Thevebioside, the Active Ingredient of Traditional Chinese Medicine, Inhibits SRC3 Mediated Signaling by Ubiquitination to Induce NSCLC Cells Apoptosis

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Research

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Abstract

Background:
The high incidence and mortality of lung cancer has been a widespread concern in the world, among which non-small cell lung cancer (NSCLC) accounts for more than 85% of lung cancer, and its 5-year prognosis is pretty poor. Accumulating studies have shown that traditional Chinese medicine (TCM) and its active ingredients have shown good anti-tumor activity. Based on the results of our previous high-throughput screening, it was the first time to find that Thevebioside (THB, an active ingredient from TCM) is sensitive to NSCLC cells, and then we will still the first to reveal the underling mechanism of its tumor suppressive activity.

Methods:
In this study, MTT and colony formation assay were used to detect cell growth. Annexin V/ propidium iodide, Hoechst 33342 were applied to test the process of cellular apoptosis or cell cycle arrest which determined by flow cytometry in NSCLC (A549 and H460) cells. qRT-PCR and Western blot was utilized to value the level of expression of SRC&(SRC1-3) and apoptosis related proteins. The ubiquitination degradation of SRC3 was through proteasome dependent pathway were examined by the usage of CHX and MG132 and the co-immunoprecipitation assay. The anticancer activity of THB, Neritaloside Odoroside H and digoxin in vivo were monitored in nude mice by subcutaneous inoculation or intrapulmonary injection of A549 cells and then administrated these agents (2 mg/ kg or 4mg/kg) by intraperitoneal injection three times a week for 30 days. Tumor growth was monitored by a luciferase living imaging system. Body weight of mice and tumor volume were measured weekly. The survival carve was recorded. Pathological histology of liver, kidney and heart were detected by H&E staining, and its functions were tested by ELISA.

Results
THB effectively inhibits the outgrowth of NSCLC cells (A549 and H460) by inducing cellular apoptosis. SRC-3 was significantly down-regulated after THB treatment dependent on ubiquitin-proteasome-mediated degradation, which subsequently inhibited the AKT signaling pathway to promte apoptosis. Animal study supported in vitro results, showing THB had a good suppressive effect on tumor growth. Besides, it also indicated that THB without overt cytotoxic effect on liver, kidney and heart.

Conclusion
THB, as an active extract from TCM, exerted inhibitory effect on tumor growth of NSCLC by down-regulating the expression of SCR-3 rely on the ubiquitin-proteasome degradation pathway, thereby inhibiting the AKT signaling to induce cellular apoptosis, with minimal toxicity.

Background
Within the global cancer burden, lung cancer is one of the most malignant tumors with the high incidence and mortality rate. Accounting for approximately 85% of lung cancer cases are classified as non-small cell lung cancer (NSCLC), which includes both non-squamous carcinoma and squamous cell carcinoma[1]. Despite recent tremendous advances with personalized therapies and immunotherapy in NSCLC treatment, the overall survival (OS) of NSCLC patients remains poor and recurrence is frequent[2]. Traditional Chinese medicine (TCM), as the most widely used complementary and alternative approach all over the world, which plays a vital role in improving the quality of life and prolonging the survival of cancer patients[3]. In addition, a growing body of evidence shows that Chinese herbs and its effective ingredients can exert tumor suppressive effects through multiple ways, including induction of apoptosis, anti-metastasis and anti-angiogenesis, proliferation inhibition, suppression of cancer stem-like cells, induction of autophagy, etc.[4–9].

Thevebioside (THB) is an active ingredient from the seed of Thevetia peruviana (Pers) K. Schum (TPKS). TPKS, is an ornamental shrub belonging to the Gentianales order, Apocynaceae family (well-known for anticancer activities), which native to China, Mexico, India, Central America. And TPKS, as a TCM, has the functions of strengthening the heart, diuretic and reducing swelling documented in Dictionary of TCM. This herb is pharmacologically recognized for containing cardiotonic glycosides such as THB, thevetin A, thevetin B, neriifolin, peruvoside, thevetoxin, and ruvoside[10]. These metabolites have a positive inotropic effect, like in digoxin. Therefore, TPKS has been applied conventionally for the treatment of heart failures. In addition, TPKS can also cure diseases like scabies, hemorrhoids, ulcers, and dissolve tumors. However, there are pretty few researches on THB, no matter in terms of cardiotonic effect, inflammatory skin diseases or in anti-cancer field.

In our previous study, we have established a new type of high-throughput screening system to search for natural products with anti-tumor activities [11]. THB is one of the natural product candidates that we used this luminescence-based screening system to find out, which sensitive to NSCLC cells. In our current study, we found that THB was capable of dramatically decreasing the expression of steroid receptor coactivators (SRC)3. SRCs, known as nuclear receptor coactivators (NCOAs), are a family of transcription coactivators, including SRC1, SRC2 and SRC3, in which SRC3 participates in the induction of cellular apoptosis and over expression of it is tightly associated with tumor growth[12]. Moreover, overexpression and amplification of SRC3 was found in 48.3% and 8.2% of NSCLCs patients[13]. Therefore, as far as we know, it's the first time to report the antineoplastic activity of THB in NSCLC. The underlying molecular mechanisms may that THB downregulate the protein expression of SRC3, and then induce cell apoptosis and inhibit the proliferation of NSCLC cells, in which SRC3 was degraded by ubiquitin-proteasome degradation pathway with THB treatment. Our study is the first to suggest that THB may be a promising complementary and alternative agent of TCM to treat lung carcinoma.

Methods

Drugs, Reagents And Antibodies
Thevebioside, Neritaloside and Odoroside H was kindly provided by National Institute for the Control of Pharmaceutical and Biological Products. Its purity was determined to be at least 95% by HPLC analysis. DMSO, Propidium iodide (PI), Rhodamine 123, Hoechst 33,342, Cycloheximide (CHX) and MG132 were obtained from Sigma-Aldrich (MO, USA). Annexin V/PI apoptosis kit and 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen (CA, USA) or Life Technologies (Carlsbad, CA, USA), respectively. Primary antibodies for SRC, pAKT, AKT, Bad, Bcl-xl, Bcl-2, cleaved-caspase 3 (Asp175), cleaved-PARP, GAPDH, SRC-1, SRC-2, SRC-3 and beta-actin were purchased from Cell Signaling Technology (MA, USA).

Cell Lines And Cell Cultures

The cell lines A549, H460, H1975, H1299, H1650, CALU-1 and BEAS-2B were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI-1640 (Gibco, NY, USA), which contained 100 U / mL penicillin-streptomycin (Hyclone, Utah, USA) and 10% fetal bovine serum (Gibco, NY, USA), incubating in 37°C with 5% CO₂.

Cell Viability Assay

Briefly, cells were seeded at a concentration of 4 × 10³ cells/well into 96-well plates. After overnight incubation, continuous concentrations of Thevebioside, Neritaloside and Odoroside H were added for the indicated times. After treatment, the cells were incubated with 10 ul 5 mg/ml MTT for 4 h. MTT formazan production was dissolved by DMSO (200 ul/well). Optical density was measured at 590 nm using a Bio-assay reader (BioRad, USA). The growth inhibition was determined using: Growth inhibition = (control O.D.-sample O.D.)/control O.D.

Cell Cycle And Apoptosis Analysis

Propidium iodide (PI) staining assay was used to analyze the cell cycle distribution. After incubating with Thevebioside at different concentrations for 48 h, the cancer cells were collected and fixed with 70% ethanol, then centrifuged (3000 rpm, 5 minutes), and incubated with 100 mg / mL RNase in PBS solution for 30 minutes at 37°C, then staining with 50 mg/mL PI in PBS. Cell cycle distribution was analyzed by a Cell Lab Quanta SC flow cytometer (Beckman Coulter, USA).

The apoptosis rate of cells was measured by using the Annexin V–FITC Apoptosis Detection Kit (BioVision). Cells (5 × 10⁵) were exposed to different concentrations of Thevebioside for 48 h. After resuspending in 500 ml binding buffer, cells were incubated with Annexin V–fluorescein isothiocyanate (FITC; 5 ml) and PI (5 ml). After 30-min incubation, cells were analyzed by fluorescence-activated cell sorting (FACS) by flow cytometer (Becton Dickinson). Annexin V–FITC–stained cells showed early apoptosis and cells with Annexin V–FITC- and PI double positive signals were combined for analysis.
**Colony Formation Assay**

Five hundred cancer cells were seeded into 6-well plates per well and then treated with Thevebioside at different concentrations for 48 h. After treatment, the cells were allowed to form cell colonies for another 7 days. Cell colonies were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. After washes and air-dried, the stained colonies were counted and photographed under a microscope (Leica, Germany).

**Hoechst Staining**

Hoechst 33342 was used to identify apoptotic cells based on previously described morphological changes in nuclear assembly. A wavelength of 350/461 nm was used. Cells were rinsed with PBS buffer and stained with Hoechst 33342 (final concentration, 18 µM) for 10 minutes. After staining with Hoechst 33342, the morphological aspects of cell nuclei were observed with a fluorescence microscope.

**Western Blot**

After treatment, the cells were collected and washed in 0.01M cold PBS and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 10% glycerol, 1 mM EDTA, 1 mM MgCl2, and 0.5% sodium deoxycholate) with a complete protease inhibitor cocktail and PMSF (Gibco, NY, USA). Following centrifugation for 15 min at 20,000 g at 4 °C. After total protein concentration was measured by Bradford method (Bio-Rad, Hercules, CA) and then all lysates were diluted to the same concentration. The cell lysates were boiled in a gel-loading buffer (20% glycerol, 10% β-mercaptoethanol, 6% SDS, 125 mM Tris–HCl, pH 6.8, 0.005% bromophenol blue; 1:5) at 95 °C for 10 min. The protein extracts were resolved by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (BioRad). Membranes were incubated in TBS blocking buffer containing 5% milk for 1 h at room temperature before an overnight incubation at 4 °C with various primary antibodies diluted in TBST (containing 0.1% Tween 20 and 2% BSA). Blots were washed and rinsed HRP-conjugated secondary antibody at a 1 : 5,000 dilution. Reactive bands were visualized by the Chemidoc Imaging System (BioRad; Hercules, CA) using ECL (Invitrogen) and analyzed by Image J software.

**Co-immunoprecipitation Assay**

Cells were washed with ice-cold phosphate-buffered saline and lysed in a lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma). Cell lysates were incubated with antibody overnight at 4 °C and precipitated with 20 µl protein G-Sepharose 4 Fast flow (Thermo, Waltham, MA, USA) for 4 h at 4 °C. The co-immunoprecipitates were washed four times with the lysis buffer and boiled for 5 min at
100 °C in protein loading buffer. Using anti-Ub (FK2) antibody and SRC-3 ubiquitination was detected by western blot using anti-SRC-3 antibody.

**Rna Isolation And Real-time Pcr**

Total RNAs were extracted using the RNeasy Mini kit (Qiagen) and were reversed transcribed into cDNAs using iScript cDNA synthesis kit (Bio Rad). For RT–qPCR analyses, KicqStart SYBR Green predefined primers (Sigma) were used for the following gene: F—5′-CTGGTGTTGCTGCTGCTGATG-3′, R—5′-GCTGCTGTTGCTGCTGCTGTTG-3′ for SRC-3. The relative expression of the mRNA level was normalized against the internal control GAPDH: F—5′-GGACCCTCATGGCTGAGAAC-3′, R—5′-GGTGGCAGTGATGGCATGGAC-3′ gene (ΔCt = Ct (target gene) – Ct (internal control gene)). Real-time PCR was performed using 1 × TaqMan Universal PCR Master Mix (Applied Biosystems). The relative fold change was measured by 2 − ΔΔCt formula compared with the control cells. Means and differences of the means with 95% confidence intervals were obtained using GraphPad Prism (GraphPad Software Inc.). Two-tailed Student’s t test was used for unpaired analysis comparing average expression between conditions.

**Animal Experiment**

All animal experiments were followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal study was approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. Nude mice (6 weeks, 18 g) were obtained from Shanghai SLAC Laboratory Animal (China). After one-week acclimation, for the orthotopic lung cancer mode, 1 × 10⁶ A549 cells in 100 ul mixture of PBS and Matrigel (Corning) (1: 1) were injected intrapulmonary in nude mice. These mice were imaged utilizing the Luminall Living Image 4.2 weekly to observe tumor growth. For subcutaneous tumor bearing model, 3 × 10⁶ A549 cells in 100 ul PBS were inoculated in the right flank of nude mice. Tumor volum were assessed every 4 days after inoculation. Different groups received relatively an intraperitoneal (i.p.) injection of 100 ul PBS as control, 2 mg/kg Digoxin as positive control, 2 mg/kg of Thevebioside, Neritaloside or Odoroside H three times a week. After 5 weeks of treatment, mice were euthanized with CO₂ to weigh the tumors. Terminal blood was collected through the inferior vena cava. Tumors and the other organs including lungs, livers, kidneys and hearts were collected, embedded in 10% formalin and then fixed in paraffin, stained with hematoxylin–eosin (H&E) to examine the changes of organ histomorphology.

**Immunohistochemical (ihc) Staining**

Paraffin-embedded sections were dewaxed in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by incubating sections in citrate buffer pH 6 (Invitrogen) using microwaves. Sections were blocked with 6% horse serum and incubated with primary antibodies. Biotinylated secondary antibody—
horseradish peroxidase conjugate (Vector Labs) was used at a dilution of 1:200. The sections were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin (Sigma-Aldrich). After dehydration and fixation in Permount, images were captured under light microscopy.

**Statistical analysis**

Comparison between two means was done by 2-tailed t-test or non-parametric 2-tailed Mann-Whitney t-test. Comparisons between three or more means were done by 1-way ANOVA test with multiple comparisons post-test. Survival test was done by Log-rank (Mantel-Cox) test. Graphs were generated and statistical analysis were performed with Prism 7 (GraphPad) and P value < 0.05 was considered to be statistically significant.

**Results**

**THB exhibits the most potent cytotoxicity to NSCLC cells through a high-throughput screening assay**

In our previous studies, we established a new luminescence-based high-throughput screening system[11], which could detect percent lysis of tumor cells and its viability with natural products treatment (Fig. 1A). After our screening for 2,880 natural candidates, there are three cardiac glycosides from Nerium oleander caught our attention, including THB, Neritaloside and Odoroside H, which possess good anti-tumor activity (Fig. 1B-C). To further investigation, we used MTT to evaluate the inhibitory effect of these three active compounds on NSCLC cells (A549) under the various concentration in 24 h, in which we found THB (a monomeric composition extracted from the Traditional Chinese Medicinal herb TPKS) showed the best suppressive ability with the dose dependent manner (Fig. 1B-C). While the results in animal study also indicated that THB had the best antitumor effect in NSCLC bearing mice, no matter in tumor volume and tumor weight, which is consistent with the in vitro data (Fig. 1D-F).

**THB inhibits NSCLC cell growth in dose and time dependent manners without overt cytotoxic effect**

To further verify our results, we applied different NSCLC cell lines (A549, H460, H1299, H1650, H1975 and CALU-1) and human normal lung epithelial cell line (BEAS-2B) to observe the viability of these cells after receiving THB treatment with 24 h, 48 h, 72 h under the same concentration (60 nM) which based on the results in Fig. 1C. However, we found that although THB had a certain inhibitory effect on the cellular activity of these NSCLC cells, A549 and H460 were the most obvious with the increasing time (Fig. 2A). At meanwhile, it was confirmed again that THB is more sensitive to NSCLC cell (A549) than the other two natural candidates Neritaloside and Odoroside H (Fig. S1). Then, based on the above results, we further tested whether there was concentration dependence with THB treatment of these NSCLC cells. Our findings showed that THB was sensitive to these different NSCLC cells (A549, H460, H1299, H1650,
H1975 and CALU-1) compared with the BEAS-2B, especially in A549 and H460 cells, as concentrations increase, which was in line with the previous data. This indicated that the inhibitory effect of THB on NSCLC cells was time as well as dose-dependent (Fig. 2B). Next, we further used the cell cloning assay to support this suppressive activity of THB and further to observe the effect of different concentration of THB (0, 30, 60 or 120 nM) on the cell clone formation of NSCLC cells (A549 and H460). Quantitative analysis further revealed that colony numbers decreased with increased THB dosage, which was reconciled with the previously mentioned concentration dependence (Fig. 2C-D). Besides, based on our results, THB did not affect the survival of normal lung epithelial cell BEAS-2B whether over time or concentration, indicating THB possess tumor inhibitory activity with minimal cytotoxic effect (Fig. 2A-B).

**THB induces the process of cellular apoptosis in NSCLC cells**

Previously, both the MTT assay and cell cloning assay proved that THB could inhibit the growth of NSCLC cells (A549 and H460). Then, we further investigated whether THB inhibits NSCLC cells growth via cell cycle disturbance detected by flow cytometry. The findings showed that THB (0, 30, 60 or 120 nM) had no significant effect on the cell cycle arrest (Fig. S2). In this case, based on the data, cell proliferation was remarkably inhibited after THB treatment, and was not associated with cell cycle arrest, so it was reasonable to hypothesize whether the administration of THB contributed to cell apoptosis. To test this hypothesis, hoechst 33342 staining was applied to detect the induction of apoptosis of these NSCLC cells. When A549 and H460 cells were exposed to various concentrations of THB (0, 30, 60 or 120 nM) for 48 h, the number of apoptotic cells, characterized by condensed and fragmented nuclei, increasing with the dose of THB (Fig. 3A-B). In addition, we also used Annexin V/PI double staining and flow cytometry to analyze the cellular apoptosis in NSCLC cells with the same concentrations of THB above mentioned. Cells in the third quadrant (Annexin V-/PI-) were viable, and in the second quadrant (Annexin V-/PI+) were necrotic, whereas those in the first (Annexin V+/PI+) and fourth quadrants (Annexin V+/PI-) represented late and early apoptotic cells, respectively. As shown in Fig. 3C-D, THB distinctly reduced the population of surviving cells and significantly increased the count of both early and late apoptosis of A549 and H460 cells with a dose dependent manner. These results implied that the augment of NSCLC cells (A549 and H460) post THB application by promoting the process of cellular apoptosis.

**THB-induced apoptosis via inhibiting SRC mediated signaling pathway in NSCLC cells**

To investigate the underlying molecular mechanism of THB induced apoptosis on A549 and H460 cells, we evaluated the expression of apoptosis-related proteins (p-AKT, AKT, cleaved-PARP, cleaved-caspase-3, Bad, and Bcl-xl) using western blot analysis after treatment of cells with various concentrations (0, 30, 60 or 120 nM) of THB for 48 h. As shown in Fig. 4A, treatment with THB resulted in the upregulation of Bad, cleaved-caspase-3 and cleaved-PARP expression, and downregulation of SRC, p-AKT, and Bcl-xl level in a
Notably, we found that the protein expression of nuclear receptor coactivators SRC which is closely related to the occurrence and development of NSCLC also decreased significantly with the increasing concentration of THB by western blot detection. Since there are three subtypes of SRC (SRC-1, SRC-2 and SRC-3), overexpression of SRC-3 has been reported to be more associated with tumorigenesis and it is closely related to the poor prognosis of NSCLC patients[14, 15]. Moreover, SRC-3 is significantly high expressed in NSCLC cells (A549 and H460) than in normal lung epithelial cells (BEAS-2B) (Fig. S3). Therefore, we went further to verify which subtype of SRC is regulated by the treatment of THB. The western blot results showed that SRC-3 (not SRC1 or SRC2) was significantly down-regulated after THB treatment and also exhibited obvious concentration dependence, which was consistent with previous research (Fig. 4B-C). What’s more, it is reported that the inhibition of SRC-3 could induce cell apoptosis[16]. Thus, we proposed the hypothesis that SRC-3 may play a vital role in THB-induced NSCLC cellular apoptosis.

**THB mediated apoptosis by down-regulating SRC-3 through the ubiquitin-proteasome degradation pathway**

In order to test our hypothesis, we will further explore how SRC-3 plays a role in inducing apoptosis. Why SRC-3 protein levels of NSCLC cells are downregulated after THB treatment? First, we detected the mRNA level of SRC-3 and found that there was no significant change in either A549 or H460 cells after THB treatment for 48 h (Fig. 5A). Then we reasoned that it may be because the stability of the protein was destroyed and protein degradation occurred after THB administration. It is well known that the ubiquitin-proteasome pathway plays an important role in protein degradation[17, 18]. What’s more, studies have reported that SRC-3 can be degraded by ubiquitin-proteasome-dependent pathways[19]. Therefore, we used CHX (cycloheximide, a broad-spectrum and nonspecific protein synthesis inhibitor) to pretreat the harvested NSCLC cells (A549 and H460) for 0 h, 6 h, 12 h, 24 h with and without THB (0 nM, 60 nM). Then, protein expression was analyzed by western blot. We found that CHX exposure alone can significantly reduce the expression of SRC-3. Meanwhile, after THB and CHX co-treatment, the decrease of the protein level of SRC-3 can be accelerated. And both of them showed a time-dependent manner (Fig. 5B-C). The result confirmed that THB-induced down-regulation of SRC-3 at the protein level. Moreover, THB mediated SRC-3 protein degradation was abrogated by the treatment with proteasome inhibitor, MG132 (Fig. 5D-E). The above findings indicated that ubiquitin–proteasome system was involved in the degradation of SRC-3. Furthermore, Annexin V/PI staining was used to detect the effect of THB-mediated apoptosis by down-regulating SRC3 expression in A549 and H460 cells after MG132 administration. The results showed that MG132 could almost completely reverse THB induced apoptosis and increase the survived cell population, which further indicated that THB induced cellular apoptosis by the degradation of SRC-3 (Fig. 5F). To further support this hypothesis, co-immunoprecipitation assay was applied to detect the level of polyubiquitination, which is a signature of protein being degraded via ubiquitin-mediated proteasome system. Consistently, SRC-3 was confirmed to constitutively ubiquitinate in response to THB treatment (60 nM) (Fig. 5F). Taken together, the results demonstrated that THB
mediated apoptosis by inducing the decreased protein expression of SRC-3 through the ubiquitin-proteasome dependent degradation pathway.

**THB inhibited the growth of NSCLC xenografts in nude mice**

The above-mentioned data are from in vitro, and we will take further exploration to verify the inhibitory effect of THB on tumor-bearing mice. The orthotopic lung cancer model was established by intrapulmonary injection with $1 \times 10^6$ A549 cells in nude mice. It is well known that THB belongs to cardiac glycosides, and many studies have shown that digoxin, the most classic representative of cardiac glycosides, has a good effect on tumor inhibition\[20\]. Therefore, digoxin as a positive control, tumor-bearing mice were received i.p. injection of digoxin and THB at the same concentration (2 mg/kg) three times a week for 30 days. Compared with the PBS control group, we found that THB had the resemble anti-tumor capability as digoxin or even better (although there was no statistical difference between the two), detecting by the luminescence live imaging system (Fig. 6A-B). To further verify these results, we rapidly collected fresh lung tissues from tumor-bearing mice and performed optical imaging. The results also showed better suppression of THB, but there was no significant difference between them, which consistent with the previous data (Fig. 6C-D). The results of H&E staining of the lung tissues from PBS control, THB and digoxin group also supported the above conclusions (Fig. 6F). In addition, although both digoxin and THB can significantly prolong the survival of tumor-bearing mice, the trend of THB is more pronounced (Fig. 6E). What's more, we also subcutaneously inoculated A549 cells, and the tumor inhibition and survival of tumor-bearing mice after THB treatment were consistent with the data of orthotopic lung cancer model (Fig. S4). All these data indicate that THB has a good effect on inhibiting tumor growth and prolonging survival of lung tumor-bearing mice.

**The tumor suppressive effect of THB in vivo via SRC-3-mediated AKT signaling pathway with minimal toxicity**

In order to further confirm whether the results of in vitro experiments are consistent with those in vivo, lung tissues of orthotopic tumor bearing mice were collected and either embedded for immunohistochemical (IHC) staining or ground for western blot analysis. According to the results, the expression of SRC-3 (not SRC-1 or SRC-2) other anti-apoptotic proteins p-AKT, Bcl-xl were significantly down-regulated after treatment with THB, while the expression levels of pro-apoptotic proteins cl-Caspase-3, Bad and cl-PARP were correspondingly increased using western blot detection (Fig. 7B). In addition, IHC was also used to observe the expression of a series of related proteins, and we found that SRC, p-AKT, Bcl-xl and Ki67 were significantly decreased, while cl-Caspase-3 was significantly increased, which was consistent with in vitro data (Fig. 7A). Next, we examined its effects on the histological structure and biochemical function of the liver, kidney, and heart after 30 days THB treatment in tumor bearing mice. The data showed that the histological structure of these organ tissues and its corresponding biochemical function indicators had no significant changes compared with the PBS control group. Also,
the weight of mice was monitored weekly, and it was found that THB administration did not affect it, indicating that THB had minimal toxicity (Fig. 7C-E). Above mentioned results suggest that THB has a good tumor-inhibiting effect in vivo through the SRC-3-mediated AKT signaling pathway, which is in line with the in vitro results (Fig. 8), and at meanwhile has little toxic effect on the liver, kidney and heart in this model.

Discussion

Accumulating reports show that TCM as an effective complementary and alternative medicine to treat cancer patients has well been accepted worldwide. Since the obvious advantages of TCM in improving the life quality of cancer patients and prolonging their survival with minimal toxicity, more and more attention has been paid to TCM formulas, herbs and their active ingredients in anti-cancer field[21, 22]. On the basis of our previous study, we used a new luminescence based high-throughput screening method, screening 2880 small molecule compound of natural products. The three kind of cardiac glycoside compounds has attracted our attention, which showed a good role in tumor suppression. Among them—THB was considered to possess the strongest inhibitory activity in NSCLC with the obvious time and concentration dependence (Figs. 1 & 2). It is well known that THB is an active component extracted from the seeds of yellow oleander—which show TCM effects of strengthening heart, diuretic, detoxification and detumescence. According to the available data, as far as we know, we are the first to explore the anti-tumor effect of THB in lung cancer.

According to our results, we found that THB had a significant inhibitory effect on the growth of NSCLC (A549 and H460) cells via inducing the process of cellular apoptosis confirmed by Hoechst 33342 staining, Annexin V/PI, and western blot (Fig. 3–5). Moreover, in the results of western blot, we found that SRC, a protein closely related to tumor progression, was also significantly down-regulated after THB administration along with apoptotic proteins p-AKT and Bcl-xl, and the corresponding the level of Bad, cleaved caspase3 and cleaved PARP were obvious up-regulated. Confirmed by our further protein detection, SRC3 (not SRC1 or SRC2) is remarkably diminished, with the increase of THB concentration. It is well known that SRC3, a member of the p160 SRC family (including SRC1, SRC2 and SRC3) regulates the transcriptional functions of nuclear receptors and other transcription factors, modulating cell proliferation, survival and growth[23, 24]. And the overexpression of SRC3 was reported to induce tumorigenesis and chemoresistance by modulating cancer-related signaling pathways, promoting the expression of anti-apoptotic proteins and inhibiting the expression of pro-apoptotic proteins[25]. In our study, it was found that the THB-induced apoptosis process is due to inhibition of the SRC-3 mediated AKT signaling pathway, which also in keeping with our animal study results (Fig. 7). Next, we further explore why the protein expression of SRC3 is significantly down-regulated with THB administration. Firstly, we tested the mRNA level of SRC3 and found that it did not alter obviously after THB treatment in NSCLC (A549 and H460) cells, indicating that SRC3 itself may have degraded. Then, we applied CHX, a protein synthesis inhibitor, to induce the decrease of SRC3 protein expression, but the co-treatment of CHX and THB could further promote the reduction of SRC3, while the proteasome inhibitor MG132 could restore the down-regulated effect of THB on SRC-3 and inhibit THB-mediated apoptosis. Subsequent co-
immunoprecipitation assay was used to confirm that SRC3 indeed undergo ubiquitin-proteasome pathway-dependent protein degradation after THB treatment (Fig. 5 & Fig. 8). However, silencing SRC-3 can be further tested in vitro as well as in vivo to further verify whether SRC-3 plays a key role in THB-mediated apoptosis. And whether the application of SRC-3 inhibitors will improve the tumor suppression efficiency when combined with THB. In addition, it has been reported that overexpression of SRC-3 will greatly increase the resistance of chemotherapeutic drugs to tumor patients[14]. Then, if THB is used in combination with chemotherapy drugs, will this resistance be reduced? These are the directions in which we will conduct in-depth research on the antitumor activity of THB mediated by SRC-3 in the future.

Due to the chemical structure of THB similar to digoxin in cardiac glycosides, it is reasoned that THB may have positive inotropic effects like digoxin. However, there are few reports on this aspect. Besides, digoxin also showed good antitumor activity in multiple cancer types [26–28]. Therefore, we used digoxin as a positive control and found that both THB and it have a strong inhibitory effect on lung cancer, but THB appeared to show better tumor suppressive effect in NSCLC mouse model without significant difference. Moreover, THB has also shown drug safety as an anti-tumor agent, which not only had little inhibitory effect on normal lung epithelial cells in vitro, but also without overt toxicity in liver, kidney and heart of mice in vivo (Figs. 6 & 7).

**Conclusion**

To our knowledge, this is the first time to reveal the tumor suppressive effect of TCM monomer THB on NSCLC which screened from our innovative high-throughput screening system. According to the results, THB treatment contributes to the process of cellular apoptosis via inhibiting the activation of the AKT signaling pathway through the ubiquitin-proteasome-dependent degradation of SRC-3, which may provide scientific research support for THB as a potent anti-tumor approach.

**Abbreviations**

**NSCLC**, Non-small cell lung cancer

**TCM**, Traditional Chinese medicine

**THB**, Thevebioside

**TPKS**, Thevetia peruviana (Pers) K. Schum

**OS**, Overall survival

**DMSO**, Dimathy1 sulfoxide

**PBS**, Phosphate buffered saline

**CHX**, Cycloheximide
IHC, Immunohistochemical
ALT, Alanine transaminase
CK, Creatine kinase
AST, Aspartate transaminase
CREA, Creatinine
MST, Median survival time

Declarations

Ethics approval and consent to participate

All studies involving animals were conducted in compliance with the Shanghai University of Traditional Chinese Medicine (SHTCM)'s ethical guidelines as approved by the Animal Ethics Committee (SHTCM, shanghai). The approvalumber obtained: Authorization: PZSHUTCM191129002.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Chao Yao: Investigation, data analysis and interpretation, project management, writing - original draft, writing - review & editing, conceptualization. Lin Su: Data acquisition, methodology, project management, manuscript writing, review and / or revision. Fei Zhang: Conception and design, methodology, project administration. Xiaowen Zhu: Investigation, methodology. Yangzhuangzhuang Zhu: Conceptualization. Xiaoning Jiao: Project administration. Yifei Hou: Project administration. Xiao Chen: Project administration. Chenyuan Gong: Project administration. Lixin Wang: Project administration. Xuewei Yan: Investigation. Wanyi Wang: Investigation. Chunpu Zou: Project administration, conceptualization, funding acquisition. Shiguo Zhu: Study supervision, conceptualization, funding acquisition. Zihang Xu: Conception and design, development of methodology, administrative, technical or material support, writing - original draft, writing - review & editing, conceptualization, funding acquisition.

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Figures
Figure 1

Schematic of high-throughput screening of natural products and the antitumor effect of THB in vivo and in vitro. A-B. A549 cells were cultured in 384-well plates treated with 2,880 natural products respectively for 24 hours. The luminescence signal was detected. Percent viability was calculated as mentioned. Thevebioside, Neritaloside and Odoroside H were found to be candidates for inhibiting NSCLC cells through 2,880 natural products. C-A549 cells were treated with various concentrations (0, 7.8, 15.6, 31.3, 62.5, 125, 250 or 500 nM) of Thevebioside, Neritaloside or Odoroside H respectively for 24 hours, cell viability was assessed by MTT assay. D-F. Tumor xenografts were established by subcutaneous implantation of 3×10^6 A549 cells into the flank of nude mice. Mice were i.p. injected with 2 mg / kg Thevebioside, Neritaloside or Odoroside H respectively at interval day 3 days after implantation. Tumor volumes were monitored every 4 days, mice were sacrificed on day 40 before tumors were excised and weighed (n=5, each). Data are means ± SD of at least three independent experiments. NS, non-statistical significance; *, p<0.05; **, p<0.01; ***, p <0.001.
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Inhibitory effect of THB on NSCLC cells and non-toxic effect on lung epithelial cells in vitro. A. A549, H460, H1299, H1650, H1975, CALU-1 and BEAS-2B cells were treated with 60 nM THB for 24, 48, and 72 h. Cell viability was assessed by MTT assay. B. A549, H460, H1299, H1650, H1975, CALU-1 and BEAS-2B cells were treated with THB (0, 7.8, 15.6, 31.3, 62.5, 125, 250 or 500 nM) for 48 h respectively. Cell viability was determined by MTT assay. C-D. A549 and H460 cells were treated with THB (0, 30, 60 or 120 nM) for 48 h and then allowed to form colonies in another 7 days. The results were observed under a microscope (×200). Data are means ± SD of at least three independent experiments. NS, non-statistical significance; *, p <0.05; **, p <0.01; ***, p <0.001.
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Figure 3

THB induces apoptosis in human lung cancer cells. A-B. Hoechst 33342 staining was used to detect cell apoptosis. A549 and H460 cells were treated with THB (0, 30, 60 or 120 nM) for 48 h. The results were observed under a microscope (× 200). C-D. Annexin V/PI staining was used to detect cell apoptosis efficacy of A549 and H460 cells after incubated with THB (0, 30, 60 or 120 nM) for 48 h. All images are representative of three independents experiments with similar results. Data are means ± SD of at least three independent experiments, *, p<0.05; **, p<0.01; ***, p<0.001.
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Figure 4

THB induces apoptosis of NSCLC cells through SRC-mediated AKT signaling pathway A-B. A549 and H460 cells were treated with THB (0, 30, 60 or 120 nM) for 48 h, western blot was applied to detect the protein expression of SRC, P-AKT, AKT, Bcl-xl, Bad, cl-Caspase 3, cl-PARP, β-actin, SRC-1, SRC-2, SRC-3.
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Figure 5

THB promotes proteasome-mediated degradation of the SRC-3 protein. A. A549 and H460 cells were incubated with THB (0, 30, 60, and 120 nM) for 48 h, Gene expression of SRC-3 was detected by q-PCR. Data are means ± SD of at least three independent experiments, NS, non-statistical significance. B-C. A549 and H460 cells were treated with cycloheximide (CHX; 1ug/mL) and THB (0, 60 nM) for 0, 6, 12, 24 hours respectively, the protein expression of SRC-3 was detected by western blot. D-E. A549 and H460 cells were changed to medium supplemented with 0.5% stripped FCS, penicillin, and streptomycin (100 U/mL) overnight, and then incubated with THB (0, 60 nM) in the absence or presence of 10 umol/L MG132 for 48 hours, the protein expression of SRC-3 was detected by western blot. All images are representative of three independent experiments with similar results. F. Annexin V/PI staining was used to detect cell apoptosis efficacy of A549 and H460 cells after incubated with THB (0, 60 nM) in the absence or presence of 10 umol/L MG132. G. A549 cells were transfected with Flag-tagged ubiquitin before stimulated with or without THB (60 nM) for 48h. Whole cell lysates (WCL) were immunoprecipitated (IPed) using an anti-Flag affinity resin, western blotting was performed in both immunoprecipitates (IPs) and WCL. Data are means ± SD of at least three independent experiments. NS, non-statistical significance; *, p <0.05; **, p <0.01; ***, p <0.001.
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Figure 6

The inhibitory effect of THB on orthotopic lung cancer mice. A-D. An orthotopic lung cancer model established by intrapulmonary injection of $1 \times 10^6$ A549 cells in nude mice, i.p. injected with digoxin (2 mg/kg), THB (2 mg/kg) or vehicle (PBS) from day 3 onwards. Both ex vivo and in vivo optical images were taken by the luminescence live imaging system. E. H&E staining was applied to detect tumor growth. The results were observed under a microscope ($\times 3.6$ and $\times 200$). F. The survival time of tumor-bearing mice in all groups was recorded. MST (median survival time) was calculated ($n=5$, each). Data are means ± SD of at least three independent experiments. NS, non-statistical significance; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
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The tumor suppressive effect of THB in vivo via SRC-3-mediated AKT signaling pathway with minimal toxicity. A. Tumor-bearing mice were treated with THB (2 mg/kg or 4 mg/kg) or vehicle at interval day. IHC was used to detect the expression of SRC, p-AKT, Bcl-xl, cl-Caspase-3 and cl-PARP in lung. The results were observed under a microscope (× 200). B. Western blot was applied to detect the protein expression of SRC-1, SRC-2, SRC-3, P-AKT, AKT, Bcl-xl, Bad, cl-Caspase 3, cl-PARP in tumor. C. H&E staining was used to detect the toxicity of THB (0, 2 mg/kg, 4 mg/kg) in heart, liver and kidney. The results were observed under a microscope (× 200). D. After treated with THB (0, 2 mg/kg, 4 mg/kg), the concentration of ALT, CK, AST and CREA in serum was detected using a chemistry analyzer (Acctue TBA-40FR TOSHIBA Japan). E. After treated with THB (0, 2 mg/kg, 4 mg/kg), the body weight of all mice was recorded every 4 days for 4 weeks (n=5, each). Data are means ± SD of at least three independent experiments, NS, non-statistical difference.
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Figure 8

THB induces lung cancer cell apoptosis by triggering the activation of SRC3-AKT-mediated signaling pathway. THB is an active ingredient from the seed of Thevetia peruviana (Pers) K. Schum (TPKS). THB treatment contributes to the process of cellular apoptosis in NSCLC cells via inhibiting the activation of the AKT signaling pathway through the ubiquitin-proteasome-dependent degradation of SRC-3, thus to inhibit lung cancer cells growth.
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