Panax notoginseng saponins improves the malignancy of chronic myeloid leukemia by inducing cell apoptosis and autophagy

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Abstract: In recent years, the anti-tumor effect of panax notoginseng saponins (PNS) has been widely acknowledged by multiple in vivo and in vitro experiments. However, few studies have focused on the effect of PNS on chronic myeloid leukemia cells. In this study, we mainly explored the effect of PNS on K562 cell apoptosis and autophagy, thereby exploring its potential application in the treatment of chronic myeloid leukemia (CML) in clinic. Firstly, we found that PNS suppressed K562 cell viability in a time- and dose-dependent manner. Then, Hoechst staining and Annexin V-PI stainning showed that PNS significantly induced K562 cells apoptosis. Meanwhile, western blot analysis indicated enhanced expression of cleaved-caspase-3 and Bax after PNS treatment. Further study showed that PNS treatment increased K562 cell autophagy. We also found the reduced levels of p210, and phosphorylated PI3K, Akt, mTOR, p70S6K, 4E-BP1 as well as enhanced Beclin1 and LC3II expression after PNS treatment. More importantly, our data showed that overexpression of p210 could significantly abolish PNS-induced suppression of PI3K/Akt/mTOR signaling. In summary, PNS induced K562 cell apoptosis and autophagy mainly by suppressing p210/PI3K/Akt/mTOR signaling, which may shed light on the treatment of CML.

Keywords: Panax notoginseng saponins, chronic myeloid leukemia, p210, apoptosis, autophagy

Introduction

Chronic myeloid leukemia (CML) is a malignant hematopoietic disease. Epidemiological survey shows that the global incidence of CML is about 1/10 million, accounting for about 20% of leukemia [1, 2]. The incidence of CML is increased with age [3]. At present, the clinical treatment of CML includes hematopoietic stem cell transplantation and the use of hydroxyurea, α-interferon drug therapy, etc. [4–6]. With the in-depth study of the pathogenesis, tyrosine kinase inhibitors has been widely used in clinical, which greatly enhance the treatment of CML [1, 7]. However, the treatment of CML in patients with blast crisis and Philadelphia chromosome-negative is still not optimistic [8, 9]. Therefore, the search for new strategies for the treatment of CML is still an important goal of CML research.

The hallmark of CML is closely related to the presence of Philadelphia chromosome, its resultant fusion transcript (BCR-ABL1), and fusion protein (p210) [10, 11]. It is suggested that the survival of CML patients can be markedly improved with the application of tyrosine kinase inhibitors which lead to long-term hematologic remissions [12]. P210 protein has strong tyrosine protein kinase activity, which can promote the activation of PI3K/Akt/mTOR, Ras/MAPK, Jun/STAT5 and other signal pathways, thereby promoting the proliferation of cancer cells, inhibiting its apoptosis and cell cycle [9, 13]. Thus, it is of great importance to suppress the expression of p210 in the progression of CML.

At present, the traditional Chinese medicine ingredients have made great progress in the screening of anti-tumor effective drugs [14]. Panax notoginseng saponins (PNS) is the main pharmacologically active ingredient of Panax notoginseng [15]. The anti-tumor effect of PNS has been widely acknowledged by multiple in vivo and in vitro experiments [16, 17]. Studies
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![Figure 1](image1.png)

Figure 1. PNS reduced K562 cell viability in a time- and dose-dependent manner. A. MTT assay showed that 20 nM PNS suppressed K562 cell viability at 24, 48, 72 h, respectively. B. K562 cell viability was suppressed by PNS at the dose of 5 nM, 20 nM, 100 nM, respectively. *p < 0.05, **p < 0.01.

have found that PNS can promote tumor cell death and apoptosis, inhibit cell proliferation, and can effectively impede tumor cell metastasis [18, 19]. For instance, PNS can significantly inhibit the proliferation of colon cancer and the migration of breast cancer cells [18, 19]. However, there are few studies in the effect of PNS on chronic myeloid leukemia cells. In this study, we used K562 cells as the in vitro model and explored the effect of PNS on K562 cell proliferation, apoptosis, and autophagy, thereby exploring its potential application in the treatment of CML in clinic.

Materials and methods

Cell culture

K562 cells were cultured in RPMI-1640 supplemented with 10% (v/v) Horse serum (Atlanta Biolabs), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.), at 37°C in a humidified atmosphere with 5% CO₂.

MTT assay

To explore the effect of PNS on cell viability, 5,000 cells per well in a 100 μl medium were seeded in 96-well plates and treated with PNS (20 nM) or DMSO (20 nM) for 12 h, 24 h, 48 h and 72 h, respectively or treated with 1 nM, 5 nM, 20 nM, 100 nM PNS for 48 h. After treatment, 20 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Solarbio, China) was added to wells and were incubated with the cells for 4 h. After removing the medium, the blue formazan was dissolved with 200 μl dimethyl sulfoxide (DMSO) and the absorbance was measured at 550 nm. Wells containing only K562 cells served as blanks.

Western blot

Proteins were extracted from K562 cells with RIPA buffer (1% TritonX-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0)) (Solarbio, China) supplemented with a protease inhibitor cocktail (Sigma). The cell lysates were separated by 10% SDS-PAGE and transferred electrophoretically onto PVDF membranes. After blocking with 8% milk in PBS (pH 7.5), the membranes were incubated with the following specific primary antibodies overnight: anti-p210, anti-cleaved-caspase-3, anti-bcl-2, anti-bax, anti-mTOR, anti-p70, anti-LC3, anti-p62 and anti-β-actin (CST). The appropriate HRP-conjugated anti-rabbit IgG secondary antibody (Abmart, all at 1:5000) was subsequently applied, and immunodetection was performed using the ECL Plus detection system (Millipore) according to the manufacturer’s instructions. β-actin was used as the internal control.

Annexin V-FITC staining

To quantify apoptotic cells, flow cytometry was performed with an Annexin V-fluorescence-5-isothiocyanate apoptosis detection kit (Biovision, USA). After treatment with 20 nM PNS or DMSO for 48 h, K562 cells were harvested in a 5 ml tube. Then, the cells were washed with cold PBS and resuspended at a final concentra-
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**Figure 2.** PNS led to apoptosis in K562 cells. A. Hoechst staining showed that PNS treatment significantly induced nuclear condensation and lysis in K562 cells compared with that of control. B. Flow cytometry analysis also indicated that cell apoptosis was increased by more than 35.6% after PNS treatment in K562 cells. C. The expression of cleaved-caspase-3 and Bax was significantly enhanced, but the protein level of Bcl-2 was markedly reduced. *p < 0.05, **p < 0.01.

tion of 1 × 10^6 cells/ml. FITC-Annexin V (5 ul) and propidium iodide were gently mixed and incubated with the cells for 15 min at a room temperature. After incubation, the samples were analyzed by flow cytometry within 1 h.

**Hoechst staining**

K562 cells were cultured in 6-well plates. After 48 h treatment with PNS or DMSO, cells were washed with PBS and stained with Hoechst 33258 (10 μg/ml) for 5 min before being washed three times with PBS.

**GFP-LC3 transfection**

K562 cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates. After 24 h, a GFP-LC3 expressing plasmid was transfected into cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Twenty-four hours post-transfection, cells were treated with 10 μM vehicle or cisplatin and incubated for 16 hours. Then, GFP-LC3-positive dots were counted under a confocal laser microscope, LSM700 (Carl Zeiss, Jena, Germany).

**Transmission microscopy**

Cell pellets were fixed in 2.3% glutaraldehyde, postfixed in 2% osmium tetroxide and 0.5% uranyl acetate, dehydrated and embedded in Spur's epoxy resin. Ultrathin sections (90 nm) were made and double-stained with uranyl acetate and lead citrate, and viewed with a Philips CM10 transmission electron microscope (Phillips Electronics, Amsterdam, the Netherlands).

**Transient transfection**

Before transfection, 1 × 10^5 cells per well were seeded in a 6-well plate with 2 ml RPMI-1640 culture medium containing serum and antibiotics. Meanwhile, a plasmid overexpressing p210, or negative control (pNC) (Genepharma) was pre-incubated with HiPerFect transfection
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Figure 3. PNS inactivates mTOR signaling and increases K562 cell autophagy. A. GFP-LC3 transfection assay indicated the enhanced cell autophagy in K562 cells after PNS treatment. B. Transmission microscopy also indicated that PNS treatment significantly increased cell autophagy. C. Western blot analysis showed that PNS significantly suppressed the activation of mTOR, p70S6K and 4E-BP1. *p < 0.05, **p < 0.01.

reagent (QIAGEN) at room temperature for 10 min. The complex was then transfected into the K562 cells at a final concentration of 50 nM. The transfected cells were incubated under normal growth conditions for another 48 h.

Statistical analysis

Data were presented as mean ± SE from 3 independent experiments. Statistical analysis was carried out with Student’s t test. P < 0.05 was considered as statistically significant difference.

Results

PNS decreases K562 cell viability in a time- and dose-dependent manner

Firstly, we evaluated the role of PNS on K562 cell viability. MTT assay showed that 20 nM PNS suppressed K562 cell viability at 24, 48, 72 h, respectively (Figure 1A). Furthermore, our data also indicated that K562 cell viability was suppressed by PNS at the dose of 5 nM, 20 nM, 100 nM, respectively (Figure 1B). These data indicated that PNS reduced K562 cell viability in a time- and dose-dependent manner.

PNS induces K562 cell apoptosis

Furthermore, Hoechst staining showed that PNS treatment significantly induced nuclear condensation and lysis in K562 cells compared with that of control (Figure 2A). Flow cytometry analysis also indicated that cell apoptosis was increased by more than 35.6% after PNS treatment in K562 cells (Figure 2B). Western blot analysis also demonstrated that the expression of cleaved-caspase-3 and Bax was significantly enhanced, but the protein level of Bcl-2 was
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markedly reduced (Figure 2C). These results indicated the pro-apoptotic role of PNS in K562 cells.

**PNS inactivates mTOR signaling and increases K562 cell autophagy**

Next, GFP-LC3 transfection assay indicated the enhanced cell autophagy in K562 cells after PNS treatment (Figure 3A). Meanwhile, transmission microscopy also indicated that PNS treatment significantly increased cell autophagy in contrast with that of control (Figure 3B). Then, we analyzed the expression of mTOR signaling. As shown in Figure 3C, the phosphorylation levels of mTOR, p70S6K and 4E-BP1, were markedly suppressed. In contrast, the protein levels of Beclin1 and the ratio between LC3II/LC3I were significantly increased after PNS treatment (Figure 3C). These data indicated that PNS induced K562 cell autophagy mainly by activating mTOR signaling.

**PNS suppresses PI3K/Akt/mTOR signaling mainly by suppressing p210 expression**

Then, we tried to elucidate whether PNS could regulate the expression of p210, which was suggested to activate PI3K/Akt/mTOR in the progression of CML. Western blot analysis demonstrated the expression of p210 was markedly suppressed by PNS treatment. Furthermore, a plasmide overexpressing p210 was applied to explore whether it could abolish PNS-induced suppression of PI3K/Akt/mTOR signaling. Our data showed that upregulation of p210 could markedly reverse PNS-induced PI3K/Akt/mTOR inactivation (Figure 4). These data suggested that PNS suppressed the progression of CML mainly by targeting p210.

**Discussion**

Philadelphia chromosome positive (Ph+) CML is a common malignant clonal disorder of he-
matopoietic stem cells which is induced by the oncogenic Bcr-Abl tyrosine kinase [20]. In recent years, the application of tyrosine kinase inhibitors in CML treatment has been greatly improved [21, 22]. However, due to drug resistance induced by the Bcr-Abl kinase mutation, it invariably relapses for most patients ceasing tyrosine kinase inhibitor therapy [23]. Thus, for these patients, they have to be treated with more complicated chemotherapy regimen, such as cytarabine, daunorubicin or doxorubicin [24, 25]. However, these chemotherapy drugs are with obvious side effects to kill normal tissue cells, thereby decreasing the immune function [26]. Therefore, searching for novel therapy to treat CML is still the research goal of CML.

Increasing evidence has indicated the key role of effective components isolated from traditional Chinese medicine in the treatment of malignant leukemia cancer [27]. In the current study, we mainly explored the role of PNS on CML cell proliferation. MTT assay indicated that PNS suppressed K562 cell viability in time- and dose-dependent manner. We also found that cell apoptosis was significantly induced after PNS treatment. In the normal status, caspase-3 usually exists in the form of proactive caspase-3, and its activation is achieved by cleavage of small fragments thereby leading to cell apoptosis [28]. Here, we found that the cleaved caspase-3 protein was increased in K562 cells after PNS, which further validated the proapoptotic effect of PNS on K562 cells.

Although autophagy is well identified in various diseases, the specific mechanism of autophagy in disease is still not well characterized. In some circumstances, it may suppress the progression of multiple diseases, including atherosclerosis, cancer, and neurodegenerative diseases [29]. However, in other situations, autophagy may prompt the development of a disease [30, 31]. Moreover, it is suggested that autophagy dysfunction is tightly related to tumorigenesis and can affect the cancer cell proliferation, cell cycle and apoptosis [32]. Thus, we evaluated the role of PNS on K562 cell autophagy. Our data showed that treatment with 20 nM PNS in K562 cells significantly induced K562 cell autophagy. According to our data, we propose that PNS could both induce K562 cell apoptosis and autophagy, thereby suppressing carcinogenesis.

Then, we explored the specific mechanism in which PNS induced K562 cell autophagy and apoptosis. It is extensively studied that mTOR signaling pathway can regulate cell proliferation, apoptosis, and autophagy in the process of tumor development [33]. In addition, mTOR signaling pathway is found to be overactivated in the Bcr/Abi-positive cell lines [34, 35]. Thus, we evaluated whether PNS induced K562 cell autophagy by modulating mTOR signaling. Western blot analysis indicated that the activation of mTOR signaling was significantly suppressed after PNS treatment. In contrast, the expression of Beclin1 and LC3II was markedly enhanced by PNS treatment. These data indicated that PNS-induced K562 cell autophagy is correlated with the inhibition of mTOR signaling.

Bcr-Abl fusion results from reciprocal translocation of chromosomes 9 and 22, and it leads to the expression of a tyrosine kinase protein p210 (bcr/abl), which then prompts the malignancy of CML through activating several survival pathways and inducing therapeutic resistance [36]. Thus, we evaluated the role of PNS on the expression of p210 and our data showed that PNS suppressed the expression of p210. More importantly, overexpressing of p210 significantly abolished PNS-induced PI3K/Akt/mTOR inactivation. These data suggested that PNS suppressed the progression of CML mainly by inhibiting p210.

In conclusion, for the first time, we showed that PNS could suppress the expression of p210 in CML cells, thereby suppressing mTOR signaling, which then triggers CML cell apoptosis and autophagy. These data validated the anti-tumor effects of PNS, which may shed light on the clinical treatment of CML.

Disclosure of conflict of interest

None.

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