

Pim-1 inhibitor SMI-4a suppresses tumor growth in non-small cell lung cancer via PI3K/AKT/mTOR pathway

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Wenjie Jiang¹
Yuan Chen¹
Xing Song¹
Yingjie Shao¹
Zhonghua Ning¹
Wendong Gu¹

¹Department of Radiation Oncology, The Third Affiliated Hospital of Soochow University, Jiangsu, Changzhou 213003, People's Republic of China

Background: In the present study, we aimed to investigate the effect of proviral integration site for moloney murine leukemia virus-1 (Pim-1) inhibitor (SMI-4a) on the progression of non-small cell lung cancer (NSCLC).

Materials and methods: The effects of SMI-4a on proliferation, apoptosis, and cell cycle of NSCLC cells were examined by in vitro experiments using human NSCLC cell lines (A549 and Ltp-a-2). The pathway regulated by SMI-4a was detected using Western blot. Furthermore, we performed in vivo experiments to assess the effects of SMI-4a on tumor growth using mouse models with NSCLC.

Results: Our data demonstrated that SMI-4a could inhibit the proliferation of A549 and Ltp-a-2 cells markedly in a dose-dependent manner ($P < 0.05$). Treatment with 80 $\mu\text{mol/L}$ of SMI-4a for 48 h significantly induced the apoptosis rate of NSCLC cells ($P < 0.05$), and blocked the cell cycle of NSCLC cells in G2/M phase ($P < 0.05$). The phosphorylation levels of PI3K, AKT, and mTOR in NSCLC cells were significantly downregulated by SMI-4a ($P < 0.05$). Result from in vivo experiments demonstrated that SMI-4a could suppress the tumor growth in mouse models with NSCLC ($P < 0.05$).

Conclusions: SMI-4a suppresses the progression of NSCLC by blocking the PI3K/AKT/mTOR pathway.

Keywords: Pim-1, SMI-4a, NSCLC, tumor progression

Introduction

In recent years, lung cancer has become the leading cause of cancer-related death in the world.¹ Non-small cell lung cancer (NSCLC) comprises 80% of all lung cancer cases.² Surgical resection is the most effective treatment of NSCLC. However, because of the lack of early and effective diagnostic methods, most NSCLC patients are diagnosed at an advanced stage and lose the opportunities of radical surgery for the early stage.³ In addition, the development and clinical application of platinum-based chemotherapy regimens have gradually entered a plateau.⁴

With the discovery of multiple oncogenes, more and more molecular targeted drugs showed satisfactory effects in NSCLC.⁵ The treatment of NSCLC has entered a new era of precision medicine. Among molecular targeted drugs, epithelial growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have been widely used in NSCLC patients.⁶ Studies have shown that EGFR-TKIs are more effective than conventional chemotherapy drugs in NSCLC patients with EGFR sensitive mutation.^{7–10} However, with the widespread use of EGFR-TKIs, resistance has emerged inevitably. Therefore,

Correspondence: Wendong Gu
Department of Radiation Oncology, The Third Affiliated Hospital of Soochow University, Jiangsu, Changzhou 213003, People's Republic of China
Tel +865 196 887 1132
Email wendonggu@163.com

it is necessary to develop more molecular drugs targeting different oncogenes of NSCLC.

Proviral integration site for moloney murine leukemia virus-1 (Pim-1), as a kind of serine threonine kinase, functions in signal transduction.¹¹ Pim-1 regulates the activities and subcellular localizations of certain proteins by phosphorylating their serine and threonine sites.¹² For example, downregulated expression level of Pim-1 induced by specific monoclonal antibody (mAb) led to decreased phosphorylation of AKT at Ser473 and increased cleavage of caspase-9, that activated the mitochondrial cell death pathway.¹³ Previous studies showed that Pim-1, acting as an oncogene, is highly expressed in various tumors and promotes their progression, and that overexpression of Pim-1 in NSCLC tissues is associated with advanced clinical parameters.^{14–16} However, the therapeutic effect of Pim-1 inhibitor in NSCLC remains unclear.

The aim of the present study is to investigate the effects of Pim-1 inhibitor (SMI-4a) on the progression of NSCLC in vitro and in vivo. We found that SMI-4a significantly suppressed the proliferation and cell cycle, and induced the apoptosis of NSCLC cells in vitro. Besides, SMI-4a could also suppress the tumor growth in mouse models with NSCLC. PI3K/AKT/mTOR pathway was involved in the anti-tumor process induced by SMI-4a. Pim-1 inhibitor may serve as a new molecular targeted drug for NSCLC patients.

Materials and methods

Cell culture

Human NSCLC cell lines (A549 and Ltp-a-2) obtained from the Shanghai Institutes of Biological Sciences Cell Bank were cultured in DMEM (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified incubator of 5% CO₂.

Proliferation assays

A549 and Ltp-a-2 cells (2×10^3 in 100 μ L/well) were seeded in 96-well plates and treated with Pim-1 inhibitor (SMI-4a; Sigma, USA) at different concentrations (0, 5, 10, 20, 40, and 80 μ mol/L) for 48 h. Then 10 μ L of CCK8 solution was added to each well for 4 h incubation. The absorbance at 450 nm was measured using Multiscan Plate Reader (Thermo Fisher Scientific).

Apoptosis assay

Cells were washed with phosphate buffered saline (PBS) twice, centrifuged at 1,000 r/min for 5 min, and then

resuspended in 500 μ L of binding buffer. Then 5 μ L of Annexin V-FITC and 5 μ L of PI were added. After incubation in dark at 37 °C for 15 min, the apoptosis of cells was analyzed by flow cytometry (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Cell cycle assay

The synchronized cells were washed with PBS twice, fixed with cold 75% ethanol, and then cultured at 4 °C overnight. The cells were again washed with PBS, followed by staining with propidium iodide (PI; 50 μ g/ml, Sigma-Aldrich®, St. Louis, MO, USA) in the presence of RNase A (100 μ g/ml; Fermentas®, Shanghai, China). After incubation in dark at 37 °C for 30 min, the cell cycle was measured by flow cytometry according to the manufacturer's instructions.

Western blotting analysis

Mammalian protein extraction agent (Thermo Fisher Scientific) mixed with alt protease inhibitor cocktail (Thermo Fisher Scientific) (100:1) was used to lyse cells. Equal amounts of proteins were loaded onto the 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Then interest proteins were transferred from the gels onto the polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at 37 °C. Then membranes were incubated in primary antibodies overnight at 4 °C and subsequently in horseradish peroxidase-conjugated secondary antibodies for 2 h at 37 °C. Protein bands were visualized with electrochemiluminescence assay (Millipore). ImageJ (National Institutes of Health, USA) was used to quantify the protein bands.¹⁷

In vivo study

Approved by Animal Ethics Committee of Soochow University, animal experiments were performed following the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication). Male C57BL/6 mice (6 weeks old) were purchased from Shanghai Experimental Animal Center (Shanghai, China). A549 or Ltp-a-2 cells (1×10^7) resuspended in PBS were subcutaneously inoculated into the armpit. After one week, mice were randomly divided into five groups treated with 5/10/20/40 mg/kg of SMI-4a or vehicle control (0 mg/kg) (5 mice per group). Every three days, the length and width of the tumors were measured. Tumor volume was calculated using the following formula: $(\text{width}^2 \times \text{height})/2$. After the last

measurement of the volume, the tumors of mice were immediately removed, weighed, and then lysed for Western blotting analysis.

Statistical analysis

Statistical analysis was performed using SPSS v21.0 (SPSS Inc., USA). Difference between two groups was assessed using Independent sample *t*-test. When comparing more than two groups, one-way analysis of variance (ANOVA) followed by Dunnett's test was used. Each experiment was repeated three times. Data were presented as the means \pm standard deviation (SD). A $P < 0.05$ was considered as statistically significant.

Results

SMI-4a inhibits the proliferation of NSCLC cells

CCK-8 assay was used to examine the cell proliferation. The results showed that SMI-4a inhibited the proliferation of A549 and Ltp-a-2 cells in a dose-dependent manner ($P < 0.05$; Figure 1). Because 80 $\mu\text{mol/L}$ of SMI-4a showed a strongest inhibitory effect on the proliferation of both cell lines, 80 $\mu\text{mol/L}$ was chosen as the SMI-4a concentration in the following in vitro experiments.

SMI-4a induces the apoptosis of NSCLC cells

Flow cytometry assay was used to examine the cell apoptosis. The results showed that treatment with 80 $\mu\text{mol/L}$ of SMI-4a for 48 h significantly induced the apoptosis rate of NSCLC cells (Figure 2). The apoptosis rate of A549 cells

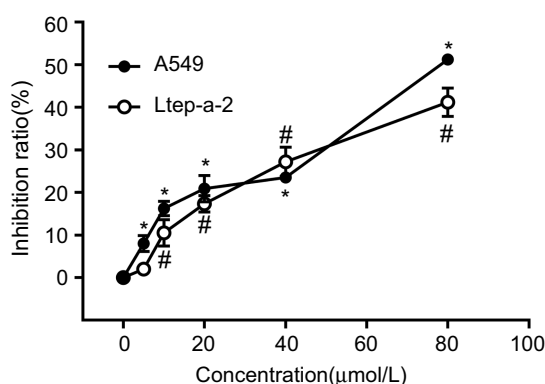


Figure 1 SMI-4a inhibits the proliferation of NSCLC cells. The inhibition ratios of A549 and Ltp-a-2 cells were detected at 48h after treatment with various concentrations of SMI-4a (0, 5, 10, 20, 40, or 80 $\mu\text{mol/L}$). Each value represents the mean of triplicate experiments; bars, SD. ANOVA; * $P < 0.05$, vs 0 $\mu\text{mol/L}$, A549 group; # $P < 0.05$, vs 0 $\mu\text{mol/L}$, Ltp-a-2 group.

increased from 1.1 ± 0.8 to 17.0 ± 0.9 ($P < 0.001$). The apoptosis rate of Ltp-a-2 cells increased from 1.3 ± 0.7 to 10.0 ± 0.8 ($P = 0.001$).

SMI-4a blocks the cell cycle of NSCLC cells

The effect of SMI-4a on the cycle distribution of NSCLC cells was also detected by flow cytometry assay. The results showed that SMI-4a blocked the cell cycle of NSCLC cells in G2/M phase (Figure 3). After 48h treatment of 80 $\mu\text{mol/L}$ of SMI-4a, the portion of A549 cells in G2/M phase increased from 19.2 ± 2.3 to 28.3 ± 3.7 ($P = 0.021$); the portion of Ltp-a-2 cells in G2/M phase increased from 19.6 ± 3.2 to 32.0 ± 5.5 ($P = 0.037$).

SMI-4a blocks the phosphorylation of PI3K/AKT/mTOR pathway

Given the complex relationship between Pim-1 and PI3K/AKT/mTOR pathway, we further investigated the role of Pim-1 inhibitor in the activation of this pathway. By Western blotting analysis, we found that the total expression of PI3K, AKT and mTOR was not altered by SMI-4a ($P > 0.05$; Figure 4). However, the phosphorylation of PI3K, AKT and mTOR was significantly downregulated by SMI-4a ($P < 0.05$; Figure 4).

SMI-4a suppresses tumor growth in vivo

Mouse models with NSCLC were established to validate the suppressive effect of SMI-4a on tumor growth. The mice were grouped according to the dose of SMI-4a. Compared with the control group (0 mg/kg), groups treated with 5/10/20/40 mg/kg of SMI-4a had lower tumor growth rates and lighter tumor weight ($P < 0.05$, Figure 5). The tumor suppressive effect of SMI-4a in vivo was also in a dose-dependent manner.

To further confirm the role of PI3K/AKT/mTOR pathway in the anti-tumor process induced by SMI-4a, we collected the mouse tumor tissues to detect the expression levels of associated proteins in the pathway. The results showed that the phosphorylation of PI3K, AKT and mTOR was downregulated by SMI-4a in a dose-dependent manner ($P < 0.05$, Figure 6), while the total expression of PI3K, AKT and mTOR was still not altered by SMI-4a ($P > 0.05$, Figure 6).

Discussion

Previous studies have confirmed the anti-tumor effects of SMI-4a in a variety of tumors, including osteosarcoma, melanoma

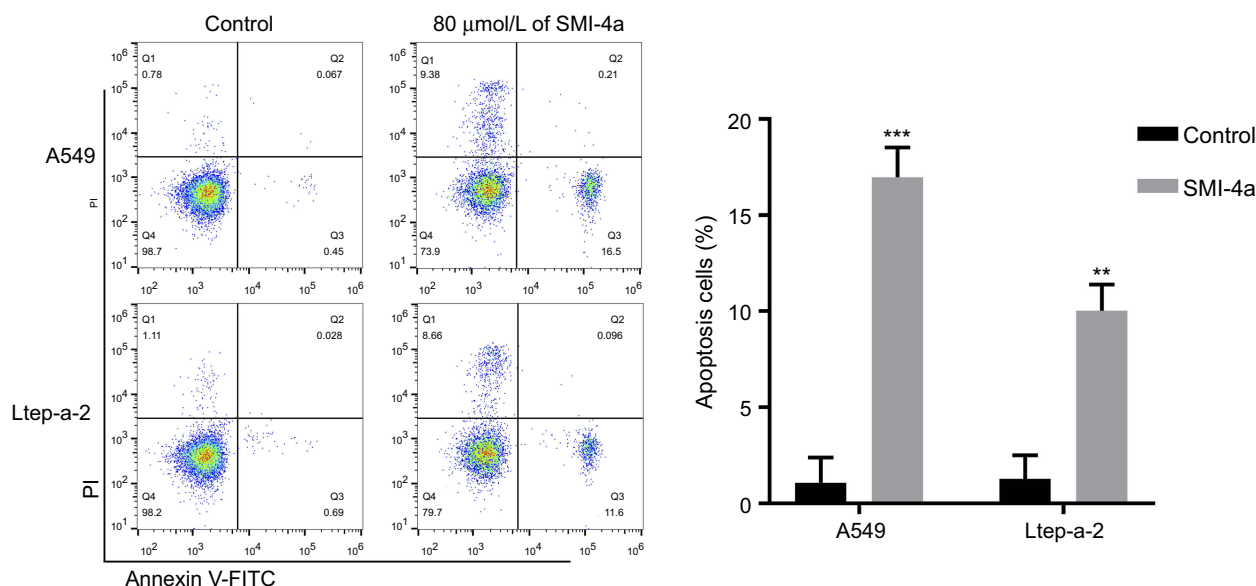


Figure 2 SMI-4a induces the apoptosis of NSCLC cells. The apoptosis ratios of A549 and Ltp-a-2 cells were detected at 48h after treatment with 80 $\mu\text{mol/L}$ of SMI-4a by flow cytometry. Representative images and quantitative data are shown. Each column is shown as the mean of triplicate experiments; bars, SD. Student's *t*-test; ** $P < 0.01$, *** $P < 0.001$.

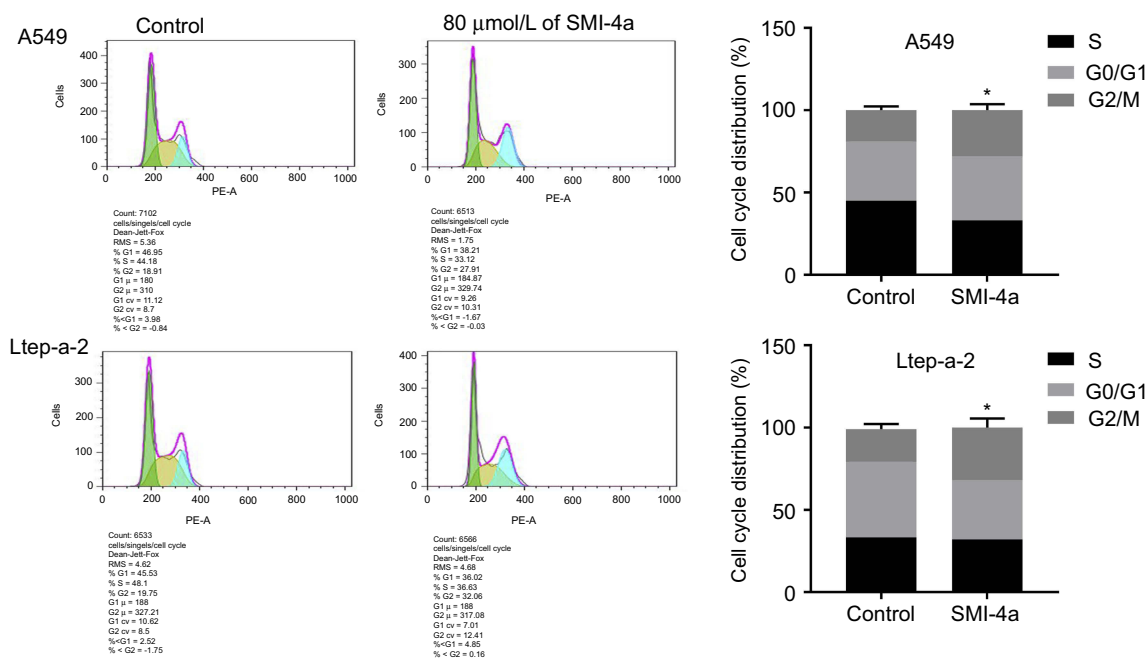


Figure 3 SMI-4a blocks the cell cycle of NSCLC cells. The cell cycles of A549 and Ltp-a-2 cells were detected at 48h after treatment with 80 $\mu\text{mol/L}$ of SMI-4a by flow cytometry. Representative images and quantitative data are shown. Each column is shown as the mean of triplicate experiments; bars, SD. Student's *t*-test; * $P < 0.05$.

and leukemia.^{18–20} Liao et al found that SMI-4a significantly downregulated the expression of anti-apoptotic protein Bcl-2, and inhibited the cell proliferation, migration, and invasion in osteosarcoma.¹⁸ Lv et al confirmed that SMI-4a inhibited tumor growth by inducing autophagy via AKT/mTOR pathway in melanoma.¹⁹ Fan et al proved that SMI-4a induced the apoptosis and inhibited the colony formation of leukemia

cells.²⁰ These findings indicated the potential of SMI-4a as a new molecular targeted drug for the treatment of tumor. The present study is the first to demonstrate the anti-tumor effects of SMI-4a in NSCLC. We found that SMI-4a inhibited the proliferation and cell cycle, and induced the apoptosis of NSCLC cells in vitro. SMI-4a also had an inhibitory effect on tumor growth in vivo in a dose-dependent manner.

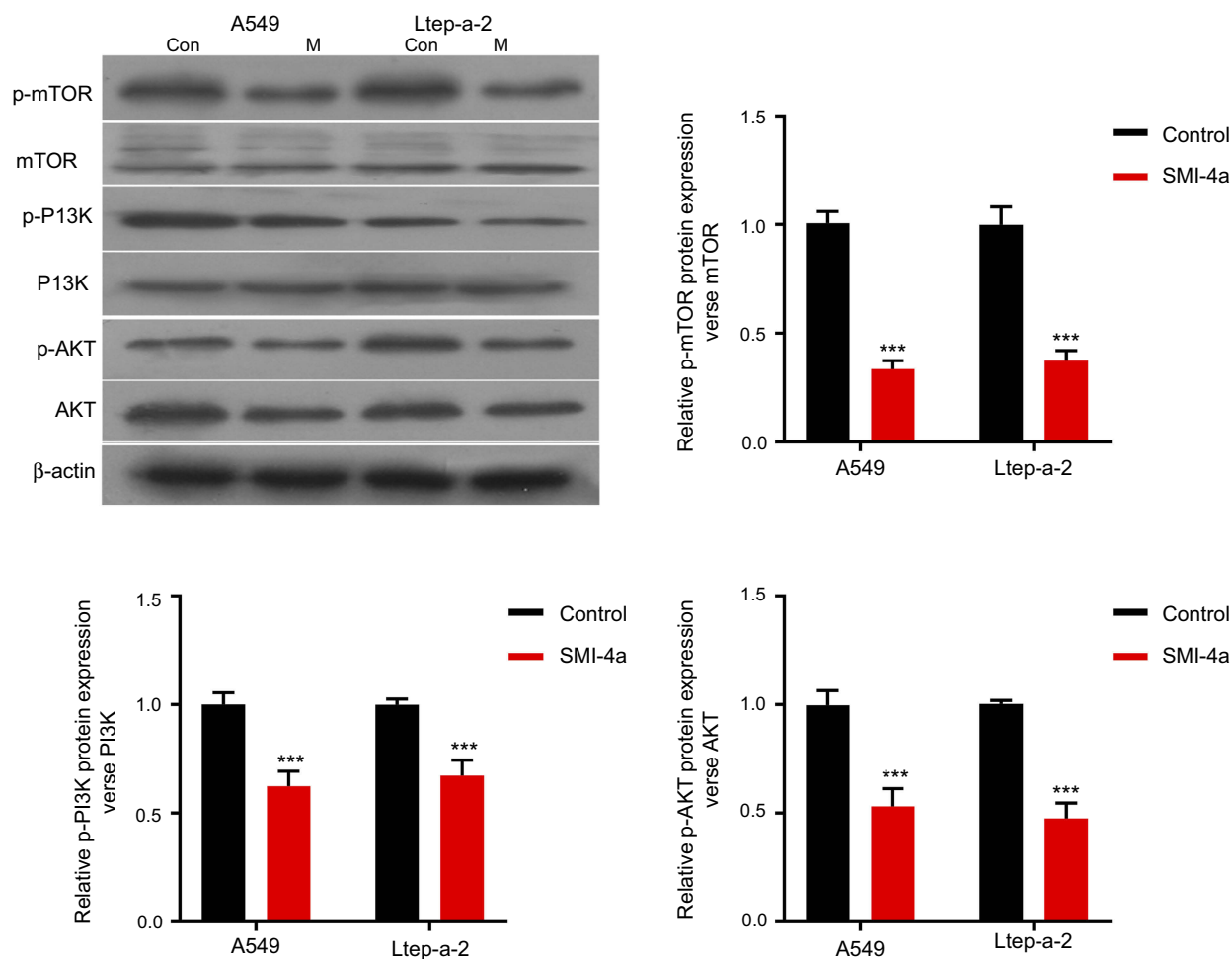


Figure 4 SMI-4a inhibits PI3K/AKT/mTOR pathway in vitro. The protein levels of PI3K/AKT/mTOR pathway in A549 and Ltp-a-2 cells were detected at 48h after treatment with 80 μ mol/L of SMI-4a. Representative images and quantitative data are shown. Each column is shown as the mean of triplicate experiments; bars, SD. Student's *t*-test; ****p*<0.001.

There are many other Pim-1 inhibitors besides SMI-4a. Lee et al proved that the combination of Pim-1 inhibitor AZD1208 and AKT inhibitor had synergism inhibitory effects on the progression of gastric cancer cells.²¹ Hu et al produced an anti-Pim-1 mAb and demonstrated that it suppressed the tumor growth by inhibiting the phosphorylation of AKT in prostate cancer.¹³ Kim et al found that Pim-1 inhibitors (SGI-1776, ETP-45299, and tryptanthrin) enhanced the radiosensitization of tumor cells by inhibiting radioresistant signaling pathway in mouse models with NSCLC.²² However, more studies are needed to compare the safety and effectiveness of different Pim-1 inhibitors.

Our results indicated that PI3K/AKT/mTOR pathway was significantly inhibited by SMI-4a in NSCLC, which is consistent with previous studies.^{13,19} In malignant tumors, the activation of PI3K/AKT/mTOR pathway breaks the balance between pro-apoptotic and anti-apoptotic proteins to exert an anti-apoptotic effect. Briefly, AKT increases the

dissociative anti-apoptotic protein Bcl-2 and blocks the activation of pro-apoptotic proteins caspase-9 and caspase-3.^{23,24} The activation of PI3K/AKT/mTOR pathway can also promote cell proliferation. For example, AKT directly phosphorylates glucose synthase kinase-3 β (GSK3 β) and increases the accumulation of cyclin D1 that accelerates the G1-S phase.²⁵ Besides, AKT inhibits the expression of p27Kip1, a cell cycle-dependent protein that blocks cell cycle in G1 phase.²⁶ However, our study showed that SMI-4a blocked the cell cycle of NSCLC cells in G2/M phase rather than G1 phase. Fan et al also revealed that SMI-4a blocked the cell cycle of chronic myeloid leukemia cells in S-phase rather than G1 phase.²⁰ Based on these inconsistent results, we hypothesized that in addition to D1 and p27Kip1, there are several other cell cycle regulatory factors involved in the SMI-4a-induced NSCLC cell cycle arrest. Further studies are needed to verify this hypothesis.

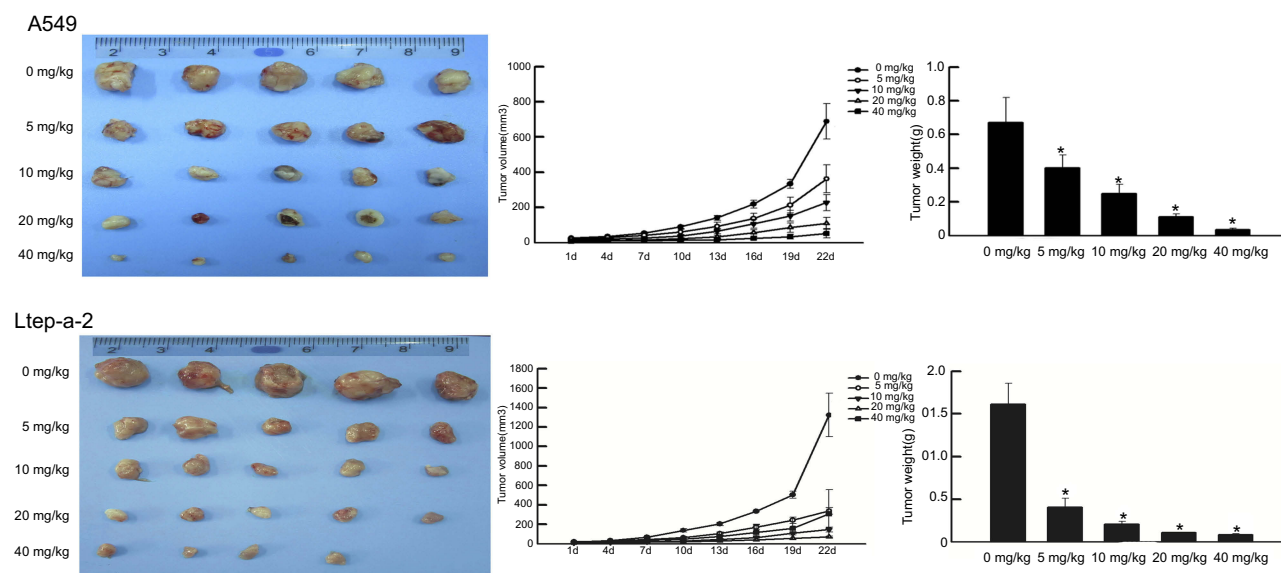


Figure 5 SMI-4a suppresses tumor growth in mouse models NSCLC. The tumor volume in A549 and Ltp-a-2 mouse models treated with various concentrations of SMI-4a (0, 5, 10, 20, or 40 mg/kg) was measured every three days. At 22 d, the tumor weight was measured. Each value represents the mean tumor volume of five mice; bars, SD. ANOVA; * $P < 0.05$, vs 0 mg/kg.

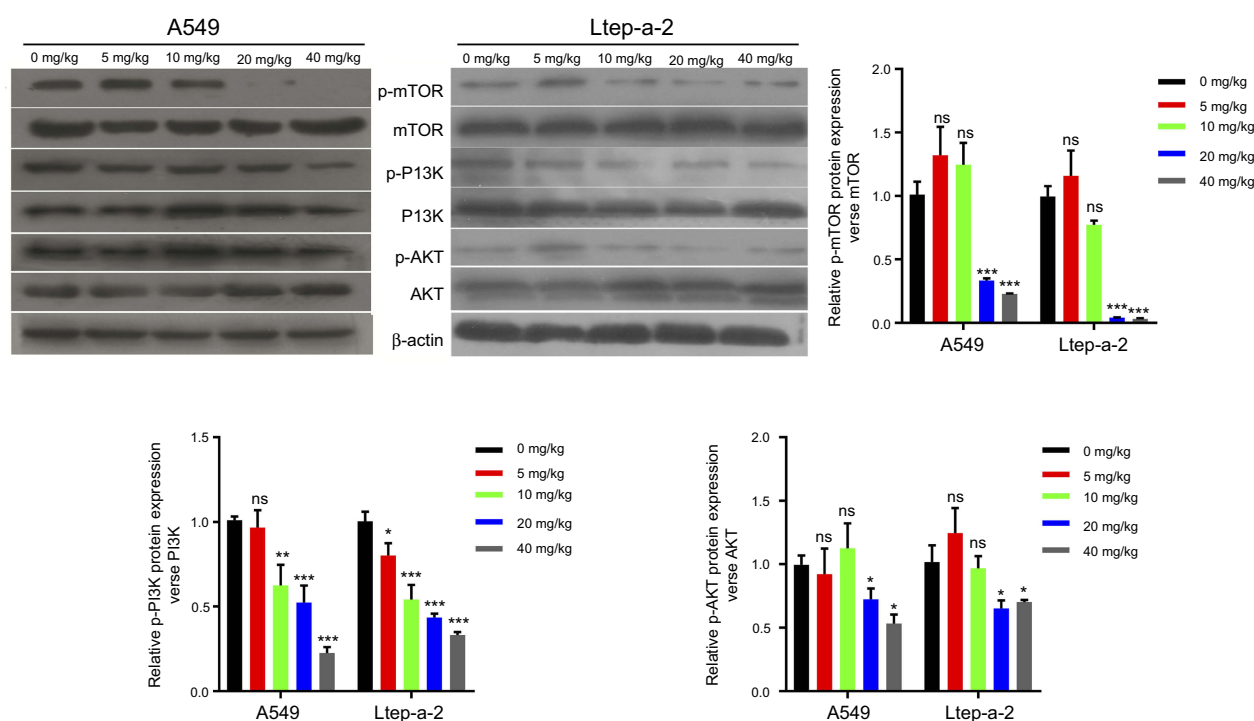


Figure 6 SMI-4a inhibits PI3K/AKT/mTOR pathway in vivo. At 22 d, the tumors of A549 and Ltp-a-2 mouse models treated with various concentrations of SMI-4a (0, 5, 10, 20, or 40 mg/kg) were lysed for Western blotting analysis. The protein levels of PI3K/AKT/mTOR pathway were examined. Representative images and quantitative data are shown. Each column is shown as the mean of triplicate experiments; bars, SD. ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs 0 mg/kg.

Abbreviation: ns, no significance.

In recent years, EGFR-TKIs have been widely used in NSCLC patients with EGFR sensitive mutation.²⁷ Although the clinical effect was significant in these patients, they will ultimately develop resistance to EGFR-

TKIs after about 10–14 months.²⁸ Moreover, targeted therapy with EGFR-TKIs can improve the quality of life in NSCLC patients, but its impact on overall prognosis is still not satisfactory.²⁹ Therefore, new drugs targeting

other molecules may be better choices for patients without EGFR sensitive mutation or resistant to EGFR-TKIs. SMI-4a selectively inhibiting Pim-1 kinase significantly inhibited tumor growth and was well tolerated in vivo in our study, so we surmised that SMI-4a has the potential to be the ideal alternative to EGFR-TKIs for NSCLC patients. More clinical trials are needed to prove the safety and effectiveness of SMI-4a.

In conclusion, the present study is the first research evaluating the therapeutic effect of Pim-1 inhibitor SMI-4a in NSCLC. The in vitro and in vivo experimental results showed that SMI-4a significantly suppresses the progression of NSCLC by blocking the PI3K/AKT/mTOR pathway. Pim-1 inhibitors may become new targeted drugs for NSCLC patients in the future.

Disclosure

The authors declare no potential conflicts of interest in this work.

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