Scutellarin Suppresses Patient-Derived Xenograft Tumor Growth by Directly Targeting AKT in Esophageal Squamous Cell Carcinoma

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Abstract

Scutellarin is a flavonoid compound that is found in Scutellaria barbata. It has been reported to exhibit anti-cancer and anti-inflammatory activities. However, the anticancer properties of scutellarin and its molecular targets have not been investigated in esophageal squamous cell carcinoma (ESCC). In the current study, we report that scutellarin is a potential AKT inhibitor that suppresses patient-derived xenograft ESCC tumor growth. To identify possible molecular targets of scutellarin, potential candidate proteins were screened by an in vitro kinase assay and Western blotting. We found that scutellarin directly binds to the AKT1/2 proteins and inhibits activities of AKT1/2 in vitro. The AKT protein is activated in ESCC tissues and knockdown of AKT significantly suppresses growth of ESCC cells. Scutellarin significantly inhibits anchorage-dependent and independent cell growth and induces G2 phase cell-cycle arrest in ESCC cells. The inhibition of cell growth by scutellarin is dependent on the expression of the AKT protein. Notably, scutellarin strongly suppresses patient-derived xenograft ESCC tumor growth in an in vivo mouse model. Taken together, our data suggest that scutellarin is a novel AKT inhibitor that may prevent progression of ESCC.

Introduction

Esophageal cancer is the sixth leading cause of cancer-related death in the world (1). Esophageal squamous cell carcinoma (ESCC) is histologically the most prevalent type of esophageal cancer that accounts for more than 50% of global ESCC cases most in China (2). Despite clinical advances in therapeutics, such as endoscopic resection, radiotherapy, and chemotherapy, the 5-year survival rate of patients with esophageal cancer is still less than 25% (3). High-throughput sequencing technologies have been investigated to identify the genomic alterations in ESCC (4, 5). Receptor tyrosine kinase (RTK) signaling pathways, including epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR) and insulin-like growth factor 1 receptor (IGF1R) are highly expressed, and these RTKs have low-frequency mutations in ESCC (4, 5). RTK inhibitors have been examined to treat patients with ESCC, but these inhibitors have shown limited responses (6). Therefore, finding novel targets against ESCC has been a priority (7).

V-Akt murine thymoma viral oncogene homolog (AKT) is a serine/threonine kinase that belongs to the AGC family of kinases (8). AKT has been shown to comprise a conserved N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory hydrophobic motif (HM) (9). The PH domain directs AKT translocation from the cytosol to the plasma membrane by binding to the membrane lipids, phosphatidylinositol-3,4-P2 and 3,4,5-P3, which are products of PI3-K (9). AKT signaling pathway regulates many cellular functions, including proliferation, survival, epithelial–mesenchymal transition (EMT), and metabolism, and directly phosphorylates a wide range of downstream substrates (9). Glycogen synthesis kinase 3β (GSK3β) is the first reported AKT substrate, which is inactivated by AKT phosphorylation. This leads to Wnt/β-catenin pathway mediation (10) and...
increases glycogen synthesis during glucose metabolism following insulin stimulation (11, 12). Recently, genomic profiling of ESCC showed that mTOR pathway genes, including phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), phosphatase and tensin homolog (PTEN), and neurogenin locus notch homolog protein 1 (NOTCH1), are altered at a high frequency in ESCC (13). The activating PIK3CA mutation and inactivating PTEN mutation promote AKT activation in various types of cancer (14, 15). In addition, the expression of phosphorylated AKT is significantly increased in ESCC tissues compared with normal esophageal mucosa and correlates highly with a shorter survival rate (16). AKT inhibitors can suppress ESCC growth and a combination of 5-fluorouracil and cisplatin enhances the inhibition of ESCC cell growth (17). Therefore, development of novel AKT inhibitors could provide effective anticancer effects for treatment of ESCC.

Scutellaria (scutellariin-7-O-beta-D-glucuronide) is a flavonoid glucuronide that is a major active compound of the traditional Chinese herb Erigeron brevicaulis (18). Scutellaria has been used clinically to treat stroke, myocardial infarction, and diabetic complications (19). In addition, scutellaria has been reported to exhibit a variety of biological effects, including antioxidant (20), anti-inflammatory (21), and anticancer (22–25) activities. Recently, scutellaria was shown to suppress proliferation and invasion of hepatocellular carcinoma cells by reducing the janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) signaling pathways (26). Scutellaria induces apoptosis and autophagy of non–small cell lung cancer cells by inducing extracellular signal-regulated kinase 1/2 (ERK1/2) activation and reducing AKT activation (27). However, the effects of scutellaria and its molecular mechanisms against ESCC have not been investigated. In the current study, we report that scutellaria is a novel AKT1 and AKT2 inhibitor that suppresses ESCC growth in vitro and in vivo.

Materials and Methods

Cell lines
Human esophageal squamous cell carcinoma (ESCC) cell lines, KYSE30, KYSE450, and KYSE510, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). JB6 mouse epidermal skin cells were obtained from the ATCC. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been authenticated and in culture for a maximum of 8 weeks. KYSE30 cells were cultured in a 1:1 mixture of RPMI1640 medium and Ham’s F12 medium, 2% FBS (Biological Industries), and 1% antibiotic–antimycotic. KYSE450 and KYSE510 cells were cultured with RPMI1640 medium, 10% FBS and 1% antibiotic–antimycotic in a 37°C, 5% CO2 environment. KYSE30, KYSE450, and KYSE510 cells were originally generated by Dr. Yutaka Shimada from the First Department of Surgery, Faculty of Medicine in Kyoto University (Kyoto, Japan) (28). Shee normal esophageal cells were obtained by Dr. Yan Zheng from the Affiliated Cancer Hospital in Zhengzhou University (Zhengzhou, Henan, China). JB6 cells were cultured in minimal Eagle’s medium (MEM) supplemented with 5% FBS and 1% antibiotic–antimycotic.

Reagents and antibodies
Scutellarin (purity > 98% by HPLC) and DMSO were purchased from Sigma Aldrich. RPMI1640 medium and FBS were from Biological Industries. Ham’s F12 medium was from Lonza and MEM (Minimum Essential Medium Eagle) medium was from Nanjing Keyjen Biotech. CNBr-Sepharose 4B beads were obtained from GE Healthcare. Active AKT1, AKT2, and inactive GSK3β recombinant proteins for kinase assays were purchased from SignalChem. The antibody to detect β-actin was from Santa Cruz Biotechnology and all the other antibodies were purchased from Cell Signaling Technology.

Cell proliferation assay
Shee (2.5 × 10^5 cells/well), KYSE30 (2.5 × 10^5 cells/well), KYSE450 (2.0 × 10^5 cells/well), or KYSE510 (1.2 × 10^5 cells/well) cells were seeded in 96-well plates with 100 μL complete growth medium (2% FBS for KYSE30 or 10% FBS for other cells) and incubated for 24 hours. Cells were treated with various concentrations of scutellarin in 100 μL of complete growth medium. After incubation for 48 hours, 20 μL of the MTT solution (Solarbio) was added to each well. After incubation for 2 hours at 37°C in a 5% CO2 incubator, the cell culture medium was removed. Then, 150 μL of DMSO was added to each well and crystal formation was dissolved. Absorbance was measured at 570 nm using the Thermo Multiskan plate-reader (Thermo Fisher Scientific).

Anchorage-independent cell growth assay
Cells (8 × 10^3 cells/well) suspended in complete growth medium (RPMI1640) supplemented with 10% FBS were added to 0.3% agar with or without different concentrations of scutellarin in a top layer over a base layer of 0.6% agar. Then, 150 μL complete growth medium (2% FBS for KYSE30 or 10% FBS for other cells) and incubated for 24 hours. The cultures were maintained at 37°C in a 5% CO2 incubator for 2 weeks, and then, colonies were counted under a microscope using the Image-Pro Plus software (v.6) program (Media Cybernetics).

In vitro kinase assay
The kinase assay was performed according to the instructions provided by Upstate Biotechnology. The active recombinant AKT1 (200 ng) or AKT2 (200 ng) protein was mixed with various doses of scutellarin in 10× buffer and kept at room temperature for 15 minutes. Then, the
in incubation with gentle rocking overnight at 4°C for 30 minutes. The reaction was stopped by adding 10 μL protein loading buffer and the mixture was separated by SDS-PAGE. AKT1 or AKT2 activity was detected by an GSK3β (S9) phosphorylation antibody.

Pull-down assay using CNBr-scuteellarin-conjugated beads
Total cell lysates (500 μg) or a recombinant human AKT1 or AKT2 protein (300 ng) was incubated with scuteellarin-Sepharose 4B (or Sepharose 4B only, as a control) beads (50 μL, 50% slurry) in reaction buffer (50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 2 μg/mL bovine serum albumin). After incubation with gentle rocking overnight at 4°C, the beads were washed 5 times with buffer (50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40) and binding was visualized by Western blotting.

Cell cycle analysis
KYSE30 or KYSE450 ESCC cells were plated into 60-mm culture dishes (2 × 10^5 cells/dish) and incubated for 24 h. Cells were treated with scuteellarin for 48 h in 2% or 10% serum-supplemented medium. Cells were collected by trypsinization and washed with phosphate buffered saline (PBS) and then fixed in 1 mL of 70% cold ethanol. After rehydration, cells were digested with RNase (100 μg/mL) and stained with propidium iodide (20 μg/mL). Propidium iodide staining was accomplished following the product instructions (Clontech). The cells were analyzed by flow cytometry.

Lentiviral infection
The pLKO.1-based lentiviral encoding shRNA against human AKT1 or AKT2 was purchased from Open Biosystems. The lentiviral packaging vectors (pMD2.0G and psPAX) were purchased from Addgene Inc. To prepare AKT1 or AKT2 viral particles, each viral vector and packaging vectors were transfected into HEK293T cells by using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s suggested protocols. The transfection medium was changed at 6 hours after transfection, and then, cells were cultured for 48 hours. The viral particles were harvested by filtration using a 0.45-mm syringe filter, then combined with 8 μg/mL of polybren (Millipore) and infected into 60% confluent ESCC cells overnight. The cell culture medium was replaced with fresh complete growth medium for 24 hours, and then, cells were selected with puromycin (1 μg/mL) for 48 hours. The selected cells were used for experiments.

In vivo patient-derived xenograft ESCC model and ethics statement
To examine the effect of scuteellarin on ESCC patient-derived xenograft (PDX) tumor growth, 16 female mice (6–9 weeks old; Vital River Labs) with SCID were maintained under “specific pathogen-free” conditions based on the guidelines established by the U.S. Common Rule. All animal studies were performed and approved by an Institutional Review Board and the Zhengzhou University Institutional Animal Care and Use Committee. Human tumor specimens of ESCC tissues were obtained from the Affiliated Cancer Hospital in Zhengzhou University. Patients with ESCC did not receive any chemotherapy or radiotherapy prior to surgery. Tissue histology was confirmed by a pathologist. Prior written informed consent was obtained from each patient. ESCC tissues were cut into pieces (3–4 mm^3) and implanted into the back of the neck of 3 individual mice. After the third generation of human ESCC tissue growth, tissues were again cut into pieces and implanted into mice. Mice were divided into 2 groups of 8 animals as follows: (i) untreated vehicle group (n = 8) and (ii) 100 mg scuteellarin/kg body weight (n = 8). Scuteellarin or vehicle (10% DMSO in 20% Tween 80) was orally administered by gavage once a day Monday through Friday. Tumor volume was calculated from measurements of 2 diameters of the individual tumor base using the following formula: tumor volume (mm^3) = (length × width × height × 0.52). Mice were monitored until average tumor volume in the vehicle group reached a volume of 1.0 cm^3, at which time mice were euthanized and tumors, liver, kidney, and spleen extracted.

Hematoxylin–cosin staining and IHC
The liver, spleen, kidney, and tumor tissues from mice were embedded in paraffin blocks and used for hematoxylin and cosin (H&E) staining or IHC. For H&E staining, the tissue sections were deparaffinized, hydrated, and stained with H&E and then dehydrated. For IHC, tumor tissue sections were deparaffinized and hydrated. After antigen retrieval with 10 mmol/L citrate acid and blocking with 5% BSA, the tumor tissue sections were hybridized with a primary antibody (Ki-67, 1:500, Cat# PA519462, Thermo Fisher Scientific) for 18 hours at 4°C and then a horseradish peroxidase–conjugated goat anti-rabbit or mouse IgG antibody (ZSGB-BIO) was added and hybridized for 30 minutes. Tissue sections were developed with 3, 3′-diaminobenzidine (ZSGB-BIO) for 10 seconds and then counterstained with hematoxylin for 1 minute. All sections were observed by microscope and analyzed using the Image-Pro Plus software (v. 6) program (Media Cybernetics).

Statistical analysis
All quantitative results are expressed as mean values ± SD or ± SE. Significant differences were compared using the Student t test or one-way ANOVA. Differences with a P < 0.05 were considered to be statistically significant.
Results
Scutellarin strongly inhibits the AKT signaling pathway

Scutellarin is a type of phenolic compound that is classified as a scutellarein-7-O-beta-D-glucuronide (Supplementary Fig. S1). Despite findings that show scutellarin exhibits anticancer properties against different types of cancers (22–25), the anticancer activity of scutellarin and its molecular target have not been

Figure 1.
Scutellarin strongly suppresses the AKT signaling pathway. A, The effect of scutellarin on the in vitro kinase activity of various kinases. The effect of scutellarin on the activity of 26 kinases was determined using active protein kinases and the specific substrate for each kinase. Data are shown as means values ± SD. B and C, Effect of scutellarin on EGF- or hydrogen peroxide (H2O2)-induced kinase signaling in JB6 cells. Serum-starved (0.1% FBS, 48 hours) cells were treated with different doses of scutellarin for 1 hour followed by treatment with EGF or H2O2 for 30 minutes. D, Effect of scutellarin on kinase signaling in KYSE30 ESCC cells. Cells were treated with scutellarin and then various signaling proteins were examined by Western blotting. For B–D, band density was measured using the Image J (NIH) software program. All results of Western blotting are shown as mean values ± SD for 3 independent experiments.
investigated in ESCC. To identify potential molecular target of scutellarin, we first screened 26 cancer-related kinases by using a recombinant active kinase protein and the specific substrate for each kinase. ASK1, GSK3β, JNK1α, JNK2α, MKK4, MKK6, AKT1, SAPK2α, PI3K, TAK1, TBK1, DNA-PK, NLK, p70S6K, PDK1, CDR2, CHK1, FAK, FGFR, HIPK2, JAK1, PKCα, BRAF, LIMK1, Src, or MET kinase and respective substrate were incubated with or without scutellarin in an in vitro kinase assay. Results indicated that scutellarin strongly suppressed the activity of the AKT1 protein, whereas the activity of other kinases was not affected (Fig. 1A). EGF or hydrogen peroxide (H$_2$O$_2$) has been reported to activate AKT and the MAPK signaling pathway in JB6 mouse epithelial cells (29, 30). To confirm the effect of scutellarin on the AKT signaling pathway stimulated by EGF or H$_2$O$_2$, JB6 cells were cultured in serum-free medium for 48 hours and then treated with scutellarin for 1 hour before treatment with EGF or H$_2$O$_2$ for 30 minutes. AKT or MAPK signaling pathway molecules were examined by Western blotting. Results indicated that the expression of phosphorylated GSK3β, a direct downstream molecule of AKT, is dose dependently inhibited by scutellarin treatment, whereas other signaling molecules are basically unchanged (Fig. 1B and C). We also investigated whether scutellarin could affect AKT signaling in ESCC cells. KYSE30 ESCC cells were treated with scutellarin for 3 hours and AKT signaling pathway molecules were detected by Western blotting. Results showed that scutellarin reduces the level of the phosphorylated GSK3β protein in a dose-dependent manner (Fig. 1D).

Scutellarin is a novel inhibitor of AKT1 and AKT2

To explain how scutellarin interacts with AKT1 and AKT2, we performed computer docking using several protocols in the Schrödinger Suite 2018. The results indicated that scutellarin nicely docks with AKT1 (Fig. 2A, left) and AKT2 (Fig. 2A, right) at the ATP-binding pocket, respectively. To confirm the computational docking results of scutellarin with AKT1 or AKT2, we performed in vitro pull-down assays with scutellarin-conjugated Sepharose 4B beads (or Sepharose 4B beads only, as a negative control) and a KYSE30 ESCC cell lysate (Fig. 2B) or a recombinant AKT1 or AKT2 protein (Fig. 2C). The results indicated that scutellarin directly binds to AKT1 and AKT2 (Fig. 2B and C). Next, to examine whether scutellarin could affect AKT activity, we performed in vitro kinase assays using a recombinant active AKT1 or AKT2 protein and an inactive GSK3β protein. The results indicated that scutellarin dose dependently suppresses the phosphorylation of GSK3β by directly targeting AKT1 (Fig. 2D, top) and AKT2 (Fig. 2D, bottom).

AKT1 and AKT2 are therapeutic targets of ESCC cells

To determine the expression levels of AKT, we performed IHC using ESCC and adjacent tissues. Results showed that the expression of phosphorylated AKT is significantly increased in ESCC tissues compared with adjacent tissues (Fig. 3A). In addition, we also examined whether phosphorylated AKT is highly expressed in normal esophagus or ESCC cells. Results indicated that the AKT protein is highly phosphorylated in ESCC cells compared with Shee normal esophageal cells (Fig. 3B). To determine the effect of AKT1 and AKT2 knockdown on ESCC cell growth, we established cells stably expressing knockdown of AKT1 and AKT2 or control. The expression of phosphorylated or total AKT1 and AKT2 was analyzed by Western blotting (Fig. 3C). We next examined the effect of AKT1 and AKT2 knockdown on anchorage-dependent or independent ESCC cell growth. Cells were seeded and incubated for 72 hours or 2 weeks, and cell growth was determined by MTT or soft agar assay. Results showed that anchorage-dependent or independent ESCC cell growth is significantly inhibited by knockdown of AKT1 and AKT2 (Fig. 3D and E).

Scutellarin suppresses ESCC tumor growth

To determine the effect of scutellarin on normal esophageal or ESCC cell growth, Shee or ESCC cells (KYSE30, KYSE450, or KYSE510) were treated with scutellarin for 48 hours. Results indicated that scutellarin has little effect on the growth of Shee cells (Fig. 4A), whereas growth of ESCC cells is dose dependently suppressed by scutellarin treatment (Fig. 4B). To examine the effect of scutellarin on cell-cycle progression, KYSE30 or KYSE450 ESCC cells were treated with scutellarin for 48 hours. Cell cycle was analyzed by flow cytometry (FACS), and also, cell cycle marker proteins were assessed by Western blotting. The results showed that scutellarin strongly reduces the G1 phase and induces G2 phase cell-cycle arrest (Fig. 4C). In addition, the expression of p27 is dose dependently increased by scutellarin treatment (Fig. 4D). We also examined the effect of scutellarin on anchorage-independent ESCC cell growth. Results showed that scutellarin significantly inhibits anchorage-independent ESCC cell growth (Fig. 4E).

Anticancer activity of scutellarin is dependent on the expression of the AKT protein

We next investigated whether the inhibition of cell growth by scutellarin is dependent on the expression of the AKT1 or AKT2 protein. Cells expressing shAKT1 and shAKT2 or shControl were treated with scutellarin for 48 hours or 2 weeks, and cell growth was determined by MTT or soft agar assay, respectively. Results indicated that cells expressing shAKT1 and shAKT2 are resistant to scutellarin’s inhibitory effect on cell growth compared with cells expressing shControl (Fig. 5A and B).

Scutellarin suppresses ESCC PDX tumor growth in vivo

We investigated whether scutellarin could inhibit ESCC tumor growth in vivo by establishing ESCC PDXs in mice. To determine the potential toxicity of scutellarin, mice...
were orally administered the compound (0, 50, or 100 mg/kg B.W.) and body weight was measured over 16 days. Results showed no significant difference between vehicle-treated group and scutellarin-treated groups (Supplementary Fig. S2). Furthermore, to select ESCC tissues for PDX studies, the expression of total or phosphorylated AKT was analyzed by Western blotting in 3 different ESCC tumor tissues (EG9, EG30, or HEG41).
Figure 3.

AKT is a therapeutic target in ESCC cells. Expression of the phosphorylated AKT (Ser473) protein in adjacent tumor tissues and ESCC tissues (A) or Shee normal esophageal and ESCC cells (B). Adjacent tumor or tumor tissues were stained with antibodies to detect phosphorylated AKT by using IHC. Cells were seeded and incubated for 48 hours and expression of the total or phosphorylated AKT protein was analyzed by Western blotting. C, Effect of knockdown of AKT1 or AKT2 on total or phosphorylated AKT protein. KYSE450 or KYSE510 ESCC cells stably expressing knockdown AKT1 and AKT2 or control were established. The expression of total AKT1, total AKT2, or phosphorylated AKT was determined by Western blotting. The expression of total AKT1, total AKT2, or phosphorylated AKT was determined by Western blotting. For B and C, similar results were observed from 3 independent experiments and band density was measured using the Image J (NIH) software program. D, Effect of AKT1 and AKT2 knockdown on growth of ESCC cells. Cells were seeded and incubated for 72 hours or 2 weeks and cell growth was determined by MTT assay (D) or soft agar assay (E). For D and E, data are shown as means ± SD of triplicate values from 3 independent experiments. The asterisk (*) indicates a significant difference (P < 0.05).
Results showed that phosphorylated AKT was highly expressed in the EG30 ESCC tissue (Supplementary Fig. S3; Supplementary Table S1), and thus, the EG30 tissue was used for the PDX study. Results indicated that the scutellarin-treated group exhibited significantly suppressed ESCC tumor volume compared with the vehicle-treated group (Fig. 6A) and showed no significant loss of body weight compared with the vehicle-treated group (Fig. 6B). In addition, to determine the potential toxic effects of scutellarin on tissue morphology, liver, spleen, and kidney tissues from PDX mice were analyzed by H&E staining. The results of H&E staining showed no morphologic changes between the scutellarin-treated group and the vehicle-treated group (Supplementary Fig. S4A–S4C). We next examined the expression of the Ki-67 tumor proliferation marker protein by using IHC, and results indicated that the expression of Ki-67 is significantly reduced in the scutellarin-treated group compared

Figure 4.
Scutellarin exerts anticancer activities. A, Effect of scutellarin on the viability of Shee normal esophageal cells. Cells were seeded for 24 hours and treated with scutellarin for 48 hours. B, Effect of scutellarin on ESCC cell growth. For A and B, cells were treated with scutellarin at various concentrations and then incubated for 48 hours and growth was determined by the MTT assay. C, Effect of scutellarin on cell cycle. Cells were treated with scutellarin for 48 hours in medium supplemented with 2% (KYSE30) or 10% (KYSE450) FBS. Cells were stained with propidium iodide (PI) and cell cycle was analyzed by FACS. For A–C, data are shown as means ± SD of triplicate values from 3 independent experiments and the asterisk (*) indicates a significant (P < 0.05) difference. D, Effect of scutellarin on the expression of cell cycle marker proteins was determined by Western blotting. Band density was measured using the Image J (NIH) software program. For D, similar results were observed from 3 independent experiments. E, Effect of scutellarin on anchorage-independent growth of ESCC cells. Cells were treated with scutellarin and incubated for 2 weeks and then colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. For E, data are shown as means ± SD of values from 5 independent experiments each with triplicate samples and the asterisk (*) indicates a significant (P < 0.05) difference.
with the vehicle-treated group (Fig. 6C). To validate whether scutellarin could suppress the AKT signaling pathway, PDX tumor tissues were analyzed by Western blotting. Results showed that the expression of phosphorylated AKT and GSK3β is strongly suppressed in the scutellarin-treated group compared with the vehicle-treated group (Fig. 6D).

**Discussion**

Polyphenols have been reported to exhibit anticancer activities including their inhibitory effects on cell proliferation, tumor growth, and inflammation (31). Scutellarin is a polyphenol compound from a traditional Chinese medicine that is clinically used to treat cardiovascular diseases and diabetic complications (19). Recently, scutellarin has been reported to possess anticancer properties against many types of cancers (22–25). However, the molecular targets of scutellarin and its potential anticancer activity have not been investigated in ESCC. The results of in vitro kinase (Fig. 2D) and binding assays (Fig. 2B and C) provided strong evidence showing that scutellarin is a novel AKT1 and AKT2 inhibitor that could inhibit AKT signaling pathway in ESCC cells (Fig. 1D). Recently, scutellarin has been reported to inhibit cisplatin resistance in glioma or ovarian cancer cells (32, 33). However, the molecular mechanisms of scutellarin action against cisplatin resistance have not been clearly investigated. The molecular functions of AKT have been consistently reported to lead to chemoresistance in various cancer cells (34). Therefore, we suggest that scutellarin is a novel AKT inhibitor that might be combined with cisplatin to overcome chemoresistance and this combination could be useful for ESCC treatment.

AKT has been reported to act on multiple key signaling mediators in several signaling pathways related to cell proliferation, survival, tumor growth, tumorigenesis, and chemoresistance (34, 35). We found that the AKT protein was strongly activated in ESCC tissues and cells (Fig. 3A and B). In addition, knockdown of AKT1 and AKT2 significantly suppressed growth of ESCC cells (Fig. 3D and E). AKT inhibitors can strongly inhibit growth of ESCC in vitro and in vivo (17). Therefore, we suggest that AKT1 and AKT2 are potential therapeutic targets of ESCC. Previously,
Figure 6.
Scutellarin inhibits ESCC PDX tumor growth in vivo. A, Effect of scutellarin on ESCC tumor growth. Tumor-bearing mice were orally administered (by gavage) scutellarin or vehicle once a day Monday through Friday for 21 days. Tumor volumes were measured on the days indicated. B, Effect of scutellarin on mouse body weight. Body weights from treated or untreated groups of mice were obtained once a week. C, Effect of scutellarin on Ki-67 expression. Treated or untreated groups of tumor tissues were stained with antibodies to detect Ki-67. The number of Ki-67–stained cells was counted from IHC results (n = 8). D, Effect of scutellarin on the AKT signaling pathway. Tumor tissues from each group were immunoblotted with antibodies to detect total AKT, total GSK3β, phosphorylated AKT, phosphorylated GSK3β, and β-actin. Band density was measured using the Image J (NIH) software program. All data are shown as means ± SE of values obtained from the experiment groups. The asterisk (*) indicates a significant difference between tumors from untreated or treated mice as determined by t test (P < 0.05).
inhibition of AKT activity by an AKT inhibitor or dominant negative mutant form of AKT induced G2–M phase cell-cycle arrest (17, 36), and inhibition of AKT increased the expression of p27 in anaplastic large cell lymphoma (37). Therefore, we investigated whether scutellarin could affect cell-cycle progression in ESCC cells. We found that scutellarin strongly induced G2 phase cell-cycle arrest and the expression of p27 through its inhibition of AKT activity (Fig. 4C and D).

We next determined the effect of scutellarin on growth of ESCC cells, and results indicated that scutellarin significantly inhibited their growth (Fig. 4B and E), but had little effect on Shee normal esophageal cell growth (Fig. 4A). This is likely because the phosphorylated AKT protein was highly expressed in ESCC cells compared with Shee normal esophageal cells (Fig. 3B). We suggest that the anticancer activities of scutellarin are dependent on the expression of the phosphorylated AKT protein. In addition, we confirmed that the inhibition of cell growth by scutellarin is dependent on the expression of the phosphorylated AKT protein (Fig. 5A and B). Therefore, we conclude that AKT is a major target of scutellarin in ESCC cells.

To overcome the low clinical efficacy of anticancer candidate drugs, researchers established the PDX model, which is the transfer of primary tumor tissues directly to xenograft mice (38). Therefore, the PDX model retains more of the histologic and genetic characteristics of patient tumors compared with the cell line xenograft (CDX) model (39). Therefore, the PDX model is a good method to predict responsiveness to anticancer agents and we established an ESCC PDX model in vivo. We first investigated the effect of scutellarin on ESCC PDX tumor growth and results indicated that scutellarin significantly suppressed ESCC tumor growth by inhibiting the AKT signaling pathway (Fig. 6A and D).

In conclusion, scutellarin is a novel AKT1 and AKT2 inhibitor that exhibits anticancer properties in vitro and in vivo. In further studies, scutellarin combined with anticancer reagents will be examined for ESCC treatment and interception.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Z. Dong, D.J. Kim
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