to determine on the inhibition of compound S4-10 on the migration of A549 cells. A549 cells were seeded into 24-well plates at an appropriate density of 1 × 10⁵ cells/well. While confluent, the cell monolayer was mechanically scratched with a 200 µL sterile pipette tip to create a linear wound. The cells were then rinsed once with PBS and cultured with serum-reduction medium (2% FBS). The cells were subsequently treated with S4-10 at a final concentration of 6 µM diluted in DMSO for 24 h. Images were taken at 0, 12, and 24 h after scarring. They were analyzed by Image J to calculate the wound closure rate. The wound-healing rate was calculated as follows: (0-h wound area - 12- or 24-h wound area)/0h wound area ×100%

**Transwell Assay**

The Matrigel (BD, 356234) was frozen at −20°C, and then placed at 4°C overnight to turn into a liquid. The liquid Matrigel was diluted with serum-free medium (1:8). Fifty microliters of the dilution was added to the upper chamber of each transwell inserts (8 µm pore size; Corning, Corning, NY, U.S.A.) for 1 h at 37°C. Precipitation was removed from the upper chamber, and it was then refilled with 100 µL serum-free suspension of A549 cells (4 × 10⁵ cells/well), with varying concentration of S4-10; 1, 6, and 10 µM were employed. The lower chamber was however filled with medium. After incubation for 24 h, the cells that had migrated through the membrane were fixed for staining with hematoxylin–eosin (H&E). A total of 50 fields were randomly observed to count the number of migrated cells. Three replication sets were performed in each group.

### Cell Apoptosis and Cell Cycle

A549 cells growing in six-well plates were treated with 6 µM of S4-10 for 15 h, each assay was performed in triplicates. The attached cells were digested with 0.25% trypsin and were suspended in cold PBS to cell solution at a final concentration of 5 × 10⁶ cells/mL.

For the cell apoptosis assays, cell solutions were then incubated with 5 µL of Annexin V-fluorescein isothiocyanate (FITC) for 10 min in the dark at ambient temperature that followed by the addition of 10 µL propidium iodide (PI) for another 15 min at 4°C. For the cell cycle assay, the suspended cells were fixed with 4% formaldehyde for 30 min and were washed three times with PBS. Then, the cell pellets were incubated with 400 µL PI (50 µg/mL) for 30 min at ambient temperature. All samples were analyzed using flow cytometry (FACS flow cytometer, BD Biosciences, San Diego, CA, U.S.A.).

**In Vivo Model**

Ten 4–5 week old female BALB/c-Foxn1null mice (purchased from the He Fei Experimental Animal Center, He Fei, China) were employed and randomly divided into two groups. The xenografts were created by subcutaneously transplanting 1 × 10⁶ numbers of A549 cells into the lower right leg of each mice. When the proliferated tumors can be visual observed, 50 µL of 6 µM S4-10 or DMSO were intratumorally injected and a second dose was injected five days later. Tumor dimensions (length, L and width, W) were measured every 2 d and tumor volumes (V) were calculated according to the formula: V = 1/2 × L × W² (mm³). The endpoint detection was performed at day 15 post treatment, and the isolated tumors were weighed and further subjected to hematoxylin–eosin (H&E) and terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. All animal experiments were performed in accordance with the institutional guidelines for the care and use of laboratory animals.
were approved by the Institutional Animal Care Committee and carried out with respect to the rules of the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (Bengbu, China).

All procedures involving patient sample collection and in vivo animal experimentation were approved by the Ethics Review Committee of the First Affiliated Hospital of Bengbu Medical College (Approval No. 2020-088).

**RNA Interference (siRNA) Assay** The silencing of squalene epoxidase (SQLE) was performed by siRNA, the sequences of siRNA for knockdown of SQLE and control were listed in Table 1. The transfection of siRNA was performed using the lipo3000 transfection reagent. Briefly, three siRNAs targeting the SQLE were designed and synthesized by GenePharma Co., Ltd. The knockdown efficacy of each siRNA was measured by RT-PCR and the most efficient siRNA was employed for SQLE-silencing studies. A549 cells cultured in 6-well plates were treated with 6 μM S4-10 for two hours and then a total of 75 pmol of siRNA was transfected into each well for another 28 h. The mRNA and protein expression of apoptotic genes were analyzed by Western blotting and RT-PCR assays. Each assay was performed in triplicates.

**Transcriptome Sequencing (RNA-Seq)** Trizol reagent was used to isolate the total RNA from A549 cells that treated with DMSO or 6 μM S4-10 for 15 h. Transcriptome sequencing and analysis were performed commercially (NovelBio, Shanghai, China). The Kyoto Encyclopedia of Genes and Genomics (KEGG) database were then used to analyze the differentially expressed genes to identify the molecular pathways affected by S4-10.

**Reverse Transcription and Real-Time-PCR (RT-PCR)** The A549 cells were treated with DMSO or 6 μM S4-10 and harvested at 15 and 24 h later, the total RNA was isolated and then converted into cDNA using Maxima reverse transcriptase (A3500; Promega, Madison, WI, U.S.A.) according to the standard protocol. The expression of Bcl2, Caspase3, P53, and SQLE was examined by RT-PCR and the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was set as an internal control. The amplification of RT-PCR was performed as follows: a denaturation step at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s ($n = 3$). The data was analyzed using the ΔΔCT method. The primers used are listed in Table 1.

**Western Blotting** The Western blotting was employed to analyze the expression of multiple proteins that related to cell apoptosis and lipid metabolism. Briefly, the cultured A549 cells were treated with 6 μM of S4-10 or DMSO for 15 and 24 h, respectively. Then the cells were harvested and lysed in RIPA buffer for 30 min on ice. Protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific, New York, NY, U.S.A.) and 30 μg protein/sample was loaded and separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels before transferred to PVDF membranes. After blocking for 1 h with 5% w/v skimmed milk in TBST, the membranes were sequentially incubated with primary and secondary antibodies (Supplementary Table 2) conjugated to HRP before visualizing an imager. Grey values of each band were quantitated with Image J software (three replicate samples per treatment). The antibodies used are listed in Table 2.

**Statistical Analysis** Data are presented as mean ± standard deviation (S.D.). A one-way ANOVA test was employed for multiple group analysis. The difference between means was compared by Tukey’s multiple comparison test. The Two-tailed $t$-test for group comparison. $0.01 < *p < 0.05$, $0.001 < **p < 0.01$, and $***p < 0.001$, and n.s. refers as no significance.

## RESULTS

**S4-10 Suppresses Cell Proliferation of A549 Cells Both in Vivo and in Vitro** The antitumor activity and clinical utility of many plant-based bioactive compounds have been widely improved by chemical derivatization. Myricetin displays inhibitory potential against numerous cancerous types and towards refining its activity. Based on the chemical structure formula of myricetin, we firstly synthesized two types of compounds, namely myricetin derivatives (S4-1/10) and piperidin-1-dithiocarboxylic acids (S2-1/2/3; Fig. 1A). We then examined that S4-10 displayed the most inhibition efficacy on cultured A549 cells, a well-characterized NSCLC cell line (Figs. 1B, C). 6 μM concentration of S4-10 could effectively attenuate the cell viability of A549 cells (Fig. 1D), which

### Table 1. The Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward (5'→3')</th>
<th>Primer Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2</td>
<td>TCCTGTGGATTTTTCGAGGTTGC</td>
<td>TGCATATTGGTTTGGGGCAGG</td>
</tr>
<tr>
<td>Caspase3</td>
<td>TGGACCAATGGACCTTGGACC</td>
<td>AGGACTCAATTCGTGGTCACC</td>
</tr>
<tr>
<td>P53</td>
<td>ACCTATGGAAACTTCTCTGAAA</td>
<td>CTGGCATTCGAGGACCTCA</td>
</tr>
<tr>
<td>SQLE</td>
<td>TGGTCTACGATTATGCTGATC</td>
<td>TACTGAACTCACCATAACAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTCTCTCTGACTTCAACAGGC</td>
<td>ACCACCTGTGTGCTGACGACCA</td>
</tr>
<tr>
<td>siNC</td>
<td>UUGUCGGAAAGCUGUGACGTT</td>
<td>ACUGGACAGGUUGGCAATTT</td>
</tr>
<tr>
<td>siRNA-1</td>
<td>GGAGUGUCCUGACUUUAUUTT</td>
<td>AUAAAGGUCAGGGAUACCTT</td>
</tr>
<tr>
<td>siRNA-2</td>
<td>GCGUGCCUGUACAACCAUACTT</td>
<td>AUGUUGAUGUACAGGCGGTT</td>
</tr>
</tbody>
</table>

### Table 2. The Antibodies and Dilutions Used in This Study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>SOURCE</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-Bax</td>
<td>Proteintech; 50599-2-ig</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-P53</td>
<td>Proteintech; 10442-1-AP</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Caspase3</td>
<td>Proteintech; 19677-1-AP</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Bcl2</td>
<td>Proteintech; 26593-1-AP</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-β-actin</td>
<td>Proteintech; BM0627</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-SQLE</td>
<td>Proteintech; 12544-1-AP</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG</td>
<td>Proteintech; SA00009-2</td>
<td>1:10000</td>
</tr>
</tbody>
</table>

**(Secondary antibody)**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>SOURCE</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Abcam; E607238</td>
<td>1:500</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Abcam; AB175473</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Fig. 1. S4-10 Was Bioactive Myricetin Derivative That Has High Anti-proliferative Activity against A549 Cells in Vitro and in Vivo

(A) Chemical structure of derivatives of myricetin for S4-1, S4-10, S2-1, S2-2, and S2-3, respectively. (B) Brightfield images of A549 cells treated with S2-1, S2-2, S2-3, S4-1 and S4-10 at 10 µM concentration for 24h (upper) and 48h (lower), respectively. The DMSO was set as negative control. (C) Relative inhibition of A549 cell proliferation after 48h treatment with the indicated compounds measured by MTT assay, the inhibition rates were calculated as [(OD (DMSO)–OD (Derivatives))/OD (DMSO)]. (D) A CCK-8 cell proliferation assay showing dose- and time-dependent inhibition of S4-10 on A549 cell growth in vitro. (E) The comparison of cell viability between original myricetin- and S4-10-treated A549 cells using CCK-8 assay. The final concentration of each drug was 6 µM and the duration for treatment was 24h, n = 8. (F) The immunostaining of Ki67 on A549 cells treated with DMSO or S4-10 for 15h, the nuclei was stained with 4',6-diamidino-2-phenylindole (DAPI). (G) The statistics data of Ki67 positive cells in total cells, which was calculated by Ki67+/DAPI+/DAPI−. (H) Images of xenografts from A549 cells treated with S4-10 (upper) and DMSO (lower). (I) Growth curve of tumor volume was measured on indicated days and (J) tumor weight at the end of the experiment. The statistical significance was performed using one-way ANOVA and the difference between means was compared by Tukey’s multiple comparison test in (C, D, I). Two-tailed t-test for group comparison in (E, G, J). Scar bars: 50 µm.
demonstrated superiority over the original myricetin (Fig. 1E). The expression of proliferative maker Ki67 of A549 cells was reduced in S4-10 treated cells (Figs. 1F, G). Moreover, the tumor size, tumor volume, and tumor weight of transplanted A549 cells in mice were significantly reduced by intratumoral injection of S4-10 (Figs. 1H–J). Collectively, a novel myricetin derivatives S4-10 was capable of inhibiting tumor growth of A549 cells both in vivo and in vitro.

S4-10 Attenuates Cell Migration and Invasiveness of A549 Cells in Vitro The local or distant metastasis of tumor cells contributes to the poor therapeutic outcome of NSCLC. Transwell and wound healing assays were performed to assess the roles of S4-10 in the migration and invasiveness of A549 cells. Transwell results showed that the migration and invasiveness of A549 cells were obviously suppressed in S4-10 treated cells compare with the control cells in concentration dependent manner (Figs. 2A, B). Meanwhile, wound healing assays showed that S4-10 impared the cell migration in A549 cells (Figs. 2C, D).

S4-10 Induces Cell Cycle Arrest and Cell Apoptosis of A549 Cells Oncogenic gene-silencing events in cancer cells always cause the disorder of the cell apoptosis and cell cycle.25) supporting this notion we observed that S4-10 treatment resulted in significant increase of the apoptosis rates in cultured A549 cells using dual staining of Annexin V and PI (Figs. 3A, B). We also established the xenograft model by transplanting the A549 cells into nude mice to create natural mimicking environment for tumor growth, similarly, the intratumoral injection of S4-10 could significantly induce cell necrosis and/or cell apoptosis on the cancer cells (Figs. 3C, D). Moreover, the cell cycle arrest in G2 stage was observed in S4-10 treated A549 cells (Figs. 3E, F).

The Lipid Metabolism-Cell Apoptosis Axis Is Critical for Anti-tumor Effects of S4-10 on A549 Cells To reveal the molecular mechanisms underlying the effects of S4-10 on A549 cells, we carried out RNA sequencing-based analyses on A549 cells treated with S4-10 or DMSO for 15 h. A total of 265 up-regulated and 87 down-regulated genes associated with S4-10 treatment were identified (Figs. 4A, B). Among the top 20 GO processes altered by S4-10, the top alterations based on the number of genes affected involved small molecule metabolic processes, oxidation-reduction processes and lipid metabolic processes (Fig. 4C). Pathway-analyses also revealed that many metabolic processes were altered, particularly sterol biosynthesis (Fig. 4D).

SQLE, a key gene related to lipid metabolism and steroid biosynthesis that was prominently upregulated in response to S4-10 treatment upon bioinformatic analysis (Figs. 4B–D), was selected for further evaluation. Indeed, interrogation of SQLE expression showed that the mRNA and protein level of SQLE were obviously increased after treatment with S4-10 for 15 h (Figs. 4E–G). Meanwhile, the expression of apoptotic associated genes, such as P53, Bax, Caspase 3, and Bcl2, were almost not changed under the treatment of S4-10 for 15 h (Fig. 4H).

Further, we observed the expression of SQLE mRNA and protein were down expressed when the treatment of S4-10 was prolonged to 24 h (Figs. 5A, B) in A549 cells. Meanwhile, the expression of apoptosis promoting genes, including Caspase 3, cleaved Caspase 3, and Bax, were upregulated (Figs. 5C–G). Consistently, the expression levels of apoptosis inhibition genes Bcl2 and P53 were reduced following the treatment of S4-10 for 24 h (Figs. 5H–L). Notably, the silencing of SQLE could attenuate the S4-10-induced apoptosis in A549 cells (Figs. 5M–S). Collectively, these results suggests that S4-10 initially induces sterol biosynthetic process disorder in A549...
cells and subsequently triggers cell apoptosis.

DISCUSSION

Chemotherapy has been employed in clinical application against NSCLC for many years and is expected to continue to work in most situations, especially for patients not suited for targeted therapies. Alternative chemotherapies are also needed to cooperate with immunotherapy approaches which are currently gaining traction in NSCLC treatment. Moreover, chemotherapies elicit potent inhibition on tumors that always accompanied with considerable side effects. The ADCs are one of the fastest growing anticancer drugs that deliver chemotherapy agents to cancer cells, which essential to bridge the gap between traditional chemotherapy and precise medicine and further promote the chemotherapy into a new era.

Plant-based natural bioactive compounds are widely used as anticancer agents and can be chemically modified for the

Fig. 3. S4-10 Induces Cell Apoptosis and Cell Cycle Arrest in A549 Cells

(A) The dot plot of apoptosis of A549 treated with S4-10 and DMSO by flow cytometry. (B) The statistics data shows the ratio of apoptotic cells in early apoptosis. (C, D) Representative H&E staining (C) and TUNEL staining (D) of sections from A549 xenografts treated with DMSO or S4-10 (D). Scale bar = 50 µm. (E) The flow cytometry curves indicate the cell cycle phase of A549 cells treated with DMSO and S4-10. (F) The statistics data presents the cell cycle frequency of G1, S, and G2 of A549 cells in S4-10 groups compared with DMSO groups. Two-tailed t-test for group comparison in (B, F).
purpose of improving their applicability and activity. In this study, we synthesized myricetin derivatives and assessed their activity against NSCLC. Three derivatives of piperidine-1-dithiocarboxylic acid displayed relatively weak inhibition on A549 cells and S4-10 harbored superior activity on tumor inhibition. We further assessed the activity of S4-10 in a range of in vitro assays measuring the proliferation, viability, migration, invasiveness, apoptosis, and cell cycle of A549 cells. S4-10 attenuated the tumor growth of and induced cell apoptosis both in in vitro and in xenograft models of A549 cells. Meanwhile, the cell cycle of A549 cells were arrested at G2 phase in response to S4-10 stimuli. Notably, the migration and invasiveness of A549 cells are more sensitive to S4-10 than that of cell proliferation, 1 μM final concentration of S4-10 could obviously alleviate the cell mobility. What's more, S4-10 has been demonstrated that myricetin impacts on ERK signaling, lipid peroxidation, and autophagy to inhibit and kill the tumor cells. Our findings added the value of providing SQLE as a novel target of myricetin. Notably, the enhanced expression of SQLE is an early indicator that response to S4-10 stimuli. Studies has shown that SQLE acts as an oncogene to promote the progression of pancreatic adenocarcinoma and hepatocellular carcinoma. Consistently, our findings also showed that the expression of SQLE was gradually dropped in A549 cells under the treatment of S4-10 (Figs. 5A, B), but the underlying mechanism was unclear.

The apoptosis pathway was induced after the activation of SQLE in A549 cells that received the treatment of S4-10. Apoptosis is an active cellular death process that is either initiated by the endogenous (mitochondrial) apoptotic pathway, and/or the exogenous (death receptor) apoptotic pathway. Both pathways converge on Caspase3, which is the main executioner caspase required to commit the cell apoptosis. We observed S4-10 strongly induced apoptosis in A549 cells that characterized by the increased expression of Caspase3 and cleaved Caspase 3 as well as the Bax at 24 h post treatment, which is differ from the observation at 15 h post treatment that the expressions of the associated genes were not altered in response to S4-10. Meanwhile, the decreased expression of the anti-apoptotic molecule Bcl2 and P53 were observed at 24 h post treatment. What's more, the apoptosis induced by S4-10

Fig. 4. Transcriptional Alteration in Response to S4-10 Stimuli on A549 Cells

(A) Hierarchical clustering comparing gene enrichment in A549 cells treated with S4-10 or DMSO (heat map). (B) Volcano plot comparing significantly up-regulated (red) and down-regulated genes (blue) following S4-10 treatment. (C) Bubble plot illustrating the top 20 changed biology processes from GO enrichment. (D) Bar plot showing top changed molecular signaling pathways affected by S4-10. Red and blue columns indicate activation or down-regulation, respectively. (E) The mRNA expression level of SQLE of A549 cells was examined by RT-PCR at 15 h post S4-10 treatment, n = 3. (F) The expression of protein level of SQLE in A549 cells treated by S4-10 for 15 h was measured by Western blotting. (G) The statistical data from the Western blotting indicate the expression level of SQLE. The values were calculated by the formula, grey value (interest genes)/grey value (relevant β-actin). Data are presented as mean ± S.D. (H) The mRNA expression level of Bcl2 of A549 cells was examined by RT-PCR at 15 h post S4-10 treatment, n = 3. Two-tailed t-test for group comparison in (E–H).
Fig. 5. The Evaluation of Lipid Metabolism-Apoptosis Axis Induced by S4-10 in A549 Cells

(A) The mRNA and (B) protein expression level of SQLE of A549 cells were measured by RT-PCR and Western blotting at 24h post S4-10 treatment, respectively. (C) The mRNA expression level of Caspase3 was measured by RT-PCR at 24h post-S4-10 treatment. \( n = 3 \). (D) The expression of protein level of Bax, Caspase3, and Cleaved Caspase3 (Caspase 3) were measured by Western blotting, cells were harvested at 24h post S4-10 treatment. \( \beta \)-Actin acts as the internal control. (E–G) The statistical data from the Western blots indicates the expression level of Caspase 3 (E), Bax (F), and SQLE (G). The values were calculated by the formula, grey value (interest genes)/grey value (relevant \( \beta \)-actin). Data are presented as mean \( \pm \) S.D. (H, I) The expression of mRNA level of P53 (H), and Bcl2 (I) were quantitated with RT-PCR, cells were harvested at 24h post-S4-10 treatment. \( \beta \)-Actin acts as the internal control. (K, L) The statistical data from the western blots indicates the expression level of P53 (K) and Bcl2 (L). The values were calculated by the formula, grey value (interest genes)/grey value (relevant \( \beta \)-actin). Data are presented as mean \( \pm \) S.D. (M) Two experimental siRNA silencing SQLE (siSQLE_1 and siSQLE_2) and control siRNA were synthesized and their knockdown efficiency was measured in A549 cells by RT-PCR. (N) The mRNA expression level was measured after siSQLE_1 treated on S4-10-interfered A549 cells. (O) The expression of protein level of cleaved Caspase 3 (Caspase 3) Bax, Bcl2, and SQLE measured by Western blotting, cells were harvested at 30h post-S4-10 treatment. \( \beta \)-Actin acts as the internal control. (P–S) The statistical data from the western blots indicate the expression level of Bax (P), Bcl2 (Q), Caspase 3 (R), and Caspase3 (S). The values were calculated by the formula, grey value (interest genes)/grey value (relevant \( \beta \)-actin). Data are presented as mean \( \pm \) S.D. Two-tailed \( t \)-test for group comparison in (A, C, E, G–I, K, L, N, P–S), One-tailed \( t \)-test for group comparison in (F), and one-way ANOVA for comparison in (M).
in A549 cells can be attenuated by the knockdown of SQLE, suggesting a possible mechanism that S4-10 induces the disorder of sterol biosynthetic process and then trigger the cell apoptosis to inhibit the progression of A549 cells.

In conclusion, our findings evaluate the inhibition of S4-10 on tumor proliferation, migration, and invasiveness of A549 cells and further reveal the sterol biosynthetic process -apoptosis axis mediated the roles of S4-10 on NSCLC cells. These findings hints the potential therapeutic value of S4-10 against NSCLC and also highlights the understanding the anti-tumor roles of myricetin and its derivatives.

Acknowledgments This work was supported by the Project of National Natural Science Foundation of China (Grant Nos. 81772493, 82072585), the Project of Excellent Youth Science Foundation of the First Affiliated Hospital of Bengbu Medical College (Grant No. 2021byfyyq05) and the Natural Science Foundation of Anhui Province (Grant No. 2208085QH1249).

Author Contributions F.C., X.W., W.X., and F.L. made the conceptual design, H.Z., and L.X. performed the major researches of the research. W.X. provided the molecule compounds. Y.S., S.G., N.W., and F.L. assisted in data collection. Y.H., W.X., and Z.Q. provided professional suggestion for this manuscript. H.Z., F.C., and F.L. wrote the manuscript. F.C., F.L., and X.W. revised the manuscript.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


