

RESEARCH ARTICLE

Saponins from *Rubus parvifolius* L. Induce Apoptosis in Human Chronic Myeloid Leukemia Cells through AMPK Activation and STAT3 Inhibition

Yu-Qing Ge¹, Xiao-Feng Xu², Bo Yang³, Zhe Chen¹, Ru-Bin Cheng^{3*}

Abstract

Background: Saponins are a major active component for the traditional Chinese medicine, *Rubus parvifolius* L., which has shown clear antitumor activities. However, the specific effects and mechanisms of saponins of *Rubus parvifolius* L. (SRP) remain unclear with regard to human chronic myeloid leukemia cells. The aim of this study was to investigate inhibition of proliferation and apoptosis induction effects of SRP in K562 cells and further elucidate its regulatory mechanisms. **Materials and Methods:** K562 cells were treated with different concentrations of SRP and MTT assays were performed to determine cell viability. Apoptosis induction by SRP was determined with FACS and DAPI staining analysis. Western blotting was used to detect expression of apoptosis and survival related genes. Specific inhibitors were added to confirm roles of STAT3 and AMPK pathways in SRP induction of apoptosis. **Results:** Our results indicated that SRP exhibited obvious inhibitory effects on the growth of K562 cells, and significantly induced apoptosis. Cleavage of pro-apoptotic proteins was dramatically increased after SRP exposure. SRP treatment also increased the activities of AMPK and JNK pathways, and inhibited the phosphorylation expression level of STAT3 in K562 cells. Inhibition of the AMPK pathway blocked the activation of JNK by SRP, indicating that SRP regulated the expression of JNK dependent on the AMPK pathway. Furthermore, inhibition of the latter significantly conferred resistance to SRP pro-apoptotic activity, suggesting involvement of the AMPK pathway in induction of apoptosis. Pretreatment with a STAT3 inhibitor also augmented SRP induced growth inhibition and cell apoptosis, further confirming roles of the STAT3 pathway after SRP treatment. **Conclusions:** Our results demonstrated that SRP induce cell apoptosis through AMPK activation and STAT3 inhibition in K562 cells. This suggests the possibility of further developing SRP as an alternative treatment option, or perhaps using it as adjuvant chemotherapeutic agent for chronic myeloid leukemia therapy.

Keywords: K562 cells - apoptosis induction - AMPK - STAT3 pathways - SRP

Asian Pac J Cancer Prev, **15** (13), 5455-5461

Introduction

Chronic myelogenous leukemia (CML) is a chronic myeloproliferative disorder with an uncontrolled production of maturing neutrophils being the predominant laboratory feature. The resulting Philadelphia chromosome (Ph) produces protein of Bcr/Abl, a constitutively active tyrosine kinase that drives expansion of leukemic progeny (Deininger et al., 2000). Presence of the Bcr/Abl tyrosine kinase renders CML resistant to traditional therapeutic approaches, which makes Bcr/Abl an important therapeutic target. The specific Abl kinase inhibitor imatinib induces complete cytogenetic responses (CCRs) in more than 80% of newly diagnosed patients in the chronic phase (Druker et al., 2006). However, resistance has been observed after various periods of imatinib treatment, especially

in advanced stages of disease (Gambacorti-Passerini et al., 2003). The formation of mutation on Bcr/Abl gene, particularly M351T mutant forms, conferred significant cell resistance toward imatinib (Chahardouli et al., 2013). In addition, imatinib could not eliminate the primitive leukemia stem cells, leading to the potential risk of initiation, drug resistance and relapse of CML (Corbin et al., 2011). Furthermore, the continuous treatment with adjuvant imatinib would lead to considerable cost and sometimes despite significant side effects for CML patients. The emergency of drug resistance created needs for the development of novel chemotherapeutic agents for CML.

Besides Bcr-Abl kinase, several other pathways are also crucial for the survival of CML cells and could be potential targets for drug screening. The AMP-activated

¹Zhejiang Provincial Hospital of Traditional Chinese Medicine, Zhejiang Chinese Medical University, ²Department of Hematology, Hangzhou Red Cross Hospital, ³College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, China *For correspondence: biothcheng@hotmail.com

protein kinase (AMPK) is an energy sensor that plays a key role in the regulation of protein and lipid metabolism in response to changes in fuel availability. Recently, interest in AMPK has grown extensively as it emerged as an attractive target molecule for cancer prevention and treatment (Kim et al., 2013). AMPK activators played significant antileukemic effects in both sensitive and resistant CML cells through apoptosis induction (Vakana et al., 2011). Activation of AMPK pathway by naturally occurring dietary constituents and plant products are considered as an alternative to the conventional chemotherapy treatment (Ji et al., 2010). For instance, results from Fan et al indicated that resveratrol inhibits Skp2-mediated ubiquitylation and 26S proteasome-dependent degradation of p27^{Kip} via AMPK activation to suppress OAC proliferation (Fan et al., 2014). Signal transducer and activator of transcription 3 (STAT3), a transcription factor, also played important roles for CML cells survival after imatinib exposure. Knock of STAT3 in K562 cells could lead to inhibition of growth and proliferation, cell cycle blockade and apoptosis induction (Ma et al., 2010). STAT3 also contributes to resistance toward Bcr/Abl inhibitors in a bone marrow microenvironment model of drug resistance (Bewry et al., 2008). Agents targeting STAT3 pathway could therefore be of therapeutic value, especially for CML patients in advanced stage.

Plant-derived phytochemicals have been used as therapeutic agents for hundreds of years (Zheng et al., 2014). Natural products from plants have been proven to be a rich resource for anticancer drug discovery. Compared to the purely synthetic drugs, some agents from plants exhibited higher activity and lower toxicity (Li et al., 2014). Chinese traditional herbs provide a guide to identifying new anticancer compounds and serve as a source of alternative cancer therapy, and they have received increased scientific attentions (Ge et al., 2012a; 2012b). *Rubus parvifolius* L. is an herb used in traditional Chinese medicine, where it is used traditionally for the treatment of angina, hepatitis and rheumatism (Gao et al., 2011). Recently, growing evidences have confirmed the anti-cancer activities of the *Rubus parvifolius* L. extracts. The saponins are the main active compounds responsible for the antitumor activities in *Rubus parvifolius* L. For instance, the isolated compound from saponins of *Rubus parvifolius* L. (SRP) ursolic acid potently disrupted cell cycle progression and induced necrosis in a clonal MMTV-Wnt-1 mammary tumor cell line (De Angel et al., 2010). Corosolic acid, another active compound from SRP, enhances the antitumor effects of chemotherapy on epithelial ovarian cancer by inhibiting STAT3 pathway (Fujiwara et al., 2013). The SRP itself significantly inhibited the proliferation of several tumor cells, suggesting that SRP was a promising agent for cancer prevention (Zhang et al., 2014). However, the effect and precise mechanisms of SRP in human chronic myeloid leukemia cells still remain unknown and require further investigations.

In this study, we systematically determined the proliferation inhibition and apoptosis induction effect of SRP in K562 cells and further investigated its

pharmacological mechanisms. Our results demonstrated that SRP could inhibit cell proliferation and induce cell apoptosis obviously in K562 cells. Further study demonstrated that SRP induced cell apoptosis dependent of AMPK activation and STAT3 inhibition. This work will not only be useful for the utilization of *Rubus parvifolius* L. but also contribute to finding novel potential natural product for CML treatment.

Materials and Methods

Cell culture

The human chronic myeloid leukemia cell line K562 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 µg/ml streptomycin in humidified 5% CO₂ at 37°C.

Preparation of SRP extract

The roots of *Rubus parvifolius* L. were used to isolate the total saponins. Briefly, the dried herbs (5 kg) were pulverized into coarse powder, and then soaked in 70% ethanol for 4 hours and extracted at room temperature by percolation. The extraction process was repeated three times to yield a concentrated ethanol extract. The obtained extractum was re-suspended with warm water and partitioned with water-saturated diethyl ether for three times. Finally, butan-1-ol was added to the reconstituted residue from above for phase separation to obtain total saponins. The dried and lyophilized SRP powder was reconstituted in DMSO to form a 50 mg/ml stock and stored at -20°C.

MTT assay

The MTT assay was used for the assessment of cell growth inhibition as described previously. Cells were seeded at a density of 8,000 cells per well in 96-well plates in RPMI 1640 containing 10 % FBS. Different concentrations of SRP were added and incubated for another 24 hours. Then, 20 µl of MTT were added into each well and the absorbance at 570nm was measured on an ELISA plate reader. The cell viabilities of each well were indicated by the measured values. Three independent sets of experiments were performed.

DAPI staining assay

K562 cells were seeded within chamber dishes and treated with or without different concentrations of SRP for 24 h. After treatment, the cells were washed with ice-cold phosphate-buffered saline (PBS) twice and then fixed with ethanol for 30 min at -20°C. The fixed cells were then washed with PBS and stained with 10 µg/ml of DAPI for 15 min at room temperature. After washing three times with PBS, the cells were observed under a fluorescence microscope (Olympus, Japan).

Apoptosis assay

Apoptotic cells were detected by FACS with the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Briefly, cells were harvested after the incubation with

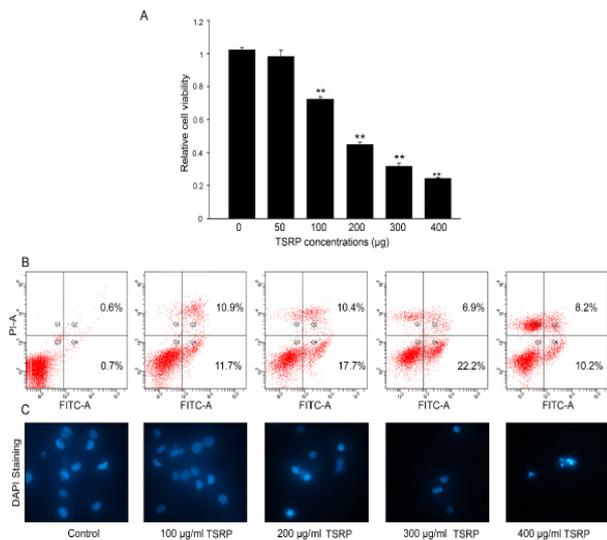


Figure 1. Effect of SRP on the Proliferation and Apoptosis of K562 Cells. Cells were treated with different concentrations of SRP for 24 h, MTT assays were then performed to determine the cell viabilities (A). Simultaneously, K562 cells were subjected to apoptosis analysis by annexin V binding (B) and DAPI staining (C). For each representative dot-plot, the percentage of cells in early and late apoptosis is indicated in the relative quadrant. Results are mean value \pm SD of three independent experiments. *indicated $p < 0.05$, while ** indicated $p < 0.01$, compared with control group

different concentrations of drugs, and washed twice with cold PBS, and resuspended in annexin-binding buffer at a concentration of 1×10^6 cells per ml. 100 μ l of the cell solution was stained with 5 μ l Annexin V and 5 μ l PI at room temperature for 20 min in the dark. The data were collected and analyzed with a FACScalibur flow cytometer using the CellQuest Software (Becton Dickinson). Three independent sets of experiments were performed.

Western blot

The general procedure for the Western blot analysis was performed as follows. Cells were collected by centrifugation at 2000g and resuspended in RIPA lysis buffer containing a mixture of protease inhibitor cocktail and phosphatase inhibitors. The mixture was sonicated for 5 seconds and protein contents were analyzed by using the Bradford assay kit. Twenty-five micrograms total protein was resolved in SDS-polyacrylamide gels on a Minigel apparatus and transferred to a nitrocellulose membrane using a semidry transfer cell. The transblotted membrane was washed 3 times and blocked with TBST containing 5% nonfat milk for 60 minutes, and then the membrane was incubated with the appropriate primary antibody. Finally, the membrane was probed with horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence. The density of the blot was further analyzed by densitometry using the Gel-Pro Analyzer. All western blot experiments were performed at least three times. Blot figures were from one representative of three independent experiments, and density data were collected from the average. The primary antibodies used were as follows: anti-cleaved caspase-3, cleaved caspase-9, cleaved PARP, Bcl-2, phospho-STAT3

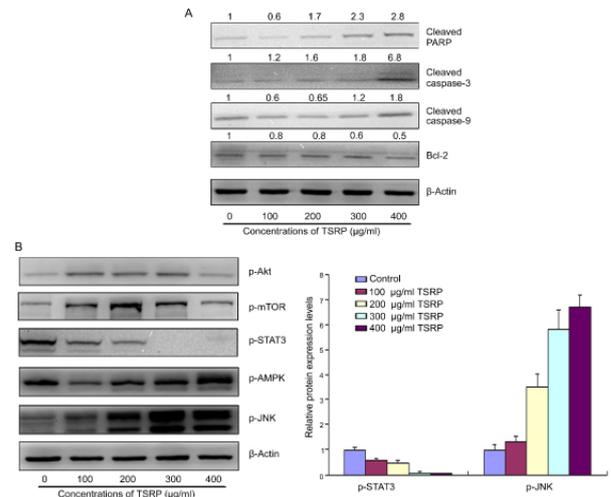


Figure 2. Western Blot Analyses of the Apoptosis and Survival Related Proteins in K562 after SRP Treated. K562 cells exposed to the different concentrations of SRP for 24 h, after which the cells were subjected to blot to determine the expressions of indicated proteins. (A) The expressions of apoptotic related proteins were determined. (B) The expressions of *p*-Akt, *p*-mTOR, *p*-STAT3, *p*-AMPK and *p*-JNK were analyzed after SRP treated

(Tyr705), phospho-mTOR (Ser2448), phospho-Akt (Thr308), phospho-AMPK, phospho-JNK and β -Actin (Cell signaling, USA).

Statistical analysis

For analysis of experimental data, comparison of categorical data was carried out by Student's *t*-test. Data are presented as the mean \pm SD. All *P* values are two-sided. A *P* value < 0.05 was considered statistically significant in all experiments. In the figures, an asterisk represents Student's *t*-test $p < 0.05$, and two asterisks represent $p < 0.01$.

Results

SRP inhibited cell proliferation through apoptosis induction in K562 cells

The effect of SRP on proliferation of human chronic myelogenous leukemia K562 cells were determined using MTT assay. As shown in Figure 1A, SRP inhibited the proliferation of K562 cells at a dose-dependent manner. The IC₅₀ value of SRP in K562 cells at 24 h was 185 μ g/ml. The relative cell viabilities decreased by 56% and 68% after the treatment of 200 and 300 μ g/ml of SRP, respectively. Our results indicated that SRP might function as a tumor suppressor for K562 cells *in vitro*.

To explore the mechanism of SRP in K562 cells, the percentage of apoptotic cells were determined by flow cytometric analysis with combined Annexin-V/PI staining after the treatment of SRP for 24 h. Compared with the control group, the apoptotic cell fractions were significantly increased after treated with SRP (Figure 1B). In the untreated control group, the percentage of apoptotic cell fraction was only 1.3%. The addition of 100 and 200 μ g/ml of SRP in K562 cells resulted in 22.6% and 28.1% apoptotic cell fractions, which were 17.4 and 21.6 folds than that of the control group, respectively. To further confirm the apoptosis induction by SRP, cells were stained

with DAPI and observed by fluorescence microscopy. As shown in Figure 1C, normal homogeneous distribution of chromatin was observed in control cells, whereas cells treated with SRP exhibited morphological features of apoptotic cells, such as chromatin condensation and marginalization or DNA fragmentation. Furthermore, SRP treatment obviously increased the activities of pro-apoptotic proteins, including PARP, caspase-3 and caspase-9. As shown in Figure 2A, after the treatment with 300 $\mu\text{g/ml}$ of SRP for 24 h, the expressions of cleaved PARP and caspase-3 were respectively 1.3 and 0.8 folds higher than that of the control. In addition, SRP also inhibited the expressions of Bcl-2, a key regulator of cell anti-apoptotic family members. The expression of Bcl-2 after 300 $\mu\text{g/ml}$ of SRP treatment reduced by 40% compared to the control group (Figure 2A). Moreover, treatment with the pan-caspase inhibitor z-VAD-fmk obviously conferred resistance to the anti-proliferative and apoptosis inductive effects of SRP, confirming that SRP induced apoptosis through a caspase-dependent manner in CML cells (Table 1). Taken together, these results demonstrated that SRP played antitumor roles by apoptosis induction in K562 cells.

Involvement of survival pathway in apoptosis induction by SRP

Besides Bcr-Abl kinase, several other regulatory pathways including Akt/mTOR, STAT3, AMPK and JNK signaling pathways are also crucial to the survival and apoptosis of CML cells. Therefore, we then determined the effect of SRP on the activities of these above-mentioned pathways. As shown in Figure 2B, SRP inhibited the expression of phospho-STAT3 and increased the expression of phospho-JNK in a dose-dependent manner. For instance, 200 $\mu\text{g/ml}$ of SRP led to about 53% fold decrease on the expression of phospho-STAT3 in K562 cells, while 300 and 400 $\mu\text{g/ml}$ of SRP almost completely inhibited the activities of STAT3 pathway. Compared to the control group, the expression levels of phospho-JNK treated with 200 and 300 $\mu\text{g/ml}$ of SRP increased to 3.5 and 5.8 folds, respectively. However, the effect of SRP on the activities of Akt/mTOR and AMPK pathways were dependent on the drug concentrations (Figure 2B). For example, the expressions of phospho-Akt and phospho-mTOR were inhibited by SRP only when the

concentrations increased to 400 $\mu\text{g/ml}$. SRP at 100 $\mu\text{g/ml}$ blocked the expression of phospho-AMKP obviously. However, the expressions of p-AMKP were significantly increased when the concentrations of SRP increased to 300 and 400 $\mu\text{g/ml}$ (Figure 2B). Our results clearly indicated the involvements of STAT3, JNK and AMPK pathways for apoptosis induction of SRP in K562 cells.

SRP increased JNK activity dependent on AMPK pathway

Since SRP exhibited global effects on the activities of survival pathways, we then determined the regulation of these pathways after SRP exposure. K562 cells were firstly treated with 300 $\mu\text{g/ml}$ of SRP for different time, the expressions of the above-mentioned proteins were determined by western blot (Figure 3A). Our results clearly demonstrated that phospho-AMPK, but not other proteins, was activated immediately after SRP exposure. The expression of phospho-JNK was increased after SRP treated for 1 h. However, SRP inhibited the activity of STAT3 only when the treated time increased to 6h. These results suggested a possibility that SRP regulated the activity of JNK pathway through AMPK activation in K56 cells. To confirm our hypothesis, we used the AMPK pathway inhibitor compound C (C. C) to determine the change of phospho-JNK expression after SRP treatment. When cells were pretreated with C. C, the expression of p-AMPK was not increased after 300 $\mu\text{g/ml}$ of SRP treatment (Figure 3C). In addition, the incubation of C. C also inhibited the activation of JNK pathways induced by SRP, suggesting that SRP increased the expression of phospho-JNK dependent on AMPK activation. However, inhibition of AMPK pathway did not alter the expression of phospho-STAT3 by SRP, indicating that SRP regulated STAT3 activity independent of AMPK expression.

SRP induced cell apoptosis via STAT3 inhibition and AMPK activation

The above results indicated that SRP induced K562 cell apoptosis by regulating the activities of AMPK and STAT3 signaling pathways. To further understand the changes of AMPK and STAT3 activities contributed anything to the anti-tumor effect of SRP, the AMPK inhibitor C.

Table 1. Effect of Caspase and JNK Inhibitors on the SRP Induced Proliferative Inhibition and Cell Apoptosis

Agents	Proliferative inhibition rates (%)	Apoptotic rates (%)
Control	-	1.4 \pm 0.13
300 $\mu\text{g/ml}$ SRP	68 \pm 5.23	28.6 \pm 0.87
20 μM z-VAD-fmk	5 \pm 1.26	1.8 \pm 0.24
20 μM SP600125	8 \pm 0.75	1.2 \pm 0.11
z-VAD-fmk+SRP	52 \pm 2.45	17.6 \pm 0.68
SP600125+SRP	38 \pm 3.21	12.8 \pm 0.42

*Cells were pretreated with the indicated inhibitors for 2 h, and then treated with SRP for another 24 h. The cell proliferation and apoptosis were determined by MTT assay and FACS respectively. The apoptotic rate here includes the early and late apoptotic cell fractions

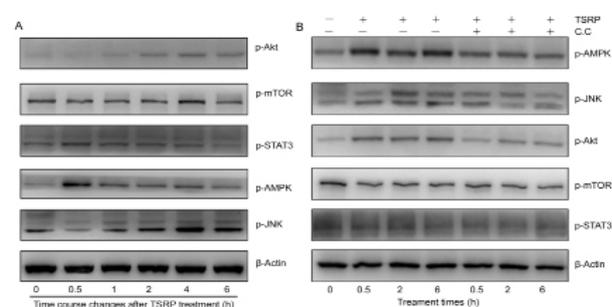


Figure 3. Time course analysis of the Survival Pathways with SRP Treatment in K562 Cells. (A) Cells were treated with SRP for the indicated times, after which the cells were collected and subjected to western blot analysis. (B) Effect of AMPK inhibitor on the survival pathways after SRP treatment. K562 cells were pretreated with the AMPK compound C for 2 h to inhibit the activity of AMPK pathway. Then, SRP was incubated with cells for the indicated times and western blot was used to determine the expressions of the indicated proteins

C and STAT3 inhibitor AG490 were used to determine the proliferation and cell apoptosis after SRP exposure. Cells were pretreated with C. C or AG490 for 2h, and then treated with 300 $\mu\text{g/ml}$ of SRP for another 24h. The cell viabilities and apoptosis rates were subsequently determined. As shown in Figure 4, the anti-proliferative inhibitory and cell apoptotic rates by single SRP were 64% and 28%, respectively. After pretreated with C. C and AG490, SRP inhibited the proliferation of K562 cells by 41% and 87%, and induced apoptotic cell fraction by 18% and 35%, respectively. Compared with cells treated with SRP alone, cells pretreated with C. C exhibited much higher cell viabilities and lower apoptotic fraction, indicating that inhibition of AMPK pathway blocked the SRP-induced cell apoptosis in K562 cells. While STAT3 inhibitor AG490 pretreatment increased the activity of SRP, resulting in significantly lower cell viabilities and higher apoptotic fraction, suggesting that STAT3 pathway plays important roles in the regulation of apoptosis induced by SRP.

To confirm the effects C. C and AG490 for the apoptosis induction of SRP, the expressions of apoptotic related proteins were then determined by western blot. Results clearly indicated that C. C pretreatment significantly decreased the cleavages of PARP, caspase-3 and caspase-9, while AG490 pretreatment dramatically increased the activities of these pro-apoptotic proteins, which was consistent with the FCAS analysis. Furthermore, the pretreatment with C. C and AG490 in K562 cells also changed the expressions of anti-apoptotic proteins bcl-2 and c-myc induced by SRP, indicating that SRP inhibited the expressions of bcl-2 and c-myc through AMPK and STAT3 pathways. More interestingly, after cells pretreated with C. C, the activation of JNK pathway was not observed by SRP, whereas a significant increased expression of phospho-JNK was observed by pretreatment with STAT3

inhibitor AG490, suggesting that the activation of JNK by SRP was at least in part, through AMPK and STAT3 pathways.

Our results clearly demonstrated that the up-regulation of JNK was involved in the apoptosis induction by SRP. To better understand the role of JNK pathway, we used the JNK inhibitor SP600125 to determine the changes in cell viability and apoptosis after SRP exposure. Inhibition of JNK significantly prevented the proliferative inhibition and apoptotic induction effect by SRP (Table 1). The apoptotic rate induced by single 300 $\mu\text{g/ml}$ of SRP was 28.6%, while it reduced to 12.8% in the presence of SP600125, further confirming the involvement of JNK pathway in the induction of apoptosis by SRP. Overall, our results demonstrated that SRP induced cell apoptosis of K562 by JNK activation, dependent on the AMPK and STAT3 signaling pathways.

Discussion

Rubus parvifolius L. is a raspberry plant in the Rosaceae family, which was used as a traditional Chinese medicine for treatment of many diseases. The saponins were the main active chemical components in *Rubus parvifolius* L. and exhibited toxicity against human cancer cell lines, suggesting saponins of *Rubus parvifolius* L. (SRP) are likely candidates for development as chemotherapeutic drugs. In the present study, we assayed the apoptotic effects of SRP as well as its apoptotic mechanism in human leukaemia cells. Our results demonstrated that SRP dose-dependently inhibited K562 cell proliferation and induced apoptosis through AMPK activation and STAT3 inhibition, suggesting that SRP could be potentially used as CML preventive or treatment agent.

Nature plants extracts are prime source of highly effective conventional anti-cancer drugs. Herbal medications are currently widespread for clinical use in therapy, as the herbs have relatively mild bioavailability and low toxicity (Kma, 2013). The crude extract from leaf of *Pereskia sacharosa* (Cactaceae) significantly induces leukemic cell death via apoptosis induction and changes in cell cycle checkpoint, suggesting potential applications in leukemia treatment (Asmaa et al., 2014). The HPLC fraction from *Voacanga globosa* (Blanco) Merr. exhibited obvious apoptosis-induction effect in human colon carcinoma cell (Acebedo et al., 2014). The ethanol extract of pendulous monkshood root also showed anti-tumor activities, suggesting therapeutic potential of Pendulous Monkshood Root for cancer treatment (Huang et al., 2013). However, insufficient scientific data are available on the efficacy of herbal therapies. The precise mechanisms for the effects of these herbs remain to be elucidated, and limited data and little convincing evidence have been provided at the molecular levels. Therefore, basic research and development with the objective of elucidating the mechanisms of action underlying the herbal effects should be a priority. The previous studies have indicated the antitumor activities of SRP in several cancer cells. However, the underlying pharmacological action of SRP against tumor cells still remained unknown, limiting further investigations of SRP as potential cancer

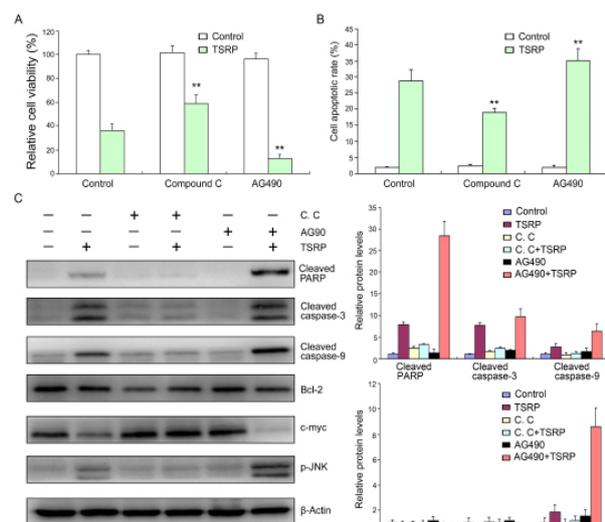


Figure 4. Effect of Inhibitors for AMPK and STAT3 Pathways on the Proliferation Inhibition and Apoptosis Induction of SRP in K562 Cells. Cells were pretreated with compound c or AG490 for 2 h, and then incubated with SRP for another 24 h. The cell viabilities (A) and apoptotic rates (B) were assayed by MTT and FACS analysis, respectively. The expressions of apoptotic related proteins were determined by western blot analysis (C)

prevention agent. Here, we demonstrated that SRP induced apoptosis of K562 cells involved the regulations of AMPK, STAT3 and JNK pathways. Our results indicated that phosphorylated-AMPK and JNK were induced by SRP in human K562 cells, and that the activity of STAT3 signaling pathway was decreased after the treatment of SRP. These results suggested the underlying mechanisms for SRP at molecular levels in human CML cells, providing potential therapeutic targets and important guides for further systematic study of SRP.

Dysregulation of apoptosis is usually considered as a major cancer hallmark (Hanahan et al., 2011). Therefore, screening agents to induce cancer cell apoptosis was an effective approach for drug development (Indran et al., 2011). The apoptosis induction effect of SRP in CML K562 cells was demonstrated by the DAPI and Annexin V-FITC staining analysis. Moreover, the observation of caspase-3 activation and PARP cleavage also confirmed that the promotion of apoptosis by SRP involves a caspase dependent pathway. A down-regulation of bcl-2 expression by SRP was also found in the present investigations. Bcl-2 plays important roles in promoting cell proliferation and regulating apoptosis in various human cancers. Over-expression bcl-2 confers resistance toward chemotherapy, and preclinical studies have shown that agents targeting anti-apoptotic bcl-2 have preclinical activity as single agents and in combination with other antineoplastic agents (Colie et al., 2009). The inhibition effect on bcl-2 expression indicated promising applications of SRP for the treatment drug resistant CML patients.

Cell apoptosis process is known to be controlled by a diverse range of cell signals. The c-Jun-N-terminal kinase (JNK) is a crucial regulator for mitochondrial dysfunction and the subsequent initiation of apoptosis (Mao et al., 2008). JNK activation could initiate release of apoptosis-regulating factors, such as cytochrome c and Smac. Our results indicated that phosphorylation of JNK was remarkably induced by the exposure of SRP, suggesting the induction of apoptosis was probably related at least in part to JNK signaling pathways. In order to better understand the molecular mechanism, we further determined the induction of apoptosis by inhibiting the JNK after SRP treatment. Pretreatment with JNK specific inhibitor partially conferred resistance of CML cells toward SRP, indicating that JNK activation was important for efficacy of SRP (Table 1). Previous studies have indicated the regulation of JNK pathway through the AMP-activated protein kinase (AMPK) in cancer cells (Chen et al., 2013). Here, we also demonstrate the activation of AMPK by SRP in CML cells. The AMPK specific inhibitor compound C prevented SRP-mediated induction of apoptosis and CML cell death, suggesting a central role of AMPK during the apoptosis induction of SRP (Figure 4). Furthermore, inhibition of AMPK also decreased the activity of JNK pathway after SRP exposure, indicating that the activation of AMPK by SRP induces apoptosis of CML cells at least by a signaling pathway involving activation of JNK. Our results suggested that JNK may be the key mediator of SRP's antileukemia activity.

Taken together, our results provide the first evidence

that SRP could inhibit the proliferation of K562 cells by inducing apoptosis. The anti-tumor effect was associated with the activation of AMPK and JNK, and inhibition of STAT3 pathways. Taking into account the wide acceptance and low toxicity of plant extracts, our study suggested the possibility of further developing SRP as an alternative treatment option, or perhaps using it as adjuvant chemotherapeutic agent in the treatment of CML. Furthermore, these results provided valuable information for further systematic investigations of SRP.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81202820 and 81303269), Zhejiang Provincial Natural Science Foundation of China (No. Q12H28005) and Zhejiang Provincial Administration of Traditional Chinese Medicine (No. 2011ZA081, 2012ZB120 and 2013ZB095).

References

- Acebedo AR, Amor EC, Jacinto SD (2014). Apoptosis-inducing activity of HPLC fraction from *Voacanga globosa* (Blanco) Merr. on the human colon carcinoma cell. *Asian Pac J Cancer Prev*, **15**, 617-22.
- Asmaa MJ, Al-Jamal HA, Ang CY, et al (2014). Apoptosis induction in MV4-11 and K562 human leukemic cells by *Pereskia sacharosa* (Cactaceae) leaf crude extract. *Asian Pac J Cancer Prev*, **15**, 475-81.
- Bewry NN, Nair RR, Emmons MF, et al (2008). Stat3 contributes to resistance toward BCR-ABL inhibitors in a bone marrow microenvironment model of drug resistance. *Mol Cancer Ther*, **7**, 3169-75.
- Chahardouli B, Zaker F, Mousavi SA, et al (2013). Evaluation of T315I mutation frequency in chronic myeloid leukemia patients after imatinib resistance. *Hematology*, **18**, 158-62.
- Chen MB, Zhang Y, Wei MX, et al (2013). Activation of AMP-activated protein kinase (AMPK) mediates plumbagin-induced apoptosis and growth inhibition in cultured human colon cancer cells. *Cellular signalling*, **25**, 1993-2002.
- Colie S, Van Veldhoven PP, Kedjouar B, et al (2009). Disruption of sphingosine 1-phosphate lyase confers resistance to chemotherapy and promotes oncogenesis through Bcl-2/Bcl-xL upregulation. *Cancer Res*, **69**, 9346-53.
- Corbin AS, Agarwal A, Loriaux M, et al (2011). Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*, **121**, 396-409.
- De Angel RE, Smith SM, Glickman RD, Perkins SN, Hursting SD (2010). Antitumor effects of ursolic acid in a mouse model of postmenopausal breast cancer. *Nutr Cancer*, **62**, 1074-86.
- Deininger MW, Goldman JM, Melo JV (2000). The molecular biology of chronic myeloid leukemia. *Blood*, **96**, 3343-56.
- Druker BJ, Guilhot F, O'Brien SG, et al (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*, **355**, 2408-17.
- Fan GH, Wang ZM, Yang X, et al (2014). Resveratrol inhibits oesophageal adenocarcinoma cell proliferation via AMP-activated protein kinase signaling. *Asian Pac J Cancer Prev*, **15**, 677-82.
- Fujiwara Y, Takaishi K, Nakao J, et al (2013). Corosolic acid enhances the antitumor effects of chemotherapy on epithelial ovarian cancer by inhibiting signal transducer and activator

- of transcription 3 signaling. *Oncol Lett*, **6**, 1619-23.
- Gambacorti-Passerini CB, Gunby RH, Piazza R, et al (2003). Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet Oncol*, **4**, 75-85.
- Gao J, Sun CR, Yang JH, et al (2011). Evaluation of the hepatoprotective and antioxidant activities of *Rubus parvifolius* L. *J Zhejiang Univ Sci B*, **12**, 135-42.
- Ge Y, Cheng R, Zhou Y, et al (2012). Cryptotanshinone induces cell cycle arrest and apoptosis of multidrug resistant human chronic myeloid leukemia cells by inhibiting the activity of eukaryotic initiation factor 4E. *Mol Cell Biochem*, **368**, 17-25.
- Gu Y, Chen T, Meng Z, et al (2012). CaMKII gamma, a critical regulator of CML stem/progenitor cells, is a target of the natural product berbamine. *Blood*, **120**, 4829-39.
- Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell*, **144**, 646-74.
- Huang XJ, Ren W, Li J, Chen LY, Mei ZN (2013). Anti-inflammatory and anticancer activities of ethanol extract of pendulous monkshood root *in vitro*. *Asian Pac J Cancer Prev*, **14**, 3569-73.
- Indran IR, Tufo G, Pervaiz S, Brenner C (2011). Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta*, **1807**, 735-45.
- Ji C, Yang B, Yang YL, et al (2010). Exogenous cell-permeable C6 ceramide sensitizes multiple cancer cell lines to Doxorubicin-induced apoptosis by promoting AMPK activation and mTORC1 inhibition. *Oncogene*, **29**, 6557-68.
- Kim I, He YY (2013). Targeting the AMP-activated protein kinase for cancer prevention and therapy. *Front Oncol*, **3**, 175.
- Kma L (2013). Roles of plant extracts and constituents in cervical cancer therapy. *Asian Pac J Cancer Prev*, **14**, 3429-36.
- Li T, Xu WS, Wu GS, et al (2014). Platycodin D induces apoptosis, and inhibits adhesion, migration and invasion in hepG2 hepatocellular carcinoma cells. *Asian Pac J Cancer Prev*, **15**, 1745-49.
- Ma LD, Zhou M, Wen SH, et al (2010). Effects of STAT3 silencing on fate of chronic myelogenous leukemia K562 cells. *Leuk Lymphoma*, **51**, 1326-36.
- Mao X, Yu CR, Li WH, Li WX (2008). Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells. *Cell Res*, **18**, 879-88.
- Vakana E, Altman JK, Glaser H, Donato NJ, Plataniotis LC (2011). Antileukemic effects of AMPK activators on BCR-ABL-expressing cells. *Blood*, **118**, 6399-402.
- Zhang XJ, Xu XF, Gao RL, Xu JF (2014). *Rubus parvifolius* L. inhibited the growth of leukemia K562 cells *in vitro* and *in vivo*. *Chin J Integr Med*, **20**, 36-42.
- Zheng L, Zhang YM, Zhan YZ, Liu CX (2014). *Momordica cochinchinensis* seed extracts suppress migration and invasion of human breast cancer ZR-75-30 cells via down-regulating MMP-2 and MMP-9. *Asian Pac J Cancer Prev*, **15**, 1105-10.