Inhibition of the STAT3 signaling pathway is involved in the antitumor activity of cepharanthine in SaOS2 cells

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Aim: To investigate the molecular mechanisms underlying the antitumor activity of cepharanthine (CEP), an alkaloid extracted from Stephania cepharantha Hayata.

Methods: Human osteosarcoma cell line SaOS2 was used. MTT assay, Hoechst 33342 nuclear staining, flow cytometry, Western blotting and nude mouse xenografts of SaOS2 cells were applied to examine the antitumor activity of CEP in vitro and in vivo. The expression levels of STAT3 and its downstream signaling molecules were measured with Western blotting and immunochemistry analysis. The activity of STAT3 was detected based on the phosphorylation level of STAT3, luciferase gene reporter assay and translocation of STAT3 to the nucleus.

Results: Treatment of SaOS2 cells with CEP (2.5–20 μmol/L) inhibited the cell growth in a concentration- and time-dependent manner. CEP (10 μmol/L) caused cell cycle arrest at G1 phase and induced apoptosis of SaOS2 cells. CEP (10 and 15 μmol/L) significantly decreased the expression of STAT3 in SaOS2 cells. Furthermore, CEP (5 and 10 μmol/L) significantly inhibited the expression of target genes of STAT3, including the anti-apoptotic gene Bcl-xL and the cell cycle regulators c-Myc and cyclin D1. In nude mouse xenografts of SaOS2 cells, CEP (20 mg·kg⁻¹·d⁻¹, ip for 19 d) significantly reduced the volume and weight of the tumor.

Conclusion: Our findings suggest that inhibition of STAT3 signaling pathway is involved in the anti-tumor activity of CEP.

Keywords: cepharanthine; anticancer drug; human osteosarcoma cell; SaOS2; cell cycle arrest; apoptosis; STAT3; nude mice

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Introduction

Cepharanthine (CEP) is a natural occurring small molecule, derived from the plant Stephania cepharantha Hayata, and has been used to treat diseases such as venomous snakebite, alopecia areata, exudative otitis media and endotoxic shock[1-4]. The pharmacological activities of CEP include the reversal of multidrug resistance[5-7], antitumor activity[8-10] and inhibiting the production of inflammatory mediators[11-14].

The mechanisms underlying the antitumor activity of CEP have drawn increasing attention. Although accumulating evidence has shown that CEP plays a potential role in inhibiting cell growth and inducing apoptosis in many tumors[3], the underlying mechanism remains elusive. Because CEP is primarily an anti-inflammatory agent, the antitumor activity of CEP is associated with inhibition of inflammation-related signaling pathways, among which NF-κB inactivation is important[8,15,16]. In addition to NF-κB, the signal transducer and activator of transcription protein 3 (STAT3) is another critical inflammatory mediator that links inflammation and cancer[17]. Constitutive activation of STAT3, as observed in multiple human cancers, plays a crucial role in cancer cell proliferation, differentiation, and survival by upregulating several genes. These genes include anti-apoptotic genes such as Bcl-xL, and cell cycle regulators such as cyclin D1 and c-Myc. As a critical mediator of oncogenic signaling, STAT3 is a promising target for cancer therapy and anti-cancer drug design[18].

It has been reported that the collaboration and crosstalk of STAT3 and NF-κB signaling are important for carcinogenesis associated with inflammation[19]. Genes that play vital roles in angiogenesis, anti-apoptosis and cell cycle regulation are regulated by both STAT3 and NF-κB in a cooperative and interdependent manner[19, 20]. In addition, although both STAT3 and NF-κB are recognized as promising targets for cancer therapy, disruption of the STAT3 or NF-κB signaling pathways...
independently does not result in cell death\(^{[19]}\). We believe that inhibition of NF-κB activation alone cannot explain the antitumor activity of CEP. Our previous study demonstrated that CEP could downregulate the expression of the STAT3 gene at the transcriptional level\(^{[21]}\). Thus, we hypothesized that inhibition of STAT3 signaling is involved in CEP-induced cell death.

In this study, cell cycle arrest and apoptosis were observed in CEP-treated SaOS2 cells, an osteosarcoma cell line with constitutive STAT3 activation\(^{[22]}\). Downregulation of STAT3 gene expression was observed in SaOS2 cells after treatment with CEP. Further analysis of the STAT3 signaling pathway demonstrated that CEP inhibited the target genes of STAT3, including the anti-apoptotic gene Bcl-xL and cell cycle regulators c-Myc and cyclin D1. In vivo experiments were performed using nude mouse xenografts of SaOS2 cells. The antitumor activity and inhibitive effect on STAT3 expression and activation by CEP were further confirmed by animal experiments. Our findings provide the first evidence that CEP can inhibit the STAT3 signaling pathway, which helps us to better understand the anti-inflammatory and anti-tumor activity of CEP.

### Materials and methods

#### Cells lines and cell culture

The SaOS2 human osteosarcoma cell line was obtained from the Global Bioresource Center (ATCC, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) in the presence of 5% CO\(_2\) in an incubator at 37°C.

#### Materials

Cepharanthine (CEP) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). MTT and Hoechst 33342 were purchased from Sigma.

#### Cell viability analysis

MTT assays were used to test cell viability as previously described\(^{[9]}\). In brief, 2.5×10\(^5\) cells were seeded in each well of a 96-well plate. Cells were serum starved for 24 h and then incubated with different concentrations of CEP for 12, 24, and 48 h. After incubation, MTT (0.5 mg/mL final concentration) was added to each well. After 4 h of additional incubation, the medium was removed, and 200 μL of dimethyl sulfoxide (DMSO) was used to dissolve the resultant crystals. Absorption at 570 nm was determined for each sample using an automatic ELISA plate reader.

#### Hoechst 33342 staining

To observe morphological changes in the nucleus, cells were stained with 40 mg/L Hoechst 33342 after incubation with CEP (10 μmol/L) for 0, 24, and 48 h. Cells were visualized under a fluorescence microscope with a blue filter. Apoptotic cells were defined as those that showed cytoplasmic and nuclear shrinkage, and chromatin condensation or fragmentation.

Flow cytometry

Cell cycle progression and apoptosis were analyzed by flow cytometry. For cell cycle analysis, SaOS2 cells were treated with CEP (10 μmol/L) for 0, 24, and 48 h. After incubation, cells were trypsinized, counted, washed, fixed by dropwise addition of 70% ethanol and stored at 4°C until analysis. Cells were washed with PBS, resuspended in 50 μg/mL propidium iodide (PI) solution and analyzed by flow cytometry. For apoptosis analysis, cells were incubated with CEP (15 μmol/L) for 0, 24, and 48 h followed by Annexin V-FITC and propidium iodide (PI) double staining performed according to the manufacturer’s instructions (Biosea, Beijing, China).

Western blotting

Protein expression was examined by Western blot analysis as previously described\(^{[23]}\). Briefly, protein was harvested and quantified at different time points after SaOS2 cells were incubated with or without CEP at different concentrations. An amount of 40 μg of total protein per sample was separated by 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred by electroblotting onto a nitrocellulose membrane. The membrane was blocked in 5% bovine serum albumin and then incubated with antibodies against caspase 3 (Cell Signaling, USA), STAT3 (Santa Cruz, USA), phospho-STAT3 (Y705) (Cell Signaling, USA), c-Myc (Cell Signaling, USA), cyclin D1 (Cell Signaling, USA), Bcl-xL (Cell Signaling, USA) and β-actin (Cell Signaling, USA) in 5% BSA overnight at 4°C. The membrane was then washed and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Bands were visualized by use of a Western super-sensitive chemiluminescence detection system (Pierce, USA). Autoradiographs were quantitated by densitometry (Science Imaging System, Bio-Rad). β-actin was used as the internal control for protein normalization.

Vector construction and luciferase gene reporter assay

Firefly luciferase reporter plasmids (APRE plasmids) with STAT3-driven promoters (2×APRE) were constructed as previously described\(^{[21]}\). Briefly, the STAT3-driven promoter (the 2×APRE sequence) was cloned into the multiple cloning site of the pGL3-TATA plasmid, which encodes a firefly luciferase gene containing a basic upstream TATA element. The internal control Renilla luciferase reporter plasmid was purchased from Promega (USA).

The luciferase gene reporter assay was performed as previously described\(^{[23]}\). Briefly, SaOS2 cells were transfected with APRE plasmids and the internal control Renilla luciferase reporter plasmid using Lipofectamine™ 2000 (Invitrogen, USA) in accordance with the manufacturer’s instructions. After 6 h of transfection, cells were treated with 0, 5, or 10 μmol/L of CEP. For the time course, cells were treated with 10 μmol/L CEP for 0, 12, and 24 h. After CEP treatment, cell extracts were prepared and assayed according to the manufacturer’s instructions (Dural Luciferase Assay System, Promega, USA). The measured firefly luciferase activity was normalized.
to the activity of Renilla luciferase in the same well.

Nude mice experiment
Eighteen four-week-old nude mice (T cell deficient nude mice) were housed and monitored in the Department of Laboratory Animal Science at the Peking University Health Science Center. The mice were randomly divided into a control group (CON) and a CEP-treated group (CEP), with nine mice in each group. Each mouse was injected subcutaneously in the right axilla with 2×10⁶ SaOS2 cells. After the longest axis of the tumors grew to between 5 and 8 mm, the nude mice were intraperitoneally (ip) injected with DMSO or CEP (20 mg·kg⁻¹·d⁻¹) for 19 d. The mice were also weighed, and the longest axis (L) and vertical axis (R) of the tumors were measured every two days after starting ip injections. Tumor volumes (V) were estimated using the following formula: \( V = 0.5 \times L \times R^2 \). On the 20th d, the tumors were removed, weighed and fixed in 4% paraformaldehyde overnight for immunohistochemistry studies.

Immunohistochemistry
Immunohistochemistry assays were performed using the peroxidase-antiperoxidase technique after a microwave antigen retrieval procedure. Coverslips with mouse graft tissue sections were exposed to antibodies against STAT3 and phospho-STAT3-Y705 (Bioworld Technology, USA) and incubated overnight at 4 °C. Secondary antibody (Dako, Germany) incubation was performed at room temperature for 30 min. 3,3'-Diaminobenzidine (DAB) staining showed that the remaining in the G1 phase significantly increased after 24- and 48-h treatments with CEP. Flow cytometry using Annexin V-FITC and PI double staining revealed that the fraction of apoptotic cells in the group treated with CEP for 48 h was significantly higher than that of the control group (Figure 2C).

The effect of CEP on the activation of caspase 3 was examined to confirm the apoptotic effect of CEP. As shown in Figure 2D, cleaved caspase 3 protein was increased significantly by treatment with CEP.

Inhibition of STAT3 expression by CEP
To investigate whether the inhibition of STAT3 gene expression was involved in apoptosis induced by CEP, Western blotting was performed. STAT3 protein levels in SaOS2 cells were significantly downregulated after incubation for 24 h with 10 μmol/L and 15 μmol/L CEP (Figure 3A). Furthermore, when treated with CEP (10 μmol/L) for 0–48 h, the level of STAT3 decreased as the incubation time increased (Figure 3B).

Inhibition of STAT3 signaling pathway by CEP
Because inhibition of the STAT3 protein was observed by Western blotting, we further investigated whether activation of the STAT3 signaling pathway was inhibited by CEP. Phosphorylation of STAT3 is a vital step in the activation of STAT3, and therefore, we first quantified the levels of phosphorylated STAT3 in SaOS2 cells after treatment with CEP using Western blotting. As the results demonstrate, phosphorylation of STAT3 was inhibited by CEP (Figure 4A). The expression of some STAT3 target genes, including c-Myc, cyclin D1, and Bcl-xL, were also assessed by Western blotting. The results demonstrate that these target genes were also significantly downregulated by treatment with CEP, indicating that the
transcriptional activating activity of STAT3 was inhibited (Figure 4A). To further confirm the inhibitive effect of CEP on the activation of STAT3, luciferase reporter gene assays were performed. SaOS2 cells were transfected with APRE plasmids, which contained STAT3-driven promoters and encoded firefly luciferase, and were treated with different concentrations of CEP for 0, 12, and 24 h. APRE luciferase activities were significantly attenuated by treatment with CEP in a time- and dose-dependent manner (Figure 4B).

**Antitumor effect of CEP and inhibition of STAT3 activation in vivo**

The in vivo antitumor activity of CEP was assessed using nude mouse xenografts of SaOS2 cells. After subcutaneous injection tumors were established in the axilla of each mouse, the mice were randomly divided into a control group treated with vehicle DMSO and a CEP group treated with CEP (20 mg·kg⁻¹·d⁻¹, ip). Tumor volumes and body weights were recorded every two days. On the twentieth day, all the mice were euthanized and tumors were weighed. The volume growth curve showed that tumors from the CEP-treated group grew significantly slower than those from the control group (Figure 5A). Moreover, after treatment with CEP for 20 d, the tumor weights and volumes of the CEP-treated group were significantly lower than those of the control group (Figure 5B and 5C). However, the body weights of the mice were not significantly influenced by treatment with CEP (Figure 5D).

We determined whether the expression of STAT3 protein was decreased and STAT3 activity was suppressed by CEP administration in the nude mouse model. SaOS2 tumors were resected and processed for immunohistochemical analyses of STAT3 and phosphorylated STAT3. As shown in Figure 6, lower levels of STAT3 and phospho-STAT3 were found in CEP-treated tumor lesions than in untreated control tumors. In addition, we observed that nuclear localization of STAT3 in the CEP-treated group was inhibited (indicated by red arrows in Figure 6), suggesting that CEP may also have affected the translocation of STAT3 to the nucleus.

**Discussion**

Cepharanthine (CEP) is a promising, natural occurring small molecule for medical applications. As an anti-inflammatory agent, CEP has been used to treat many inflammatory diseases such as exudative otitis media and endotoxic shock. Similar to other widely studied natural products such as curcumin, CEP has multiple targets and multiple effects. In recent years, the antitumor activity of CEP has drawn the
most attention among its multiple pharmacological activities. CEP has shown the potential to inhibit growth and induce apoptosis in many tumors such as leukemia, lymphoma, lung carcinoma, myeloma, cholangiocarcinoma, oral squamous cell carcinoma and hepatocellular carcinoma. In this study, treatment with CEP induced cell growth inhibition and apoptosis in SaOS2 cells, an osteosarcoma cell line (Figure 1 and 2). Notably, cell cycle arrest occurred earlier and at a lower dose than apoptosis in SaOS2 cells treated with CEP. This indicates that the antitumor activity of CEP is attributed to both cell cycle arrest and apoptosis, and cell cycle arrest might play a more important role (Figure 2). The ip administration of CEP in nude mouse grafts of SaOS2 cells demonstrated the tumor-suppressing activity of CEP (Figure 5).

According to previous studies, the mechanisms underlying the antitumor activity of CEP include the following. First, CEP stabilizes the cell membrane by interacting with P-glycoprotein and increases the intracellular accumulation of anticancer drugs, thus reversing multidrug resistance in cancer cells. Second, CEP might indirectly inhibit tumor growth by enhancing the immunological responses of the host.
Third, CEP could induce tumor cell apoptosis by interfering with vital signaling pathways such as the nuclear factor kappa B (NF-κB) and JNK1/2 signaling pathways\cite{8–10}. Because CEP is primarily an anti-inflammation agent, the mechanisms underlying its antitumor activity are closely related to the inhibition of inflammatory signaling\cite{3}. The inflammatory pathways that are important in carcinogenesis include NF-κB, STATs and COX-2, and they are promising targets for cancer therapy\cite{31,32}. Previous studies have shown that inactivation of NF-κB is an important mechanism for the antitumor activity of CEP\cite{8,15,16}.

SaOS2 is an osteosarcoma cell line in which STAT3 is constitutively activated and plays a crucial role in cell proliferation and survival\cite{10,22,33}. STAT3 is a critical inflammatory mediator that links inflammation and cancer\cite{17,32}. It relays signals from the cell membrane directly to the nucleus and activates the transcription of many important genes, including anti-apoptotic genes such as Bcl-xL, and cell cycle regulators such as cyclin D1 and c-Myc\cite{34}. According to Western blotting results, STAT3 expression in SaOS2 cells was suppressed by CEP (Figure 4). Because downregulation of STAT3 expression was observed, we asked whether the inactivation of the STAT3 signaling pathway was involved in the antitumor activity of CEP. The activation of STAT3 requires many steps. Briefly, to be activated, cytoplasmic STAT3 protein is first phospho-

**Figure 5.** In vivo experiments. Eighteen nude mice were randomly grouped as a control group (CON) and a CEP-treated group (CEP). When the xenograft model of SaOS2 cells was established, mice in CON group were injected with DMSO while those in CEP group with CEP (20 mg·kg\(^{-1}\)·d\(^{-1}\), ip). Body weight, long axon (L) and vertical axon (R) of each mouse were measured every two days. (A) Tumor volume growth curve of SaOS2 grafts in mice. The volumes of tumors were estimated using the formula: \(V=0.5\times L\times R^2\). \(P<0.01\). (B) The tumor weights of the two groups. On the twentieth day of CEP treatment, mice were sacrificed and the tumors were weighed after being sected. \(P<0.01\) vs control. (C) Photograph of the tumors. (D) Record of body weights of mice.

**Figure 6.** Immunohistochemical analyses of STAT3. After treatment of CEP for 20 d, the tumor samples were taken from the sacrificed mice and immunostained with antibodies against total and phosphorylated STAT3. (A) Immunohistochemical staining of total STAT3. STAT3 nuclear localization in control group was indicated with the red arrows. (B) Immunohistochemical staining of phospho-STAT3. Original magnification: \(\times400\).
Phosphorylated STAT3 forms homodimers that translocate to the cell nucleus where they bind to the promoters of target genes and activate the transcription of oncogenes[34]. In our study, we showed that the phosphorylation of STAT3 was inhibited, the vital target genes of STAT3 such as c-Myc, cyclin D1 and Bcl-XL were downregulated, and the luciferase activity of APRE plasmids were attenuated by CEP (Figure 4). Immunohistochemistry further demonstrated that the expression of STAT3 was inhibited by CEP treatment (Figure 6A). It also indicated that activation of STAT3 was blocked by CEP because the phospho-STAT3 level in the CEP group significantly decreased compared to the control group (Figure 6B), and STAT3 translocation to the nucleus decreased with the administration of CEP (Figure 6A, indicated by red arrows). We showed through these results that inhibition of the STAT3 signaling pathway might play an important role in the antitumor activity of CEP. Our results also indicated that CEP inhibited STAT3 signaling by downregulating the expression of the STAT3 gene. However, further experiments are necessary to determine how STAT3 gene expression is downregulated by CEP, and to confirm the causal relationship between inhibition of the STAT3 signaling pathway and cell death.

In summary, CEP is an anti-inflammation and antitumor agent with multiple targets and effects. NF-κB signaling aside, our findings demonstrated that inhibition of another vital signaling pathway, the STAT3 signaling pathway, is involved in the antitumor activity of CEP. This helps us to better understand the anti-inflammation and antitumor activity of CEP. Moreover, our discovery of STAT3 signaling as a new mediator of the antitumor activity of CEP provides novel insight into the treatment of tumors with multi-target, naturally occurring small molecules.

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Author contribution
Ming XU and You-yi ZHANG designed the research; Zan CHEN performed the research and analyzed the data; Zan CHEN, Chen HUANG, Yan-ling YANG, Yi DING, and Han-qiang OU-YANG contributed new analytical tools; and Zan CHEN and Ming XU wrote this paper.

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