Benzoyl-xanthone derivative induces apoptosis in MCF-7 cells by binding TRAF6

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Abstract. TNF receptor-associated factor 6 (TRAF6) has been reported to be associated with the development of cancer. Nevertheless, the exact role of TRAF6 in cancer remains unclear. The purpose of the present study was to explore the mechanism of 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one leading to the inhibition of the activation of AKT and TGF-β-activated kinase 1 (TAK1), and to the apoptosis of MCF-7 cells. Using a computational docking program and examination of AKT and TAK1 level changes, a new small molecule was identified, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one, which competitively bound to TRAF6. Next, the effect of this new compound on MCF-7 cells' biological behavior was studied *in vitro*. MTT assays were used to investigate cell viability; flow cytometry and invasion assays were performed to detect early apoptosis and invasion in MCF-7 cells, respectively. Immunoprecipitation, western blotting and caspase-3/9 activity assays were carried out to explore changes in protein expression. Briefly, the present data indicated that 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one could suppress proliferation, induce early apoptosis and inhibit invasion in MCF-7 cells by suppressing the expression of Bcl-2 and promoting the expression of Bax, caspase-9, and caspase-3. These findings indicated that 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one could induce apoptosis by inhibiting the activation of AKT and TAK1, and affecting the Bcl-2/Bax-caspase-9-caspase-3 pathway by competitively binding with TRAF6.

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Introduction

TNF receptor-associated factors (TRAFs) were initially identified as adaptor proteins in the TRAF family signaling pathways (1). TRAF6, which can activate IL-1 receptor/Toll-like receptor (TLR) superfamilies, was indicated to play an essential role in cell survival and apoptosis (2,3). TRAF6 consists of a RING finger domain, a series of zinc finger motifs, a coiled-coil domain and a highly conserved TRAF-C domain (4). The RING domain performs an essential function in ubiquitin ligase activity (5-7), while the TRAF-C domain regulates CD40 binding in the immune response (8). Together with ubiquitin-conjugating enzyme E2 13/ubiquitin-conjugating enzyme E2 variant 1A (Ubc13/Uev1A), TRAF6 was reported to regulate AKT and TGF-β-activated kinase 1 (TAK1) activation, and induced cancer cell apoptosis (9-11).

AKT promotes cell survival against several apoptotic stimuli through growth factors, and plays a significant role in tumor development and its potential response to cancer treatment (12,13). In cancer cells, AKT is activated by phosphorylation at Thr-308 of the catalytic domain by phosphoinositide-dependent kinase (PDK)-1 and at Ser-473 of the C-terminal hydrophobic region by PDK-2 (14). Blocking AKT activity with LY294002 induced cell death and cell cycle arrest in HTLV-1-transformed cells (15).

TAK1 is a serine/threonine kinase playing a critical role in pro-inflammatory cytokine- and TLR-mediated signaling pathways (16,17). A previous study indicated that ubiquitin-activated TAK1 phosphorylates mitogen-activated protein kinase kinase (MKK), leading to the activation of the JNK and p38 kinase pathways (18). Upon activation, TAK1 was indicated to phosphorylate the IKK complex, p38 and JNK, leading to activation of the NF-κB and MAPK signaling pathways (19).

AKT and TAK1 can also facilitate the activation of downstream NF-κB via the phosphorylation of NF-κB inhibitor, thus subsequently affecting the expression levels of apoptosis-related Bax/Bcl-2 (20), and activating caspase-9 and downstream caspase-3. Therefore, AKT and TAK1 are involved in the initiation and mediation of cell apoptosis (21).

Previous studies have suggested that TRAF6 may directly catalyze AKT ubiquitination, which is essential for AKT membrane recruitment and its phosphorylation at Thr-308 and Ser-473 (22,23) TRAF6 deficiency was indicated to lead

to constitutive inactivation of the crucial downstream targets of AKT such as NF-κB and Bax/Bcl-2 (20,21,24). TAK1 is activated in a polyubiquitin and TRAF6-dependent manner. The complex formed by TRAF6, Ubc13 and Uev1A induces Lys-63-dependent ubiquitination on TAK1 binding protein and MAP3K7-binding protein 2, which results in TAK1 autophosphorylation (23). Moreover, the polyubiquitin chains, TRAF6 and Ubc13/Uev1A synthesize, which can promote the autophosphorylation of TAK1 at Thr-184/187, resulting in its activation (18).

Considering the important associations between TRAF6 and activations of both AKT and TAK1, the present study further examined the role of TRAF6 on cell survival and oncogenic signaling through the changes in AKT and TAK1 expression. Through computer-assisted drug screening, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one (L18722) was reported to compete with TRAF6. The suppressive effect of L18722 on the activation of AKT and TAK1 was further explored.

Materials and methods

Materials. MCF-7 cells were provided by the Tianjin International Joint Academy of Biomedicine, while normal human dermal fibroblast (NHDF) cells were gifted by Professor Lijun Zhou (Tianjin University, Tianjin, China). RPMI-1640 medium, DMEM and FBS were purchased from Corning Life Sciences. L18722 (Xi'Ensi Biochemical Technology Co., Ltd.) was dissolved in DMSO (Millipore Sigma). MTT reagent was obtained from Millipore Sigma. *Cis*-platinum was obtained from Jiangsu Hanson Pharmaceutical Co., Ltd. Protease inhibitors and phosphatase inhibitors were purchased from Millipore Sigma. PVDF membranes were acquired from Millipore Sigma. Protein A-Agarose beads were obtained from Pierce (Thermo Fisher Scientific, Inc.). ECL chemiluminescence detection kit (SuperSignal HRP) was purchased from Pierce (Thermo Fisher Scientific, Inc.). The caspase-3 detection assay kit (cat. no. C1116) and the caspase-9 detection assay kit (cat. no. C1158) were obtained from Beyotime Institute of Biotechnology.

The following antibodies were used in the present study: Mouse ubiquitination antibody (cat. no. SC8017) and mouse anti-TRAF6 polyclonal antibody (cat. no. SC8409) (Santa Cruz Biotechnology, Inc.); rabbit polyclonal antibody against total AKT (cat. no. 4685), phosphorylated (p)-AKT (Thr-308) (cat. no. 8205), p-AKT (Ser-473) (cat. no. 8200), total TAK1 (cat. no. 4505) , p-TAK1 (Thr-184/Thr-187) (cat. no. 4508), Bax (cat. no. 5023), Bcl-2 (cat. no. 3498), p65 (cat. no. 8242), p-p65 (cat. no. 3033), caspase-3 (cat. no. 14220) and caspase-9 (cat. no. 9054) (all Cell Signaling Technology, Inc.); rabbit anti-β-actin polyclonal antibody (cat. no. K101527P), corresponding secondary HRP-conjugated antibodies (cat. no. SE205) (all Beijing Solarbio Science & Technology Co., Ltd.).

Cell culture. MCF-7 cells were cultured in RPMI-1640 medium, while NHDF cells were cultured in DMEM; both media were supplemented with 10% FBS. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. STR profiling was performed on NHDF cells to confirm their authenticity.

Docking study. Firstly, the structure of the RING and zinc finger domains of TRAF6 was obtained from the Protein Data Bank database (25). A CHARMM-like force field was used to screen suitable small molecules which could bind to the RING domain of TRAF6. Subsequently, AutoDock 4.10 software (The Scripps Research Institute) with the default parameters was used to dock TRAF6 and the available compounds from the structure library. The compounds were then filtered according to the predicted binding free energy.

Cell proliferation assay. The effect of L18722 on MCF-7 cell viability and proliferation was determined using an MTT assay. Briefly, cells were cultured in 96-well plates overnight at density of $4.1x10^3$ cells/well. overnight. After cellular adhesion, different concentrations (1, 5, 10, 25, 50, 100, 120, 150, 180, 200, 250 and 500 μM) of L18722 were added into the wells; *cis*-platinum (16.7 μM) was used as a positive control. After incubating cells at 37˚C with L18722 for 48, 72 and 96 h, 20 μl of MTT (5 mg/ml) was added to each well, and the plates were incubated at 37˚C for an additional 4 h. The medium was then removed, and formazan crystals were dissolved in 150 μl DMSO. Optical density was measured at 490 nm. The inhibition ratio was calculated using the following formula: Inhibition ratio (%) = (A control-A treated/A control) x100%. A regression curve was used to calculate the half-maximal inhibitory concentration.

Determination of early apoptosis via flow cytometry. The effect of L18722 (100, 150 and 200 μM) on the early apoptosis of MCF-7 cells ($2x10^4$ cells/ml) was investigated via flow cytometry. At 48 h post-treatment with L18722 or *cis*-platinum (16.7 μM) at 37˚C, the cells were harvested and washed twice with cold PBS. Subsequently, each sample was resuspended in 100 μl 1X binding buffer, in which 5 μl Annexin V-FITC and PI (BD FITC Annexin V Apoptosis Detection kit) were added according to the manufacturer's instructions. The mixture was incubated for 15 min in the dark at room temperature. After the addition of 400 μl Annexin-V binding buffer per sample, the cells were analyzed using a FACScalibur flow cytometer and CellQuest Pro 5.1 (BD, Biosciences).

Determination of invasive ability of MCF-7 cells. The invasive ability of MCF-7 cells was assessed via Transwell assay based on the number of cells passing through the polycarbonate membrane. A serum-free RPMI-1640 was used to wash the upper and lower chambers. Matrigel (1:7) was added to the upper chamber of the insert at 37˚C and stand for 2 h. Subsequently, $4x10^5$ cells were seeded in the upper chamber with RPMI-1640 containing 10% FBS, and he same medium was added into the lower chamber. Then, the chamber was placed into the incubator at 37˚C for 48 h with 150 mM L18722. and *cis*-platinum-treated (16.4 μM) group was used as the positive group. Subsequently, the Transwell chamber was taken out, and the culture medium in the hole was discarded. The cells were fixed with methanol or formaldehyde for 30 min. Subsequently, 1% crystal violet was used to stain the cells present in the lower chamber at room temperature for 30-60 min. A light microscope was used to observe the cells in five fields (magnification, x400). Crystal violet-stained cells and the quantified results are presented as the mean ± SD, and the experiment was repeated three times for each group.

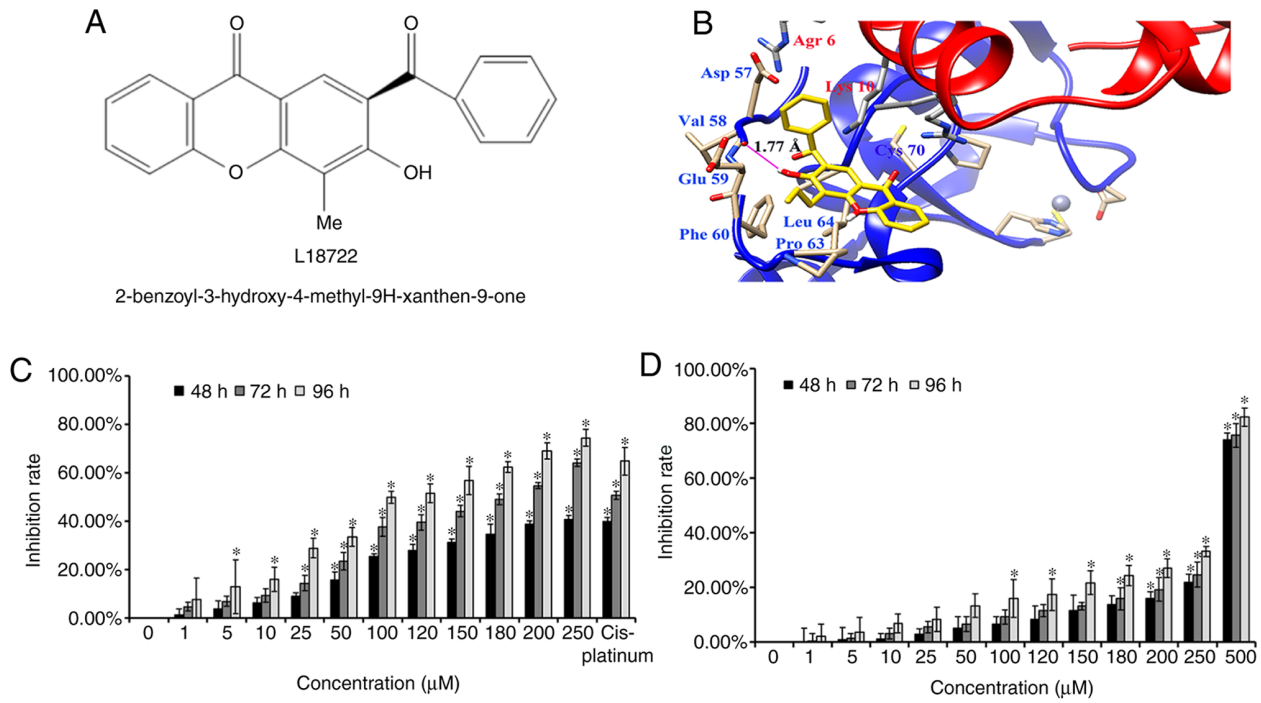

Figure 1. Molecular docking results and assessment of apoptosis by MTT assay. (A) Chemical structure of L18722. (B) Binding of L18722 (in yellow) with TRAF6 (in blue). Inhibition rate of (C) MCF-7 and (D) NHDF cells treated with different concentrations of L18722 for 48, 72 and 96 h. *Cis*-platinum (16.7 μM) was used as a positive control. Quantitative analysis of MTT represented as the mean ± SD of three independent experiments. *P<0.05 vs. untreated control. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one; TRAF6, TNF receptor-associated factor 6.

Immunoprecipitation. MCF-7 cells were cultured with L18722 (200 μM) 37˚C for 24 h, after which the total cellular protein was extracted with lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP-40. The protein was harvested after centrifugation at 12,000 x g for 20 min at 4˚C. The samples were incubated with antibodies against AKT (1:1,000) and TAK1 (1:1,000) overnight at 4˚C. Subsequently, 20 μl of protein A-agarose beads (Pierce; Thermo Fisher Scientific, Inc.) were added, and samples were incubated for 4-6 h at 4˚C with gentle rotation. The supernatant was discarded after centrifuging three times at 800 x g for 3 min at 4˚C. Western blotting was performed to visualize the protein bands, as reported by Schnetzke *et al* (26).

Western blotting. MCF-7 cells were treated with L18722 at a concentration of 200 μM for different durations (1, 2, 3, 6, 12, 24, 48 and 72 h). Subsequently, total proteins of MCF-7 cells were extracted and homogenized in a lysis buffer (containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP-40) and BCA Protein Assay kit was used to quantify the protein. Total proteins were separated via 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk at room temperature for 2 h, which was supplemented with TBS containing 0.1% Tween-20. Subsequently, the membranes were incubated with antibodies against TRAF6 (1:1,000), AKT (1:1,000), p-AKT (1:1,000), TAK1 (1:1,000), p-TAK1 (1:1,000), ubiquitin (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), caspase-3 (1:1,000), caspase-9 (1:1,000), and β-actin (1:1,000) at 4˚C overnight. Next, the membranes were probed with their corresponding secondary HRP-conjugated antibodies (1:5,000) for 1 h at room temperature. Finally, an ECL chemiluminescence kit (Thermo Fisher Scientific, Inc.) was used to detect the expression of the proteins. The density of the blots was quantified using ImageJ software v.1.48u (National Institutes of Health), with β-actin as a loading control.

Caspase-3 and caspase-9 activity assay. MCF-7 cells were firstly treated with L18722 at a concentration of 200 μM for 48 h. The cells were collected and treated with ice-cold lysis buffer after 0, 24, 48 or 72 h of L18722 treatment. The supernatants were then collected and centrifuged at 20,000 x g for 15 min at 4˚C. According to the manufacturer's instructions for the caspase-3 and caspase-9 detection assay kit (Beyotime Institute of Biotechnology), 10 μl of supernatant and 10 μl of acetyl-DEVD-p-nitroanilide were added to 80 μl of reaction buffer. The mixed samples were incubated at 37˚C for 2 h, and the enzyme-catalyzed release of p-nitroanilide was quantified at 405 nm using a Microplate Reader (Tecan Group, Ltd.).

Statistical analysis. Data analyses were performed using SPSS 18.0 software (SPSS Inc.), and the present results represent at least three independent experiments, presented as the mean ± SD. Data were log-transformed to detect differences between groups, using one-way ANOVA and Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Selection of L18722 through docking study. The present data indicated that L18722 could bind to the RING domain of TRAF6 (Fig. 1B). After analyzing all possible areas and comparing the free energies, the suitable binding mode for L18722 was discovered, which possessed the lowest free energy of all the possible compounds. As presented in Fig. 1B,

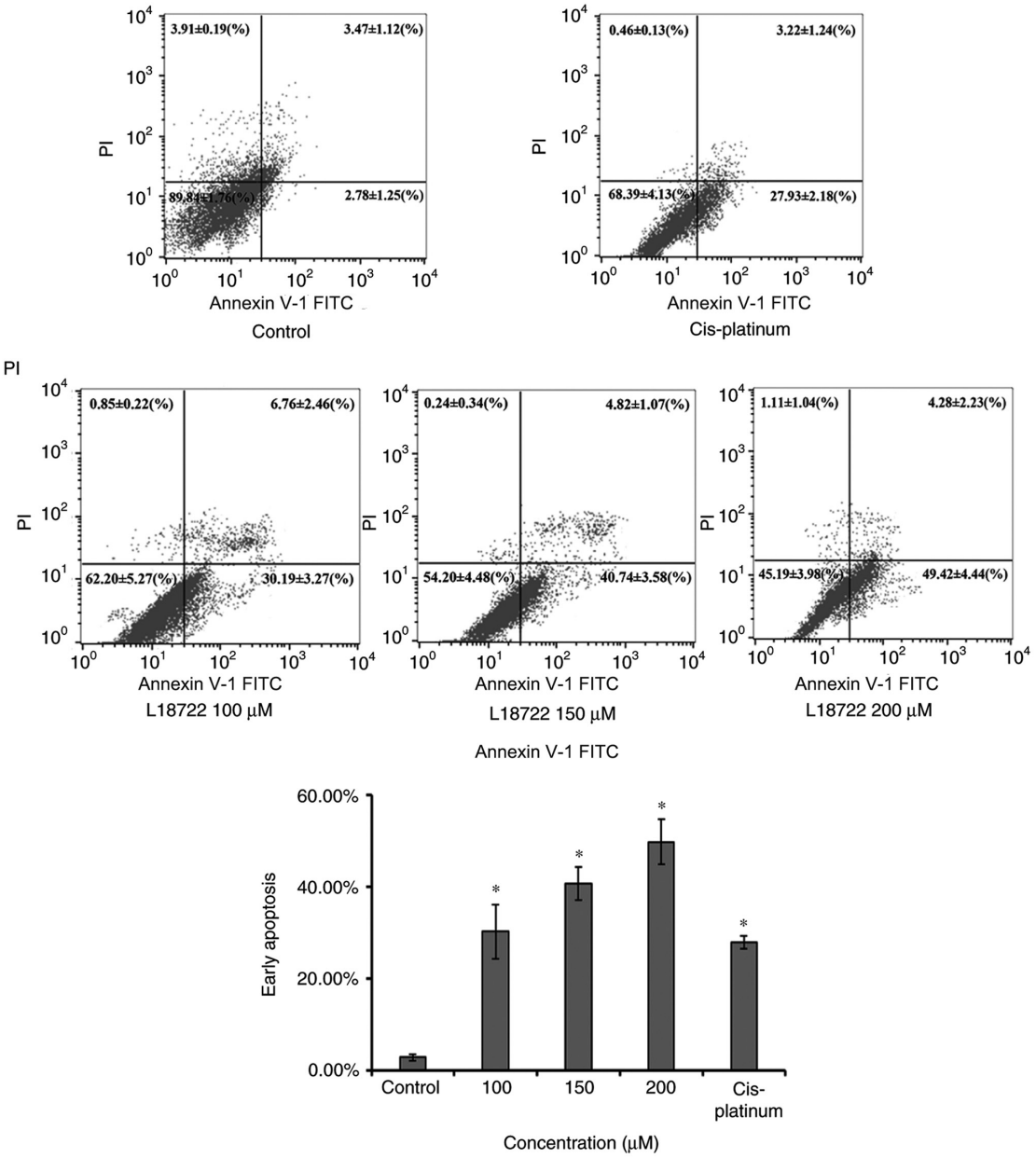

Figure 2. Evaluation of the early apoptosis induced by L18722. The apoptosis level of MCF-7 cells was measured using the Annexin V-FITC and PI assay at 48 h after L18722 treatment. L18722 induced early apoptosis of cells in a concentration-dependent manner. *Cis*-platinum (16.7 μM) was used as a positive control. Quantitative analysis of flow cytometry results represented as the mean ± SD of three independent experiments. *P<0.05 vs. control group. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one.

L18722 was surrounded by amino acid residues Asp-57, Val-58, Glu-59, Phe-60, Pro-63 and Leu-64, thereby suggesting interactions with residues (54-66) preceding TRAF6 RING domain (67-124); the free energy (ΔG) was -5.37 kcal/mol. More notably, the present results reported that the hydroxy group (-OH) of L18722 formed a key hydrogen bond with Val-58, which could effectively bind with TRFA6 (Fig. 1B).

L18722 inhibits MCF-7 cell proliferation. Western blotting results reported high levels of endogenous TRAF6 in MCF-7 cells compared with NHDF cells (Fig. S1). MTT assays revealed that L18722 could inhibit MCF-7 cell proliferation in a dose- and time-dependent manner (Fig. 1C). However, this effect was not observed after treating NHDF cells with the same concentrations of L18722 (Fig. 1D). The IC_{50} of L18722 in MCF-7 cells at 48 h was >250 μM. The IC_{50} of L18722 at 72 h was 200 μM, and the IC_{50} of L18722 at 96 h was 180 μM. In order to obtain more satisfying experimental results by stimulating cells with compounds, we use IC_{50} at 72 h, and the concentration was 200 μM. The inhibition rate of NHDF cells treated with 200 μM of L18722 was 13.81%, 14.74%, 24.34% for 48, 72 and 96 h, respectively (Fig. 1D). The inhibition ratio in NHDF cells was significantly lower than in MCF-7 cells.

L18722 induces the early apoptosis and inhibits the invasion of MCF-7 cells. The apoptosis of MCF-7 cells was measured via flow cytometry, using the Annexin V-FITC and PI assay 48 h after L18722 treatment. As indicated in Fig. 2, the early apoptosis rate of MCF-7 cells treated with L18722 increased

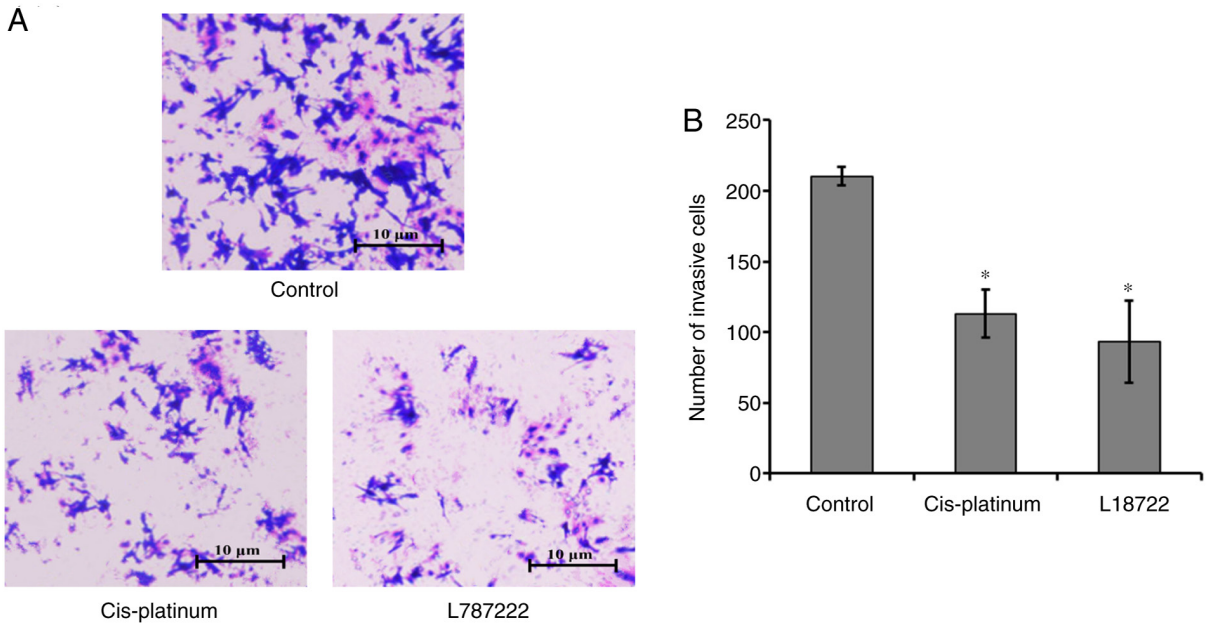

Figure 3. Effect of L18722 on MCF-7 cell invasion ability. (A) Crystal violet staining of the MCF-7 cells that passed through the Transwell polycarbonate membrane. Scale bar=10 μm. (B) Number of MCF-7 cells passed through the Transwell polycarbonate membrane. Quantitative analysis of the results represented as the mean ± SD of three independent experiments. *P<0.05 vs. control group. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one.

compared with the control group. The fraction corresponding to early apoptosis increased in a dose-dependent manner; 2.78±1.25 to 30.19±3.27% were observed after treating cells with 100 μM of L18722; 40.74±3.58% after treatment with 150 μM L18722; 49.42±4.44% after treatment with 200 μM L18722, all significantly higher compared with *cis*-platinum (16.7 μM) treatment (27.93±2.18%).

The Transwell invasion experiment results indicated that the number of MCF-7 cells that passed through the membrane in the L18722-treated group and the *cis*-platinum (16.7 μM) treated group were significantly lower compared with in the control group (Fig. 3). These results suggested that L18722 reduced invasion and induced apoptosis in MCF-7 cells.

Changes in AKT and TAK1 pathway activation. It has been reported that activation of the AKT and TAK1 signaling pathways can regulate cell proliferation, apoptosis and migration (27-29). Thus, AKT and TAK1 signaling pathway expression levels were studied in MCF-7 cells. The cells were exposed to L18722 (200 μM) for 0, 3, 6, 12 and 24 h. Immunoprecipitation was performed to test the level of ubiquitination of AKT. Results showed that ubiquitination of AKT was downregulated after being treated with L18722 for 1, 2 and 3 h. (Fig. 4A). Western blotting results suggested that, after L18722 treatment, the phosphorylation level of AKT at Thr-308 and Ser-473 significantly decreased. (Fig. 4B). In addition, TAK1 phosphorylation and ubiquitination levels were also reduced (Fig. 4C and D).

Effect of L18722 treatment on Bax, Bcl-2, caspase-3 and caspase-9 expression. To further confirm the inhibitory effect of L18722 on cell proliferation and apoptosis in MCF-7 cells, the protein expression of Bcl-2, Bax, caspase-3 and caspase-9 was examined via western blotting. The present results demonstrated that, after treating cells with L18722, the expression of Bcl-2 was decreased. Conversely, the expression of Bax, caspase-3 and caspase-9 was increased (Fig. 5A and B).

L18722 can downregulate the expression of p-p65 in MCF-7cells. To further detect the mechanism of apoptosis induced by L17822 in MCF-7 cells, the expression of p-p65 was examined. The obtained results showed that after treatment with L18722, the expression of p-p65 was decreased. (Fig. 5C and 5D).

L18722 can increase caspase-3 and 9 activities in MCF-7 cells. To further detect the mechanism of apoptosis induced by L17822 in MCF-7 cells, the activities of caspase-9 and caspase-3 were examined. The obtained results showed that after treatment with L18722, the actions of caspase-3 and caspase-9 were significantly increased (Fig. 6).

Discussion

TRAF6 has been indicated to be involved in carcinogenesis in numerous cancers; overexpression of TRAF6 resulted in the malignant transformation of fibroblasts and tumor formation (30), whereas its knockdown reduced cell proliferation and tumor formation (31). The RING domain of TRAF6 is well-known to possess ubiquitin E3 ligase activity, while the TRAF domain serves as a protein-protein interaction domain (32). Previous studies have demonstrated that the E3 ubiquitin ligase activity of TRAF6 exerted a pivotal function in tumorigenesis (7,32). Furthermore, Liu *et al* (33) have indicated that suppression of TRAF6 could rescue cell proliferation and induce apoptosis in myeloma cells. Moreover, previous studies have suggested that TRAF6 played a critical role in regulating a number of genes involved in cell proliferation and apoptosis, as well as immune responses to invasion through NF-κB (16,34).

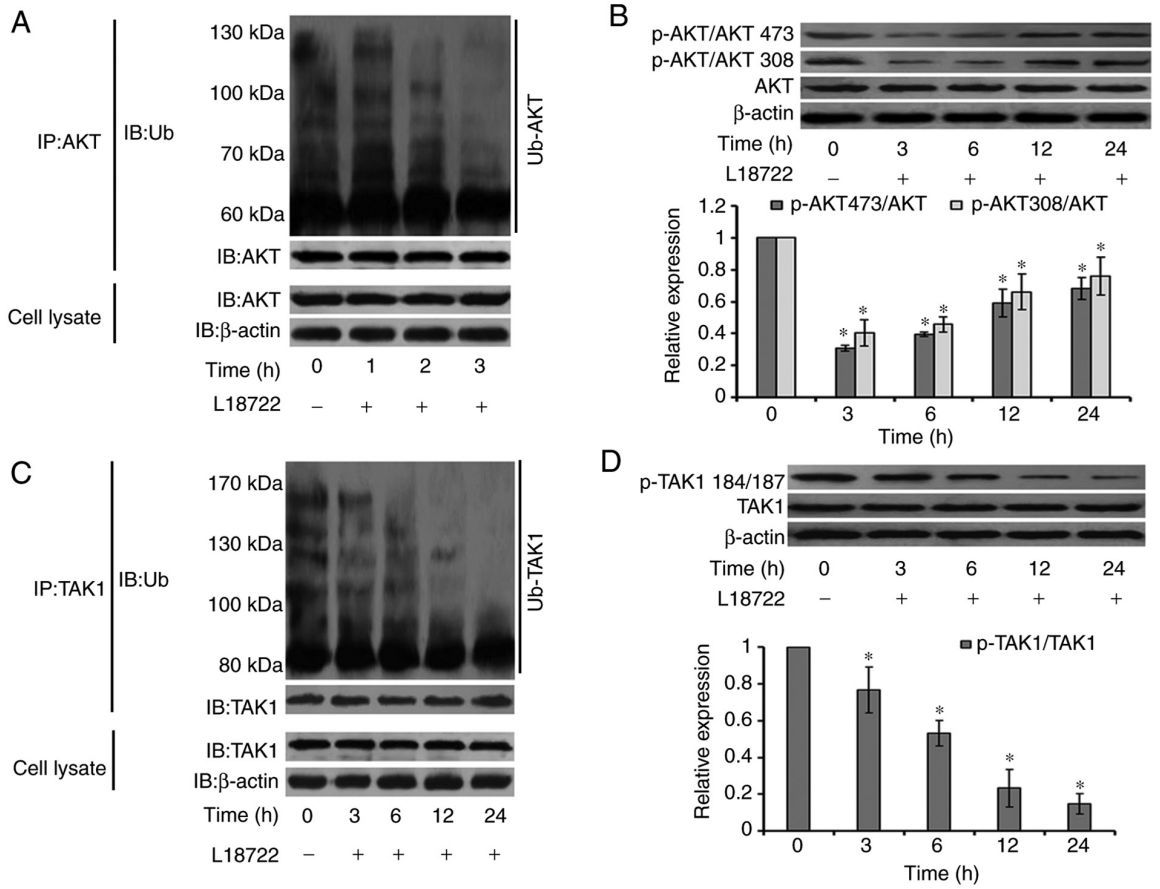

Figure 4. Effect of L18722 treatment on AKT and TAK1 activation. (A) MCF-7 cells were exposed to L18722 (200 μM), and immunoprecipitation was performed to test the expression level of the ubiquitination of AKT. (B) MCF-7 cells were pre-treated with L18722 for the indicated durations, and western blotting was performed to quantify p-AKT and AKT. (C) MCF-7 cells were exposed to L18722 (200 μM), and immunoprecipitation was carried out to determine the expression level of the ubiquitination of TAK1. (D) MCF-7 cells were pre-treated with L18722 for the indicated durations, and western blotting was performed to quantify p-TAK1 and TAK1. The reported results are the mean ± SD of three independent experiments. *P<0.05 vs. control group. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one; TAK1, TGF-β-activated kinase 1; p, phosphorylated; Ub, ubiquitin.

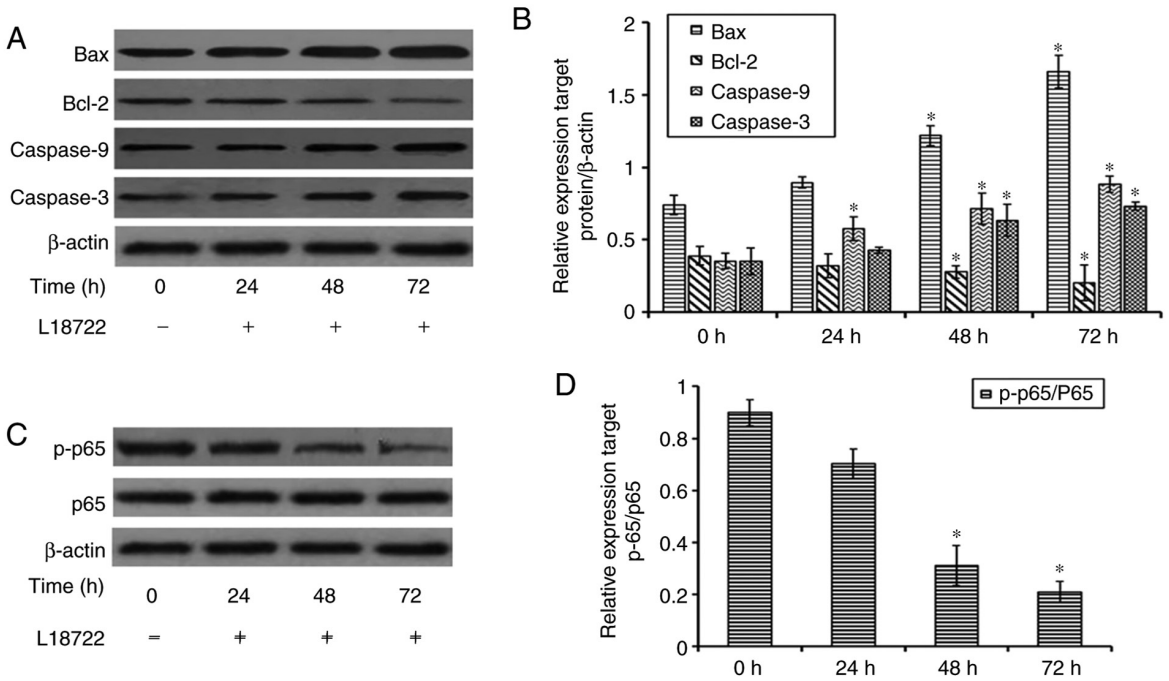

Figure 5. Effect of L18722 on Bax, Bcl-2, caspase-9, caspase-3, p65 and p-p65 expression. (A and B) The expression of Bax, Bcl-2, caspase-9 and caspase-3 protein in MCF-7 cells after treatment with L18722. (C) and (D) The expression of p65 and p-p65 in MCF-7 cells following treatment with L18722. The results shown are the mean ± SD of three independent experiments. *P<0.05 vs. control group. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one; p, phosphorylated.

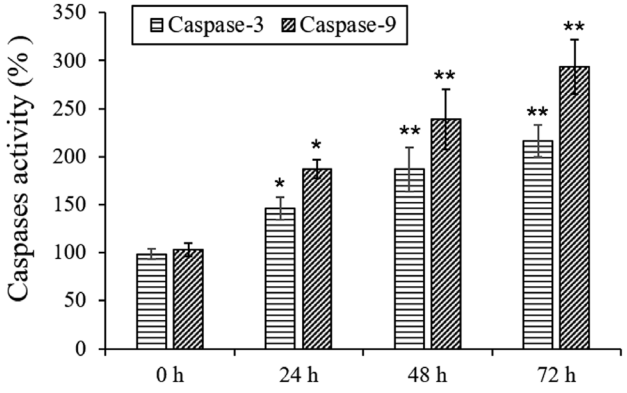

Figure 6. The caspase-3 and 9 activities in MCF-7 cells. MCF-7 cells were exposed to L18722 with 200 μM for 48 h. The results shown are the mean ± SD of three independent experiments. *P<0.05 vs. control group, **P<0.01 vs. control group. L18722, 2-benzoyl-3-hydroxy-4-methyl-9H-xanthen-9-one.

Based on the vital function of TRAF6 in tumorigenesis, growth and apoptosis, the present study used a computational docking program to screen small molecules that could competitively and effectively bind with the RING domain of TRAF6. Yin *et al* (35) identified a salt bridge between Glu-69 of the RING domain and Arg-14 of Ubc13, and demonstrated that the salt bridges between Asp-57 and Lys-10 of Ubc13 were essential for the interaction between TRAF6 and Ubc13. Through computational analysis, L18722 was identified in the present study to bind with Val-58 of TRAF6, and could block the interaction between TRAF6 and Ubc13 by preventing the formation of a salt bridge. Without the salt bridge between TRAF6 and Ubc13, Ubc13 could not bind with TRAF6 and activate its downstream signaling pathway.

Next, the effects of L18722 on cell proliferation, apoptosis and invasion ability were examined *in vitro*. L18722 inhibited proliferation of MCF-7 cells in a concentration- and time-dependent manner; however, this effect was not observed in non-tumoral NHDF cells with concentration of <250 μM. The present results suggested that L18722 had low cytotoxicity when used at lower concentrations. Furthermore, flow cytometry indicated that treatment with L18722 for 48 h could induce early apoptosis without causing cell death.

Chaudhry *et al* (36) demonstrated that TRAF6 was essential in promoting squamous cell carcinoma invasion. In the present study, Transwell invasion assay results revealed reduced invasion by cells treated with L18722 compared with the control group, suggesting that L18722 may affect TRAF6 activity and subsequently inhibit the invasive ability of the cells.

As a ubiquitin E3 ligase, TRAF6 mediates numerous apoptosis-related signaling pathways (37-39). Additional activation of AKT regulates a wide range of target proteins that control cell proliferation, survival and growth (12). Downregulation of TRAF6 using a short hairpin RNA resulted in a significant decrease of AKT ubiquitination (22) and phosphorylation at both Thr-308 and Ser-473 (26). In addition, a previous report has indicated that TRAF6, together with Ubc13-Uev1A, could rapidly active TAK1 and subsequently cause a tumorigenic response (16). The activated TAK1 complex can also phosphorylate members of the MKK family, leading to JNK and p38 kinase activation (40). Furthermore, in TRAF6-deficient mouse embryonic fibroblasts, phosphorylation levels at Thr-184 and Thr-187 of TAK1 were found to be reduced, which affected the activation of downstream signals (18,41). Therefore, the present results demonstrated that L18722 could competitively bind with the RING domain of TRAF6 and affect the activation of AKT and TAK1 by targeting TRAF6.

The Bcl-2 and caspase protein families are well-known regulators of cell apoptosis. The Bcl-2 protein family comprises anti-apoptotic Bcl-2 and pro-apoptotic Bax (42) . Wu *et al* (21) reported that caspase-3 and caspase-9 activity measurements were important in determining apoptosis factors. Bcl-2 could regulate apoptosis through caspase-9 and caspase-3-dependent pathways (43-45). In addition, inhibition of Bax and Bcl-2 could activate caspase-9 (46), as well as promote the activation of caspase-3, leading to apoptosis (47).

To further confirm the inhibitory effect of L18722 on cell proliferation and apoptosis, the expression of Bcl-2, Bax, caspase-3 and caspase-9 was examined in MCF-7 cells via western blot analysis. After L18722 treatment, the expression of Bcl-2 decreased, while Bax, caspase-3 and caspase-9 expression levels increased.

Compounds that could bind to the ubiquitin ligase active region of TRAF6 were screened from a compound database (a small library of chemical compounds established by Jkchemical Sigma and Alfa Aesar that includes 1,792 commercially available compounds) (48), and L18722 was identified within this database through computer-aided drug design and molecular docking studies. Simultaneously, MTT assays in MCF-7 and NHDF cells indicated that L18722 had a strong inhibitory effect on the proliferation of MCF-7 cells, but not on NHDF cell (it is a limitation to the present study that these cells were not both sourced from breast tissues). Therefore, subsequent experiments were conducted to verify if L18722 could induce apoptosis by inhibiting the E3 ubiquitin ligase activity of TRAF6. Future research will be conducted to uncover precise mechanisms associated with L18722 in MCF-7 cells.

Furthermore, the present research aimed to find small molecule compounds that could target TRAF6 and inhibit tumor cell proliferation. Western blot assay results indicated that MCF-7 cells presented a high expression level of TRAF6 compared with NHDF cells. It was previously demonstrated that small molecule compounds could significantly inhibit tumor cells with increased expression of TRAF6, and with little effect on healthy tissue cells (4). In the present study, the use of MCF-7 and NHDF cells demonstrated the antitumor activity of the small molecule compound L18722. However, analysis of the overexpression and knockdown of TRAF6 would also be essential for the study of compounds and their effect on downstream signal pathways. In future research, small interfering RNAs will be used to knock down TRAF6 in MCF-7 cells. L18722 will be used to treat the cells to certify that the compound has little effect on knockout of TRAF6 cells. In addition, TRAF6 overexpression using a recombinant plasmid into cells may be considered, in order to verify if high-expressing TRAF6 cells may have a heightened sensitivity for L18722.

In conclusion, the present data suggested that the compound L18722 could competitively bind with TRAF6 and inhibit its ubiquitination activity. Immunoprecipitation and western blot assays demonstrated that L18722 could decrease

AKT and TAK1 phosphorylation levels, thus inactivating them. The decrease in AKT and TAK1 activity could lead to subsequent suppression of anti-apoptotic protein Bcl-2, while elevating pro-apoptotic protein Bax. In addition, caspase-3 and caspase-9 expression levels increased, suggesting that L18722 could play key roles in cell apoptosis. The RING domain of TRAF6 could be considered a potentially viable antitumoral target, and future research will investigate its potential for a practical approach for treating tumors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YQ conceived and designed the experiments. YQ, XZ and QY participated in the design of the study and drafting of the manuscript. XZ carried out most of the experiments. XZ, XW and GH participated in the cell culture and MTT assays. LR and SW participated in the flow cytometry and detection of signaling pathways. YQ and XZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The requirement for ethics approved for the use of NHDFs initially derived from the National Biomedical Experimental Cell Resource Bank was waived by the Ethics Committee of Shijiazhuang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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