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A thiopyran derivative with low murine toxicity with therapeutic potential on lung cancer acting through a NF-κB mediated apoptosis-to-pyroptosis switch

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Abstract

Pyroptosis is a novel manner of cell death that can be mediated by chemotherapy drugs. The awareness of pyroptosis is significantly increasing in the fields of anti-tumor research and chemotherapy drugs. Invoking the occurrence of pyroptosis is an attractive prospect for the treatment of lung cancer. Here, the compound L61H10 was obtained as a thiopyran derivative to compare its activity with curcumin. It was indicated that L61H10 exhibited good anti-tumor activity both in vitro and in vivo via the switch of apoptosis-to-pyroptosis, which was associated with the NF-κB signaling pathway. In addition, L61H10 had no obvious side effects both in vitro and in vivo. In brief, L61H10 is shown to be a potential anti-lung cancer agent and research on its anti-tumor mechanism provides new information for chemotherapy drug research.

Keywords Anti-lung cancer · Toxicity · Apoptosis · Pyroptosis · NF-κB

Introduction

Lung cancer remains the one of the most harmful malignant tumors among human neoplastic diseases [1, 2]. Because of the poor early detection, many patients are diagnosed at an advanced stage, which is responsible for the limited efficacy and low survival rates [3]. Currently, conventional chemotherapy continues to be the standard regime for lung cancer patients when operations have been unsuccessful [4]. However, the efficacy underlying chemotherapy to lung cancer is limited due to its side effect and partially ambiguous molecular mechanisms [5, 6]. Therefore, it is urgent to uncover new therapeutic mechanisms and find highly potent candidates with a lower toxicity against lung cancer.

Pyroptosis, a form of programmed cell death, manifests itself as a continual swelling of the cells until the cell membrane ruptures, which results in the release of intracellular contents which in turn activates a strong inflammatory response with roles in antagonizing infection and endogenous danger signals [7, 8]. In addition, as a new breakthrough, it was observed that pyroptotic bubbles occurred by overriding the apoptotic signals or switching from apoptosis following the treatment of cancer cells with chemotherapy drugs [9], which offered a new insight into chemotherapy drugs and cell death.

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Curcumin, a naturally occurring compound, has traditionally been taken into consideration as a treatment for cancer [10]. Nevertheless, the clinical application of curcumin is still limited because of its low biological activity [10]. To circumvent this limitation, there have been significant efforts to modify its structure [10–12]. Thereinto, curcumin-related heterocyclic ketone derivatives have been demonstrated to possess an improved activity in comparison to curcumin [12]. Based on the relevant literature, we screened a new thiopyran derivative, L61H10 which was a heterocyclic ketone derivative and exhibited higher anti-tumor activity than curcumin. It was further found that the treatment with L61H10 caused an apoptosis-to-pyroptosis switch, which was associated with NF-κB inhibition. Additionally, L61H10 even exhibited no obvious toxicity both in vitro and in vivo.

Materials and methods

Cell culture

The human lung cancer cell lines H460 and A549 were purchased from ATCC (Manassas, VA), the normal human liver cell line HL7702 was obtained from the Chinese academy of sciences, a typical cell library culture preservation committee (China, Shanghai). All cells were cultured in 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin (Gibco) in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 °C.

MTT assay

Cells were trypsinized and seeded on a 96-well plate with 3×10^3 cells per well. Each sample had three replicates. The viability of cells at 48 and 72 h after drug treatment was evaluated by the MTT. MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was added to each well at a concentration of 500 μg/mL and the cells were incubated for 4 h. After 4 h, the medium was discarded and the crystal was lysed with 150 μL DMSO (Sigma). Absorbance was measured using a computer-controlled microplate analyzer at 490 nm.

Clonogenic assay

H460 cells (1000 cells in 2 mL medium) were planted in 6-well dishes and allowed to adhere overnight. The next day, the cells were treated with various concentrations of L61H10. After 24 h, the medium containing drugs was replaced with regular growth medium. The cells were continued to be cultured for 14 days. Following incubation, the medium was removed, the cells were washed with PBS, fixed with 4% paraformaldehyde and stained with crystal violet. Finally, all cells were photographed by a camera.

Acute toxicity experiment

BALB/C mice purchased from SHANGHAI SLAC were randomly divided into three groups and each group consisted of six mice. The medicated groups were given the same dose (0.1 g/kg) dissolved in 6% castor oil while the control group was injected with 6% castor oil. After the intraperitoneal injection of the drugs, all mice were free to eat and drink water. The weights of the mice in each group were closely observed once a day and recorded for 10 days.

Cell cycle analysis

2 mL H460 cell suspension (6×10^5) was spread on Petri dishes with a diameter of 60 mm and cultured overnight. The cells were then incubated with the compound. About 20 h later, the H460 cells were trypsinized, washed in cold PBS and fixed with a 75% ethanol solution at − 20 °C overnight. Then, all cells were stained with propidium iodide (PI) containing RNase A (BD Biosciences, San Jose, California, USA) for 10 min in the dark. The cell cycle distribution was determined using a flow cytometer (BD Biosciences).

Western blot analysis

H460 cells were plated in 6-well plates (3×10^5 cells per well). After incubation with the compounds, the cells were homogenized with lysis buffer and harvested. The total protein was separated by polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts, USA), and then blocked by 5% non-fat milk. After 90 min, the protein samples were incubated with the primary antibody overnight and then incubated with the appropriate secondary antibody for 60 min and finally detected using the ECL (Bio-Rad, Hercules, California, USA) reagent. The primary antibodies for IκBα, Bcl-2, Bax, IKKβ and GAPDH as well as the secondary antibody goat anti-rabbit IgG-HRP were all purchased from Santa Cruz Biotechnology (California, USA). The anti-caspase 3 antibody was obtained from Cell Signaling Technology (CST). The primary antibody for GSDME was obtained from Abcam. The optical density of the bands was quantified by Quantity One v4.62 (Bio-Rad). The protein levels were normalized to GAPDH and fold changes were determined.

Microscopy imaging of cell pyroptosis

To detect the morphology of pyroptotic cells, cells were planted in 6-well plates and incubated at 37 °C in a 5%

CO_2 atmosphere. The cells were then treated with the compounds. The phenomenon was observed continuously under an inverted microscope.

NF-κB screening system

RAW264.7-NFκB-RE-EGFP cell lines were constructed by lentiviral transfection and generously donated by Dr. Wu Luo. The system could be activated after LPS stimulation. The system was used to confirm whether the drug had the ability to inhibit NF-κB by reversing the system activated by LPS stimulation. RAW264.7-NFκB-RE-EGFP cells (3×10^5 cells/well) were seeded in Petri dishes with a diameter of 3 cm overnight. After 2 h of L61H10 action, LPS (0.5 μg/mL) was added. About 9 h later, the cells were collected by trypsin, washed with PBS and measured with a FACSCalibur flow cytometer (BD Biosciences, CA).

Cell transfection

At the beginning of the cell transfection experiment, the cell culture medium was replaced with serum-free medium. Then, the DNA-liposome complex and empty vector were added to the cell culture in each well. After 6 h, the serum-free medium was removed and replaced with the 1640 medium containing 10% FBS and 1% penicillin–streptomycin solution. After further incubation for 12 h, cells were washed twice with PBS, harvested by cell lysate and detected using a western blot.

Anti-tumor research in vivo

Female BALB/c nude mice (18–22 g) were purchased from the SLRC Laboratory Animal LLC (Shanghai, China). The H460 cells were harvested and subcutaneously injected into the right flank (2×10^6 cells in 100 μL of PBS) of the mice. Once the tumor volumes had reached 100–200 mm^3, the mice were injected intraperitoneally (i.p.) with L61H10 in 6% castor oil at a dosage of 5 mg/kg/day, whereas the control mice were injected with the vehicle in 6% castor oil (n = 8 in each group). The length and width of the tumors were measured using a vernier caliper. The volumes of the tumors were calculated using the formula $V=0.52\times L\times W^2$. The weights of mice were recorded weekly and the tumor sizes were recorded on the day the mice were killed. After 29 days, the nude mice were dissected and the tissue samples were removed and further investigated.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 5 μm. The tissue sections were stained primarily with antibodies. The signal was detected by staining with biotinylated secondary antibody and 3,3-diaminobenzidine (DAB). The antibodies of IκBα, Bcl-2 were purchased from Santa Cruz Biotechnology. The antibody of COX-2 was obtained from Bioworld Technology. The immunostainings of the slides were evaluated under an optical microscope (Nikon, Tokyo, Japan).

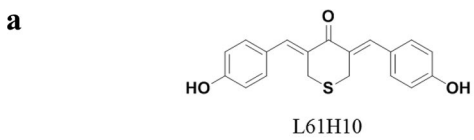
Data analysis

All experiments were repeated at least twice. Statistical significance was assessed by comparing mean (± SD) values, which were normalized to the control group with a student's *t* test for the independent groups. GraphPad Prism 5.0 software (SanDiego, California, USA) was used for all statistical analysis. p Values < 0.05 were considered significant.

Results

L61H10 inhibited the growth of lung cancer cells in vitro

As shown in Fig. 1a, the thiopyran derivative named L61H10 was obtained in our previous study. To identify whether the compound possessed anti-tumor activity and exhibited improved activity with respect to curcumin, the lung cancer cell lines H460 and A549 were tested for viability using a MTT assay. In addition, the normal cells HL7702 were tested for toxicity at the same time. It was found that the



b

Compound.	IC_{50} (μM)		
	H460	A549	HL7702
L61H10	12.1 ± 0.5	27.5 ± 1.6	42.2 ± 6.5
Curcumin	23.5 ± 4.1	21.6 ± 2.7	22.2 ± 0.6

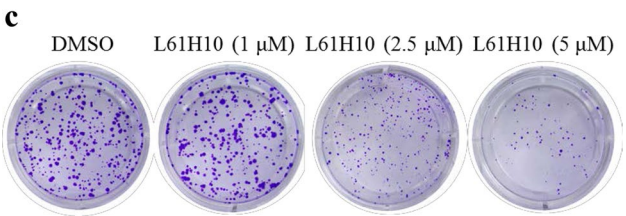


Fig. 1 Anti-tumor activity of L61H10 in vitro. **a** Chemical structure of L61H10. **b** The IC_{50} value of L61H10 and curcumin for lung cancer cells and normal cells after 72 h treatment was detected by MTT assay. **c** Clonogenic assay of H460 cells treated with increasing concentrations of L61H10 as indicated

lung cancer cell line H460 was sensitive to L61H10-induced cytotoxicity (Fig. 1b). In addition, the activity of L61H10 against H460 cells surpassed curcumin, and the cytotoxicity of L61H10 towards normal cells was far below curcumin (Fig. 1b). To further determine whether L61H10 inhibited the proliferation of human lung cancer cells, H460 cells were used to perform follow-up research work. As shown in Fig. 1c, L61H10 could concentration-dependently inhibit the formation of colony.

L61H10 arrested the cell cycle and caused an apoptosis-to-pyroptosis switch in H460 cells

The inhibition of cell growth could be mediated by cell cycle arrest [13, 14]. To find out the molecular mechanisms involved in L61H10, we detected the changes in cell cycle distribution. As shown in Fig. 2a, L61H10 led to a cell cycle arrest in G2/M phase. In addition, as a classical manner of cell death, apoptosis was also considered for explaining the inhibitory effects of L61H10 [15]. For that reason, the expression of Bcl-2 and Bax, which play an important role in apoptosis [16], were assessed by western blot. The expression levels of Bcl-2 and Bax are usually relatively stable, while in most tumors, the expression of Bcl-2 is increased and the expression of Bax is decreased. When the Bax is overexpressed in cancer cells, the number of Bax/Bax homodimers is significantly increased, and the cells are more responsive to death signals. However, when Bcl-2 is highly expressed, it will produce a more stable Bcl-2/Bax heterodimer, which counteracts the effect of inducing cell apoptosis and prolongs cell survival. Therefore, the study suggests that the ratio between the two opposite proteins in the cancer cells is the key to determining cell survival. Down-regulation of Bcl-2 or overexpression of Bax can promote cell apoptosis in a variety of cancer cells induced by various factors. The results in Fig. 2b indicated that L61H10 significantly increased the expression of Bax and decreased the expression of Bcl-2 in a concentration-dependent manner compared with the control treatment, which suggested that L61H10 possessed the capacity of inducing cell apoptosis. But beyond that, benefiting from a recent report of *Nature* [9], we further hypothesized that L61H10 could also promptly change the way programmed cell deaths takes place from apoptosis to pyroptosis and thereby exerted its anti-tumor effects. Through continuous observation after drug application, the occurrence of pyroptosis was unexpectedly observed after 28 h of L61H10 (20 μM) treatment (Fig. 2c). The protein GSDME [9], which could switch chemotherapy drugs-induced apoptosis to pyroptosis, was analyzed by western blot (Fig. 2d). GSDME could be cleaved to generate a GSDME-N fragment upon the activation of caspase 3. It was first reported in *Nature* that caspase 3 was no longer a unique hallmark of apoptosis, but also a hallmark of pyroptosis. The expression of caspase 3 was detected and shown in Fig. 2d. It was observed that L61H10 could decrease the expression of caspase 3 in a concentration-dependent manner (Fig. 2d). All results revealed that L61H10-induced inhibition was related to the cell cycle arrest and the apoptosis-to-pyroptosis switch.

L61H10 induced apoptosis and pyroptosis via NF-κB pathway

Since the report that cell pyroptosis occurred in cancer cells upon the treatment of chemotherapy drugs was first proposed in 2017, the related mechanisms for inducing cell pyroptosis have remained unclear. Therefore, it inspired us to investigate the mechanism of L61H10 for pyroptosis induction.

The NF-κB pathway is a classical apoptotic pathway in lung cancer [17, 18]. Heterocyclic ketone derivatives exhibit good anti-tumor activity by inhibiting the NF-κB pathway [19]. Additionally, the correlation between the NF-κB pathway and cell pyroptosis has been reported in inflammation [20, 21]. As a consequence, it was hypothesized that L61H10 might inhibit the NF-κB pathway and possibly mediated the shift from cell apoptosis to pyroptosis. Thereupon, the following work was carried out. The results in Fig. 3a showed that L61H10 reversed the activation of the NF-κB system induced by LPS stimulation in RAW264.7-NFκB-RE-EGFP cells. L61H10 also inhibited the degradation of IκBα (an inhibitor of NF-κB) induced by TNFα in H460 cells (Fig. 3b). The above results confirmed that L61H10 possessed the capacity of inhibiting NF-κB. Then, we investigated whether there was a link between NF-κB and apoptosis as well as pyroptosis. IKKβ is an important kinase of the NF-κB signaling pathway, and its up-regulation leads to the activation of the NF-κB pathway. After transfection with the IKKβ plasmid (Fig. 3c), the effect of L61H10 on cell viability was attenuated (Fig. 3d). Conversely, L61H10-induced apoptosis was found decreased through detecting the expression of the apoptosis-related protein Bax (Fig. 3e) while pyroptosis was also significantly reduced compared to the vector-transfected control group (Fig. 3f). In conclusion, the inhibition of NF-κB induced by L61H10 probably mediated the transformation of the cell death pathway, however, further study is required (Fig. 4).

L61H10 exerted anti-tumor effects in vivo

To evaluate the in vivo anti-tumor effects of L61H10, the therapeutic potential of L61H10 on the growth of nude mice bearing xenografts was examined. As shown in Fig. 5b, treatment with L61H10 resulted in a significant reduction in both tumor volume and weight, which suggested that L61H10 exhibited excellent activity during therapy. Then, we investigated its in vivo anti-tumor mechanisms to certify that

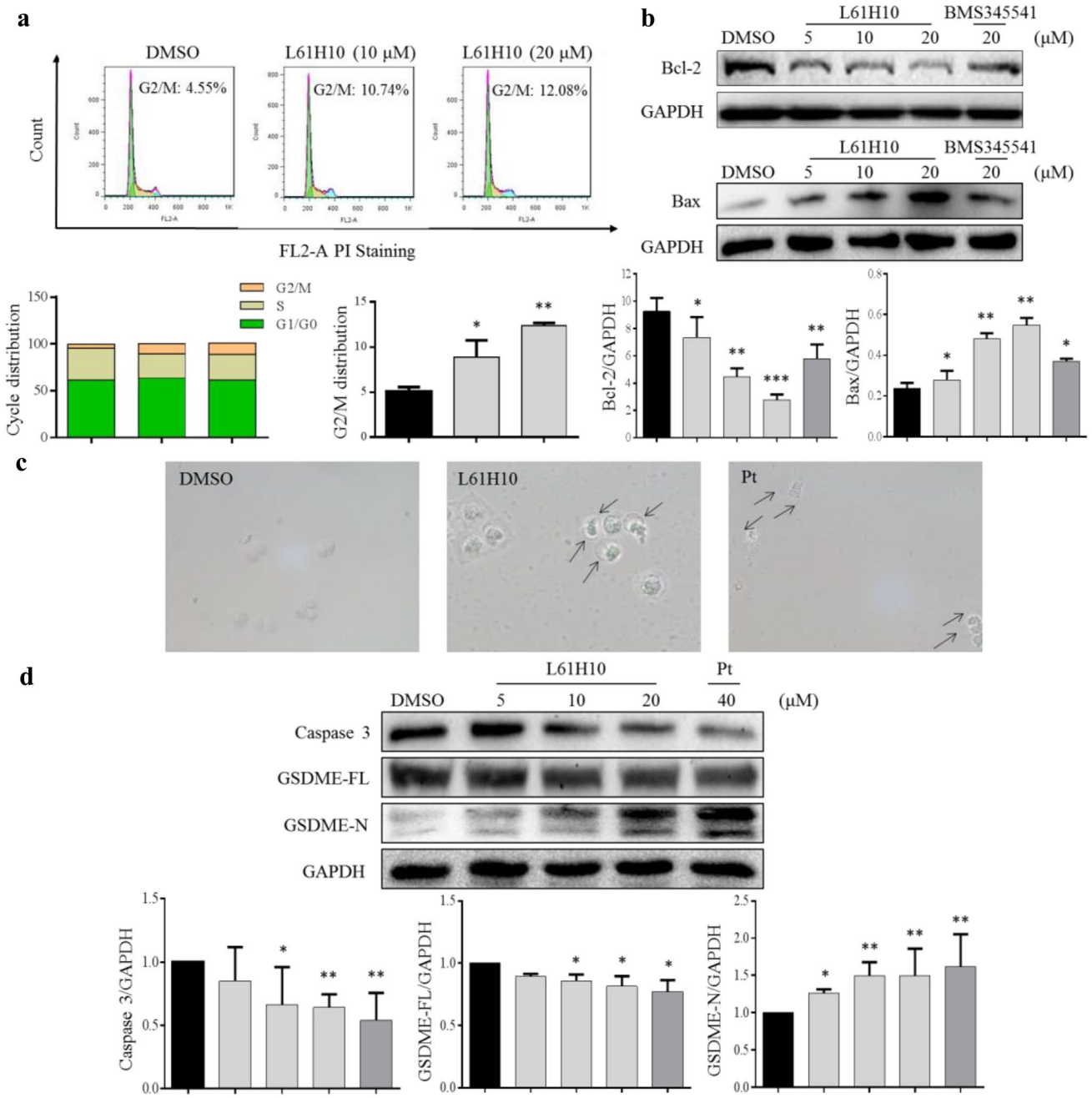


Fig. 2 L61H10 induced cell cycle arrest, cell apoptosis and cell pyroptosis. **a** The effect of L61H10 in cell cycle was detected using flow cytometry. The frequency distribution of G1/G0, S, G2/M and DNA content of G2/M were calculated and analyzed. **b** Cells were treated with different concentrations of L61H10 for 24 h. BMS345541 was used as positive control. The expression of Bcl-2, Bax were analyzed by western blot. **c** The morphology of pyroptosis was observed after 28 h of L61H10 (20 μM) or cisplatin (Pt, 40 μM) action. Pt was chosen as positive control. **d** The expression of pyroptosis-related protein caspase 3, GSDME were analyzed. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ relative to DMSO group

the in vivo results were consistent with the in vitro results. Accordingly, the expression of IκBα (involved in NF-κB), COX-2 (involved in proliferation) and Bcl-2 (involved in apoptosis) were detected. It was observed that the expression of IκBα increased and the others decreased in the nude mice injected with L61H10 (Fig. 5c). In addition, we found that there was no significant change in body weight among the control and L61H10-treated groups, suggesting that L61H10 exerted less obvious toxicity within the 29 days treatment (Fig. 5a). In conclusion, L61H10 possessed the ability to inhibit proliferation, to arrest the cell cycle and induce apoptosis prior to switching into pyroptosis with no significant

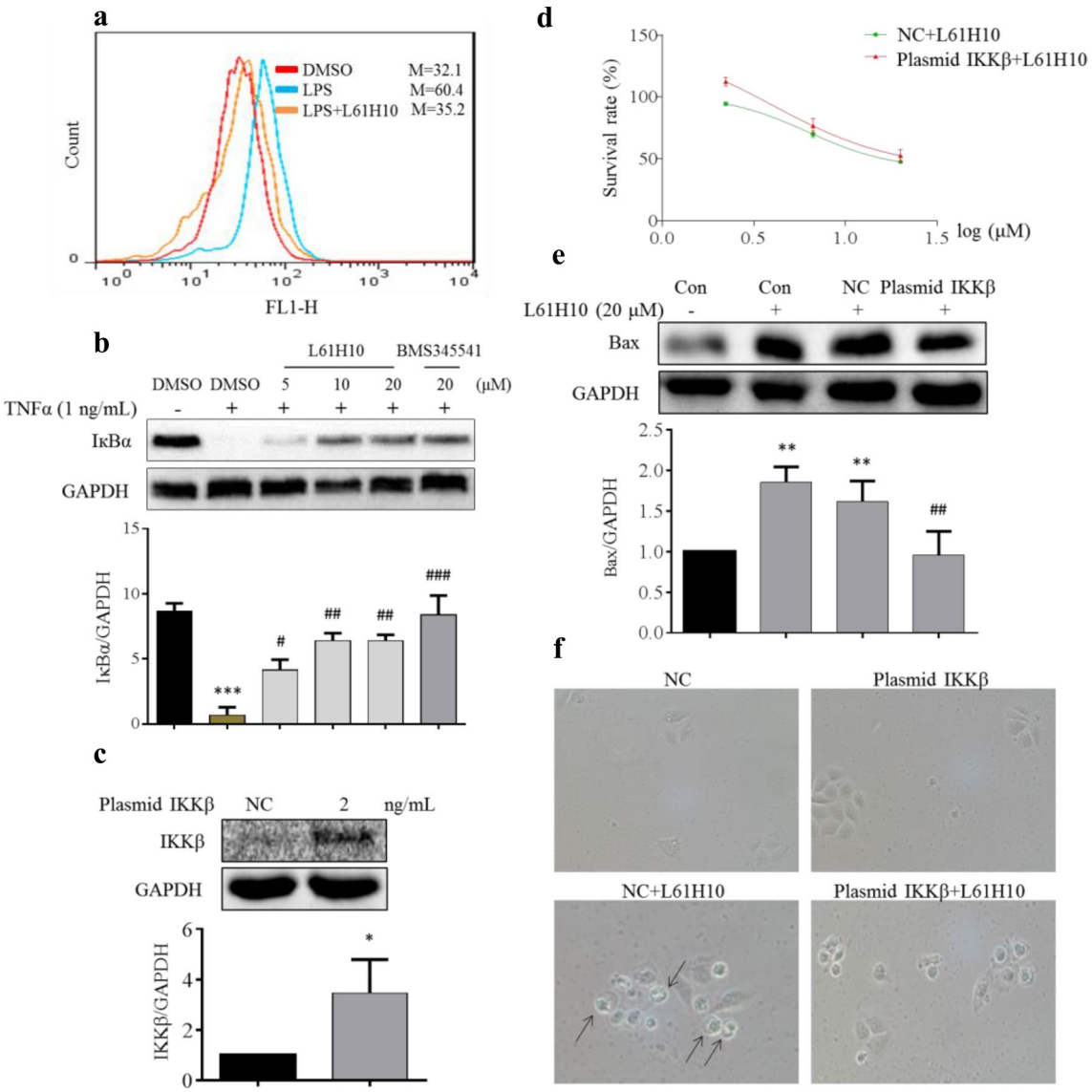


Fig. 3 L61H10 induced cell apoptosis and pyroptosis via NF-κB singaling pathway. **a** Flow cytometry was performed to detect the effect of L61H10 on the RAW264.7-NFκB-RE-EGFP cells. **b** H460 cells were pre-incubated with L61H10 (5, 10, 20 μM) or BMS345541 (20 μM) before TNFα action for 15 min. Western blot was used to detect the expression of IκBα and internal control GAPDH. **c** H460 cells were transfected with plasmid IKKβ-liposome complex (2 ng/mL) or separate liposome. The transfection effect was confirmed by western blot. **d** H460 cells transfected with plasmid IKKβ-liposome complex or separate liposome were treated with L61H10 (20, 6.67, 2.22 μM) for 48 h. The effect of L61H10 on cell vitality was determined by the MTT. **e** Western blot was carried out to show the impact of L61H10 on the expression of apoptosis related protein (Bax) in the transfection group, non-transfection and solvent groups after 24 h. GAPDH was used as internal control. **f** Treatment with L61H10 (20 μM) in the blank load transfection group increased the occurance of pyroptosis compared with plasmid IKKβ transfection group and non-drug groups. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ were compared to control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ relative to (DMSO + TNFα) or (NC + L61H10)

toxicity. It was expected to become a promising lead compound for lung cancer treatment.

The in vitro and in vivo toxicity of L61H10

At the cellular level, it was found that L61H10 showed less toxicity to normal cells than curcumin (Fig. 1b). In the subsequent experiment of tumor xenograft models, L61H10 exhibited no significant changes in body weight as well (Fig. 5a). To further evaluate whether L61H10 exerts lower toxicity compared to the much-studied heterocyclic ketone derivative EF24 in vivo and to see if it compensates for the defect that is the toxicity of EF24, which would likely be one of the limiting factors for clinical development [22], an acute toxicity experiment was carried out. It was shown in Table 1 that in the later stage of the experiment, the mice treated

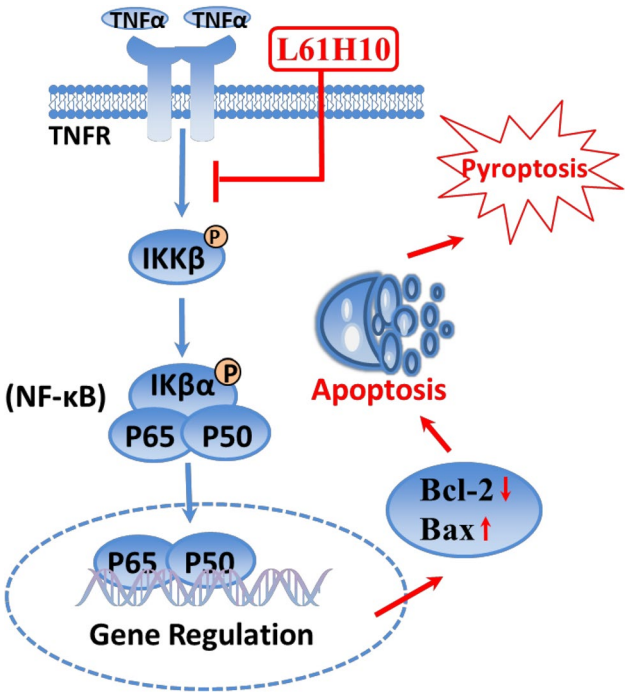


Fig. 4 Schematic illustration of the underlying mechanism of L61H10's anti-tumor activity

with L61H10 and EF24 began to exhibit a certain weight difference. Given the above, there was no significant toxicity under the action of L61H10. As for whether L61H10 really possessed less toxicity than EF24, there still need studies undertaken to evaluate this.

Discussion

As a lytic programmed cell death manner, pyroptosis is regarded as a new direction of anti-tumor research. The morphology of pyroptosis could be observed in some cancer cells under the influence of chemotherapy drugs [9]. The in-depth understanding of pyroptosis is very important for the anti-tumor research of chemotherapy drugs.

In this article, we found that the compound L61H10 showed good antitumor activity with no obvious toxicity, and moreover, its activity was higher than curcumin. It was further found that L61H10 possessed the capacity to arrest the cell cycle in the G2/M phase and mediate the expression of apoptosis-related proteins. Additionally, L61H10 was observed to induce pyroptosis, which was consistent with other reported chemotherapy drugs [9]. However, the relevant signaling pathway regarding the switch from apoptosis to pyroptosis remains unclear. Up to now, research on apoptosis and pyroptosis are mainly concentrated on the Erk1/2-Nrf2/Bach1 pathway [23], inflammasome [24], reactive oxygen species (ROS) [25, 26], GSDME protein [27], autophagy

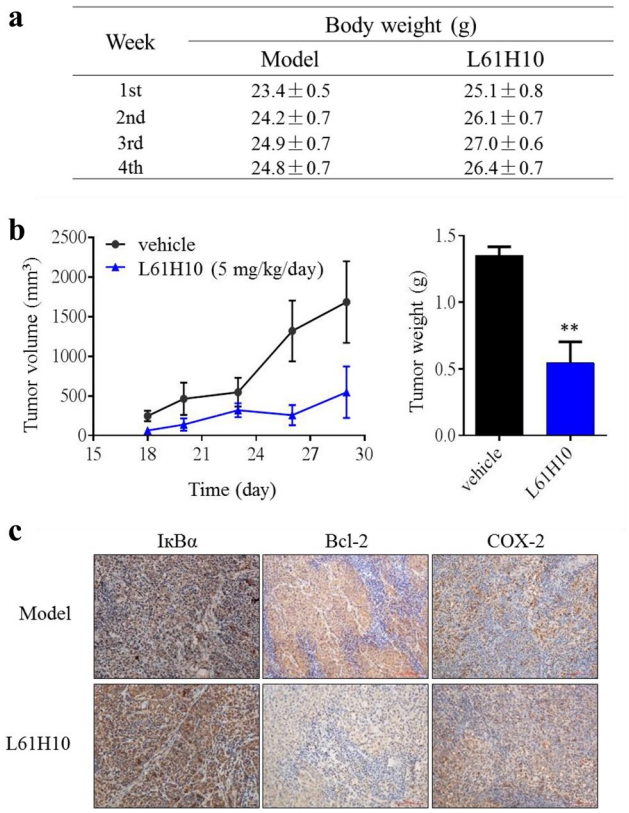


Fig. 5 Effect of L61H10 in vivo. **a** Body weight of the nude mice was measured every week. **b** The tumor volume of control group and L61H10 group was monitored for 29 days. The tumor was weighted on the day the mice were sacrificed and tumors were excised. **c** Tumor tissues were stained with primary antibodies of IκBα, Bcl-2 and COX-2. The date of IHC as indicated. $^{**}p < 0.01$ was compared to vehicle group

Table 1 Changes in body weight of BALB/C mice administered with vehicle, L61H10 and EF24

Day	Body weight (g)		
	Control	L61H10	EF24
1	27.7 ± 3.3	27.8 ± 4.7	27.7 ± 5.4
2	28.8 ± 3.1	28.9 ± 3.7	28.5 ± 4.6
3	28.6 ± 3.1	28.9 ± 3.6	28.3 ± 4.7
4	28.7 ± 3.2	27.7 ± 4.3	28.4 ± 4.8
5	28.6 ± 3.2	28.3 ± 3.6	27.9 ± 4.5
6	28.6 ± 2.7	28.5 ± 3.9	28.1 ± 4.7
7	28.3 ± 2.5	28.5 ± 3.4	28.0 ± 4.9
8	28.5 ± 2.6	29.0 ± 3.5	27.8 ± 4.6
9	28.6 ± 2.6	28.9 ± 3.2	27.3 ± 3.4
10	28.1 ± 2.7	28.8 ± 3.4	25.7 ± 3.2

Each group consisted of six BALB/C mice. All mice were administered by intraperitoneal injection. Medicated groups were given the same dose (0.1 g/kg) dissolved in 6% castor oil, control group was injected with 6% castor oil

[26], oxysterols [28], caspases [29, 30], endoplasmic reticulum stress [31] and NF-κB signaling pathway [20, 21]. Nevertheless, the relationship between NF-κB and pyroptosis is only reported in inflammation and there is no prior research work in cancer. In the research of L61H10, it was found that L61H10 exhibited similar activity in inhibiting the NF-κB signaling pathway as other heterocyclic ketone derivatives. More unexpectedly, it was not just cell apoptosis that was induced by L61H10 associated with NF-κB inhibition, but the link between cell pyroptosis and NF-κB existed as well, which deserved to be further studied. Ultimately, L61H10 also showed excellent anti-tumor activity in vivo. The function of suppressing proliferation and mediating apoptosis induced by L61H10 in vivo agreed with the in vitro experiment. In conclusion, as an efficient and low-toxic thiopyran derivative, L61H10 could exert anti-tumor effects through the transformation of the cell death pathway. The transformation mentioned above has been proven to be associated with NF-κB inhibition preliminarily. It is therefore of great significance to further study L61H10 in depth.

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