

Cryptotanshinone inhibits lung tumor growth by increasing CD4⁺ T cell cytotoxicity through activation of the JAK2/STAT4 pathway

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Abstract. Cryptotanshinone is one of the fat-soluble phenanthrene quinone components. *In vitro* studies have shown that tanshinone compounds can inhibit the proliferation of various tumor cells and affect cell cycle distribution. The aim of the present study was to better understand the effect of cryptotanshinone on the inhibition of small cell lung cancer by cytotoxic cluster of differentiation (CD)4⁺ T cells through activation of the Janus kinase 2/signal transducer and activator of transcription 4 (JAK2/STAT4) pathway. The Cell Counting kit-8 assay and the lactate dehydrogenase assay were used to analyze the cell proliferation of H446 and CD4⁺ T cells, and the cell cytotoxicity of CD4⁺ and CD8⁺ T cells, respectively. JAK2 and STAT4 protein expression was measured by western blot analysis. Cryptotanshinone effectively inhibited the tumor growth of the H446 cells and the cell proliferation of the CD4⁺ T cells. Treatment with cryptotanshinone increased the cytotoxicity of the CD4⁺ T cells, but could not affect the cytotoxicity of the CD8⁺ T cells. Meanwhile, cryptotanshinone induced phosphorylated (p)-JAK2 and p-STAT4 protein expression in the CD4⁺ T cells. These results suggest that cryptotanshinone inhibits the cell growth of lung tumors by increasing CD4⁺ T cell toxicity through activation of the JAK2/STAT4 pathway.

Introduction

Lung cancer is a highly malignant disease and the incidence is increasing annually (1). With the enhancement and popularity of efforts aimed at educating individuals about not smoking, this incidence rate of smoking has shown a declining trend. According to the Centers for Disease Control and Prevention

statistics, there were ~172,570 new cases of lung cancer in the United States in 2005, of which ~93,010 were men and ~79,560 were women; for the two genders, the disease was ranked in second place among all the newly added tumor diseases (prostate cancer was ranked first for men and breast cancer for women) (2). At that time, among all cancer-related mortality cases in the United States, men with lung cancer accounted for 31% of cases and women for 27%. At present, the annual number of newly diagnosed lung cancer cases has reached 210,000. However, the incidence rate of male lung cancer in the United States has declined for the first time, with the female incidence unchanged. In addition to a reduction in the overall incidence of lung cancer, the proportion of small cell lung cancer (SCLC) has decreased by 13-20% (3).

The occurrence and development of SCLC involves many factors, including environmental microbiology and inflammation, accompanied by the participation of the immune response and the disorder of ultimate immune surveillance mechanisms. Cluster of differentiation (CD)4⁺ regulatory T cells are a class of T-cell subsets with immune function, closely associated with the tumor immune escape process (4). In recent years, it has generally been considered that transcription factor forkhead box P3 (Foxp3) is a characteristic sign of CD4⁺ regulatory T cells and is a key transcription factor for obtaining immunomodulatory properties (5). Foxp3 transforms primary CD4⁺ T cells into CD4⁺ regulatory T cells, and the normal expression of the gene is built around the premise that CD4⁺ regulatory T cells play a role (6). The Janus kinase 2/signal transducer and activator of transcription 4 (JAK2/STAT4) signaling pathway is activated continuously and abnormally activated in a variety of tumor tissues and cell lines, which promotes tumor growth, invasion, angiogenesis and metastasis (1). Increased activation of the the JAK2/STAT4 signaling pathway has been demonstrated in a variety of tumors, including stomach and liver cancer (2).

STAT4 is the direct substrate of JAKs, which transmits signals directly to the nucleus to regulate the expression of specific genes (2). STAT4 is closely associated with tumor formation and development (3). The STAT4 protein consists of ~770 amino acids and is involved in cell growth, development, cell division and differentiation (3). The JAK2/STAT4 signaling pathway is important for cytokine signal transmission following the activation of Ras, which exhibits an important function in the regulation of various pathological

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and physiological processes, including cell proliferation, differentiation, apoptosis, immune regulation and inflammation (4).

Cryptotanshinone is a diterpene quinone compound, separated and extracted from the dried roots and rhizomes of all *Salvia* plants (Labiatae family) (7). The total tanshinone extract, with cryptotanshinone and tanshinone IIA as the main components, not only has pharmacological cardiovascular (8), anti-oxidation (9), anti-bacteria (10) and anti-inflammation (7) effects, but also has significant anti-tumor effects (11). However, to the best of our knowledge, there have been no published studies regarding the inhibition of lung tumor cell growth by cryptotanshinone through regulation of the immune system. Thus, the present study investigated whether the anticancer effect of cryptotanshinone inhibits the tumor growth of human SCLC H446 cells by affecting CD4⁺ T cell cytotoxicity through activation of the JAK2/STAT4 pathway.

Materials and methods

Materials. Cell Counting kit 8 (CCK-8), anti-interferon (IFN)- γ and concanamycin A (CMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The Enhanced Bicinchoninic (BCA) Acid Protein assay kit was purchased from KeyGen Biotech Co., Ltd. (Nanjing, China).

Cell culture and cell proliferation. Human SCLC H446 cells and splenocytes of wild-type (WT) C57BL/J6 mice were obtained from the Center for Animal Experiments of Wuhan University (Wuhan, Hubei, China). The H446 cells were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. CD4⁺ T cells were freshly isolated from WT mice and then seeded at 1x10⁴ cells/ml (CD4⁺ T cells/splenocytes/H446 cells) into a 96-well plate, and treated with 5, 10 or 20 μ M of cryptotanshinone (purity, >90%; Sigma-Aldrich) for 24, 48 or 72 h. CCK-8 solution (10 μ l) was added to the cells, which were incubated for 4 h in a humidified atmosphere of 5% CO₂ at 37°C; the optical density at 450nm was detected after 4 h using a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA).

In vitro cytotoxicity assay. CD4⁺/CD8⁺ T cells were acquired from the splenocytes of untreated tumor-bearing C57BL/J6 mice and H446 cells were prepared at 1x10⁴ cells/ml, with effector-target ratios from 1:1 to 50:1. Cell solution (~100 μ l) was seeded into a 96-well plate and incubated for 48 h in a humidified atmosphere of 5% CO₂ at 37°C. Next, cells were assigned randomly into four groups: i) CD4⁺ T cell group (CD4⁺; n=6), CD4⁺ T cells were treated with complete medium for 48 h; ii) CD4⁺ T cell + cryptotanshinone group (CD4⁺ + Cry; n=6), CD4⁺ T cells were treated with cryptotanshinone (10 μ M) for 48 h; iii) CD8⁺ T cell group (CD8⁺; n=6), CD8⁺ T cells were treated with complete medium for 48 h; and iv) CD8⁺ T cell + cryptotanshinone group (CD8⁺ + Cry; n=6), CD8⁺ T cells were treated with cryptotanshinone

(10 μ M) for 48 h. Meanwhile, 1x10⁴-5x10⁵ cells/ml cells were assigned randomly into four groups: i) CD4⁺ T cell group (CD4⁺; n=6), CD4⁺ T cells were treated with complete medium for 48 h; ii) CD4⁺ T cell + cryptotanshinone group (CD4⁺ + Cry; n=6), CD4⁺ T cells were treated with cryptotanshinone (10 μ M) for 48 h; iii) CD4⁺ T cell + cryptotanshinone + anti-IFN- γ group (CD4⁺ + Cry + IFN- γ ; n=6), CD4⁺ T cells were treated with cryptotanshinone (10 μ M) and anti-IFN- γ (5 μ g/ml) for 48 h; and iv) CD4⁺ T cell + cryptotanshinone + CMA group (CD4⁺ + Cry + CMA; n=6), CD4⁺ T cells were treated with cryptotanshinone (10 μ M) and CMA (50 ng/ml) for 48 h. Cell cytotoxicity was detected by lactate dehydrogenase assay (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocols. Optical density was read at 490 nm after 1 h. Percentage of cells killed was calculated using the following formula: Cytotoxicity (%) = (test sample - low control) / (high control - low control) x 100.

Western blot analysis. CD4⁺ T cells were acquired from splenocytes of untreated tumor-bearing mice and H446 cells were prepared at 1x10⁴ cells/ml, with effector-target ratios from 1:1 to 50:1. Cell solution (~0.5 ml) was seeded into 6-well plates and incubated for 48 h in a humidified atmosphere of 5% CO₂ at 37°C. Next, 1x10⁴-5x10⁵ cells/ml were assigned randomly into two groups: i) CD4⁺ T cell group (CD4⁺; n=6), CD4⁺ T cells were treated with complete medium for 48 h; and ii) CD4⁺ T cell + cryptotanshinone group (CD4⁺ + Cry; n=6), CD4⁺ T cells were treated with cryptotanshinone (10 μ M) for 48 h. All cells were washed with cold phosphate-buffered saline (PBS) and incubated with ice-cold lysis buffer for 30 min on ice. Lysates were centrifuged at 14,000 rpm (12,000 x g) for 10 min at 4°C. Protein concentration was determined using the Enhanced BCA Protein assay kit (KeyGen Biotech Co., Ltd.). An equivalent amount of protein was separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Membranes were incubated with PBS containing 0.5% skimmed dry milk to block non-specific binding. Next, the membranes were incubated with polyclonal rabbit anti-mouse JAK2 (1:1,000; #sc-294), polyclonal rabbit anti-human phosphorylated (p)-JAK2 (1:1,000; #sc-16566-R), polyclonal rabbit anti-mouse STAT4 (1:1,000; #sc-486), polyclonal rabbit anti-mouse p-STAT4 (1:1,000; #sc-22160-R) and monoclonal mouse anti-human β -actin (1:1,000; #sc-47778) (all Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibodies overnight at 4°C. The membranes were washed twice with Tris-buffered saline plus Tween 20 for 2 h at room temperature, and then incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (1:1,000; Santa Cruz Biotechnology Inc.) for 2 h at room temperature. Immunoreactive bands were visualized using Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific Inc.).

Statistical analysis. Statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean \pm standard deviation. Differences between the control and experimental groups were analyzed by analysis of variance followed by Tukey's range test. P<0.05 was used to indicate a statistically significant difference.

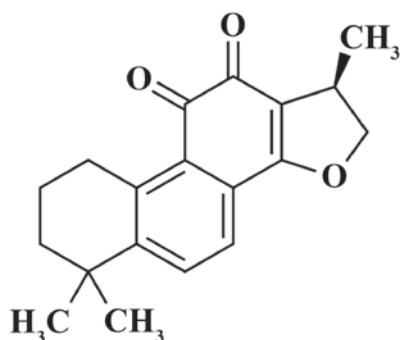


Figure 1. Chemical structure of cryptotanshinone.

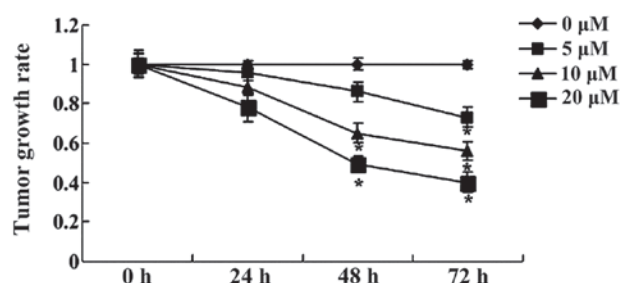


Figure 2. Cryptotanshinone inhibits the tumor growth of H446 cells *in vivo*. * $P < 0.01$ vs. 0 μM group.

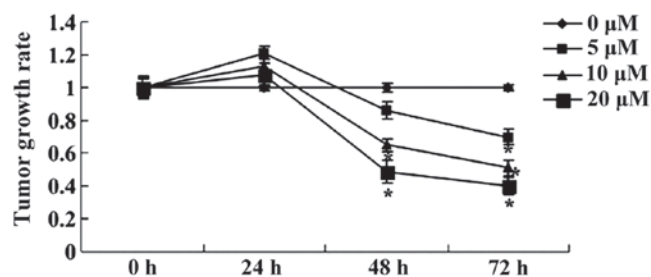


Figure 3. Cryptotanshinone inhibits cluster of differentiation (CD)4⁺ T cell proliferation. * $P < 0.01$ vs. 0 μM group.

Results

Cryptotanshinone inhibits tumor growth of H446 cells *in vivo*. The chemical structure of cryptotanshinone is indicated in Fig. 1. In order to investigate the anticancer effect of cryptotanshinone in the H446 cells, different concentrations (5, 10 and 20 μM) of cryptotanshinone were used to treat the H446 cells for 0, 24, 48 and 72 h. As shown in Fig. 2, treatment with cryptotanshinone significantly inhibited the growth of the H446 cells in a concentration- and time-dependent manner. Considering that after 48 h cryptotanshinone inhibited H446 cell growth by 40% at 10 μM ($P < 0.05$), this was chosen as the experimental concentration.

Cryptotanshinone inhibits CD4⁺ T cell proliferation. In order to probe the anticancer effect of cryptotanshinone in the CD4⁺ T cells, different concentrations (5, 10 and 20 μM) of cryptotanshinone were used to treat the CD4⁺ T cells for 0, 24, 48 and 72 h. As shown in Fig. 3, treatment with

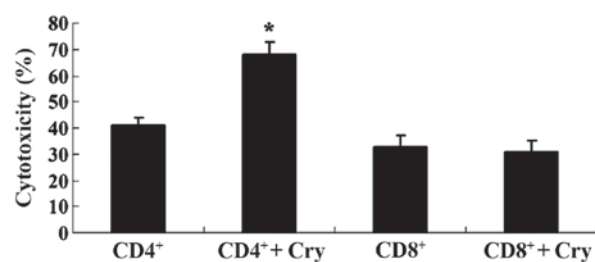


Figure 4. Cryptotanshinone (Cry) exploits cytotoxicity in CD4⁺/CD8⁺ T cells. * $P < 0.01$ vs. CD4⁺ group. CD, cluster of differentiation.

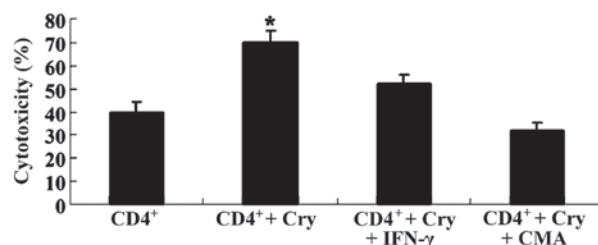


Figure 5. Cryptotanshinone (Cry) exploits cytotoxicity in CD4⁺ T cells. * $P < 0.01$ vs. CD4⁺ group. CD4, cluster of differentiation 4; IFN, interferon.

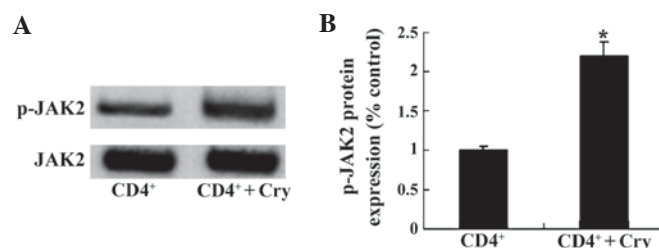


Figure 6. Cryptotanshinone induces p-JAK2 protein expression in CD4⁺ T cells. (A) Western blot analysis of cryptotanshinone showing induced p-JAK2 protein expression, and (B) statistical analysis of p-JAK2 protein level in CD4⁺ T cells. * $P < 0.01$ vs. CD4⁺ group. CD4, cluster of differentiation 4; p-JAK2, phosphorylated Janus kinase 2.

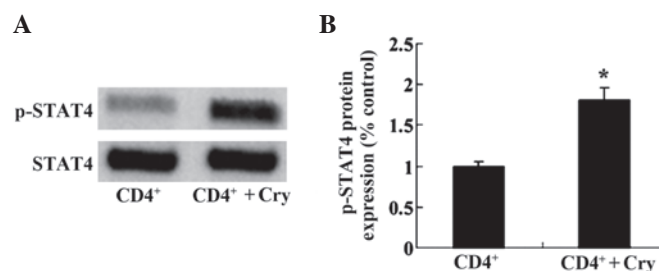


Figure 7. Cryptotanshinone (Cry) induces p-STAT4 protein expression in CD4⁺ T cells. (A) Western blot analysis of cryptotanshinone showing induced p-STAT4 protein expression, and (B) statistical analysis of p-STAT4 protein level in CD4⁺ T cells. * $P < 0.01$ vs. CD4⁺ group. CD4⁺ group. CD4, cluster of differentiation 4; p-STAT4, phosphorylated signal transducer and activator of transcription 4

cryptotanshinone significantly suppressed the growth of the CD4⁺ T cells in a concentration- and time-dependent manner. Considering that after 48 h cryptotanshinone inhibited CD4⁺ T cell growth by 40% at 10 μM ($P < 0.05$), this was chosen as the experimental concentration.

Cryptotanshinone exploits cytotoxicity in CD4⁺/CD8⁺ T cells. To expound the anticancer effect of cryptotanshinone on the cytotoxic CD4⁺T/ CD8⁺ T cells, a 10- μ M concentration of cryptotanshinone was used to treat the CD4⁺/CD8⁺ T cells for 48 h. As shown in Fig. 4, treatment with cryptotanshinone significantly increased the cytotoxicity of the CD4⁺ T cells ($P<0.05$). However, cryptotanshinone could not affect the cytotoxicity of the CD8⁺ T cells ($P>0.05$; Fig. 4).

Cryptotanshinone exploits cytotoxicity in CD4⁺ T cells. To analyze the anticancer effect of cryptotanshinone in the cytotoxic CD4⁺ T cells, a 10- μ M concentration of cryptotanshinone was used to treat the CD4⁺ T cells for 48 h. As shown in Fig. 5, treatment with cryptotanshinone significantly increased the cytotoxicity of the CD4⁺ T cells ($P<0.05$). However, the cytotoxicity of the CD4⁺ + Cry + IFN- γ and CD4⁺ + Cry + CMA groups were inhibited when compared with the CD4⁺ + Cry group.

Cryptotanshinone induces p-JAK2 protein expression in CD4⁺ T cells. To elucidate the anticancer effect of cryptotanshinone on the p-JAK2 protein expression of CD4⁺ T cells, a 10- μ M concentration of cryptotanshinone was used to treat the CD4⁺ T cells for 48 h. Administration of cryptotanshinone significantly increased the p-JAK2 protein expression of the CD4⁺ T cells ($P<0.05$; Fig. 6).

Cryptotanshinone induces p-STAT4 protein expression in CD4⁺ T cells. To elucidate the anticancer effect of cryptotanshinone on the p-STAT4 protein expression of the CD4⁺ T cells, a 10- μ M concentration of cryptotanshinone was used to treat the CD4⁺ T cells for 48 h. Administration of cryptotanshinone significantly induced the p-STAT4 protein expression of the CD4⁺ T cells ($P<0.05$; Fig. 7).

Discussion

SCLC grows rapidly and is extremely aggressive, and if the disease is not treated effectively in time, the patient will succumb within a few months (12). SCLC has the features of a high growth score and short doubling time, often with distant metastases at the diagnosis of the disease. SCLC tumor cells are sensitive to chemotherapy and radiotherapy (13), but decades of clinical trials have shown no effective way to completely cure SCLC, and the majority of patients will relapse or develop metastases following first-line therapy (14). Therefore, it is difficult to achieve long-term survival for patients with SCLC (15). In the present study, cryptotanshinone was found to significantly reduce the cell growth of H446 cells and the cell proliferation of CD4⁺ T in a concentration- and time-dependent manner. Li *et al* confirmed that cryptotanshinone suppressed the cell growth of breast cancer (16), mucoepidermoid carcinoma (17) and hepatoma (18).

For SCLC patients with lymph node metastasis, the positive degree of Foxp3 expressed in the lymphocytes is significantly higher than that of the non-metastatic lymph nodes (19). This may be since tumor growth promotes the proliferation and differentiation of CD4⁺ regulatory T cells in the lymph nodes, while at the same time, increased regulatory T cells enhance the immunosuppressive effects,

thus inhibiting the killing effect of various immune cells within the lymph nodes, thus contributing to the invasion of tumor cells (20). In the process of tumor immunity, tumor antigens can be recognized and eliminated by the immune system in the body, conferring an important protective effect on the human body (19). Regulatory T cells can inhibit the maturation and antigen-presenting ability of dendritic cells by inhibiting the proliferation of CD4⁺ T cells and other effector T cells, and can inhibit the antitumor immune response of the body by participation in interactions with various cytokines, thereby promoting the growth and invasion of tumor cells (21,22). In the present study, treatment with cryptotanshinone significantly increased the cytotoxicity of the CD4⁺ T cells, but did not affect the cytotoxicity of the CD8⁺ T cells. Zhou *et al* suggested that cryptotanshinone inhibited breast tumor growth by increasing the cytotoxicity of CD4⁺ T cells through activation of the JAK2/STAT4/perforin pathway (23).

JAK/STAT is a novel signal transduction pathway within cells, which is composed of JAK protein family (JAK1, JAK2, JAK3 and tyrosine kinase 2) and STAT protein family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) components (24). A number of cytokines [including IFN, interleukin (IL)-2, IL-4, IL-6 and ciliary neurotrophic factor] and growth factors (including epidermal growth factor, platelet-derived growth factor and colony-stimulating factor) induce cell proliferation, differentiation or apoptosis using the signal transduction pathway (25,26). These molecules have specificity and pleiotropic biological functions in the regulation of immune function, blood cell production, tumorigenesis, and nerve and embryonic development (27). The present study showed that cryptotanshinone significantly promoted p-JAK2 and p-STAT4 protein expression in the CD4⁺ T cells. Jung *et al* reported that cryptotanshinone induced apoptosis of chronic myeloid leukemia K562 cells via JAK/STAT3/5 signaling (28). Shin *et al* suggested that cryptotanshinone suppressed JAK2 phosphorylation and STAT3 phosphorylation in prostate cancer DU145 cells (29).

In conclusion, the current study showed that cryptotanshinone inhibited the tumor growth of H446 cells and CD4⁺ T cell proliferation, and that it increased the cytotoxicity of CD4⁺ T cells through activation of the JAK2/STAT4 pathway. These findings will be important in further understanding the anticancer effect of cryptotanshinone on lung cancer by immunotherapy.

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