Scutellaria Extract Decreases the Proportion of Side Population Cells in a Myeloma Cell Line by Down-regulating the Expression of ABCG2 Protein

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Abstract

Background and Aims: Scutellaria is one of the most popular traditional Chinese herbal remedies against various human diseases, including cancer. In this study, we examined the active effects of Scutellaria extract and its main flavonoid constituents on the proportion of side population cells within human multiple myeloma cell line RPMI8226 in vitro and explored the potential molecular mechanisms involved. Materials and Methods: The contents of flavonoids in ethanolic extract of Scutellaria baicalensis Georgi were determined using high performance liquid chromatography. The antiproliferative effect of the ethanolic extract on RPMI-8226 was determined by CCK assay. Apoptosis was measured by annexin combining with propidium iodide in a flow cytometer. Cell cycle analysis was performed by propidium iodide staining in combination with flow cytometry analysis. Hoechst 33342 exclusion assay was used for the identification of side population within RPMI8226 cells. The expression of ABCG2 protein was assessed by Western blotting assay. Results: The content of major flavonoids constituents of Scutellaria extract was baicalin (10.2%), wogonoside (2.50%), baicalein (2.29%), and wogonin (0.99%), respectively. The crude Scutellaria extract did not show significant anti-proliferative effect, apoptosis induction and cell cycle arrest in RPMI-8226 within the concentrations of 1-75µg/mL. However, the ethanolic extract, baicalein, wogonin and baicalin reduced the side population cells in RPMI-8226, and data showed that baicalein and wogonin had stronger inhibitory effects. Correspondingly, they also exhibited significant effects on decreasing the expression level of ABCG2 protein in RPMI-8226 in vitro. Conclusions: Our results for the first time demonstrated a novel mechanism of action for Scutellaria extract and its main active flavonoids, namely targeting SP cells by modulating the expression of ABCG2 protein. This study provides an insight for new therapeutic strategies targeting cancer stem cells of multiple myeloma.

Keywords: Multiple myeloma - Scutellaria - side population cells - ABCG2 protein

Introduction

Multiple myeloma (MM), one of the most common hematological malignancies among older people, remains largely incurable and fatal (Jemal et al., 2008; Taniguchi et al., 2009). The MM patients are prone to quickly relapse with an average survival time of 4-7 years, despite advance in treatment with new therapeutic agents, such as thalidomide, lenalidomide, and bortezomib (Dmoszynska et al., 2008). It has been postulated that current anti-MM strategies are effective in targeting the bulk of tumor cells, however, the persistence of a tumor-initiating subpopulation or cancer stem cells may be responsible for eventual relapses and poor prognosis (Matsui et al., 2008). Side population (SP) cells are enriched cancer-initiating cells with stem cell properties, which have been identified in MM in our previous studies (Mo et al., 2011), as well as in other hematopoietic malignancies and solid tumors (Kabashima et al., 2009; Feng et al., 2010; Hu et al., 2010; Salcido et al., 2010; Mo et al., 2011; Hiraga et al., 2011; Van et al., 2012).

SP cells are first described as a subset of adult mouse bone marrow with enriched hematopoietic stem cells (Goodell et al., 1996; Goodell et al., 1997). This subset is characterized by its ability to rapidly efflux the Hoechst 33342 DNA-binding dye and therefore shows a distinct “low-staining profile” with the Hoechst 33342 dye and locates sideways from the diagonal on flow cytometry (FCM) profile. Recent studies have shown the presence of SP cells in various human tumor including ovarian cancer (Moserle et al., 2008), glioblastoma (Wu et al., 2008), lung cancer (Wu et al., 2008; Shi et al., 2012),
Scutellaria Extract Decreases the Proportion of Side Population Cells in Myeloma Cells by Down-regulating ABCG2 Protein
Mei-Gui Lin et al

Preparation of Extract and HPLC Analysis
The dried roots of Scutellaria baicalensis Georgii (1000g) were obtained from TCM pharmacy of the First Affiliated Hospital, Sun Yat-sen University. The dried roots were ground to powder and extracted with 70% ethanol for 2 h. The filtrate of extraction was collected and stored at 4°C prior to use. Scutellaria extract, and stored at 4°C prior to use. Scutellaria extract was examined by analytical high-performance liquid chromatography (HPLC) as described (Wang et al., 2010). Briefly, the Scutellaria extract was carried out on a Phenomenex Prodigy ODS (2) column (150×3.2 mm, 5 μm) in HPLC system (Milford, MA, USA). A binary gradient solvent system of acetonitrile (eluent A) - 0.03% (v/v) phosphoric acid in water (eluent B) was used as follows: 15% A and 85% B (0 min), 28% A and 72% B (12 min), 35% A and 65% B (23 min), 50% A and 50% B (30 min), 95% A and 5% B (32-34 min), 15% A and 85% B (37-42 min). The flow rate of 0.8 ml/min was used and absorbance was detected at 280 nm. The tested solution was filtered through Millex 0.2 μm nylon membrane syringe filters (Bedford, MA, USA) before use. The contents of the constituents were calculated using standard curves of flavonoids.

Chemicals
Flavonoid standards baicalin, wogonoside, baicalein and wogonin were obtained from Sigma (St Louis, MO). All standards were of biochemical-reagent grade and at least 98% pure as confirmed by HPLC. HPLC grade methanol, ethanol, n-butanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Cell culture medium RPMI-1640 was obtained form Invitrogen, Inc. (Carlsbad, CA, USA). Adriamycin, Hoechst 33342, fumitremorgin C (FTC) and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA).

Cell Culture
The human myeloma cell line RPMI-8226 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). RPMI-8226 cells were cultured in RPMI-1640 containing 100 μg/mL of penicillin (Invitrogen), 100 μg/mL of streptomycin (Invitrogen), and 10% fetal bovine serum (FBS) (Sigma, St Louis, MO). Cells were maintained at 37°C in a humidified atmosphere, with 5% carbon dioxide and 95% air.

Cell Viability Assays Using Cell Counting Kit (CCK) Method
To evaluate the growth inhibitory effect of Scutellaria extract on myeloma cells, cell counting kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan) colorimetric assay was performed according to the manufacturer’s instructions. Briefly, cells were seeded into 96-well plates. Various concentrations of Scutellaria extract or Adriamycin were added to the wells. Controls were exposed to culture medium containing 0.2% DMSO without drugs. After treatment for 44 h and 68 h, 10 μL of CCK-8 solution was added to each well of the plate and cells were incubated for 4 h. This was followed by measurement of absorbance at a wavelength of 492 nm using a microplate reader (Thermo Labsystems, Waltham, MA, USA). To calculate the cell viability, the absorbance readings were plotted and analyzed. Results were expressed as a percentage of the control.

Cell Cycle Analysis by Flow Cytometry
Cells (3×10⁶/well) were plated into 35-mm dishes to yield 90% confluence within 3 h and then treated with Scutellaria extract in various concentration (10 and 50 μg/mL) for 24 h. Both the adherent and floating cells were harvested, and the cells were resuspended in PBS, fixed with 70% ethanol at 4°C overnight. The cells were first incubated with Rhose A (50 μg/mL, Sigma Co. St. Louis,
MO) at 37°C for 30 min and then labeled with propidium iodide (PI, 0.1 mg/ml) and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using a FACScan instrument equipped with FACStation running cell Quest software (Becton-Dickinson). For each measurement, at least 20,000 cells were counted.

Apoptosis Assay
Cells were seeded in 24-well tissue culture plates for 3 h to yield 90% confluence and then treated with Scutellaria extract in various concentrations (10 and 50 µg/mL). After treatment for 48 h, cells were collected by centrifugation. The cells were stained with Annexin V-FITC and PI according to the manufacturer’s instructions. Untreated cells were used as a control for double staining. The cells were analyzed immediately after staining using a FACScan flow cytometer.

Hoechst 33342 Staining and Flow Cytometric Analysis
Cells were seeded in 100-mm culture dish for 3 h to yield 90% confluence and then treated with Scutellaria extract or Adriamycin at the desired concentrations. After incubation for 24 h, the cells were harvested and stained with Hoechst 33342 dye as described previously (Salcido et al., 2010). Briefly, after discarding culture medium, cells were suspended in the staining medium RPMI1640+ containing 2% FBS and 10mM HEPES buffer. Live cell number was counted at least twice and adjusted to a final cell density of 1×10⁶ cells/mL by adding appropriate volume of warm staining medium. Hoechst 33342 water solution (1 mg/mL) was then added to make a final concentration of 3 µg/mL followed by incubation in a water bath at 37°C for 90 min with shaking every 30 min. Samples treated with 1 µg/mL FTC, an ABCG2 transporter inhibitor, were included during the entire staining procedure as controls for the SP gating. Once incubation finished, samples were immediately put on ice to stop dye efflux. Subsequently, the cells were centrifuged for 5 min at 300g at 4°C and resuspended in 300µL of ice-cold Hanks’ Balanced Saline Solution (HBSS) (Invitrogen) containing 2% FBS, 100 µg/mL of penicillin, 100 µg/mL of streptomycin, and 10 mM HEPES. The samples were kept on ice before flow cytometry analysis. PI solution was added at a final concentration of 2 µg/mL to exclude dead cells just before flow analysis.

Western Blot Analysis
The cells were seeded at a 1×10⁶ per 100-mm culture dish for 3 h to yield 90% confluence and then treated with Scutellaria extract or adriamycin at the desired concentrations. After treatment for 24 h, the cells were washed with ice-cold PBS and lysed with lysis buffer (50 mMol/L HEPES, pH 7.6, 150 mMol/L NaCl, 1% Triton X-100, 10 mMol/L NaF, 20 mMol/L sodium pyrophosphate, 20 mMol/L β-glycerol phosphate, 1 mMol/L sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mMol/L phenylmethanesulfonyl fluoride). The cell lysates were incubated on ice for 10 min and then centrifuged at 14000g for 10 min at 4°C. The supernatants were mixed with equal volumes of 2×SDS-PAGE sample loading buffer. After heating at 95°C for 4 min, the proteins were separated using a SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected with mouse monoclonal ABCG2 antibody (Santa cruz biotechnology, Santa Cruz, CA) antibodies. The membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (1:2,000). The immunoreactive protein bands were developed by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Freiburg, Germany). Relative optical density (ROD, ratio to GAPDH) of each blot band was quantified by using National Institutes of Health (NIH) image software (Image J 1.36b).

Statistical Analyses
All cell proliferation and flow cytometry experiments were performed in triplicate. Data were presented as means±SD. Data were analyzed using SPSS 13.0 software by one-way ANOVA with Dunnett’s post hoc test and Turkey’s post hoc test for multigroup comparisons. Student’s t-test was used for paired data. A P value of 0.05 or less was considered as significant.

Results

HPLC Analysis of Scutellaria Extract
The HPLC chromatograms of Scutellaria extract recorded at 280 nm. There were four major constituents found in Scutellaria extract: baicalin (1), wogonoside (2), baicalein (3), and wogonin (4). (B) The percentage of content of flavonoids in Scutellaria extract. (C) The chemical structures of these four flavonoids in Scutellaria extract

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Figure 1. HPLC analysis of Scutellaria Extract. (A) HPLC chromatograms of Scutellaria extract recorded at 280 nm. There were four major constituents found in Scutellaria extract: baicalin (1), wogonoside (2), baicalein (3), and wogonin (4). (B) The percentage of content of flavonoids in Scutellaria extract. (C) The chemical structures of these four flavonoids in Scutellaria extract

56.3
20.3
38.0
12.8
6.3

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Results

HPLC Analysis of Scutellaria Extract
The HPLC chromatograms of Scutellaria extract showed that the major constituents in Scutellaria extract were baicalin, wogonoside, baicalein and wogonin (Figure 1A). The content of baicalin, wogonoside, baicalein, and wogonin in Scutellaria extract were 10.22%, 25.0%, 2.92% and 0.99% (Figure 1B), respectively. The chemical structures of these four flavonoids in Scutellaria baikalenisa were shown in Figure 1C.

Effect of Scutellaria Extract on Proliferation of RPMI-8266
The anti-proliferative effects of Scutellaria extract...
Scutellaria Extract Decreases the Proportion of Side Population Cells in Myeloma Cells by Down-regulating ABCG2 Protein

Mei-Gui Lin et al


and adriamycin were evaluated by CCK-8 method. As shown in Figure 2A, Scutellaria extract did not inhibit cell growth. Contrarily, when the cells were treated for 24 h, Scutellaria extract actually increased cell growth mildly, although there was no significant difference found when compared to the control. However, adriamycin showed potent effect on cell growth inhibition. 2.5 µg/mL of adriamycin could inhibit cell growth by 95% (*P<0.001 vs. control) (Figure 2B).

Effect of Scutellaria Extract on Cell Apoptosis of RPMI-8226

An apoptosis assay was performed by FCM after staining with annexin V and PI. Early apoptotic cells located at lower right quadrant because they were positive for annexin V and negative for PI. After treatment for 48 h, the percentage of early apoptotic cells did not change obviously under the various concentration of Scutellaria extract (Figure 3A). Scutellaria extract showed no significant effect on cell apoptosis in vitro at the concentration of 10 and 50 µg/mL respectively.

Effect of Scutellaria Extract on Cell Cycle of RPMI-8226

An apoptosis assay was performed by FCM after staining with annexin V and PI. Early apoptotic cells located at lower right quadrant because they were positive for annexin V and negative for PI. After treatment for 48 h, the percentage of early apoptotic cells did not change obviously under the various concentration of Scutellaria extract (Figure 3A). Scutellaria extract showed no significant effect on cell apoptosis in vitro at the concentration of 10 and 50 µg/mL respectively.

Figure 2. Effects of Scutellaria Extract on Proliferation of Myeloma Cells.

Cells were treated with 5, 10, 25, 50, and 75 µg/mL of Scutellaria extract (A) and 0.01, 0.05, 0.5, 2.5 and 10 µg/mL of adriamycin (B) for 24, 48 and 72 h, and then assayed by CCK-8 method. Data were derived from three independent experiments.

Figure 3. (A) Effects of Scutellaria extract on cell apoptosis of myeloma cells. Myeloma cells were treated with 10 and 50 µg/mL of Scutellaria extract for 48h and stained with annexin V-FITC/PI. (B) Myeloma cells were treated with 10 and 50 µg/ml of Scutellaria extract for 24h. There was no significant difference in G1-, S- and G2/M- phase between control and test groups (*P>0.05).

Figure 4. Effect of Scutellaria Extract and Adriamycin on SP in RPMI-8226. (A) Representative FCM profiles of cells treated with Scutellaria extract and adriamycin. (B) Scutellaria extract–treated cells showed significantly decreased in proportion of SP cells in dose-dependent manner. *P<0.01 vs. blank or vehicle group; #P<0.01 vs. other concentrations of drug in same test group and adriamycin were evaluated by CCK-8 method. As shown in Figure 2A, Scutellaria extract did not inhibit cell growth. Contrarily, when the cells were treated for 24 h, Scutellaria extract actually increased cell growth mildly, although there was no significant difference found when compared to the control. However, adriamycin showed potent effect on cell growth inhibition. 2.5 µg/mL of adriamycin could inhibit cell growth by 95% (P<0.001 vs. control) (Figure 2B).

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Effect of Scutellaria Extract on Cell Cycle of RPMI-8226

Analysis of the cell cycle phase by FCM revealed that there was no significant difference in the cell cycle between the Scutellaria extract–treated RPMI-8226 cells and the control groups (Figure 3B).
Effect of Scutellaria extract on SP in RPMI-8226
We continued to investigate the effect of Scutellaria extract on the proportion of SP cells using FCM-based Hoechst 33342 staining. As whether the alternation of cell viability, cell cycle or apoptosis would influence SP yield remains poor understood, hence we chose the non-cytotoxic concentration of Scutellaria extract for SP study. In this study, there was no statistically significant difference in cell viability among all the groups (One-way ANOVA, \( P > 0.05 \), data not show). A portion of cells (from vehicle control) treated with 1 \( \mu \)g/mL FTC was as control group for SP gating. The results showed that SP% was significantly decreased when RPMI-8226 cells were treated with Scutellaria extract at desired concentrations for 24 h (Figure 4). Moreover, the proportion of SP cells decreased in a dose-dependent manner \( (P < 0.01) \) (Figure 5B). However, adriamycin-treated cells showed no significant difference in the proportion of SP cells only in higher dosage, and wogonoside did not show an effect on proportion of SP cells. These results indicated that baicalein and wogonin were two main constituents in Scutellaria extract contributing to the inhibition of SP cells.

Effect of Flavonoid Standards on SP Cells
To further characterize the potential effect of four main constituents in Scutellaria extract on proportion of SP cells, we carried out an SP cells assay by flow cytometry after treatment with four flavonoid standards. The results showed that baicalein and wogonin significantly decreased proportion of SP cells in RPMI-8226 in a dose-dependent manner (Figure 5, \( P < 0.01 \)), baikalin inhibited SP cells only in higher dosage, and wogonoside did not show an effect on proportion of SP cells.

Discussion
Since most tumors generated from human being are heterogeneous containing a spectrum of phenotypically different cell types, the complex characteristics of tumors may also require some alternative management to improve
the therapeutic efficacy of conventional treatment, including surgery, chemotherapy, and radiotherapy. It has been postulated that tumor-initiating cells or cancer stem cells (CSCs) resist current drug therapies and repair DNA after radiation treatment more efficiently than their differentiated, daughter cells (Dean et al., 2009; Moitra et al., 2011). CSCs can both undergo self-renewal and give rise to the entire tumor population, therefore, they are responsible for the recurrence of tumors after systemic treatment even in patients who achieved complete clinical remission (Alison et al., 2008; Jordan et al., 2009; Saigal et al., 2011). From this point, it is likely that a feasible new strategy to cure tumors is to target against the CSCs.

SP cell is considered as an enriched source of CSCs with stem cell properties, associated with chemoresistance and tumorigenicity in vivo. It has been identified in a variety of tumor types, including lung, gastric, esophageal, squamous, and ovarian carcinoma cell lines. Several recent reports and our previous study have also demonstrated the presence of SP cells in multiple myeloma, and these SP cells could survive in standard chemotherapeutics for MM (Loh et al., 2008; Matsui et al., 2008; Jakubikova et al., 2011). In this study, SP cells in RPMI-8226 were identified by FCM-based Hoechst 33342 staining, which was optimized in our previous study (Mo et al., 2011). We found that adriamycin (doxorubicin), a first-line chemotherapeutant for hematopoietic tumors, did not affect the percentage of SP cells in the MM cell line RPMI-8226, although it showed potent effect on inhibition of cell proliferation. In the present study, we did not examine the effects of adriamycin on cell cycle and cell apoptosis because it has long appreciated that adriamycin can induce DNA damage and arrest cell cycle in various tumors (Di et al., 2008; Marshall et al., 2008; Xu et al., 2011). Therefore, we postulate that adriamycin targets non-SP cells in tumor, which make up of the bulk of tumor, to inhibit the tumor growth and result in clinical remission after treatment with systemic chemotherapy. However, small proportions of SP cells persist after treatment and contribute to the recurrence of disease, as well as the drug-resistant behavior of tumors. Recently, some new therapeutic agents, such as thalidomide and lenalidomide, have been used in clinical for MM. However, only lenalidomide significantly decreased the percentage and clonogenicity of SP cells, as well as their repopulation ability, at clinically achievable concentrations (Jakubikova et al., 2011). In contrast, thalidomide did not change the proportion of the SP fraction and did not affect the clonogenicity of SP cells (Jakubikova et al., 2011). This difference implies intriguing possibility that lenalidomide acts as a new treatment strategies targeting presumptive MM stem/tumor-initiating cells.

Numerous effective anticancer drugs have been developed from botanical sources, however, the potentially effective anticancer compounds of herbs and involved mechanisms still needs to be investigated. The root of Scutellaria baicalensis Georgii is a widely recognized herb in the traditional medical systems of China and Japan in the treatment of various inflammatory diseases and hypertension with positive results for these diseases (Li-Weber et al., 2009). Accumulating evidence demonstrates that Scutellaria also possesses potent anticancer activities in various human cancers, including MM (Ma et al., 2005; Kumagai et al., 2007; Li-Weber et al., 2009). In the present study, we did not find significantly anti-proliferative effects of Scutellaria extract on human MM cells within a concentration range of 5-75 µg/mL. Furthermore, Scutellaria extract was not observed to modulate the cell cycle and induce the cell apoptosis of MM cells within this concentration range. These results were in accord with the effect of Scutellaria extract on human breast cancer cells, although it has demonstrated the anti-proliferative effects on prostate cancer in concentration range of 25-200µg/mL and hepatoma cells in another concentration range of 50-800 µg/mL (Adams et al., 2006; Ye et al., 2009).

On the other hand, being the major active constituents of Scutellaria baikalensis, baikalin, baicalein and wogonin are not only cytostatic but also cytotoxic to various human tumor cell lines in vitro and inhibit tumor growth in vivo (Li-Weber et al., 2009). Baicalein showed the strongest inhibitory effect on the proliferation of MM cells among these three components (Jakubikova et al., 2011). However, baikalin showed weakly anti-proliferative effect on MM cells and insensitivity on breast cancer cells (Ikezoe et al., 2001). In our study, we found that the content of baikalin was 5-fold more than that of baicalein and wogonin in Scutellaria extract. We hypothesize that the anti-proliferative effect of Scutellaria extract may be reduced by baikalin, although baicalein possessed a stronger cell growth inhibitory effect in isolated flavonoid form.

Our present study found that Scutellaria extract could modify the proportion of SP cells in myeloma cell line. Furthermore, we identified several major components of Scutellaria extract as responsible for the modified percentage of SP cells. We found that baikalin and wogonin had stronger effect on decreasing the percentage of SP cells. Baicalein showed weak activity on SP cells percentage only at higher concentration, and wogonoside showed no effect on SP cells in vitro. These results suggest that Scutellaria extract and some of its isolated components may perform their anticancer effect mainly targeting CSCs of tumors. Despite they also exhibit mild inhibition effect on cell growth in bulk of tumor cells. That might be an important reason why Scutellaria baikalensis and its active constituents were found to target specifically to malignant cells with minor toxicity to corresponding dormant or normal plasma cells, which have undergone differentiation. From this point, Scutellaria baikalensis Georgii or baikalin, wogonin may be used as adjuvant agents for conventional chemotherapeutic drugs for the management of multiple myeloma. To our knowledge, this is the first study to evaluate the effect of Scutellaria baikalensis on SP cells in multiple myeloma cell lines.

The ATP-binding cassette (ABC) transporter superfamily comprises membrane proteins that translocate a variety of substrates across extra- and intra-cellular membranes, and act as efflux proteins. The efflux of Hoechst dye is attributed to members of the ABC family of membrane pumps, with ABCG2 and ABCB1 identified as likely mediators of Hoechst efflux (Pfister et al., 2008; Dean et al., 2009). ABCG2 protein plays an important role.
in the drug resistance of normal stem cells and tumor stem cells, and is a molecular determinant of the SP phenotype (Zhou et al., 2001; Dean et al., 2009). To date, effects of Scutellaria baicalensis on ABCG2 protein in tumors are largely unknown. In our study, we found for the first time that Scutellaria extract, baikalenin and wogonin decreased expression of ABCG2 in myeloma cells. The baikalin also showed similar effect at high concentration, but wogonoside had no effect on ABCG2 protein in myeloma cells. These results were in accordance with percentage alteration of SP cells in our study, and indicated that Scutellaria extract and some of its active flavonoids might modify the proportion of SP cells through the way of modulating the ABCG2 expression in myeloma cells. It has been demonstrated that ABCG2 activity could be regulated by Akt and PTEN/PI3K/Akt pathway in various tumor cells (Bleau et al., 2009; Li et al., 2011). Scutellaria baikalenin and its active constituents were also observed to modulate cellular functions by diverse signaling pathways, including phosphorylation changes in Akt, GSK-3beta, MEK/ERK, and c-Jun (Chang et al., 2011; Huang et al., 2012; Liang et al., 2012). Further investigations need to be performed to clarify the molecular mechanism linked between Scutellaria baikalenin and ABCG2 expression.

In summary, the highlight of the study is that Scutellaria extract and its active flavonoids can inhibit the SP cells and decrease the expression of ABCG2 protein in MM celline RPMI-8226. Our results suggest that Scutellaria extract and its active flavonoids may be potential SP inhibitors targeting CSCs, thereby enhancing the effect of conventional chemotherapy.

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References


