Received: 20 March 2022 | Accepted: 19 April 2022

DOI: 10.1111/1759-7714.14453

ORIGINAL ARTICLE

WILEY

# ACT001 inhibits the proliferation of non-small cell lung cancer cells by upregulating NKTR expression

**Min Zhao** | **Tingting Qin** | **Dingzhi Huang**

Department of Thoracic Oncology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin, Key Laboratory of Cancer Prevention and Therapy, Tianjin, Tianjin's Clinical Research Center for Cancer, Tianjin, China

**Correspondence**
Dingzhi Huang, Department of Thoracic Oncology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin, Key Laboratory of Cancer Prevention and Therapy, Tianjin, Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China.
Email: dingzhih72@163.com

**Funding information**
National Natural Science Foundation of China, Grant/Award Numbers: 81802296, 82172635

## Abstract

**Background:** Lung cancer, the primary cause of cancer-related deaths worldwide, is diagnosed at an advanced stage and has a poor prognosis. Non-small cell lung cancer (NSCLC) is a major histological type of lung malignancy. This study investigated the effect of ACT001, a novel sesquiterpene lactone derivative, on the proliferation of NSCLC cells and explored the underlying mechanism.

**Methods:** The effect of ACT001 on cell proliferation was examined by clone formation and MTT assay. Differentially expressed genes and enrichment pathways were analyzed by RNA-seq. Flow cytometry and cell cycle-related protein expression analysis were performed to study the cell cycle. Phosphorylated AKT was detected to explore the mechanism in natural killer cell triggering receptor (NKTR) KD cells with AKT activator and/or inhibitor. The therapeutic effect of ACT001 in vivo was studied in the xenograft tumor model.

**Results:** ACT001 inhibited the proliferation and G1/S transition in NSCLC cell lines. By RNA-seq analysis, NKTR may be the target of ACT001. Moreover, knockdown NKTR promoted cell proliferation and reversed the effects of ACT001. In addition, ACT001 inhibited AKT phosphorylation, but NKTR knockdown promoted AKT phosphorylation.

**Conclusion:** Our results suggested NKTR may be the target of ACT001 in NSCLC. ACT001 holds promise as a novel method for the treatment of NSCLC.

**KEYWORDS**
ACT001, AKT, NKTR, non-small cell lung cancer

## INTRODUCTION

Lung cancer exhibits the highest morbidity and mortality of cancers, with non-small cell lung cancer (NSCLC) being the predominant histological subtype.[1,2] The five-year survival rate for patients with advanced disease is only 5%.[3] Over the past two decades, considerable efforts have been made to identify the molecular mechanisms underlying cancer development.[4] Remarkable advances in the therapeutic regimens of lung cancer have been achieved, in particular, molecularly targeted therapies and immunotherapies such as antiprogrammed cell death ligand 1 antibodies and immune-checkpoint inhibitors.[5,6] Despite efforts to improve the treatment of NSCLC, the available options remain limited, and patient survival rates have not markedly improved.[7,8] Therefore, new treatment modalities are urgently needed for patients with advanced disease to control this deadly disease, expand clinical benefits, and improve survival and prognosis.

Micheliolide (MCL) is found in Michelia compressa and Michelia champaca plants. MCL is a derivative of parthenolide (PTL), a natural guaianolide sesquiterpene lactone, which can suppress leukemia and colitis-associated cancer.[9,10] Micheliolide has the same anticancer structure as parthenolide but it is more stable.[11,12] ACT001, a derivative of micheliolide developed by Accendatech, has shown potent anticancer and anti-inflammatory activities.[13,14] ACT001 has also been reported to inhibit the inflammatory response and activation of the NLRP3 inflammasome in the

midbrain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice.[13] Because ACT001 can pass through the blood–brain barrier, considerable research has been conducted on its antiglioblastoma (GBM) activity. It has been shown that ACT001 inhibits GBM via NF-κB suppression.[15] ACT001 targets glioma stem-like cells[16] and synergizes with cisplatin against gliomas through inhibition of the phosphoinositide 3-kinase/AKT pathway.[17] The United States Food and Drug Administration has certified it as an orphan drug for GBM in 2017, and it is currently in phase II clinical trials (ACTRN12616000228482 in Australia, ChiCTR-OIC-17013604 in China).[18]

Some data have shown the potential effectiveness of ACT001. For example, a patient with relapsed and drug-free GBM maintained complete remission for 40 months after receiving ACT001. By inhibiting phospho-NF-κB and IκB-α in triple-negative breast cancer cells, ACT001 decreases granulocyte-macrophage colony-stimulating factor (GM-CSF), promotes apoptosis, and reduces angiogenesis.[19] In addition, ACT001 also induces cell cycle arrest, apoptosis, and the generation of reactive oxygen species in hepatocellular carcinoma cells by inhibiting the PI3K/AKT pathway.[21] However, the mechanism of ACT001 is less well understood, especially in NSCLC. This has significantly limited its clinical application, and further research is needed to understand its pharmacological mechanism.

Herein, we demonstrate, for the first time, that ACT001 inhibits the proliferation of NSCLC. These results may serve as a preliminary basis for clinical trials of ACT001 in lung cancer.

## METHODS

### Cell lines, drugs, and antibodies

Human squamous cell carcinoma cell lines NCI-H1703, EBC-1, NCI-H226, and human adenocarcinoma cell lines NCI-H1975, PC9, LTEP-A2, and HCC827 were obtained from American type culture collection (ATCC) with STR analysis. All cells were cultured in a standard medium containing 10% fetal bovine serum and penicillin/streptomycin. ACT001 was purchased from Accendatech Ltd. Anti-NKTR antibody was purchased from Santa Cruz Biotechnology (19978-1-AP) for Western blotting and immunohistochemistry. Antibodies for cyclinD1 and CDK6 were purchased from Abcam (ab228528) for Western blotting. Antibodies for AKT and Phosphorylated AKT were from CST (9272,4060) for Western blotting. All antibodies were diluted in antibody diluent (beyotime P0256FT). The AKT activator SC79 (HY-19749) and inhibitors MK-2206 (HY-108232) were from MCE.

### Transfection

Researchers used shRNA carried by a lentiviral vector to knock down NKTR in H1703 and H1975 cells with lipofectamine 3000 (Invitrogen) and selected by puromycin (Invitrogen). NKTR expression was validated by WB.

### CancerSEA analysis

The URL for CancerSEA is http://biocc.hrbmu.edu.cn/CancerSEA/. This cancer database allows exploration of gene expression and function at the single-cell level. The database contains datasets from the SRA, GEO, and ArrayExpress websites. A single gene can be searched directly. The database allows analysis of the functional status of 41 900 single cells from 25 tumor types including NSCLC.

### GEPIA analysis

GEPIA (http://gepia.cancer-pku.cn/index.html) is a web server based on the TCGA database and GTEx projects. Researchers can easily analyze RNA sequencing data from tumor/normal samples without programming. With GEPIA, researchers can analyze NKTR differential expression.

### Colony formation

Experiments were performed on six-well plates. A total of 500 cells were inoculated in each well. After the cells had adhered to the wall, different treatments were carried out. The number of clones was counted after 10–14 days. Phosphate-buffered saline (PBS) was used to wash the clones. After that, paraformaldehyde was used to fix the six-well plates. The plates were set aside for 30 min. Crystalline purple was used to stain six-well plates. The plates were then set aside for 20 min.

### Cell viability analysis

An MTT assay was performed to detect the proliferation of NSCLC cells. Experiments were performed using 96-well plates and 5 000 cells were inoculated in each well. Different treatments were performed after cell adhesion, and 10 μl MTT was added to each well after an appropriate time. After 3 h, purple crystals were visualized by the experimenter, the medium and MTT were discarded, and 150 μl DMSO was added to each well to dissolve the crystals. The absorbance values were measured at 490 and 570 nm using an absorbance reader.

### RNA extraction and quantitative RT-PCR

Trizol was used to extract total RNA. After measuring the concentration, 500 ng RNA was reverse transcribed to cDNA. Real-time quantitative PCR (RT-qPCR) was performed using SYBR PCR mix (Takara rr820) following the

instructions. Data were standardized to the GAPDH level for at least three repetitions.

The primer sequence of NKTR:

forward primer: 5GCA GTG GCA GTG CTT TCT CT3.
reverse primer: 5TAA TGC GAC CAA CCG GCT C3.

## Western blot

According to the procedure, this study utilized a buffer of Sigma (C2978) and a mixture of protease and phosphatase inhibitors (Sigma, P8340) to extract proteins. After proteins quantitative detection (BCA, Solarbio, PC0020), 8%–15% SDS-PAGE gels were used to separate the same mass of proteins and protein blots were transferred to PVDF membranes. Blocking fluid (beyotime p0235) was used to block the membrane and the whole process took 30 min. Membranes were later fully immersed in the primary antibody at 4°C overnight. The next day, membranes were washed three times for 25 min using PBS. Then, secondary antibody was used to bind to the primary antibody for 1 h. PBS was used to wash off the secondary antibody for 25 min. Finally, a positive protein signal was detected using an Immobilon Western HRP kit (Millipore).

## RNA sequencing

After ACT001 treatment, RNA was extracted from H1703 cells and stored at −80°C. RNA sequencing (RNA-seq) was accomplished using CapitalBio Technology.

## Flow cytometry

A DNA content quantitation assay (cell cycle) kit (Solarbio, CA1510) was used to analyze the cell cycle. The cell concentration was adjusted to $1 \times 10^6$/ml. After the corresponding treatments, 1 ml of cell suspension was harvested. Then, 70% cold ethanol was used to fix the cells. The whole process was carried out at 4°C overnight. Following overnight fixation, the cells were treated with RNase for 30 min at 37°C for removing RNA contamination. Finally, propidium iodide (PI) dye liquor was added before incubation of the cells in the dark at 4°C for 30 min. The cell cycle was detected with grouped prepared samples.

## Immunohistochemistry and hematoxylin–eosin (HE) staining

NSCLC tissues from 2014–2015 were provided by the Tianjin Medical University Cancer Institute and Hospital. The Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital consented to this experiment. On the first day, paraffin sections were dewaxed, hydrated, antigen repaired, incubated with 3% hydrogen peroxide ($H_2O_2$) protected from light, and blocked in a 5% lamb serum solution for 15 min. Sections were then treated with primary antibodies overnight at 4°C. On the second day, the sections were incubated with DAB enhancers, secondary antibodies, and DAB chromogen successively.The results were observed under a microscope and determined independently by two observers using a blinded method.

The degree of expression was defined by staining shade and percentage of stained cells in the observations. No staining, light yellow, brownish-yellow and brownish brown were scored as 0, 1, 2, and 3. The proportion of stained cells in the field of view defined as <5% was scored as 0, 5%–25% as 1, 26%–50% as 2, 51%–75% as 3 and 76%–100% as 4. The final result was the product of the two figures. High expression was defined as a score of 5 and above.

HE staining was done using a conventional method. The dewaxed sections were incubated with hematoxylin and stained with eosin.

## Xenograft tumor model

This experiment was conducted at the Cancer Institute and Hospital of Tianjin Medical University. Consent for all operations was obtained from the Ethics Committee. The results of all animal experiments were approved by the ethics committee of the Tianjin Medical University Cancer Institute and Hospital. To establish the lung cancer model, 5–6 weeks old BALB/c nude mice were inoculated subcutaneously into the groin with $1 \times 10^7$ H1703 cells suspended in 100 μl sterile PBS. Tumor volume (½ [length × width$^2$]) was measured every two days. After the average tumor volume reached 100 mm$^3$, mice received 200 mg/kg ACT001 or sterile saline solution orally every day. On day 13, one mouse in the saline group died because the tumor was too large. After 12 days of administration, the mice were killed under anesthesia. The tumors, livers and kidneys were carefully taken, fixed with 10% formalin and embedded in paraffin.

## Statistical analysis

Statistical differences were analyzed by a Student's *t*-test. The criteria for statistically significant differences were: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. $p < 0.05$ was considered to be statistically significant.

# RESULTS

## ACT001 inhibited NSCLC cell proliferation in vitro

The molecular formula of ACT001 is $C_{17}H_{27}NO_3 \cdot C_4H_4O_4$, with a molecular weight of 409.47 Da[24] (Figure 1a). To

investigate the effect of ACT001 in NSCLC, this study initially determined its IC50 in NSCLC cell lines using an MTT assay. The viability of NSCLC cells decreased as the concentration of ACT001 increased (0.0625–800 μM) (Figure 1b). Calculations showed that ACT001 had a relatively pronounced inhibitory effect on H1703 (IC50 = 9.21 μM) and H1975 (IC50 = 14.42 μM) cells; the IC50s of the other cell lines tested ranged from 50 to 100 μM (Table 1). For subsequent experiments, H1703 $IC_{50}$ concentrations of 0, 100%, and 200% were used, with an incubation time of 72 h. This study performed clone formation using H1703 and H1975 cells to observe the effects of ACT001. The clone formation assay showed that increasing concentrations of ACT001 inhibited the proliferation ability of these cells (Figure 1c,d). These results suggest that ACT001 inhibits NSCLC cell proliferation.

## ACT001 induced G1/S arrest in NSCLC cell lines

To investigate the effect of ACT001 on NSCLC, this study performed RNA-seq on H1703 cells treated with or without ACT001. Pathway enrichment analysis revealed that RNAs associated with the cell cycle pathway changed the most significantly (Figure 2a). To verify the RNA sequencing results, the experimenter performed flow cytometry. The ratios of cells in the G1, S, and G2/M phases indicated that ACT001 induced G1/S arrest in the NSCLC cell lines(Figure 2b). At classical G1/S phase checkpoints, the expression of cyclinD1 and CDK6 decreased following ACT001 administration (Figure 2c). These results indicate that ACT001 inhibits G1/S transition.

## ACT001 upregulated NKTR expression, and high NKTR expression favored patient survival and prognosis

To further explore the mechanism of action of ACT001 in NSCLC cells, this study determined differentially expressed genes following ACT001 treatment by RNA-seq (false discovery rate <0.05 and a folder change >2). *NKTR* is a top one in ACT001 upregulated genes (Figure 3a). WB and

**TABLE 1** Inhibitory effect of ACT001 on non-small cell lung cancer cells

| | ACT001 72 h IC50 (μM) |
|---|---|
| H1703 | 9.21 |
| H1975 | 14.42 |
| EBC-1 | 58.75 |
| H226 | 91.58 |
| PC-9 | 53.62 |
| LTEP-A2 | 70.93 |
| HCC827 | 52.79 |

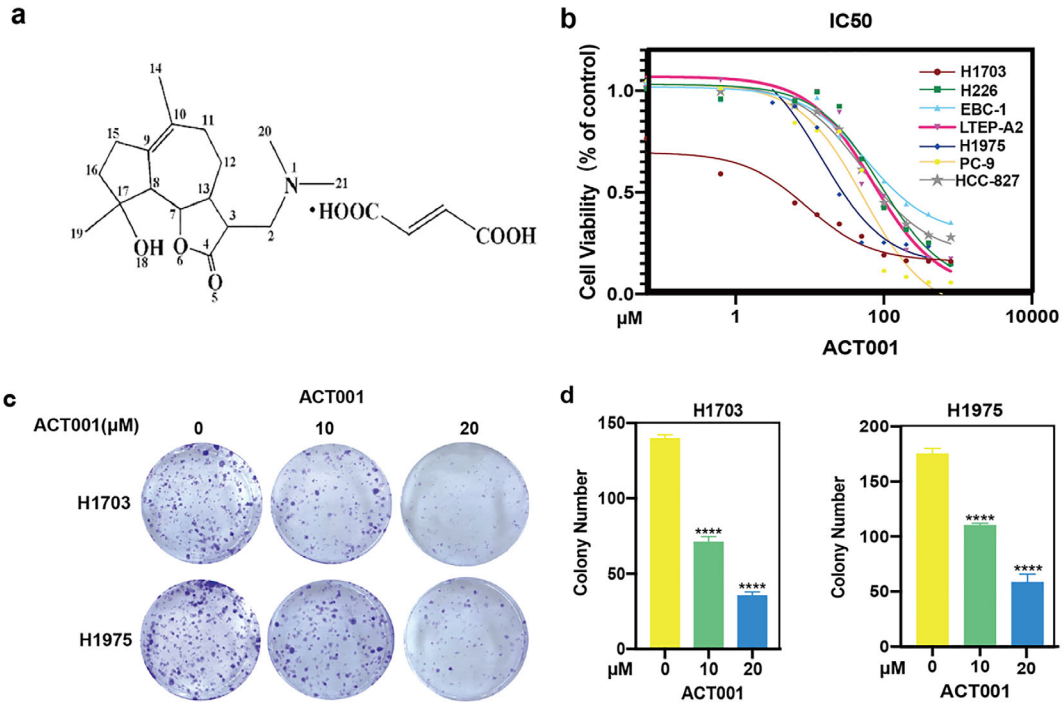


**FIGURE 1** ACT001 inhibited non-small cell lung cancer (NSCLC) cell proliferation in vitro. (a) Molecular structure formula of ACT001. (b) The ACT001 72 h IC50 in NSCLC cells. (c) A clone formation assay was performed to investigate the effect of different concentrations of ACT001 on H1703 and H1975 cell lines. (d) Quantification of the clone formation assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

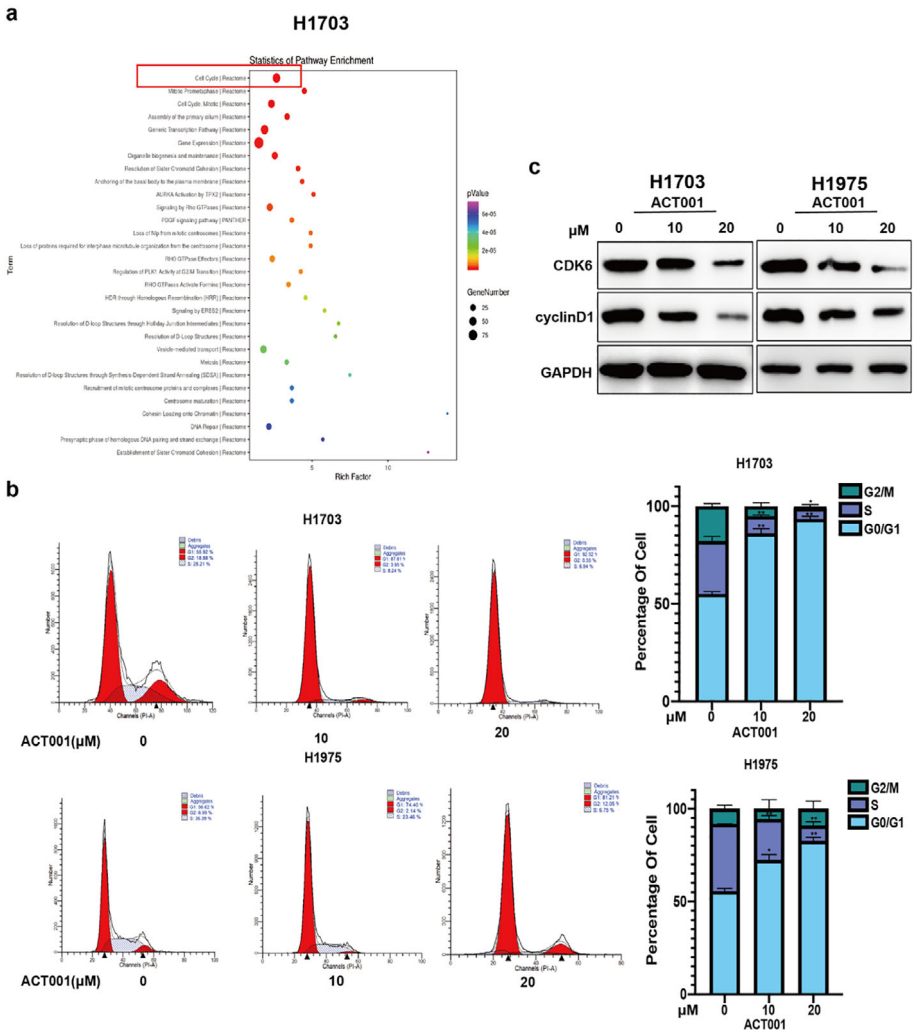


**FIGURE 2** ACT001 induced G1/S arrest in non-small cell lung cancer (NSCLC) cell lines. (a) The bubble chart shows the up-/downregulation pathway of ACT001 treatment after analyzing the RNA-Seq database. (b) Cell cycle analysis. Results of cell cycle analysis were obtained for the H1703 and H1975 cell lines with ACT001. The percentages of G1, S, and G2/M phase cells were analyzed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. (c) Western blot analysis of cyclin D1 and CDK6 in H1703 and H1975 cell lines after treatment with different concentrations of ACT001

qPCR assays demonstrated that ACT001 increased NKTR expression (Figure 3b,c).

To investigate the effect of NKTR expression on the diagnosis and survival of patients with NSCLC, this study utilized the GEPIA database (http://gepia.cancer-pku.cn/index.html). The results showed that NKTR expression was lower in NSCLC tissue samples than in normal lung tissue (Figure 3d). The CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/) database showed that NSCLC cells with low NKTR expression tended to cluster together, suggesting that low NKTR expression promotes the malignant progression of NSCLC (Figure 3e). This study then used IHC to detect NKTR expression in NSCLC tissue from patients and found that its expression was associated with the clinical stage; specifically, late-stage patients had lower NKTR expression levels (Figure 3f) (Table 2). This study investigated the prognostic impact of NKTR using the Kaplan–Meier analysis. Patients had longer overall survival with NKTR overexpression. (Figure 3g). These results suggest that high NKTR expression is beneficial for survival and prognosis.

## ACT001 induced G1/S arrest by upregulating NKTR expression

To further investigate the mechanism of ACT001 in NSCLC, this study used shRNA carried by a lentiviral vector to knock down NKTR in H1703 and H1975 cells. (Figure 4a). This was followed by an analysis of the effect on cell proliferation viability and clone formation. Cell proliferation data from the MTT assay are shown in Figure 4b. The proliferation of tumor cells was significantly enhanced after NKTR knockdown. The clone formation assay also showed that decreased NKTR expression promoted tumor cell proliferation (Figure 4c, d). These results show that NKTR inhibits the proliferation of tumor cells. The effect of NKTR knockdown on the cell cycle was measured using flow cytometry. These results and the statistical chart showed knockdown of NKTR resulted in fewer cells in the G1 phase in H1703 and H1975 cells. The researcher also observed that the S phase cells became more (Figure 4e). CDK6 and cyclinD1 protein expression was measured by WB in the NKTR

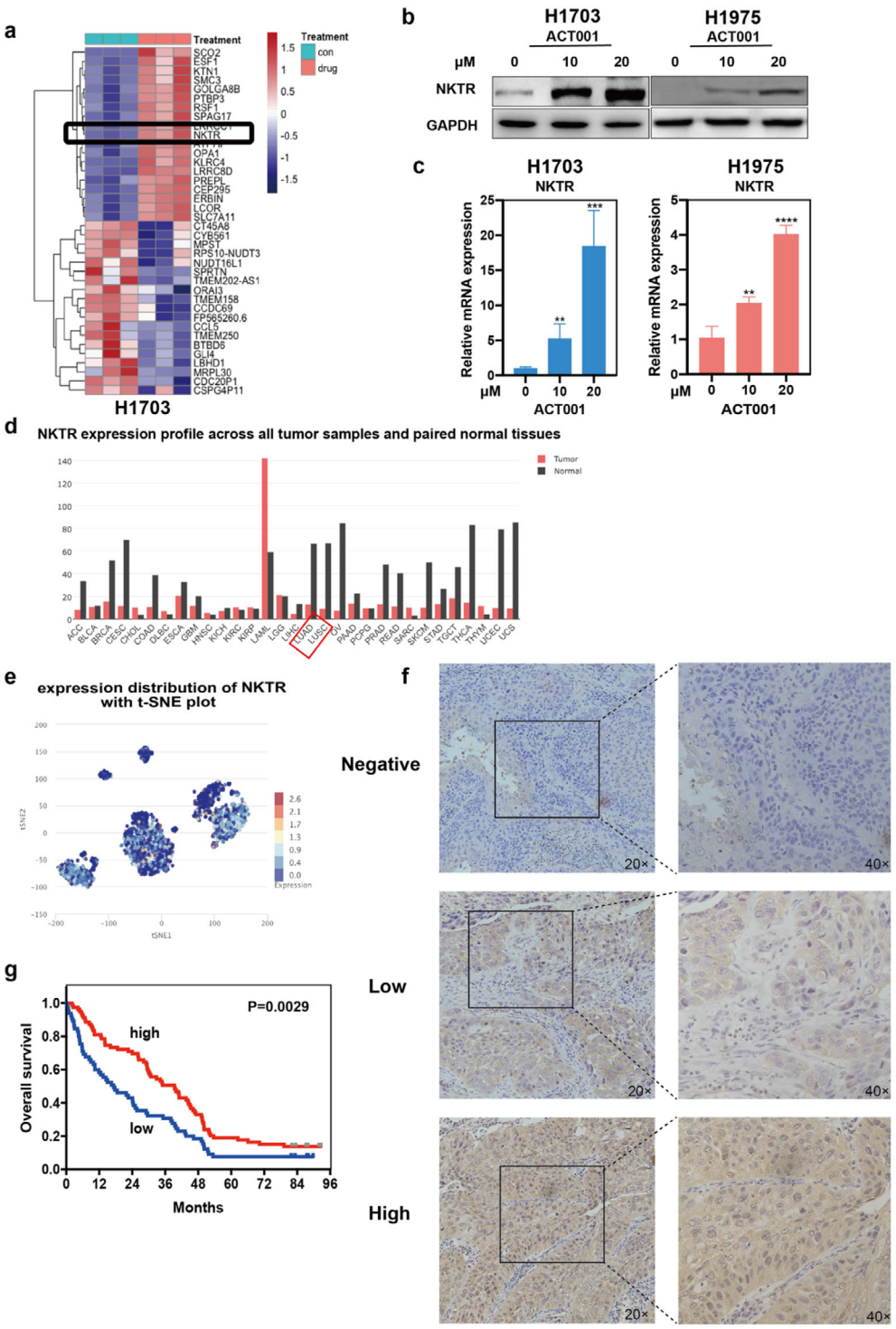


**FIGURE 3** ACT001 upregulated natural killer cell triggering receptor (NKTR) expression, and high NKTR expression favored patient survival and prognosis. (a) The data were RNA-sequenced after dosing ACT001 in the H1703 cell line and processed to obtain a heat map. Red, upregulated DEGs; blue, downregulated DEGs ($p < 0.05$, fold change >2). (b, c) The RNA-Seq results were verified. The expression of NKTR protein and mRNA in H1703 and H1975 cells were detected by Western blot (WB) and qPCR. (d) In most types of cancer, NKTR expression was lower in tumor tissues compared to normal tissues. (e) Distribution of NKTR expression in non-small cell lung cancer (NSCLC) cells from the CancerSEA database. (f) Representative images of NKTR IHC staining at different expression levels in NSCLC. (g) Survival analysis of NKTR in NSCLC patients. The red line represents patients with high NKTR expression and the blue line represents patients with low NKTR expression. The x-axis indicates overall survival time (months) and y-axis indicates survival rate. A Kaplan–Meier test was performed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

knockdown cell lines and found to be upregulated (Figure 4f). This indicated that NKTR promoted G1/S arrest in tumor cells.

Based on previous results showing that ACT001 induced higher NKTR expression and NKTR inhibited tumor cell proliferation, it was hypothesized that ACT001 induced G1/S arrest and inhibited NSCLC cell proliferation by upregulating NKTR expression. To validate this conjecture, we performed MTT, clone formation, and cell cycle assays. After NKTR knockdown in H1703 and H1975 cells, the ACT001 IC50 values increased (Figure 5a), suggesting that NKTR may be the target of ACT001 in NSCLC cells. To further support our conjecture, 20 μM ACT001 was added to the NKTR knockdown cell lines, and the NKTR mRNA and protein levels were determined. The results showed ACT001 upregulated NKTR in the NKTR-knockdown cell lines (Figure 5b,c).

**TABLE 2** Stratified analysis of NKTR expression in non-small cell lung cancer

| Characteristics | *N* (%) Total | Low NKTR scores | High NKTR scores | *p*-value |
|---|---|---|---|---|
| Gender | | | | |
| Male | 130 (90.3) | 58 (44.6) | 72 (55.4) | 0.700 |
| Female | 14 (9.7) | 7 (50.0) | 7 (50.0) | |
| Age (year) | | | | |
| <60 | 59 (41.0) | 27 (45.8) | 32 (54.2) | 0.900 |
| ≥60 | 85 (59.0) | 38 (44.7) | 47 (55.3) | |
| Smoking history | | | | |
| Yes | 129 (89.6) | 57 (44.2) | 72 (55.8) | 0.500 |
| No | 15 (10.4) | 8 (53.3) | 7 (46.7) | |
| AJCC eighth TNM stage | | | | |
| I/II | 122 (84.7) | 43 (35.2) | 79 (64.8) | $p < 0.001^{***}$ |
| III/IV | 22 (15.2) | 20 (90.9) | 2 (9.1) | |

Abbreviation: NKCT, natural killer cell triggering receptor.

In order to additionally test this hypothesis, 20 μM ACT001 was added to the NKTR knockdown cell lines to analyze the proliferation and cell cycles. The proliferation of cells subjected to each treatment was measured using a clone formation assay. The results showed that NKTR knockdown in vitro reversed the effects of ACT001 (Figure 5d,e), implying that the effects of ACT001 on cell proliferation are related to NKTR.

To verify that the influence of ACT001 on the cell cycle was mediated by high NKTR expression, NC treatment, ShNKTR treatment, NC + ACT001 treatment, ShNKTR+-ACT001 treatment groups were examined for changes in the cell cycle and CDK6 and cyclin D1 levels. The most distinct G1/S cell cycle arrest was observed in the NC + ACT001 group, followed closely by the ShNKTR + ACT001 group (Figure 5f,g). These results indicate that the inhibitory effect of ACT001 on the cell cycle is achieved by increasing NKTR expression. NKTR is the target of ACT001 in NSCLC.

## ACT001 inhibited AKT phosphorylation by increasing NKTR expression

It has been documented that ACT001 inhibits the AKT pathway in glioma and hepatocellular carcinoma[16,17,21]; therefore, this study speculated that ACT001 inhibited AKT phosphorylation and NKTR inhibited cell proliferation by inhibiting AKT phosphorylation in NSCLC. To verify this hypothesis, this study treated H1703 and H1975 cells with ACT001 and measured the levels of total and phosphorylated AKT protein. ACT001 inhibited the phosphorylation of AKT. There was no change in total AKT (Figure 6a). This study also examined the total and phosphorylated AKT protein levels after NKTR knockdown. Unsurprisingly, total AKT protein levels were unchanged and phosphorylated AKT was elevated (Figure 6b). NKTR knockdown in vitro reversed the effects of ACT001 (Figure 6c). In general, ACT001 inhibited the phosphorylation of AKT by increasing NKTR expression. To further verify the mechanism by which NKTR affects cell proliferation, NKTR and P-AKT levels were observed in NC, ShNKTR treated cells with/without the addition of AKT activator and inhibition. It was found that SC79 and MK-2206 could not change the protein expression of NKTR, suggesting that the AKT signaling pathway could not regulate NKTR. Following NKTR KD, MK-2206 was able to reduce P-AKT levels and SC79 was able to increase the levels of P-AKT in H1975 and H1703 cells (Figure 6d). These data suggested that NKTR inhibited cell proliferation by inhibiting AKT phosphorylation. In conclusion, ACT001 inhibited AKT phosphorylation by upregulating NKTR expression.

## ACT001 inhibited the growth of tumors in vivo

This study verified whether ACT001 inhibited tumor growth and induced high expression of NKTR in vivo by establishing a xenograft mouse model using H1703 cells. After the tumor volume reached the standard size, the mice were treated with saline or ACT001 (200 mg/kg) daily. Volume changes in the subcutaneous tumors ($n = 5$) showed that the ACT001-treated group had a significantly smaller volume than the saline-treated group (Figure 7a-c). HE staining of the liver and kidney tissue demonstrated no detectable hepatic or renal toxicity after ACT001 treatment (Figure 7d). In addition, an IHC analysis of NKTR and Ki67 expression levels in the tumor xenografts showed that ACT001 significantly increased the expression of NKTR in vivo, which was consistent with the in vitro results. Moreover, ACT001 reduced Ki67 expression(Figure 7e).

## DISCUSSION

ACT001, a novel sesquiterpene lactone derivative, has shown excellent antitumor effects in many studies. ACT001 has been studied clinically in glioma, neuromyelitis optica, diffuse intrinsic pontine glioma, pulmonary fibrosis, and brain metastases. This experiment investigates the effect of ACT001 on NSCLC.

Some studies have previously shown that ACT001 is an adjuvant that improves the efficacy of chemotherapy[17] or other anticancer treatments such as endocrine therapy for breast cancer.[20] In the current study, ACT001 monotherapy was highly effective against NSCLC. In vitro, ACT001 significantly inhibited tumor cell growth and cell cycle transition; in vivo, the ACT001-treated group had a significantly smaller xenograft tumor volume than the saline-treated group ($p < 0.05$). These findings reflect ACT001 monotherapy has potential therapeutic value and good application prospects for patients with NSCLC and provides

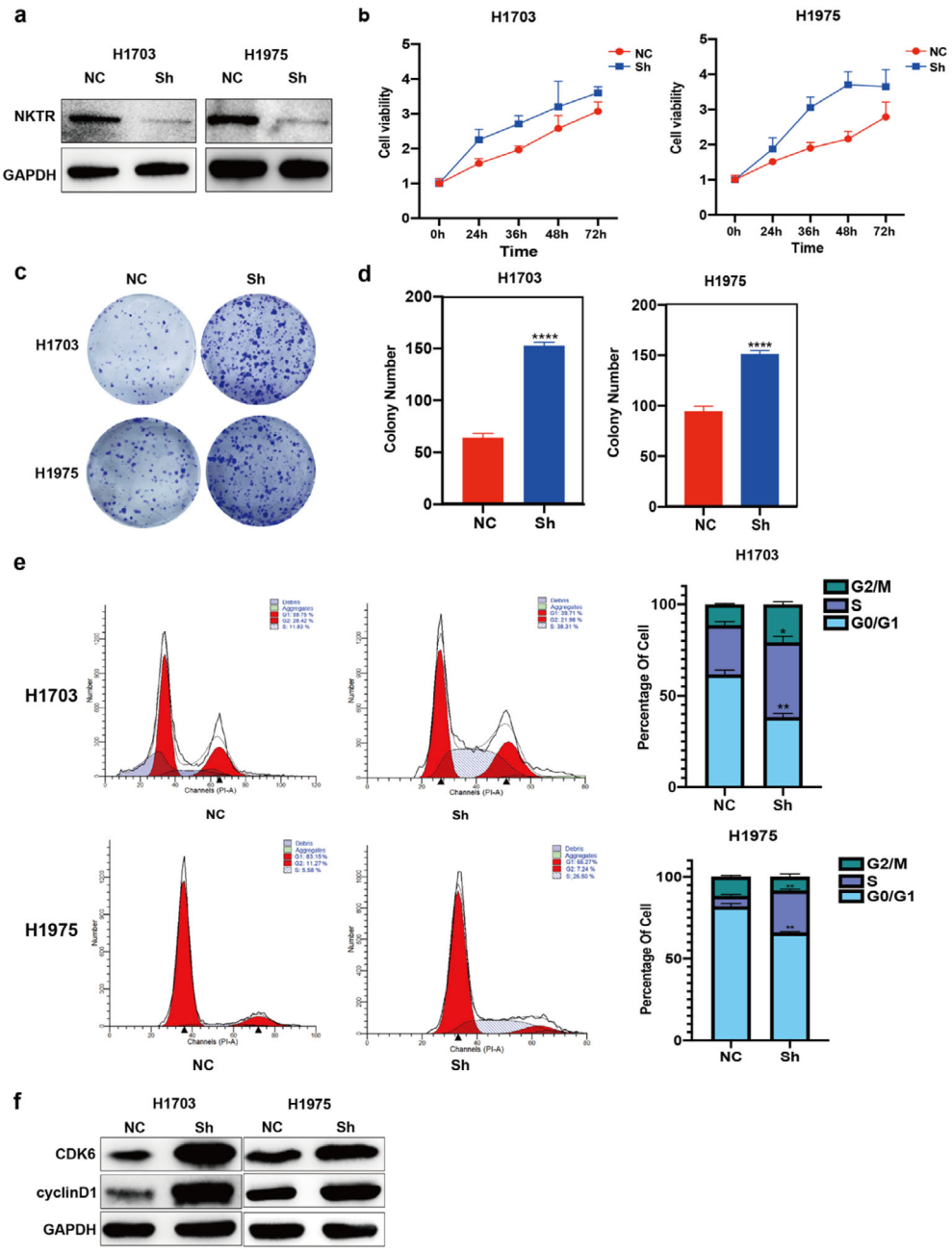


**FIGURE 4** Natural killer cell triggering receptor (NKTR) inhibited tumor cell proliferation and promoted G1/S arrest. (a) Knockdown NKTR in H1703 and H1975 cells. NC, empty carrier; Sh, NKTR KD. (b) Proliferation viability was assayed with MTT in NKTR KD cells. (c) Clone formation assays were conducted to investigate the influence of knockdown NKTR on H1703 and H1975 cells. (d) Quantification of the clone formation assay. (e) Flow analysis of the cell cycle. Cell number at the G1, S and G2/M stages was assessed after NKTR KD. (f) Western blots were performed to detect cyclin D1 and CDK6 expression after NKTR KD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

relevant evidence supporting clinical trials of ACT001 in lung cancer.

NK cells can lyse tumor and virus-infected cells without prior sensitization. T cells can exhibit analogous NK-like activity that is independent of the major histocompatibility complex. This activity is thought to be mediated by an independent structure, NKTR.[22,23] NKTR is a member of the protein complex responsible for target recognition and signal transduction on NK cells, part of the target recognition contractile complex located on chromosome 9 (3–21-3p23) that is necessary for NK-like killing.[24–26] NKTR inhibits tumor growth in the SP2/0 myeloma model.[27] This study found NSCLC cells also express NKTR. NKTR expression may be a prognostic marker and treatment target of NSCLC patients, providing a relevant basis for further research on new diagnostic indicators and treatments of this disease. However, this finding is in contrast to that in a previous study in which a database analysis showed that NKTR was upregulated in lung cancer.[28] There are some reasons to account for this discrepancy. First, and most likely, the authors of the above study used the A549, H1299, and H1650 cell lines, which were different from those used in the current study. Another possible reason for the discrepancy is that previous microarray data were developed from a small number of samples of 19 patients. Finally, NKTR gene expression can be controlled at the level of mRNA splicing to produce three NKTR isoforms[29] which could be a secondary reason.

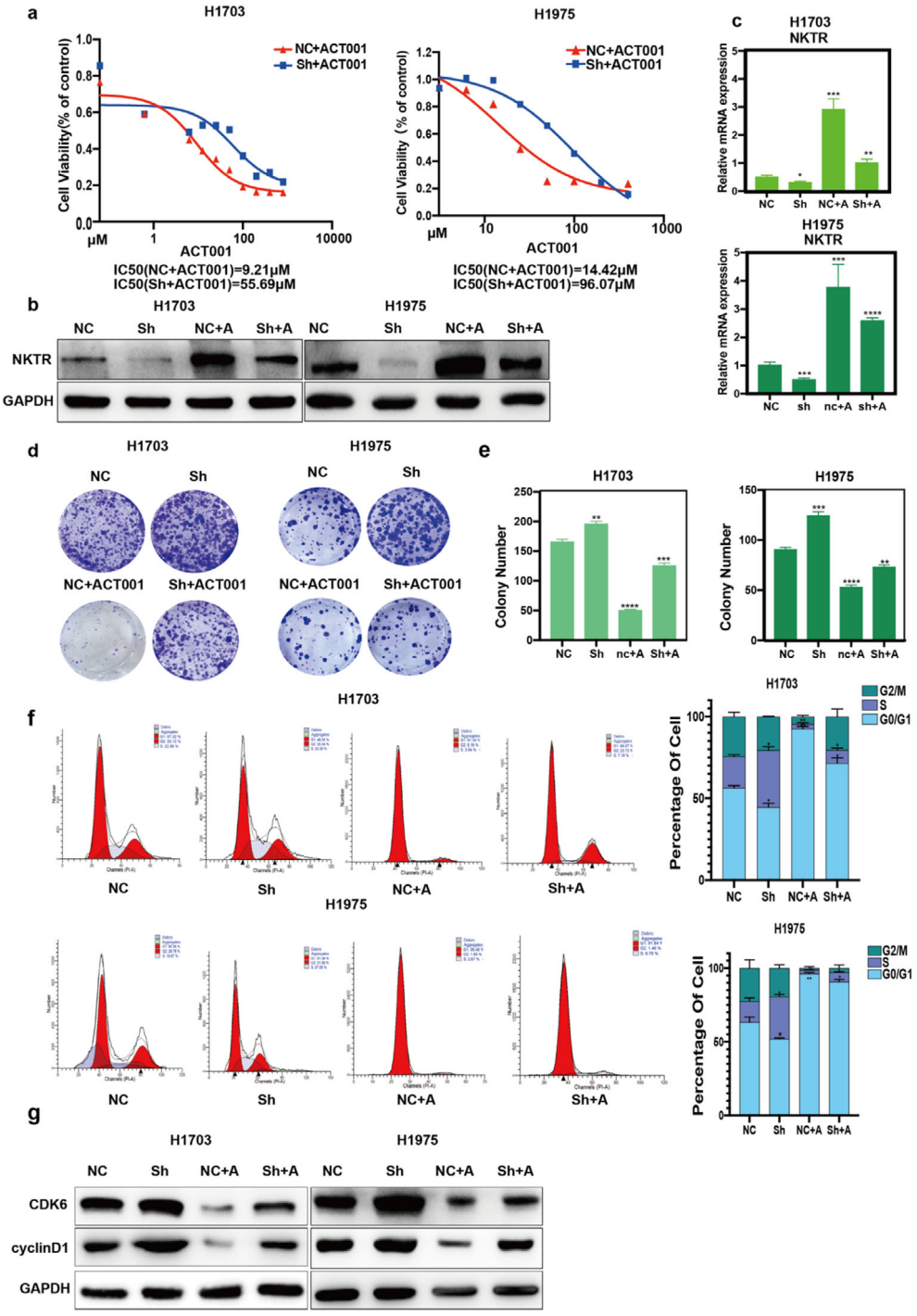


**FIGURE 5** ACT001 induced G1/S arrest by upregulating natural killer cell triggering receptor (NKTR) expression. (a) The ACT001 72 h IC50 in NKTR KD cells. (b, c) Expression of NKTR was detected by Western blot (WB) and qPCR after 20 μM ACT001 treatment in NC or NKTR KD cells. The empty carrier with the ACT001 group is called NC + A, the NKTR KD with ACT001 group is called Sh + A. (d, e) The proliferation ability of different treatments was determined by clone formation. (f, g) Flow cytometry and western blots were used to detect the cell cycle after 20 μM ACT001 treatment in NC or NKTR KD cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

Based on the RNA sequencing results, this study confirmed that NKTR inhibited NSCLC cell proliferation and induced G1/S arrest in this paper. In conjunction with previous studies, it has been determined that NKTR can function as a tumor suppressor in NSCLC cells while also being an important activator of NK cells. Based on the study of NKTR, we reach the conjecture that NKTR may affect the differentiation of precursor cells into NK cells.[29] NKTR might have other roles, apart from activation, which should be studied further. Throughout the study, this study conjectured that NKTR expression was important for maintaining the lytic activity of NK cells and was inextricably linked to immunity.[30] This is supported by a study showing ACT001 reduced the expression of programmed cell death ligand 1 in glioblastoma.[18] In addition, in the previously mentioned drug-free patients with GBM treated with ACT001, the pathology showed immunological changes. Therefore, the interaction between ACT001 and NKTR, as well as the effect of ACT001 on the activation and killing ability of T and NK cells, are questions requiring further investigation.

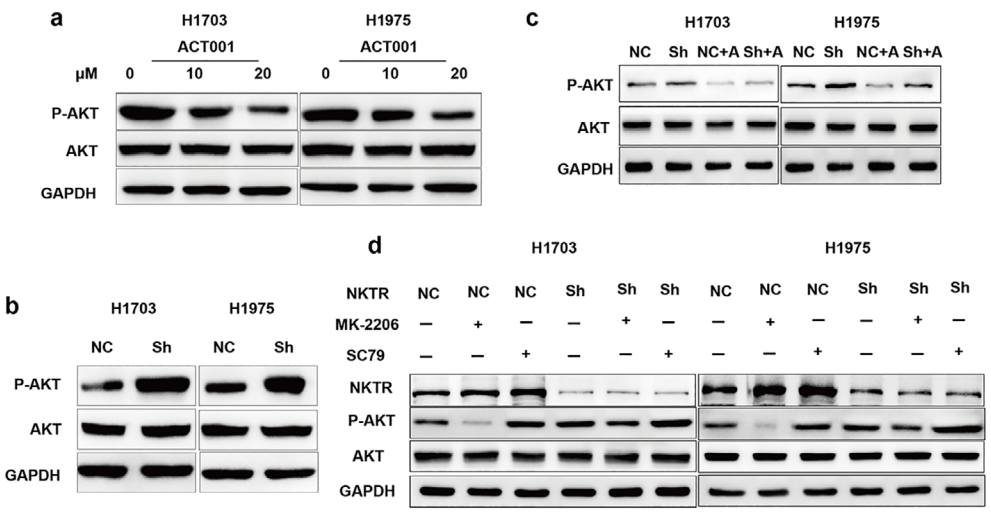


**FIGURE 6** ACT001 inhibited AKT phosphorylation by increasing natural killer cell triggering receptor (NKTR) expression. (a) The total AKT and phosphorylated AKT protein were detected by Western blot (WB) after different concentrations of ACT001 treatment. (b) The effect of NKTR on AKT phosphorylation was measured by WB. (c) The total AKT and phosphorylated AKT protein were detected after 20 μM ACT001 treatment in NC or NKTR KD cells. (d) The total AKT, phosphorylated AKT and NKTR protein were detected after MK-2206 (10 μM) and/or SC79 (10 μM) treatment in NC or NKTR KD cells

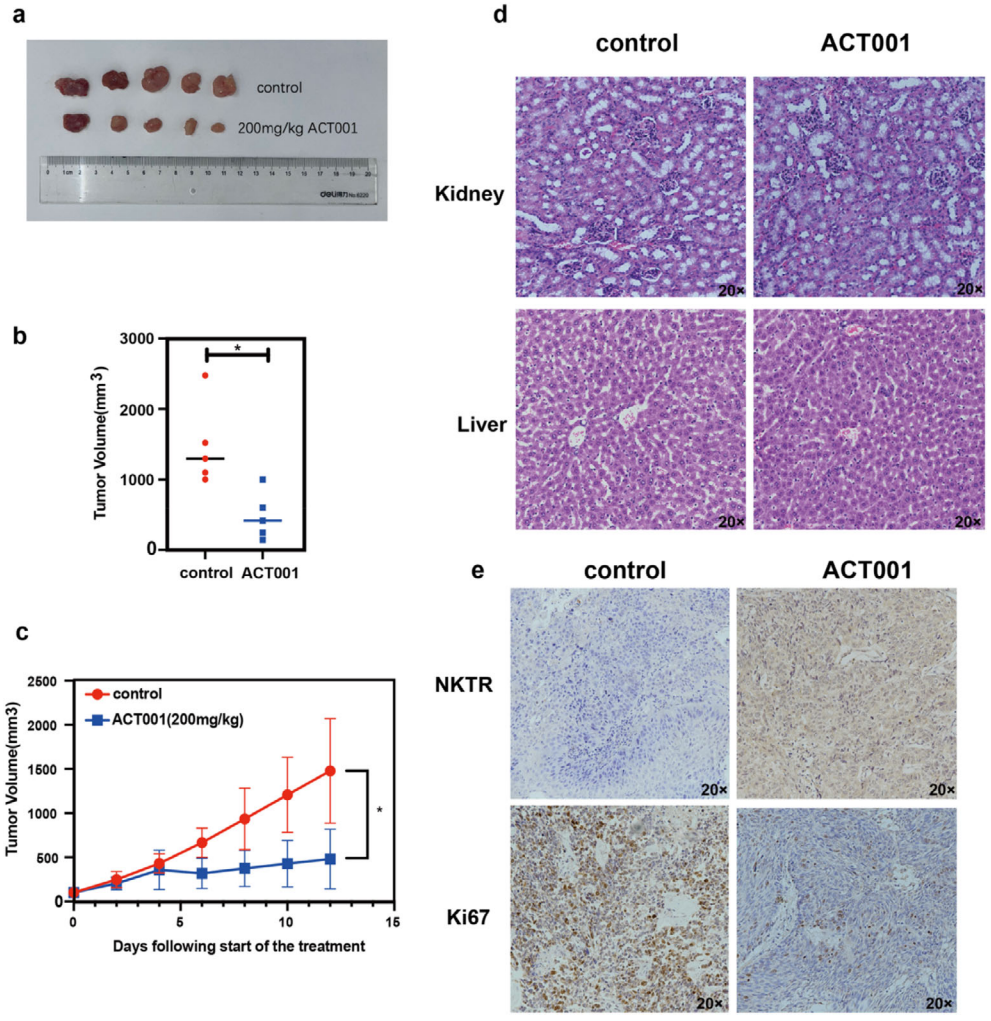


**FIGURE 7** ACT001 inhibited the growth of tumors in vivo. (a–c) Xenograft tumor experiments with H1703 cells, and the tumor volume changes after being treated with saline and ACT001, respectively. (d) Liver and kidney HE staining of mice after ACT001 administration. (e) Immunohistochemical (IHC) analysis of natural killer cell triggering receptor (NKTR) and Ki67 in H1703 tumor xenograft. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

The current study has some limitations. For example, ACT001 affected the expression profile of numerous genes in NSCLC, not just NKTR. These changes, and their potential role in the effects of ACT001, need to be further explored. An additional limitation is that the variety of cell lines used may not have been sufficiently comprehensive. Overall, the mechanism by which ACT001 affects the NKTR requires further investigation.

In conclusion, this study verified the inhibitory activity of ACT001 monotherapy in NSCLC cells both in vivo and

in vitro. This study also found that ACT001 inhibited AKT phosphorylation to induce G1/S arrest by increasing NKTR expression. ACT001 could be an effective monotherapy for patients with NSCLC.

## ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (no. 82172635) awarded to D. Z. Huang and National Natural Science Foundation of China (no. 81802296) awarded to T. T. Qin.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest in this work.

## ORCID

*Dingzhi Huang* https://orcid.org/0000-0002-2798-9459

## REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70(1):7–30.
2. Lee W, Jiang Z, Liu J, Haverty PM, Guan Y, Stinson J, et al. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature. 2010;465(7297):473–7.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
4. Heigener DF, Reck M. Lung cancer in 2017: Giant steps and stumbling blocks. Nat Rev Clin Oncol. 2018;15(2):71–2.
5. Hang T, Yang L, Zhang X, Li J, Long F, Zhu N, et al. Peroxisome proliferator-activated receptor γ improves pemetrexed therapeutic efficacy in non-squamous non-small cell lung cancer. Am J Transl Res. 2021;13(4):2296–307.
6. Ma SC, Tang XR, Long LL, Bai X, Zhou JG, Duan ZJ, et al. Integrative evaluation of primary and metastatic lesion spectrum to guide anti-PD-L1 therapy of non-small cell lung cancer: results from two randomized studies. Onco Targets Ther. 2021;10(1):1909296.
7. Kim MJ, Cervantes C, Jung YS, Zhang X, Zhang J, Lee SH, et al. PAF remodels the DREAM complex to bypass cell quiescence and promote lung tumorigenesis. Mol Cell. 2021;81(8):1698–1714.e6.
8. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature. 2018;553(7689):446–54.
9. Viennois E, Xiao B, Ayyadurai S, Wang L, Wang PG, Zhang Q, et al. A new sesquiterpene lactone that inhibits intestinal inflammation and colitis-associated cancer. Lab Invest. 2014;94:950–65.
10. Li J, Li S, Guo J, Li Q, Long J, Ma C, et al. Natural product micheliolide (MCL) irreversibly activates pyruvate kinase M2 and suppresses leukemia. J Med Chem. 2018;61:4155–64.
11. Alwaseem H, Giovani S, Crotti M, Welle K, Jordan CT, Ghaemmaghami S, et al. Comprehensive structure-activity profiling of micheliolide and its targeted proteome in leukemia cells via probe-guided late-stage C-H functionalization. ACS Cent Sci. 2021;7(5):841–57.
12. Zhang Q, Lu Y, Ding Y, Zhai J, Ji Q, Ma W, et al. Guaianolide sesquiterpene lactones, a source to discover agents that selectively inhibit acute myelogenous leukemia stem and progenitor cells. J Med Chem. 2012;55:8757–69.
13. Liu Q, Guo X, Huang Z, He Q, Zhu D, Zhang S, et al. Anti-neuroinflammatory effects of dimethylaminomylide (DMAMCL, i.e., ACT001) are associated with attenuating the NLRP3 inflammasome in MPTP-induced Parkinson disease in mice. Behav Brain Res. 2020;383:112539.
14. Xu N, Hua Z, Ba G, Zhang S, Liu Z, Thiele CJ, et al. The anti-tumor growth effect of a novel agent DMAMCL in rhabdomyosarcoma in vitro and in vivo. J Exp Clin Cancer Res. 2019;38:118.
15. Li Q, Sun Y, Liu B, Li J, Hao X, Ge W, et al. ACT001 modulates the NF-κB/MnSOD/ROS axis by targeting IKKβ to inhibit glioblastoma cell growth. J Mol Med (Berl). 2020;98(2):263–77.
16. Hou Y, Sun B, Liu W, Yu B, Shi Q, Luo F, et al. Targeting of glioma stem-like cells with a parthenolide derivative ACT001 through inhibition of AEBP1/PI3K/AKT signaling. Theranostics. 2021;11(2):555–66.
17. Xi X, Liu N, Wang Q, Chu Y, Yin Z, Ding Y, et al. ACT001, a novel PAI-1 inhibitor, exerts synergistic effects in combination with cisplatin by inhibiting PI3K/AKT pathway in glioma. Cell Death Dis. 2019;10(10):757.
18. Tong L, Li J, Li Q, Wang X, Medikonda R, Zhao T, et al. ACT001 reduces the expression of PD-L1 by inhibiting the phosphorylation of STAT3 in glioblastoma. Theranostics. 2020;10(13):5943–56.
19. Liu Y, Wang L, Liu J, Xie X, Hu H, Luo F. Anticancer Effects of ACT001 via NF-κB Suppression in Murine Triple-Negative Breast Cancer Cell Line 4T1. Cancer Manag Res. 2020;12:5131–9.
20. Jin XH, Jia YS, Shi YH, Li Q-Y, Bao S-Q, Lu W-P, et al. ACT001 can prevent and reverse tamoxifen resistance in human breast cancer cell lines by inhibiting NF-κB activation. J Cell Biochem. 2019;120(2):1386–97.
21. Yao S, Ye J, Yin M, Yu R. DMAMCL exerts antitumor effects on hepatocellular carcinoma both in vitro and in vivo. Cancer Lett. 2020;483:87–97.
22. Koide J, Rivas A, Engleman EG. Natural killer (NK)- like cytotoxicactivity of allospecific T cellreceptor-y.6′ T cell clones. J Immunol. 1989;142:4261.
23. Chambers CA, Gallinger S, Anderson SK, Giardina S, Ortaldo JR, Hozumi N, et al. Expression of the NK-TR gene is required for NK-like activity in human T cells. J Immunol. 1994;152(6):2669–74.
24. Anderson SK, Gallinger S, Roder J, Frey J, Young HA, Ortaldo JR. A cyclophilin-related protein involved in the function of natural killer cells. Proc Natl Acad Sci U S A. 1993;90(2):542–6.
25. Frey JL, Bino T, Kantor RR, Segal DM, Giardina SL, Roder J, et al. Mechanism of target cell recognition by natural killer cells: characterization of a novel triggering molecule restricted to CD3- large granular lymphocytes. J Exp Med. 1991;174(6):1527–36.
26. Young HA, Jenkins NA, Copeland NG, Simek S, Lerman MI, Zbar B, et al. Localization of a novel natural killer triggering receptor locus to human chromosome 3p23-21 and mouse chromosome 9. Genomics. 1993;16:548–9.
27. Deng B, Gong P, Li J, Cheng B, Ren W, Yang J, et al. Identification of the differentially expressed genes in SP2/0 myeloma cells from Balb/c mice infected with Trichinella spiralis. Vet Parasitol. 2013;194(2–4):179–82.
28. Wang J, Song J, Gao Z, Huo X, Zhang Y, Wang W, et al. Analysis of gene expression profiles of non-small cell lung cancer at different stages reveals significantly altered biological functions and candidate genes. Oncol Rep. 2017;37(3):1736–46.
29. Rinfret A, Anderson SK. IL-2 regulates the expression of the NK-TR gene via an alternate RNA splicing mechanism. Mol Immunol. 1993;30(14):1307–13.
30. Simons-Evelyn M, Young HA, Anderson SK. Characterization of the mouse Nktr gene and promoter. Genomics. 1997;40(1):94–100.

**How to cite this article:** Zhao M, Qin T, Huang D. ACT001 inhibits the proliferation of non-small cell lung cancer cells by upregulating NKTR expression. Thorac Cancer. 2022;13(12):1772–82. https://doi.org/10.1111/1759-7714.14453