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# Mitochondrial apoptosis contributes to the anti-cancer effect of *Smilax glabra Roxb.*

Yujing Gao[a,1,2], Yahui Su[a,1], Like Qu[a], Shuo Xu[b], Lin Meng[a], Shao-Qing Cai[b,*], Chengchao Shou[a,**]

[a] *Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, Peking University Cancer Hospital & Institute, Beijing 100142, China*
[b] *State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China*

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ABSTRACT

*Smilax glabra Roxb.* (SGR), a member of the Smilacaceae family and a rhizome of the Liliaceae plant, has shown anti-inflammation and detoxification properties, and a few studies reported its anti-cancer effect. In this study, we showed that SGR inhibited growth of human breast cancer cell line MCF7, colon carcinoma cell line HT-29, and gastric cancer cell line BGC-823 in a dose-dependent manner. Furthermore, SGR could inhibit tumor growth of HT-29 in Balb/c nude mice and murine hepatoma H22 cells in ICR mice. SGR elicited apoptotic cell death, as confirmed by DNA ladder formation, changes in nuclear morphology, and the increased FITC-Annexin-V/PI staining. Permeabilization of mitochondrial membrane (MMP), production of reactive oxygen species (ROS), elevation of intracellular [$Ca^{2+}$], relocation of cytochrome c, and the activation of caspase-3 were found to be associated with the initiation of apoptosis by SGR treatment. Using microarray analysis, we found the changes in expression profiles of genes related to apoptosis, proliferation and cell cycle control in the cells treated with SGR. Our results demonstrated the mitochondrial regulation of apoptosis by which SGR exerts the anti-cancer effect.



## 1. Introduction

Until now, cancer remains a significant health problem and poses a huge economic burden all over the world. The WHO (World Health Organization) predicts that by 2030 twelve million of all deaths worldwide will be due to cancer (Bode and Dong, 2009). Despite of considerable efforts devoted to the study of the tumor biology, current therapies still largely rely on the cytotoxic agents (Cotter, 2009). Due to the less toxicity and cost-effectiveness, and the progress in herbogenomics, traditional herb medicine increasingly attracts more attentions as alternative cancer therapies. *Smilax glabra Roxb.* (SGR) is a member of the Smilacaceae family and its rhizome has been used for anti-inflammation and detoxification in traditional Chinese medicine. Moreover, it was used in the preparation of traditional medication administered to cancer patients in Sri Lanka and Thailand (Iddamaldeniya et al., 2006; Itharat et al., 2004). Few studies had reported its anti-cancer effect, mainly for hepatoma cell lines in vitro (Sa et al., 2008; Thabrew et al., 2005), and EAC, S180, and H22 cells in vivo (You et al., 2001). In addition, a group of glycoproteins isolated from *Smilax glabra* (named as SGPFs) showed antiviral activity against respiratory syncytial virus (RSV) and Herpes simplex virus type 1 (HSV-1), and had the ability to suppress the proliferation of MCF7 cells through enhanced apoptosis (Ooi et al., 2008). All these findings indicated the potential of SGR for cancer therapy.

Apoptosis plays an important role in maintaining the tissue homeostasis and elimination of damaged cells, therefore it is tightly related with cell proliferation and differentiation. Cell shrinkage, chromatin condensation, and nuclear fragmentation are the morphological hallmarks of apoptosis (Saraste and Pulkki, 2000). The process of apoptosis is highly complex and sophisticated, involving a large number of genes, such as BCL-2, TNF, NF-κB, p53, and caspase. To date, two major apoptotic pathways had been found: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The best-characterized death ligands and their cognate receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5. In addition, the

* Corresponding author at: Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, No. 38, Xueyuan Road, Beijing 100191, China. Tel.: +86 10 82801693; fax: +86 10 82801693.
** Corresponding author at: Department of Biochemistry and Molecular Biology, Peking University Cancer Hospital & Institute, 52 Fucheng Road, Beijing 100142, China. Tel.: +86 10 88196766; fax: +86 10 88122437.
*E-mail addresses:* sqcai@bjmu.edu.cn (S.-Q. Cai), scc@bjcancer.org (C. Shou).
[1] Both authors contributed equally to this work.
[2] Present address: Key Laboratory of Fertility Preservation and Maintenance (Ministry of Education), Key Laboratory of Reproduction and Genetics in Ningxia, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Ningxia Medical University, Yinchuan 750004, China.

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endoplasmic reticulum (ER) stress, T-cell mediated cytotoxicity, and perforin-granzyme-dependent pathway also contribute to apoptotic execution of target cells (Elmore, 2007). There should be a fine balance between pro-apoptotic and anti-apoptotic mechanisms, and disturbances in the cell death process may lead to uncontrolled cell growth and tumorigenesis (Cotter, 2009; Zhivotovsky and Orrenius, 2006). Therefore, molecules involved in apoptotic control are considered to be targets for cancer therapy.

Despite that SGR had been found to induce apoptotic cell death, the molecular mechanism remains elusive. In this study, we showed that SGR inhibited the growth of tumor cells in vitro and in vivo mainly through regulating the mitochondrial apoptosis pathway.

## 2. Materials and methods

### 2.1. Crude drug, cell lines, and reagents

The dried rhizome of SGR was purchased from the Beijing Tong Ren Tang Pharmacy (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen (Grand Island, NY). Hoechst 33342 was obtained from Sigma Chemical (St Louis, MO). JC-1, Fluo-3 AM, DCFH-DA, and DAPI were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The human breast cancer MCF7 cell line, human colon cancer HT-29 cell line, and murine hepatoma H22 cell line were from the American Type Culture Collection (ATCC). The human gastric cancer BGC-823 cell line was maintained in our laboratory. All the cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Invitrogen).

### 2.2. Preparation of SGR

SGR was smashed and extracted with 95% ethanol under reflux for three times, 2 h each time. After leaching, the filtrate was combined. The dregs were then extracted with 50% ethanol under reflux for three times, 2 h each time. The extracted liquids were centrifuged at 4980 rpm for 10 min, and then the supernatants were combined with the 95% ethanol extracts. After evaporation of the combined extracts, the residue precipitate was dissolved in Milli-Q water, lyophilized, and stored at room temperature. The extraction efficiency of SGR is 12.19%, and the concentration used in the experiment was based on the dry weight of SGR rhizome (mg/mL). SGR extract was dissolved in PBS containing DMSO when used, and further diluted in cell culture medium so that the final DMSO concentration did not exceed 0.2% (v/v).

### 2.3. Cell survival assays

Cell viability was determined by the MTT assay. MCF7, HT-29 and BGC-823 cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells/well in RPMI1640 plus 5% FBS, allowed to attach for 12 h, then treated with vehicle (control) or indicated concentrations of SGR for 24 h. MTT was added to each well at a final concentration of 0.5 mg/mL for additional 4 h incubation at 37 °C. The medium containing the MTT solution was aspirated off and DMSO (150 μl) was added to solubilize the formazan salt formed. The amount of formazan salt was determined by measuring the optical density at 492 nm using a Bio-Rad microplate reader (Hercules, CA). The cytotoxicity of SGR was expressed as IC50 (the concentration for 50% inhibition of cell growth), which was extrapolated from linear regression analysis of experimental data.

### 2.4. Colony formation assay

MCF7, HT-29 and BGC-823 cells were plated in 12-well culture plate (500–600 cells per well) respectively and allowed to attach for 12 h, then treated them with vehicle (control) or indicated concentrations of SGR. After incubation for another 7 days, cells were washed with PBS, fixed with methanol for 30 min, and stained with 0.5% crystal violet solution. Colonies (>50 cells per colony) were counted under an inverted microscope.

### 2.5. Animal studies

Animal studies were approved and supervised by the Biomedical Ethical Committee of Peking University Cancer Hospital & Institute. For HT-29 xenograft mouse model, 5–6 week old nude mice weighing approximately 20 g were divided into two groups, five each group. HT-29 cells were subcutaneously injected ($5 \times 10^6$ per mouse). After the tumors had established (about 100 $mm^3$ in size), the mice were orally administered with or without 1 mL SGR (1 g/mL) everyday. After 15 days, the mice were sacrificed, and body weight and tumor weight were measured. For H22 allograft model, ICR mice weighing approximately 20 g were used. H22 cells were maintained by weekly intraperitoneal injection passages in ICR mice. 7 days after the tumor inoculation, 0.2 mL 1:6 diluted ascites from H22 tumor-bearing mice were subcutaneously injected into the right armpit region of ICR mice. 24 h later, the mice were randomly divided into two groups of ten each (half female and half male), and orally administered with or without 1 mL SGR (1 g/mL) or PBS daily. After 7 days, the mice were sacrificed, and body weight and tumor weight were measured.

### 2.6. Nuclear staining with Hoechst 33342

MCF7, HT-29 and BGC-823 cells were seeded onto glass coverslips in 6-well culture plate. After treatment with SGR for 48 h, cells were fixed with 4% paraformaldehyde in PBS for 30 min, and stained with Hoechst 33342 (5 μg/mL) in PBS for 10 min at room temperature. The cells were then washed with PBS for two more times and examined under a Leica TCS SP5 fluorescence microscope.

### 2.7. DNA fragmentation assays

SGR treated or untreated cells were lysed in the buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM NaCl, 5 mM EDTA and 1% Triton X-100 for 30 min on ice. The lysates were vortexed and cleared by centrifugation at 10,000 rpm for 20 min. The DNA in the supernatant was extracted using equal volume of neutral phenol and chloroform, followed by precipitation with two volumes of dehydrated alcohol, and analyzed electrophoretically on 2% agarose gels containing 0.1 mg/ml ethidium bromide.

### 2.8. Flow cytometric analysis of apoptotic cells

After cells were exposed to the indicated concentration of SGR for 24 h, the apoptotic cell death was determined using an FITC-Annexin V/PI Apoptosis Detection Assay according to the manufacturer's protocol (Beijing Biosea Biotechnology, China).

### 2.9. Assays for mitochondria transmembrane potential (MMP), reactive oxygen species (ROS) production, and intracellular [$Ca^{2+}$] ([$Ca^{2+}$]i)

To measure the mitochondrial transmembrane potential, the dual-emission potential sensitive probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) was used. Briefly, the indicated cells were loaded with 1×JC-1 at 37 °C for 20 min, then washed, and analyzed by Leica TCS SP5 fluorescence microscope. JC-1 is green, whereas the membrane potential of energized mitochondria promotes the formation of red-fluorescent JC-1 aggregates. Mitochondrial permeability transition was assessed with decreased red fluorescence, which indicates the presence of mitochondria with a lower membrane potential ($\Delta\psi_m$). The intracellular generation of ROS was measured using 10 μM cell-permeable probes 29, 79-dichlorofluorescein diacetate (DCFH-DA). Intracellular [$Ca^{2+}$] was measured using 2 μM Fluo-3 AM. After probing for 20–30 min, cells were analyzed by Leica TCS SP5 fluorescence microscope. Fluorescence intensity was quantified by Leica Microsystems LAS AF simulator software.

### 2.10. Immunofluorescence

Cells were grown on the coverslips and treated with SGR for 24 h. The treated cells were fixed in cold methanol for 30 min, and permeabilized with 0.2% Triton X-100/PBS at room temperature for 5 min. After blocking with 5% goat serum, cells were incubated with anti-cytochrome c (A-8, Santa Cruz Inc., Santa Cruz, CA) in 1% BSA/PBS overnight, then washed, and stained with FITC-conjugated anti-mouse secondary antibody for 60 min at room temperature. Nuclei were counterstained with DAPI. Cells were examined by Leica TCS SP5 fluorescent microscope.

### 2.11. Immunoblotting

Cells were treated with indicated concentrations of SGR for 24 h, and lysed in cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protein inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were subjected to SDS–PAGE and immunoblotting as previously described (Hernandez-Vargas et al., 2006). The polyclonal antibody against caspase-3 (#9662, Cell Signalling Technology, Beverly, MA) was used at 1:1000 dilution, polyclonal antibody against β-actin (N-21, Santa Cruz Inc.) was used at 1:500 dilution.

### 2.12. Gene expression microarray analysis

Total RNA from SGR-treated (20 mg/ml) and untreated (vehicle control) MCF7 and HT-29 cells were isolated using TRIZOL Reagent (Invitrogen) for expression analysis as described by the provider. Whole human genome microarray chip (Agilent) was used to identify the changes in mRNA expression. Microarray hybridization, data acquisition and analysis were performed by OE Bio-tech (Shanghai, China).

### 2.13. Quantitative real-time PCR

The same preparation of RNA used for microarray experiments was also subjected to quantitative real-time PCR (qPCR). qPCR was composed of 10 pmol of sense and antisense primers and 12.5 μl of 2× SYBR Green Supermix (TOYOBO) to make a