


Article

A Ferulic Acid Derivative FXS-3 Inhibits Proliferation and Metastasis of Human Lung Cancer A549 Cells via Positive JNK Signaling Pathway and Negative ERK/p38, AKT/mTOR and MEK/ERK Signaling Pathways

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Abstract: Lung cancer is one of the most common malignancies and is an increasing cause of cancer-related deaths. In our previous study, a series of ferulic acid (FA) derivatives were designed and synthesized; they exhibited positive anti-cancer activities, especially for a compound labelled FXS-3. In this study, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed, wherein it revealed the inhibitory effect of FXS-3 on the proliferation and metastasis of human lung cancer A549 cells. The further flow cytometry assay showed that FXS-3 induced apoptosis of A549 cells induced cell cycle arrest at the G0/G1 phase. The trans-well migration and Matrigel invasion assays revealed that FXS-3 inhibited the migration and invasion of A549 cells. By the western blotting analysis, FXS-3 increased the expression of B-cell lymphoma-2 (Bcl-2) associated X protein (Bax)/Bcl-2 ratio, inhibited matrix metalloproteinase (MMP)-2 and MMP-9, and regulated the extracellular signal-regulated kinase (ERK)/p38, c-Jun N-terminal kinase (JNK), protein kinase B (AKT)/mechanistic target of rapamycin (mTOR), as well as mitogen-activated protein kinase (MEK)/ERK signaling pathways. The subsequent A549 xenograft-bearing mouse model and tail vein injection of A549 cells induced pulmonary tumor metastasis model showed that FXS-3 significantly restrained the tumor growth and metastasis. In conclusion, FXS-3 might inhibit proliferation and metastasis of human lung cancer A549 cells by positively regulating JNK signaling pathway and negatively regulating ERK/p38, AKT/mTOR, and MEK/ERK signaling pathways, which provides important scientific basis for the development of anti-cancer drugs about FA derivatives.

Keywords: FA derivatives; lung cancer A549 cells; ERK/p38; JNK; AKT/mTOR; MEK/ERK

1. Introduction

4-Hydroxyl-3-methoxyl phenylpropenoic acid, also known as ferulic acid (FA, Figure 1), is one of the most bioactive ingredients in various foods and medicines. Further, it serves dual purposes in plants such as *Ligusticum chuanxiong*, *Angelica sinensis*, *Cimicifuga foetida*, and so on. FA has a wide range of pharmacological activities, such as anti-oxidation, anti-atherosclerosis, anti-inflammation, anticoagulation, detoxification, hepato-protection, and immune-regulation [1]. Since FA has the advantages of clear pharmacological activities, stability, low toxicity, and is widely present in nature, FA and its derivatives have received considerable attention. Recently, FA and sodium ferulate have proved to possess significant anti-cancer activities [2,3].

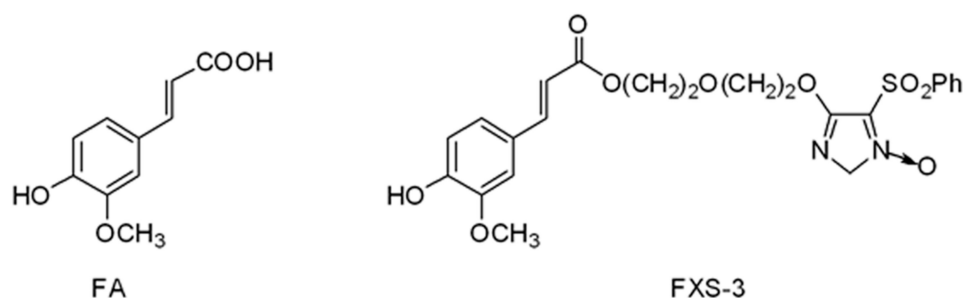


Figure 1. Chemical structures of ferulic acid (FA) and FXS-3.

In our previous study, a series of FA derivatives were designed and synthesized. By using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a compound labelled as FXS-3 (Figure 1) was screened out, which significantly inhibited the proliferation of human lung cancer A549 cells [4]. To further explore its underlying mechanism, we evaluated the potential effects of FXS-3 on proliferation and metastasis of A549 cells both in vitro and in vivo. Our data showed that FXS-3 could inhibit the proliferation of A549 cells by up-regulating the B-cell lymphoma-2 (Bcl-2) associated X protein (Bax)/Bcl-2 ratio mediated by c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)/p38 signaling pathway. Furthermore, FXS-3 suppressed the migration and invasion of A549 cells by down-regulating expressions of matrix metalloproteinase (MMP)-2 and MMP-9, which may be mediated by protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinase kinase (MEK)/ERK signaling pathways.

2. Results

2.1. FXS-3 Inhibited A549 Cell Proliferation

An MTT assay was performed in order to evaluate the effect of FXS-3 on the proliferation of A549 cells. After the treatment of FXS-3 at concentrations ranging from 0.2–50 μ M for 24 h, the cell viability decreased from 92.27% to 14.63% (Figure 2), indicating that FXS-3 inhibited the growth of A549 cells in a dose-dependent manner. The survival rate of A549 cells in cisplatin (DDP, 50 μ M) group was 30.68%. Besides, FXS-3 could inhibit cell proliferation of other cancer cells, including HepG2 and MCF-7 cells (Figure S1).

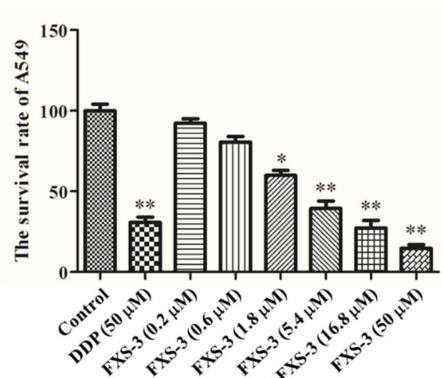


Figure 2. FXS-3 inhibited the proliferation of lung cancer A549 cells ($n = 3$). DDP: Cisplatin; Statistical significance relative to the control group is indicated: *, $p < 0.05$; **, $p < 0.01$.

2.2. FXS-3 Induced A549 Cell Cycle Arrest in G0/G1 Phase

Further, we examined the cell cycle distribution of A549 cells by flow cytometric analysis. The treatment of FXS-3 (50 μM) for 24 h induced a significant cell cycle arrest in the G0/G1 phase, which was comparable with DDP at 50 μM (Figure 3A,B).

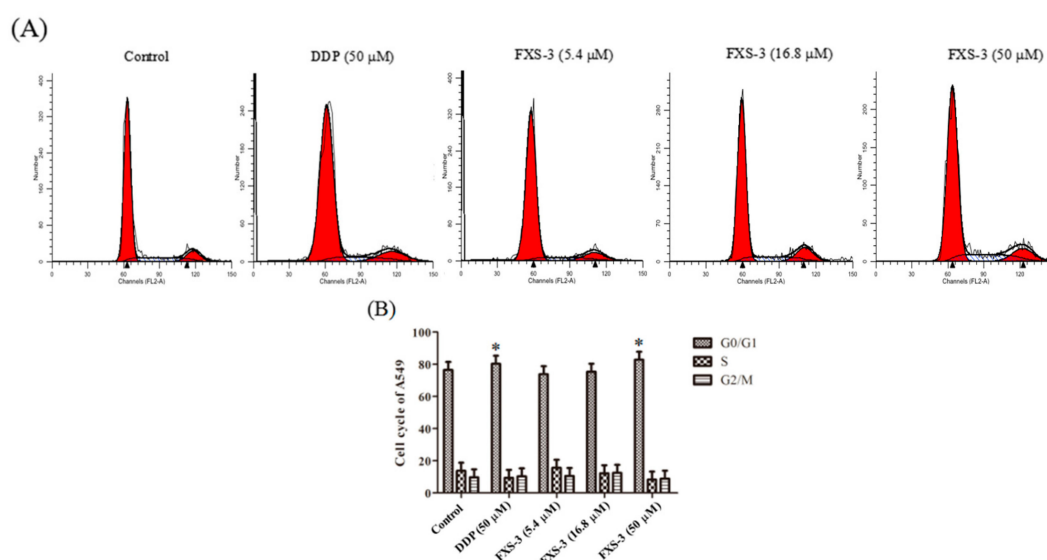


Figure 3. FXS-3 induced A549 cell cycle arrest. (A) Flow cytometric plots showed that FXS-3 treatment at 50 μM for 24 h induced cell cycle arrest of A549 cells in the G0/G1 phase. (B) Bar graphs showed that 24 h incubation of cell with FXS-3 induced marked cell cycle arrest of A549 cells ($n = 3$). DDP: Cisplatin; Statistical significance relative to the control group is indicated: *, $p < 0.05$.

2.3. FXS-3 Induced the Apoptosis of A549 Cells

Apoptosis of A549 cells was examined using flow cytometry and FXS-3 dose-dependently inhibited cell growth (Figure 4A,B). The apoptosis rate of A549 in DDP (50 μM) group was 9.66%. To elucidate the mechanism of the pro-apoptotic effect of FXS-3 on A549 cells, the expression levels of two key pro- and anti-apoptotic proteins (i.e., Bax and Bcl-2) were determined after the treatment. As shown in Figure 4C, the protein expression level of Bcl-2 was decreased, while Bax was increased in the FXS-3-treated group compared with the control group. Therefore, the results indicated that FXS-3 induced the apoptosis of A549 cells associated with the increased Bax/Bcl-2 ratio.

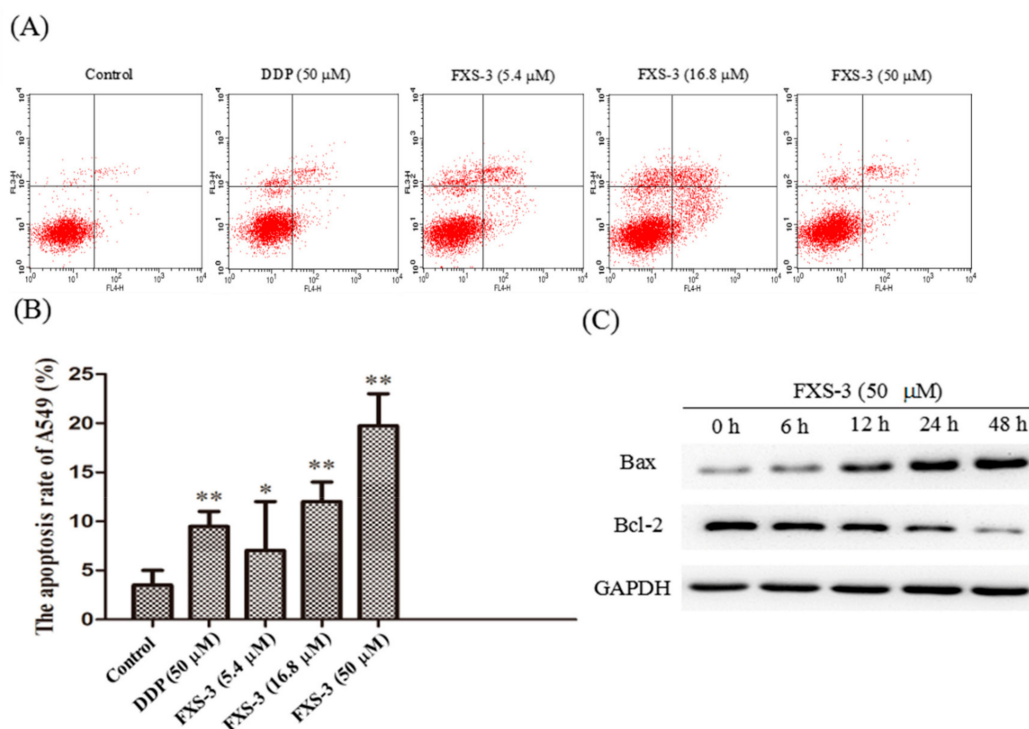


Figure 4. FXS-3 induced the apoptosis of A549 cells. (A) Flow cytometric plots showed that treatment with FXS-3 at 5.4, 16.8, and 50 μM for 24 h induced apoptosis of A549 cells ($n = 3$). (B) Bar graphs showed that 24 h incubation of cell with FXS-3 induced marked apoptosis of A549 cells ($n = 3$). (C) Representative blots of Bcl-2 and Bax in A549 cells treated with FXS-3 at 50 μM for 0, 6, 12, 24, and 48 h ($n = 3$). DDP: Cisplatin; Statistical significance relative to the control group is indicated: *, $p < 0.05$; **, $p < 0.01$.

2.4. FXS-3 Suppressed A549 Cell Migration and Invasion

Further, we assessed the effect of FXS-3 on A549 cell migration and invasion, which are the key determinants of malignant progression and metastasis. Compared with the control group, the migration and invasion ability of A549 cells decreased dose-dependently after FXS-3 treatment (Figure 5A–C). DDP (50 μM) also significantly inhibited the migration and invasion ability of A549 cells. Furthermore, as an indicator of the cell migration and invasion capacity, MMP-2 and MMP-9 protein expression levels were obviously decreased in A549 cells treated with FXS-3 (50 μM), as detected using the western blotting analysis (Figure 5D). These results demonstrated that FXS-3 critically regulated cell migration and invasion in A549 cells.

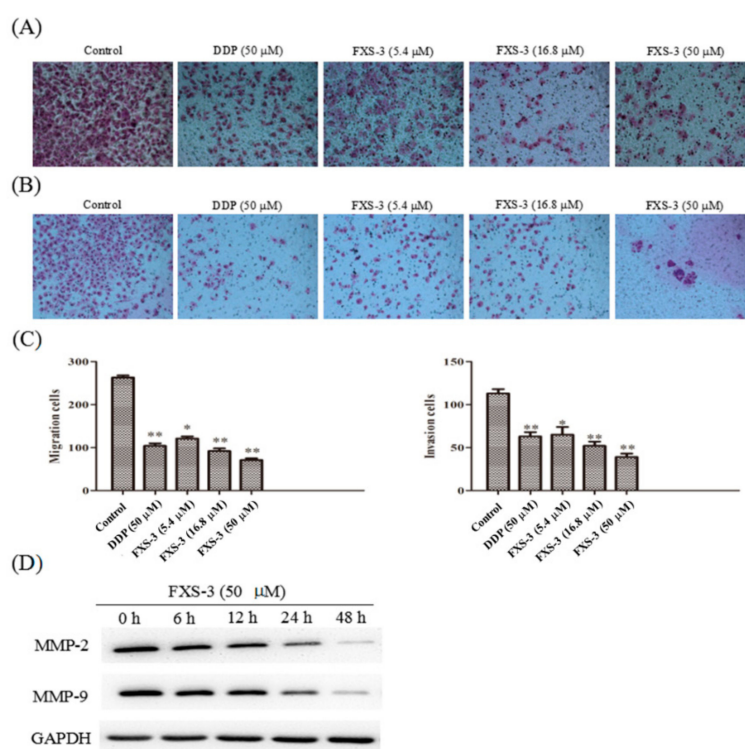


Figure 5. FXS-3 inhibited A549 cell migration and invasion. A549 cell migration (A) and invasion (B) by FXS-3 at 5.4, 16.8, and 50 μM for 24 h. (C) The migration and invasion cells were assayed in lung cancer cell treated with FXS-3 at 5.4, 16.8, and 50 μM for 24 h. (D) Representative blots of MMP-2 and MMP-9 in A549 cells treated with FXS-3 at 50 μM for 0, 6, 12, 24, and 48 h ($n = 3$). DDP: Cisplatin; Statistical significance relative to the control group is indicated: *, $p < 0.05$; **, $p < 0.01$.

2.5. FXS-3 Regulated JNK, ERK/p38, AKT/mTOR, and MEK/ERK Signaling Pathways in A549 Cells

The mitogen-activated protein kinase (MAPK) signaling pathway plays a crucial role in lung cancer development and its downstream molecules (ERK, JNK, and p38) are associated with tumor growth, migration, and invasion [5]. To further characterize the mechanism underlying the inhibition of cell proliferation by FXS-3, we examined whether it affected ERK, JNK, and p38 expression using the western blotting analysis. As shown in Figure 6A, while FXS-3 suppressed the phosphorylation of ERK and p38, it increased JNK expression compared with the vehicle control, which suggested that FXS-3 suppressed A549 cell migration and invasion by positive regulating JNK pathway and negative regulating ERK/p38 pathway. Furthermore, we treated A549 cells with SP600125, the inhibitor of JNK, and then the Bax/Bcl-2 ratio was measured. As shown in Figure 6B, SP600125 obviously suppressed the increased the Bax/Bcl-2 ratio by FXS-3. To characterize the mechanism underlying the inhibition of metastasis by FXS-3, we examined whether FXS-3 affects the activation of AKT or MEK using the western blotting analysis. As shown in Figure 6C, FXS-3 suppressed AKT, mTOR, MEK, and ERK phosphorylation compared with the effects of the vehicle. These phenomena suggested that FXS-3 suppressed A549 cell migration and invasion by down-regulating the AKT/mTOR and MEK/ERK pathways.

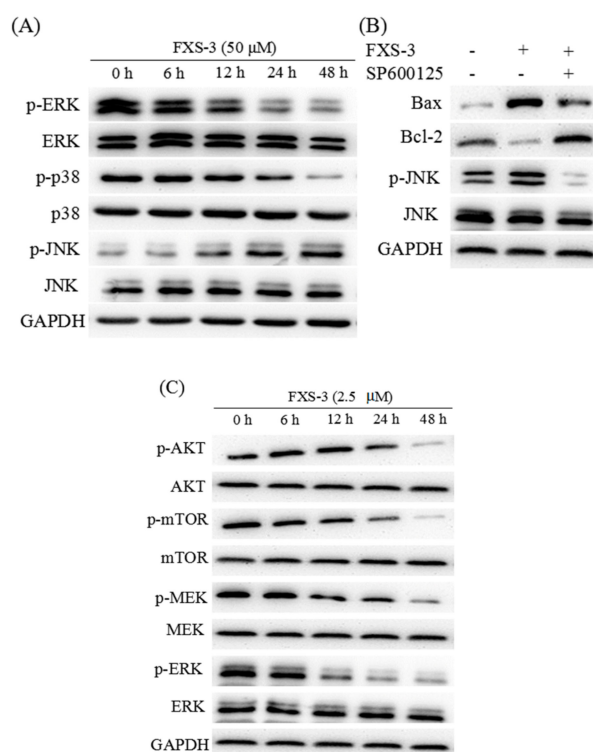


Figure 6. FXS-3 regulated c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK)/p38, AKT/ mechanistic target of rapamycin (mTOR), and mitogen-activated protein kinase kinase (MEK)/ERK signaling pathways in A549 cells. **(A)** Representative blots of phosphorylated (p)-ERK, ERK, p-p38, p38, p-JNK, and JNK in A549 cells treated with FXS-3 at 50 μM for 48 h ($n = 3$). **(B)** Representative blots of p-JNK, JNK, Bax, and Bcl-2 in A549 cells treated with FXS-3 and JNK inhibitor, SP600125 ($n = 3$). **(C)** Representative blots of p-AKT, AKT, p-mTOR, mTOR, p-MEK, MEK, p-ERK, and ERK in A549 cells treated with FXS-3 at 2.5 μM for 48 h ($n = 3$).

2.6. FXS-3 Inhibited the Growth of A549 Xenograft Tumors In Vivo

To further confirm the *in vivo* inhibitory activity of FXS-3, a xenograft tumors A549 xenograft-bearing mouse model was developed. After treatment, a significant inhibition of the tumor volume was observed in the FXS-3-treated groups and paclitaxel group in comparison with the control group (Figure 7A,B). Figure 8 displayed the effects of compound FXS-3 on tumor growth in nude mice presented as tumor volume over time. There was a statistically significant dose-dependent decrease in tumor volume with increasing compound FXS-3 dose. Besides, there was no significant differences between the control and FXS-3-treated groups in the body weight of A549 xenograft-bearing mouse models (Table S1), suggesting that FXS-3 possessed no obvious adverse effect.

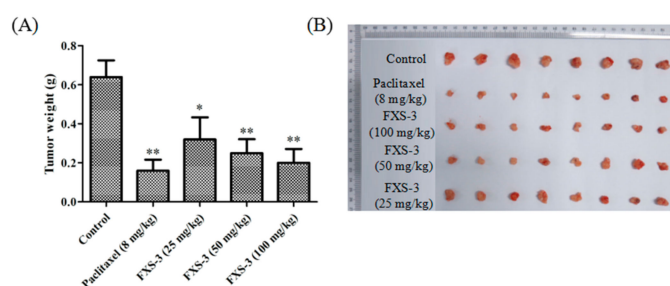


Figure 7. FXS-3 inhibited the growth of A549 xenograft tumors *in vivo*. **(A)** Tumor weight. **(B)** Image showing tumor volume. Statistical significance relative to the control group is indicated: *, $p < 0.05$; **, $p < 0.01$; $n = 8$ mice/group.

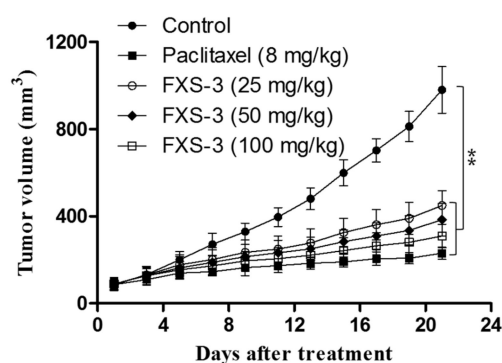


Figure 8. Effects of FXS-3 on tumor growth of A549 xenograft in nude mice ($\bar{x} \pm s$, $n = 8$). ** $p < 0.01$, versus the control group.

2.7. FXS-3 Inhibited Pulmonary Tumor Metastasis In Vivo

To determine the anti-metastatic effect of FXS-3 in vivo, A549 cells were intravenously injected into C57BL/6 mice via the tail vein and then pulmonary metastatic nodules were analyzed. In Figure 9, FXS-3 could significantly dose-dependently decrease pulmonary metastatic nodules so as to inhibit pulmonary tumor metastasis in mice. The efficacy of FXS-3 at 100 mg/kg was comparable with cyclophosphamide at 50 mg/kg.

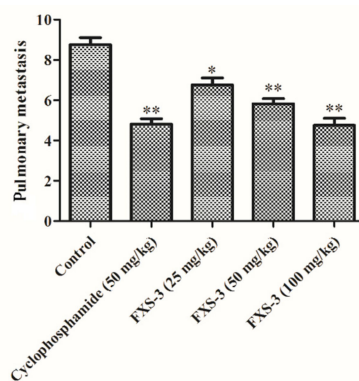


Figure 9. FXS-3 exhibited anti-metastasis effects in vivo. Statistical significance relative to the control group is indicated: *, $p < 0.05$; **, $p < 0.01$; $n = 8$ mice/group.

3. Discussion

Lung cancer is a common malignancy in numerous countries, in part due to cigarette smoke-induced lung tissue hypoxia and carcinogenesis [6,7]; it is becoming an increasing cause of cancer-related deaths. In our previous study, FA derivatives obviously exhibited anti-cancer activity [4]. Here, we performed an MTT assay, flow cytometry assay, trans-well and matrigel invasion assays, as well as animal studies to reveal the underlying mechanism of FXS-3 on proliferation and metastasis of A549 cells.

Recent studies indicate that ERK is activated by growth factors and plays a key role in cell proliferation and survival. On the contrary, inhibition of ERK activity down-regulates the expression of c-myc, which promotes cell apoptosis. The p38 MAPK molecule contributes to the cancer cell apoptosis by mediating Fas/FasL-induced Bax translocation. Moreover, the apoptotic mechanism promoted by JNK is related to the increased expression of Fas, regulation of Bcl-2, change of intracellular Ca^{2+} concentration, and then the activation of caspase family. Thus, the ERK, p38, and JNK family members are closely related to cancer cell apoptosis [8]. We discovered that the A549 cell proliferation was inhibited and a significant apoptotic effect was observed in the treatment of FXS-3. Meanwhile, the expression of Bax was significantly increased while that of Bcl-2 was obviously decreased after FXS-3

treatment, shifting the balance towards the pro-apoptotic members of the Bcl-2 family. In addition, FXS-3 could activate the JNK signaling pathway but inhibit the ERK/p38 signaling pathways, which might be an important mechanism underlying the apoptosis of lung cancer cells induced by FXS-3. Recently, microRNAs play a significant role in the MAPK signaling cascade [9]. It will be interesting to explore the potential microRNAs modulated by FXS-3 in A549 cells. Furthermore, anti-apoptotic Bcl-2 family proteins control commitment to programmed cell death and targeting these proteins via small molecule inhibitors has thus been a longstanding goal in the oncology community [10,11]. BH3-only proteins are the linchpins in the Bcl-2 family network and compete with Bcl-2 homologs to inactivate anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) or activate pro-apoptotic homologs (BAX and BAK) to determine if apoptosis commences [12]. Activation of p53, an important tumor suppressor gene, inhibits Bcl-2 to trigger mitochondrial apoptotic signaling [13]. Therefore, it will be meaningful to investigate the direct/indirect action of FXS-3 on Bcl-2 family proteins. Since the inhibitor of apoptosis pathway is upregulated in cancer cells [14], whether FXS-3 could inhibit the inhibitor of apoptosis proteins (e.g., cIAP1, cIAP2, XIAP, and Survivin) remains to be discovered.

The high mortality associated with malignant tumors often derives from their strong metastatic ability. As to the signaling pathway of metastasis, the activation of AKT/mTOR and MEK/ERK signaling pathways plays an important role in tumor migration [15,16]. As shown in our results, FXS-3 reduced the expression of p-AKT and inhibited the AKT/mTOR signaling pathway in A549 cells. As mentioned before, ERK is an important member of the MAPK family and most ERK substrate molecules are the proteins that regulate the cell cycle and cell differentiation, which are key factors in the transmission of cell proliferation signals [17]. Our study exhibited that FXS-3 reduced the expression of p-MEK and inhibited the MEK/ERK signaling pathway in A549 cells so as to inhibit the migration of cancer cells. All of the above results provided important scientific basis for the development anti-cancer drugs about FXS-3.

4. Materials and Methods

4.1. Cell Line and Culture

The human lung cancer A549 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 IU/mL penicillin in a 5% CO₂ atmosphere at 37 °C. DDP was used as a positive drug. FXS-3 was dissolved in dimethyl sulfoxide (DMSO), which had a final concentration of 50 mmol/L. All of the cell culture reagents were purchased from Gibco Thermo Fisher Scientific (Waltham, MA, USA).

4.2. Cell Viability Assay

The effect of FXS-3 on cell viability was examined using an MTT assay. Briefly, A549 cells were seeded in 96-well culture plates at a density of 1×10^5 cells/well. After a 24-h incubation, the cells were treated with DDP (50 µM) and FXS-3 at concentrations ranging from 0.2–50 µM for another 24 h. The absorbance was then measured by using a microplate reader (Bio-Rad, Hercules, CA, USA) to detect the MTT formazan at a wavelength of 570 nm.

4.3. Cell Cycle Analysis

A549 cells were seeded in a six-well plate at a concentration of 1.0×10^5 cells/mL, incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO₂, and then were treated with DDP (50 µM) and FXS-3 at different concentrations (5.4, 16.8, and 50 µM). After an incubation of 48 h, A549 cells were collected, washed with phosphate-buffered saline (PBS), and then fixed with cold 70% ethanol for at least 24 h. Then, A549 cells were washed twice with PBS, stained with PI/RNase staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min in the dark, and then analyzed using a FACSCalibur flow cytometer (BD INSTRUMENTS INC., San Jose, CA, USA).

4.4. Cell Apoptosis Assay

Cell apoptosis was examined using double staining with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). A549 cells were seeded in six-well plates at a concentration of 1.0×10^5 cells/mL, incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO₂, and then treated with DDP (50 μM) and FXS-3 at various concentrations of 5.4, 16.8, and 50 μM. After incubation for 48 h, A549 cells were detached, washed with PBS, and centrifuged at 1000 rpm for 5 min. Annexin V-FITC and PI (5 μL each) were added to 500 μL of the cell suspension, mixed and incubated at room temperature for 5 to 15 min in the dark, and then the cells were analyzed using a FACScalibur flow cytometer.

4.5. Cell Migration and Invasion Assay

Trans-well chambers (Corning Inc., Corning, NY, USA) with an 8-μm pore size were used for the migration and invasion assays. In brief, A549 cells were harvested and re-suspended in a serum-free medium. For the migration assay, 4×10^4 cells were directly added to the upper chamber. For the invasion assay, the melted Matrigel (BD Biosciences, San Jose, CA, USA) was diluted with the medium and 30 μL was added to the upper chamber of the trans-well chamber, which was incubated at 37 °C for 4 h to allow the Matrigel solidify. Then 1×10^5 cells in serum-free medium were added to the upper chamber. Medium containing 20% FBS was added to the lower chamber to serve as a chemoattractant. After incubation in an incubator at 37 °C and 5% CO₂ condition for 24 h, cotton swabs were used to gently wipe off the cells adhered on the membrane surface without passing through. The cells migrating to or invading the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 10% crystal violet and washed with PBS. Then cells in five randomly selected fields of each chamber were microscopically counted and used to indirectly reflect the tumor cell migration and invasion ability.

4.6. Western Blotting Analysis

Total cell lysates were extracted from A549 cells using a radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) with repetitive pipetting and lysis on ice for half an hour. Then, the contents were collected into centrifuge tubes and centrifuged at 12,000 rpm. The loading buffer was added to the supernatant, which were then treated at 100 °C for 5 min. Afterwards, 10 μL samples were loaded into each lane, and the proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% non-fatted milk, washed four times (15 min each time) with Tris-buffered saline plus Tween (TBS-T), and then incubated with the following primary antibodies: rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-ERK, rabbit anti-phosphorylated p-ERK, rabbit anti-human p38, rabbit anti-human p-p38, rabbit anti-human JNK, rabbit anti-human p-JNK, rabbit anti-MMP-2, rabbit anti-MMP-9, rabbit anti-human AKT, rabbit anti-human p-AKT, rabbit anti-mTOR, rabbit anti-human p-mTOR, rabbit anti-MEK, and rabbit anti-human p-MEK (Cell Signaling Technology, MA, USA). After incubation for 2 h at room temperature, the PVDF membrane was washed thrice with TBS-T and then incubated with their respective HRP secondary antibodies for another 2 h. The relative band density was determined by using the Tanon 5200 Multifunctional Imaging System (Beijing, China) with an enhanced chemiluminescence (ECL) western blotting substrate kit (Millipore, MA, USA) and GAPDH as the internal control.

4.7. Xenograft Mouse Model

The animal experimental protocol was approved by the Ethics Committee of Nanjing University of Chinese Medicine. Male BALB/C nude mice (16–22 g and 4–5-week-old) were provided by Nanjing University of Chinese Medicine Animal Center and the animals were randomly divided into the

following five groups ($n = 8$ mice/group) and treated as indicated: model control, positive drug paclitaxel (8 mg/kg), and FXS-3 (25, 50, and 100 mg/kg) groups. The A549 cells were suspended in PBS and subcutaneously injected into the right axilla of the nude mice ($1-5 \times 10^5$ cells/mice). When the tumor volumes had grown to a 50-mm³ size, the mice were administered via tail vein injections of saline, paclitaxel or FXS-3 every two days for three consecutive weeks. Both paclitaxel and FXS-3 were dissolved in normal saline with 8% Cremophor EL and 8% ethanol. The tumor volume was calculated by the formula $(W^2 \times L)/2$, where W is the tumor measurement at the widest point and L is the tumor dimension at the longest point.

4.8. Murine Experimental Metastasis Assay

Four-week-old C57BL/6 male mice were purchased from the Experimental Animal Center of the Chinese Academy of Science (Shanghai, China) and maintained in a specific pathogen-free environment at Nanjing University of Chinese Medicine Animal Center. The animal study was conducted according to protocols approved by the Ethics Committee of Nanjing University of Chinese Medicine. The mice were randomly divided into five groups ($n = 8$ mice/group) and administered via tail vein injections of saline, cyclophosphamide (50 mg/kg) or FXS-3 (25, 50, and 100 mg/kg) every two days for three consecutive weeks. The murine metastasis model was established by intravenously injection with 1×10^6 A549 cells via the tail vein as previously described [18]. Both cyclophosphamide and FXS-3 were dissolved in normal saline with 8% Cremophor EL and 8% ethanol.

4.9. Statistical Analysis

The GraphPad Prism 5.0 statistical software was used to analyze the experimental data. The measured data were presented as the mean \pm standard deviation (SD) and an analysis of variance (ANOVA) and paired t -test were used to analyze the inter-group differences. Furthermore, a difference with a p -value < 0.05 was considered statistically significant.

5. Conclusions

In this study, we demonstrated that FXS-3 could inhibit the proliferation and metastasis of lung cancer A549 cells by positively regulating JNK signaling pathways and negatively regulating ERK/p38, AKT/mTOR, and MEK/ERK signaling pathways. These results provide important scientific basis for the development of anti-cancer drugs about FA derivatives. Therefore, FXS-3 could be a promising anticancer therapeutic.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1420-3049/24/11/2165/s1>, Figure S1: FXS-3 inhibits proliferation of other cancer cells, Table S1: Effects of FXS-3 on body weight of A549 xenograft in nude mice.

Author Contributions: Conceptualization, Y.-P.T. and J.-A.D.; methodology, S.-J.Y., P.-X.Z., Y.Z., and N.-G.L.; formal analysis, S.-J.Y., P.-X.Z., Y.-Y.C., and J.-J.L.; investigation, S.-J.Y. and P.-X.Z.; data curation, R.-Y.J.; writing—original draft preparation, S.-J.Y., P.-X.Z., and Y.-P.T.; writing—review and editing, S.-J.Y., S.Z., H.Y., and X.-Q.S.; supervision, Y.-P.T.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Srinivasan, M.; Sudheer, A.R.; Menon, V.P. Ferulic acid: Therapeutic potential through its antioxidant property. *J. Clini. Biochem. Nutr.* **2007**, *40*, 92–100. [[CrossRef](#)] [[PubMed](#)]
2. Kampa, M.; Alexaki, V.I.; Notas, G.; Nifli, A.P.; Nistikaki, A.; Hatzoglou, A.; Bakogeorgou, E.; Kouimtoglou, E.; Blekas, G.; Boskou, D.; et al. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells, potential mechanisms of actions. *Breast Cancer Res.* **2004**, *6*, 63–74. [[CrossRef](#)] [[PubMed](#)]
3. Lee, Y.-S. Role of NADPH oxidase-mediated generation of reactive oxygen species in the mechanism of apoptosis induced by phenolic acid in HepG2 human hepatoma cells. *Arch. Pharm. Res.* **2005**, *28*, 1183–1189. [[CrossRef](#)] [[PubMed](#)]
4. Li, W.-X.; Li, N.-G.; Tang, Y.-P.; Li, B.-Q.; Liu, L.; Zhang, X.; Fu, H.-A.; Duan, J.-A. Biological activity evaluation and structure-activity relationships analysis of ferulic acid and caffeic acid derivatives for anticancer. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6085–6088. [[CrossRef](#)] [[PubMed](#)]
5. Nquyen, T.T.; Tran, E.; Nquyen, T.H.; Do, P.T.; Huynh, T.H.; Huynh, H. The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* **2004**, *25*, 647–659.
6. Tonini, G.; D’Onofrio, L.; Dell’Aquilal, E.; Pezzuto, A. New molecular insights in tobacco-induced lung cancer. *Future Oncol.* **2013**, *9*, 649–655. [[CrossRef](#)] [[PubMed](#)]
7. Aldo, P.; Elisabetta, C. Role of HIF-1 in cancer progression: Novel insights. A review. *Curr. Mol. Med.* **2018**, *18*, 343–351.
8. Wang, W.-Z.; Li, L.; Liu, M.-Y.; Jin, X.-B.; Mao, J.-W.; Pu, Q.-H.; Meng, M.-J.; Chen, X.-G.; Zhu, J.-Y. Curcumin induces FasL-related apoptosis through p38 activation in human hepatocellular carcinoma Huh7 cells. *Life Sci.* **2013**, *92*, 352–358. [[CrossRef](#)] [[PubMed](#)]
9. Bhardwaj, V.; Mandal, A.K.A. Next-generation sequencing reveals the role of epigallocatechin-3-gallate in regulating putative novel and known microRNAs which target the MAPK pathway in non-small-cell lung cancer A549 cell. *Molecules* **2019**, *24*, 368. [[CrossRef](#)] [[PubMed](#)]
10. Tao, Z.F.; Hasvold, L.; Wang, L.; Wang, X.; Petros, A.M.; Park, C.H.; Boghaert, E.R.; Catron, N.D.; Chen, J.; Colman, P.M.; et al. Discovery of a potent and selective BCL-X_L inhibitor with in vivo activity. *ACS Med. Chem. Lett.* **2014**, *5*, 1088–1093. [[CrossRef](#)] [[PubMed](#)]
11. Zeitlin, B.D.; Joo, E.; Dong, Z.; Warner, K.; Wang, G.; Nikolovska-Coleska, Z.; Wang, S.; Nör, J.E. Antiangiogenic effect of TW37, a small-molecule inhibitor of Bcl-2. *Cancer Res.* **2006**, *66*, 8698–8706. [[CrossRef](#)] [[PubMed](#)]
12. Huska, J.D.; Lamb, H.M.; Hardwick, J.M. Overview of BCL-2 Family Proteins and Therapeutic Potentials. In *BCL-2 Family Proteins*; Humana Press: New York, NY, USA, 2019; pp. 1–21.
13. Beberok, A.; Wrześniok, D.; Rok, J.; Rzepka, Z.; Respondek, M.; Buszman, E. Ciprofloxacin triggers the apoptosis of human triple-negative breast cancer MDA-MB-231 cells via the p53/Bax/Bcl-2 signaling pathway. *Int. J. Oncol.* **2018**, *52*, 1727–1737. [[CrossRef](#)] [[PubMed](#)]
14. Vellanki, S.H.; Cruz, R.G.B.; Jahns, H.; Hudson, L.; Sette, G.; Eramo, A.; Hopkins, A.M. Natural compound Tetrocarcin-A downregulates Junctional Adhesion Molecule-A in conjunction with HER2 and inhibitor of apoptosis proteins and inhibits tumor cell growth. *Cancer Lett.* **2019**, *440–441*, 23–34. [[CrossRef](#)] [[PubMed](#)]
15. Lee, W.-J.; Wu, L.-F.; Chen, W.-K.; Wang, C.-J.; Tseng, T.H. Inhibitory effect of luteolin on hepatocyte growth factor/scatterfactor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K/Akt pathways. *Chem. Biol. Interact.* **2006**, *160*, 123–133. [[CrossRef](#)] [[PubMed](#)]
16. Schmitz, K.J.; Lang, H.; Wohlschlaeger, J.; Sotiropoulos, G.C.; Reis, H.; Schmid, K.W.; Baba, H.A. AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma. *World J. Gastroentero.* **2007**, *13*, 6470–6477. [[CrossRef](#)] [[PubMed](#)]
17. Kruger, J.S.; Reddy, K.B. Distinct mechanisms mediate the initial and sustained phases of cell migration in epidermal growth factor receptor-overexpressing cells. *Mol. Cancer Res.* **2003**, *1*, 801–809. [[PubMed](#)]

18. Chen, R.-S.; Song, Y.-M.; Zhou, Z.-Y.; Tong, T.; Li, Y.; Fu, M.; Guo, X.-L.; Dong, L.-J.; He, X.; Qiao, H.-X.; et al. Disruption of xCT inhibits cancer cell metastasis via the caveolin-1/beta-catenin pathway. *Oncogene* **2009**, *28*, 599–609. [[CrossRef](#)] [[PubMed](#)]

Sample Availability: Samples of the compound FXS-3 are available from the authors.



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