Antitumor and anti-angiogenic activities of *Scutellaria barbata* extracts *in vitro* are partially mediated by inhibition of Akt/protein kinase B

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Abstract. The Akt pathway is considered a pivotal player in regulating cell survival, growth, migration and angiogenesis. Disruption of normal Akt/PKB/PTEN signaling frequently occurs in numerous types of human cancers. Therefore, this signaling pathway is regarded as an important target for effective cancer therapeutic strategies. In the present study, methanol extracts from *Scutellaria barbata* (*S. barbata*) were determined to be Akt/protein kinase B inhibitory, after screening a panel of 40 traditional Chinese herbs with the Fast Activated Cell-based ELISA (FACE) assay. *S. barbata* extracts were found to suppress the phosphorylation levels of Akt. This inhibition was Akt kinase-specific as it had no effect on PI3K, the upstream kinase of Akt, whereas the levels of phosphorylated Bad and FHKR, the two downstream targets of Akt, changed as the levels of Akt changed. *S. barbata* extracts also exhibited cytotoxicity against LoVo and human umbilical vein endothelial cells (HUVECs). Furthermore, this extract inhibited the process of *in vitro* angiogenesis of HUVECs on Matrigel. *S. barbata* may be a suitable alternative source with which to isolate small molecules for use as Akt kinase inhibitors.

Introduction

The serine/threonine kinase Akt/PKB, a downstream target of phosphatidylinositol 3- kinase (PI3K), has been found to play a key role in the regulation of cell survival, migration, proliferation, metabolism, tumor growth and angiogenesis (1-5). Akt is a subfamily of the mammalian cAMP-dependent, cGMP-dependent, protein kinase C (AGC) family of kinases. To date, there are 3 Akt family members identified in mammals, designated as Akt1/PKBα, Akt2/PKBβ and Akt3/PKBλ (6,7). Disturbed activation of the PI3K-Akt pathway has been associated with the development of diseases, such as cancer, diabetes mellitus and autoimmunity (8-13). Akt is targeted by genomic aberrations including mutations, amplifications and rearrangements more frequently than any other pathway in human cancer, with the possible exception of the p53 and retinoblastoma (Rb) pathways (14). The activation of Akt has been reported in many types of human cancers, including breast, prostate, gastric, lung, ovary, pancreas and thyroid carcinomas, as well as in glioblastoma and various hematological malignancies (15). As the PI3K pathway is activated in cancer, this makes it an ideal target for therapy as it is easier to inhibit activation events than to replace lost tumor-suppressor function. A number of inhibitors of proteins involved in the PI3K/Akt signaling pathway have been under development, and some agents have now entered clinical trials or have been approved for clinical practice, including inhibitors that directly inhibit the PI3K/Akt/mTOR pathway and those that do so indirectly, by suppressing the upstream regulators of PI3K/Akt, such as membrane growth factor receptors (16).

In the present study, a Fast Activated Cell-based ELISA (FACE) assay was applied to screen Akt kinase inhibitors against a panel of traditional Chinese herbs believed to have anticancer properties. *Scutellaria barbata* (*S. barbata*) was identified as the most suitable among them to suppress the phosphorylation level of Akt kinase in LoVo cells. *S. barbata* also disrupted the process of *in vitro* angiogenesis by human umbilical vein endothelial cells on Matrigel.

Materials and methods

Herbal materials. All herbal powders were purchased from a local Chinese medicine distributor (Atlanta, GA, USA) (Table I). LoVo cells were obtained from ATCC (Manassas, VA) and human umbilical vein endothelial cells were acquired from Lonza. LY294002 was from Calbiochem. The FACE kit was from Active Motif (Carlsbad, CA, USA) and CellTiter-Glo Luminescent Cell Viability Assay kit was from Promega (Madison, WI, USA).

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Table I. List of herbs used for screening in the present study.

	English name	Chinese name
1	*Andrographis*	Chuan Xin Lian
2	Herba Scutellariae Barbatae	Ban Zhi Lian
3	Japanese Honeysuckle	Jin Yin Hua
4	*Abrus cantoniensis*	Ji Gu Cao
5	*Gyunra divancata* (L.) D.C	Bai Zi Cao
6	*Artemisia scoparia* Waldst. et Kit.	Yin Chen Hao
7	Spreading Hedvotis Herb	Bai Hua She She Cao
8	Common Lantana Leaf	Wu Se Mei
9	Yunnanmanyleaf Paris Rhizome	Sao Xiu
10	*Stephania tetrandra* S. Moore	Fen Fang Ji
11	*Pittoaporum tobira* (Thunb.) Ait.	Hai Tong
12	*Ardisia crenata*	Zhu sha Gen
13	Knoxia Root	Da Ji
14	Herb of Chinese Lobelia	Ban Bian Lian
15	Black Nightshade Herb	Long Kui
16	Uniflower Swisscentaury Root	Loulu
17	Airpotato Yam Rhizome	Huang Yao Zi
18	Common Monkshood Mother Root	Chuan Wu Tou
19	Java Brucea Fruit	Ya Dan Zi
20	Agaric	Zhu Ling
21	*Curcuma kwangsiensis* S.G.Lee & C.F. Liang	E Zhu
22	Leaf of *Appendiculate cremastra*	Shan Ci Gu
23	Philippine Flemingia Root	Qian Jin Ba
24	*Ehretia microphylla* Lam.	
25	Common Selfheal Fruit- Spike	Xia Ku Cao
26	Lagundi or Fruit of Hempleaf Negundo Chastertree (*Vitex negundo*)	Mu Jing Zi
27	Herb of Hairyvein Agrimonia	Xian He Cao
28	Herb of Spanishneedles	Gui Zhen Cao
29	Akebia Fruit	Ba Yue Zha
30	*Gynostemma pentaphyllum*	Jiaogulan
31	Lightyellow Sophora Root	Ku Shen
32	Tender Catchweed Bedstraw Herb	Ba Xian Cao
33	Herba Bidentis Pilosae	Mang Chang Cao
34	*Rabdosia rubescens*	Dong Ling Cao
35	*Cephalotaxus fortunei* Hook.f.	San Jian Shan
36	Root of Maire Sophora	Wu Dou Gen
37	*Catharanthus roseus* (L.) G. Don	Chang Chun Hua
38	Motherwort Herb	Yi Mu Cao
40	*Plumbago zeylanica* L.	Bai Hua Dan

Preparation of extracts. Powdered plant materials (2.0 g) were extracted with 100% ethanol overnight at room temperature. Supernatants were filtered through a funnel with glass wool and dried in a vacuum using a rotary evaporator at 45˚C. All dried herbal materials were weighed and dissolved in methanol to give a final concentration of 10 mg/ml.

Antibodies. Phospho-Akt (Ser473) (193H12) rabbit mAb #4058, Akt (pan) (C67E7) rabbit mAb #4691, phospho-PI3K p85 (Tyr458)/p55 (Tyr199), phospho-Bad (Ser136) (185D10) rabbit mAb #5286, and phospho-FoxO1 (Ser256) antibody #9461 were obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). HRP-conjugated goat anti-rabbit IgG secondary antibody was from Pierce (Rockford, IL, USA).

Cell culture and treatment. Human colorectal adenocarcinoma LoVo cell line (ATCC no. CCL-229) was cultured in F-12K medium with 10% FBS and 1% penicillin-streptomycin at a temperature of 37˚C in a humidified incubator with a 5% CO_2 atmosphere. For the assays, LoVo cells were seeded in 96-well plates at a concentration of 70,000 cells/well. After overnight culture, cells were placed in medium containing different herbal extracts all at a final concentration of 1 μg/ml.

FACE assay. Cells cultured in medium supplemented with phosphate buffered saline (PBS), ethanol, LY294002 (final concentration 1.4 μM), or herbal extracts (10 μg/ml) were processed, according to the manufacturer's instructions. Briefly, cells were fixed by replacing the growth medium with 100 μl of 4% formaldehyde in PBS and incubated at room temperature for 20 min. After washing the cells 3 times with 200 μl wash buffer (5 min x 3), 100 μl quenching buffer was added to each well and incubation was carried out for 20 min at room temperature. Cells were washed 2 times (5 min x 2) with 200 μl wash buffer and then 100 μl antibody blocking buffer was added to each well and incubation was carried out for 1 h at room temperature. After removing the antibody blocking buffer and washing cells 2 times with 200 μl wash buffer, 40 μl of diluted primary antibody (or antibody dilution buffer for negative control wells) was added to each well and the plate was sealed with sealing tape. The plate was then covered with a lid and incubated overnight at 4˚C. After removing the primary antibody and washing cells 3 times with 200 μl wash buffer, 100 μl diluted secondary antibody was added to each well and the plate was covered with sealing tape and incubated 1 h at room temperature. After removing the secondary antibody, cells were washed 3 times with 200 μl wash buffer and then 2 times with 200 μl 1X PBS. Developing solution (100 μl) was added to each well and incubation was carried out for 2-20 min at room temperature. Absorbance was measured on a spectrophotometer within 5 min at 450 nm with an optional reference wavelength of 655 nm. After reading the chemiluminescence, cells were stained with crystal violet and read at OD595. These numbers were used to normalize the cell numbers among different wells. The change in phosphorylation status was calculated by dividing the chemiluminscence detected using the phospho Akt-specific antibody with that of the total Akt-specific antibody.

Western blot analysis of the inhibition of Akt phosphorylation in LoVo cells by S. barbata extracts. For the Western blot analysis, $6x10^6$ LoVo cells were plated into a T-25 flask and cultured overnight. Cells in different flasks were then treated with or without *S. barbata* herbal extracts for 3 h. Cells were washed with PBS and lysed with 400 μl lysis buffer as described (41). Samples were resolved by 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose

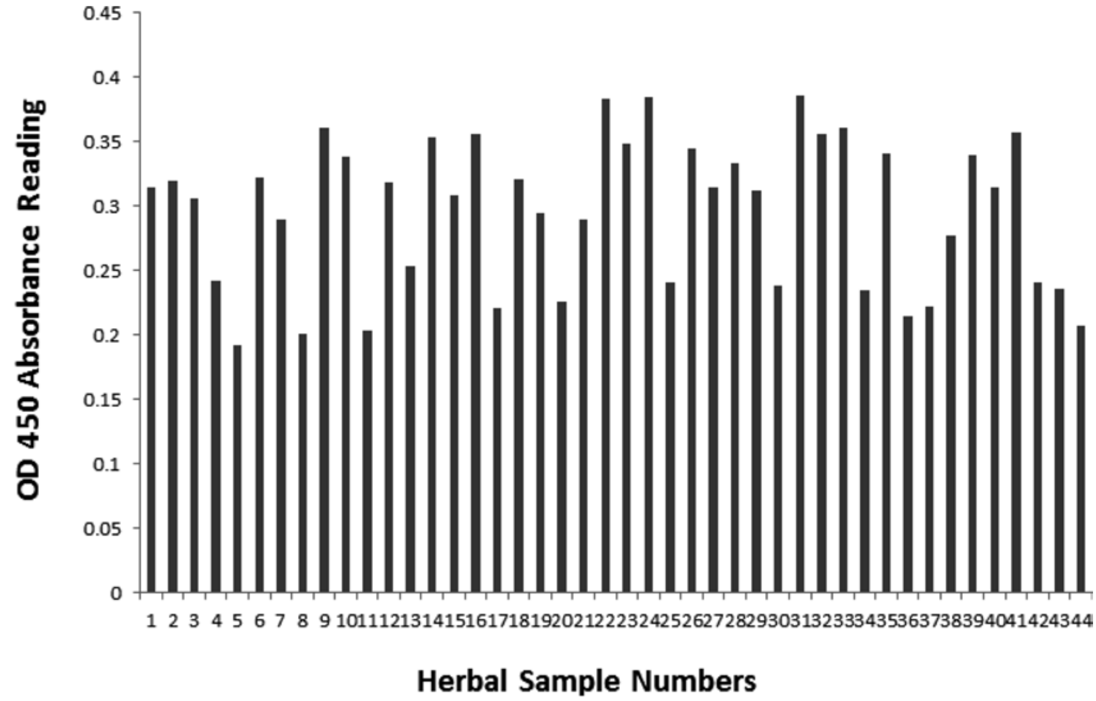

Figure 1. Screening results of the herbal extracts with FACE kits against Akt kinase. Sample nos. 1, 2, 3, and 44 are the readings for cells treated with PBS, methanol, DMSO and LY294002, respectively. Nos. 4-43 represents readings from cells treated with herbal extracts and no. 5 is the reading for *S. barbata* extracts.

membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h at room temperature with a nonfat dry milk solution (5% in Tris-buffered saline) containing 0.1% Tween-20. Blots were incubated overnight at 4˚C with anti-phospho Akt primary antibodies (1:750) followed by incubation for 1 h with the secondary antibody (horseradish peroxidase-conjugated; 1:2000). After an extensive wash, bands were detected by enhanced chemiluminescence (ECL) (Pierce). Subsequently, the same membrane was stripped and reprobed for total protein loading using an anti-Akt antibody. For the Western blot analysis of PI3K, FKHR and Bad, the procedures were essentially the same as for Akt.

Cytotoxicity of S. barbata extracts on LoVo cells. LoVo cells were plated in a 96 well opaque-walled tissue culture plate at 70,000 cells/well in 100 µl culture medium. Following overnight culture, cells were treated with *S. barbata* extracts in DMSO at different concentrations of 0.6, 1.25, 2.5, 5 and 10 µg/ml for 3 h. Cells treated with DMSO (2 µl) were used as the negative control. After 3 h, cell viability was measured by the CellTiter-Glo Luminescent Cell Viability Assay kit from Promega, according to the manufacturer's instructions. Cytotoxicity was calculated as the percent decrease in ATP levels of cells treated with *S. barbata* extracts as compared with that of cells treated with DMSO.

In vitro anti-angiogenesis assay. HUVECs (42,000 viable cells/cm^2) were seeded on a 24-well polystyrene plate coated with Matrigel (BD Biosciences) (50 µl/cm^2) in HUVEC medium supplemented with or without *S. barbata* herbal extract at a concentration of 5 µg/ml and incubated overnight at 37˚C and 5% CO_2.

Results

FACE screening results. Data from the FACE screening showed that 6 herbal extracts had values similar to LY294002 (Fig. 1). A second round of FACE screening was conducted for those 6 extracts with a series of different concentrations. The results showed that *S. barbata* extracts were the most effective at much lower concentrations, and thus was chosen for further analysis.

Inhibition of Akt phosphorylation in LoVo cells by S. barbata extracts. Western blot analysis showed that the level of phosphorylated Akt decreased with increasing concentrations of *S. barbata* extracts in the culture medium (Fig. 2A). At 10 µg/ml, *S. barbata* achieved approximately the same level of Akt phosphorylation inhibition as that of LY294002. There was no change in the phosphorylation level of PI3K kinase after treatment with the *S. barbata* extracts (Fig. 2C). The phosphorylation level of Bad correlated with the level of Akt. In contrast, the level of phosphorylated FKHR increased with a decreasing level of phosphorylation of Akt (Fig. 2B).

Cytotoxicity of S. barbata extracts against LoVo cells. Fig. 3 showed the percent decrease in ATP levels of cells treated with *S. barbata* extracts as compared with cells treated with DMSO. Cell ATP levels decreased as *S. barbata* extract levels in the culture increased, and at 10 µg/ml, the cell ATP level was barely less than 25% of that in the DMSO-treated cells.

In vitro anti-angiogenesis assay. As shown in Fig. 4, the *in vitro* tube formation process of HUVECs on Matrigel was completely inhibited when the *S. barbata* extract was added to the culture at a concentration of 5 µg/ml.

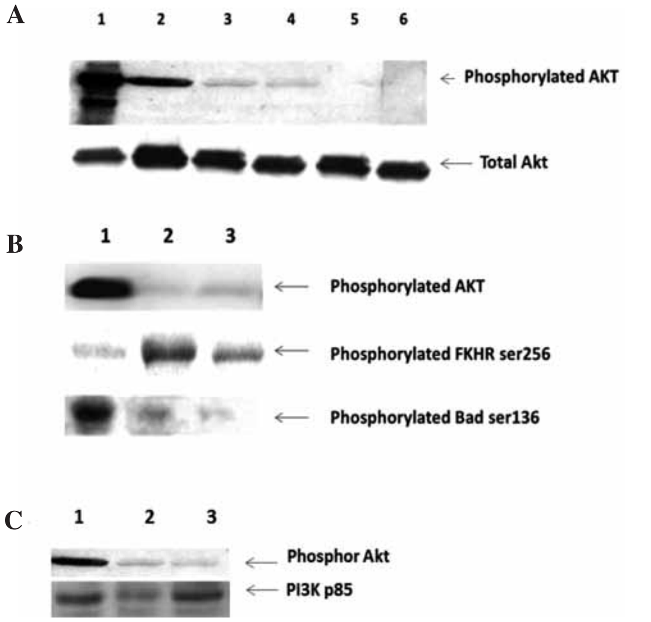

Figure 2. Western blot analysis of phosphorylation levels of Akt, PI3K, Bad, and FKHR in LoVo cells treated with *S. barbata* extracts. (A) Lane 1, positive control; 2, LoVo cells treated with methanol; 3, LoVo treated with *S. barbata* (2.5 μg/ml); 4, LoVo cells treated with *S. barbata* (5 μg/ml); 5, LoVo cells treated with S. *barbata* (10 μg/ml); 6, LoVo cells treated with LY29002 (1.4 μg/ml). (B) Lane 1, untreated cells; 2, LoVo cells treated with *S. barbata* at a concentration of 5 μg/ml for 3 h; 3, LoVo cells treated with *S. barbata* at a concentration of 10 μg/ml for 3 h. (C) Lane 1, untreated LoVo cell lysates; 2, LoVo cells treated with *S. barbata* (2.5 μg/ml); 3, LoVo cells treated with *S. barbata* (5 μg/ml).

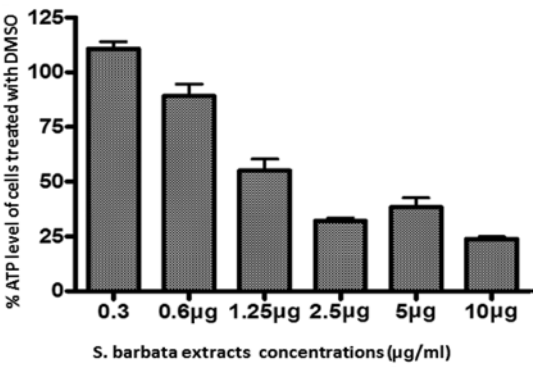

Figure 3. Cell viability assay of LoVo cells after treatment with different concentrations of *S. barbata*. Cytotoxicity was calculated as the percent decrease in ATP levels of cells treated with *S. barbata* extracts as compared with cells treated with DMSO.

Discussion

The phosphatidylinostitol-3-kinase (PI3K)/PTEN/Akt pathway is involved in a number of human cancers, and Akt is a key player in cancer cell proliferation and survival (15,17-22). Great effort has been made in designing small-molecule Akt inhibitors, particularly following the availability of the X-ray structure of the active form of Akt. Current Akt inhibitors mainly bind to the ATP-binding site, allosteric sites and the PH domain. Moreover, studies exist involving Akt inhibitors composed of pseudo-substrates and antisense oligonucleotides as well as inhibitors with unknown action mechanisms (22).

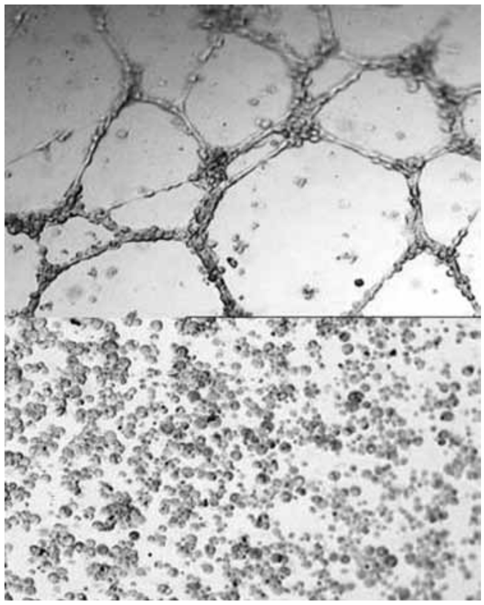

Figure 4. Angiogenesis of human umbilical vein endothelial cells (HUVECs) *in vitro* on Matrigel. Upper panel: HUVECs on Matrigel. Lower panel: HUVECs on Matrigel supplemented with 5 μg/ml of *S. barbata* extract.

S. barbata has been used in traditional Chinese medicine as an anti-inflammatory and antitumor agent, and also as a diuretic. Extracts of *S. barbata* have been shown to exhibit *in vivo* growth inhibitory effects on a number of cancer types (23-27). This herbal agent is known to contain a large number of alkaloids and flavones, frequently found as glucoside and other constituents, including phenethyl alcohols, sterols, essential oils and amino acids. It was found that a 30% ethanol extract of *S. barbata* induced apoptosis in the lung cancer A549 cell line (28), the methylene chloride fraction of *S. barbata* induced apoptosis in human U937 leukemia cells via the mitochondrial signaling pathway (29), and the chloroform fraction exhibited strong cytotoxicity on the cancer cell line Bel-7402 with a lower cytotoxic effect on a normal liver cell line (30). It has been suggested that the extracts of *S. barbata* exert their antitumor and antiproliferative effects through different mechanisms. *S. barbata* was also found to inhibit the proliferation of myometrial and leiomyomal smooth muscle cells through the induction of α-SMA, calponin h1 and p27 (31). It also enhanced apoptosis via the suppression of ERK-mediated autophagy in estrogen receptor-negative human breast adenocarcinoma MDA-MB-231 cells through photo-activated pheophorbide-a (32), and in mouse hepatoma H22 cells through the release of cytochrome C and activation of caspase-3 (33). Significant apoptosis induction has been observed in TRAMP-C1 and LNCaP cells treated with *S. barbata* (1 mg/ml) (34). An aqueous extract from *S. barbata*, BZL101, induced the production of reactive oxygen species in tumor cells, and therefore caused extensive oxidative DNA damage leading to necrotic death (35).

In the present study, a FACE method was used to screen an Akt inhibitor against a panel of 40 traditional Chinese herbs. Among 6 herbal extracts with anti-Akt phosphorylation activity based on a preliminary screening, *S. barbata* was chosen for further analysis. Western blot analysis and the cell viability assay suggest that the antitumor and antiproliferative properties of the *S. barbata* extracts may be attributed to the inhibition of Akt kinase phosphorylation. This Akt inhibition was found to be specific as PI3K kinase, the upstream kinase of Akt, was not inhibited, whereas the levels of phosphorylated Bad and FHKR, the two downstream targets of Akt, changed as the level of Akt changed. It is known that certain synthetic small-molecule Akt inhibitors are associated with unexpected toxicities, such as increased blood glucose and insulin levels, hyperglycemia, and acute systemic hypotension (36-38). Thus, *S. barbata* may be a suitable alternative source to isolate small molecules for use as Akt kinase inhibitors, as this herb has been safely used for a long time.

The expression of PI3K and Akt in cancer cells is required for tumor angiogenesis, and down-regulation of PI3K or Akt by their siRNA constructs in human ovarian cancer cells greatly decreases tumor growth and angiogenesis (39). Treatment with LY294002 was found to decrease glioma tumor growth and the tumor-induced angiogenic response (40). It is evident from our study that *S. barbata* extracts completely blocked the process of HUVEC angiogenesis *in vitro* on Matrigel at a concentration of 5 μg/ml. Furthermore, *S. barbata* extracts also inhibited Akt phosphorylation and cell proliferation in HUVECs.

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