ExcisaninA, a diterpenoid compound purified from *Isodon MacrocalyxinD*, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of its signal pathway

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Abstract

Isodon **diterpenoids have received considerable phytochemical and biological attention for their strong antitumor activity with low toxicity. In this study, ExcisaninA, a diterpenoid compound purified from *Isodon MacrocalyxinD*, was tested on human Hep3B and MDA-MB-453 cell lines and Hep3B xenograft models. The results showed ExcisaninA could inhibit the proliferation of Hep3B and MDA-MB-453 cells via induction of apoptosis, with the evidence of increasing AnnexinV–positive cells and characteristic morphologic changes of apoptosis in the nucleus. Also, ExcisaninA sensitized Hep3B cells to 5-fluorouracil treatment or MDA-MB-453 cells to ADM treatment *in vitro*. In Hep3B xenograft models, ExcisaninA at 20 mg/kg/d remarkably decreased the xenograft tumor size and induced tumor cells apoptosis using transferase-mediated FITC-12-dUTP nick-end labeling assay. More importantly, we found that ExcisaninA could inhibit AKT activity and block its signal pathway *in vitro* and *in vivo*. And treatment with ExcisaninA significantly reduced the number of viable cells in Hep3B/myr-AKT1 cells more than that in control cells. Together, ExcisaninA might be a potent inhibitor of AKT signaling pathway in tumor cells. These data provide validation for the development of ExcisaninA to treat cancers displaying elevated levels of AKT. [Mol Cancer Ther 2009;8(4):873–82]**

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Introduction

Herbal drugs have been widely used for thousands of years in traditional Chinese medicine for the treatment of human diseases (1). Given the complexity of the chemical composition and the multiple potential targets of herbs, many of them are claimed to exhibit anticancer activity. After the development of the traditional Chinese medicine, many effective natural monomer products are extracted from these herbs and their antitumor molecular mechanisms are under elucidating. It could offer a new paradigm in future drug development for the prevention and treatment of cancer, and many lead compounds are subsequently used as templates for the design of novel compounds with enhanced biological properties (2). There are many good examples such as vinblastin and paclitaxel.

PKB/AKT kinase, a serine/threonine kinase, is the core component of the phosphoinositide 3-kinase /AKT signaling pathway and therefore involved in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis, glucose metabolism, and tumorigenesis (3–5). It is well-established that hyperactivation of AKT kinase is a common event in many human cancers (6), and increased AKT activity can also be detected in preneoplastic lesions (7). Loss or mutation of tumor suppressor *PTEN*, amplification or mutation of phosphoinositide 3-kinase, activation or mutation of growth factor receptors and oncogenes, and amplification of *AKT* itself are involved in activation of AKT in tumors (8, 9). Activation of AKT promotes the development or progression of cancer as well as resistance to treatment with chemotherapy and/or radiation therapy. Also, immunohistochemical analyses have shown that AKT activation is a poor prognostic factor in various cancers (10). Therefore, AKT is an attractive target for cancer therapy, and it has been postulated that inhibition of AKT alone or in combination with conventional chemotherapeutics or radiotherapy will reduce the apoptotic threshold and preferentially kill cancer cells (11, 12).

Isodon species (Labiatae) are widely distributed plants in China, many of which have long been used in Chinese popular folk medicine for their antibacterial and anti-inflammatory activities (13). Over the past 20 years, *Isodon* diterpenoids have received considerable phytochemical and biological attention for their strong antitumor activity with

low toxicity (14). Oridonin and ponicidin, two major constituents of *Isodon rubescens*, are the most frequently studied compounds among the *Isodon* diterpenoids. They are reported to exhibit cytotoxity and induce apoptosis against various cancer cell lines, and have significant antiangiogenic activity at subcytotoxic concentrations (15–17). ExcisaninA is also a kaurane diterpenoid compound that can be purified from many plants of genus *Isodon* and has been reported to exhibit cytotoxic effect in cancer cells, but the molecular mechanisms of its antitumor effect is little known. In our previous work, we successfully extracted ExcisaninA (an effective natural product) from *Isodon MacrocalyxinD* in Jiangxi province of China (18). In the present study, we found that it could induce human breast cancer MDA-MB-453 cell and human hepatocellular carcinoma Hep3B cell apoptosis, sensitize tumor cells to other anticancer drug treatment, and significantly reduce tumor growth of Hep3B xenografts. Furthermore, we observed that inhibition of AKT kinase activity and blockade of AKT signal pathway were involved in antitumor activity of ExcisaninA.

Materials and Methods

Cell Culture and Reagents

Human hepatocellular carcinoma cell line Hep3B and breast cancer cell line MDA-MB-453 were cultivated in RMPI 1640 medium supplemented 10% fetal bovine serum in a 5% CO_2 humidified atmosphere at 37°C. Glyceraldehyde-3-phosphate dehydrogenase, AKT, phospho-AKT (Ser473), phospho-AKT(Thr308), phospho-FKHR, GSK3α/β, and horseradish peroxidase–conjugated second antibodies were purchased from Santa Cruz Biotechnology. AKT Kinase Assay kit, chemiluminescence reagents, and phospho-GSK3β, phospho-mammalian target of rapamycin were obtained from Cell Signaling. The eukaryotic expression plasmid pUSEamp containing myr-AKT1 (activated) was bought from Upstate Biotechnology. Annexin V–FITC Apoptosis Detection kit and AKT Inhibitor V (Triciribine) were purchased from Calbiochem. Transferase-mediated FITC-12-dUTP nick-end labeling (TUNEL) Assay kit was bought from KeyGEN. DMSO, 4,6-diamidine-2-phenylindole (DAPI), and 3-(4,5-dime-thylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Lipofectamine 2000 and Alexa Fluor 488–labeled antifluorescein antibody were obtained from Invitrogen Corporation. ExcisaninA, a natural monomer product extracted from *Isodon MacrocalyxinD*, was initially dissolved in 100% DMSO at 50 mg/mL and stored at –20°C. Its structure is shown in Fig. 1A.

MTT Assay

Cells were seeded in 96-well plate (Falcon) at the 8,000 to 10,000 density per well. Then different concentrations of compounds were added to the medium and incubated for an indicated period. Cell viability was determined by MTT assay. Briefly, MTT was dissolved and sterilized in PBS at 5 mg/mL and 10 μL was added into each well. The plates were incubated in an incubator (37°C, 5% CO_2) for 4 h, then all the medium was removed. DMSO (100 μL) was into each well to dissolve the dark blue crystal, and the plate was shaken gently for 5 min. Absorbance values with a test wavelength of 570 nm and a reference wavelength of 650 nm was read.

Moreover, to study the effect of ExcisaninA in combination with other anticancer agents, Calcusyn software (Biosoft) was used to calculate the combination index values for each concentration tested, whereby combination index values of <1 indicate synergy, those equal to 1 indicates additivity, and those >1 indicate antagonism in the interaction of the drugs (19).

Detection of Cell Apoptosis

ExcisaninA–induced apoptosis in tumor cells was evaluated by Annexin V–FITC Apoptosis assay and DAPI nuclear staining assay. Cells were cultured in six-well plate and exposed to ExcisaninA for indicated times. For AnnexinV–FITC Apoptosis assay, the cells were collected and resuspended with 10% 1640 medium at the density of ~1 × 10^6 cells/mL. Then the cells were incubated at room temperature in the presence of media binding reagent and Annexin V–FITC for 15 min in the dark. After being washed in PBS, the cells were resuspended in cold 1 × binding buffer and added 10 μL propidium iodide (30 μg/mL), placing samples on ice and away from light. Apoptosis was analyzed by flow cytometry (BD Company) at the wavelength of 488 nm immediately.

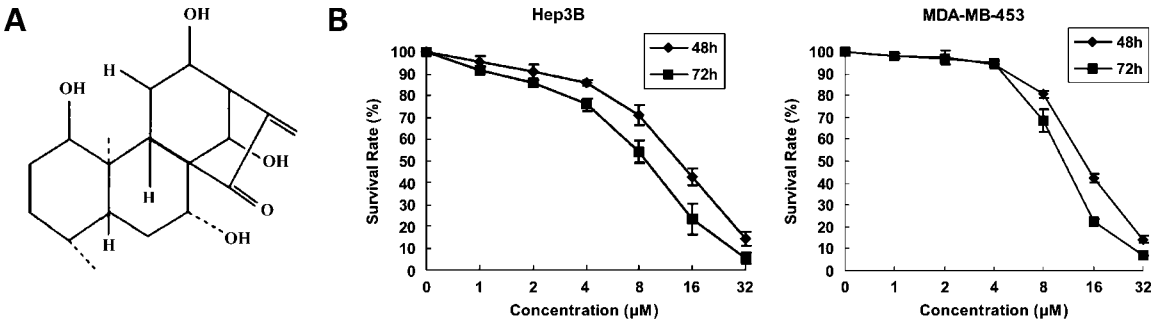
For DAPI nuclear staining assay, the cells were collected and centrifuged onto slides and fixed in 0.1% paraform for 30 min. After being washed in PBS, the slides were soaked in DAPI staining solution (100 μg DAPI dissolved in 100 mL PBS that contain 0.1% TritionX-100) for 10 min in the dark. The morphologic changes of apoptosis characteristic nuclei were examined by confocal microscopy (Olympus).

AKT Kinase Assay

Nonradioactive AKT Kinase Assay kit (Cell signaling) provides all the reagents necessary to this experiment. Cells were lysed using the 1× cell lysis buffer plus 100 mmol/L phenylmethylsulfonyl fluoride. Protein (1,000 μg) was added to 100 μL immobilized AKT antibody slurry, then incubated with gentle rocking overnight at 4°C. Immunoprecipitation were washed twice with 1× lysis buffer and twice with 1× kinase buffer. Pellet in 200 μL 1× kinase buffer were suspended and allocated to 5 tubes equally. Then three different concentrations of ExcisaninA were added into tubes. Triciribine was used as a positive control and 0.1% DMSO was used as a negative control. The kinase assay was done in the presence of 200 μmol/L ATP and 1 μg GSK-3 fusion protein for 30 min at 30°C, then terminated with 20 μL 3 × SDS sample buffer. The tubes were boiled for 5 min at 100°C, and the protein levels of phospho-GSK-3α/β (Ser21/9) and total AKT were measured using Western blot analysis.

Western Blot Analysis

After treated with ExcisaninA, cells were harvested and lysed in lysis buffer [20 mmol/L Na_2PO_4 (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenymethysulfonyl fluoride, 10 mg/mL



C

CIs generated from isoblogram at increasing concentrations of Excisanin A and 5-FU in Hep3B cells

Excisanin A (μM)	5-FU (μM)	Fa	CI
1	0.2	0.348	0.531
2	0.4	0.538	0.568
4	0.8	0.635	0.831

CIs generated from isoblogram at increasing concentrations of Excisanin A and ADM in MDA-MB-453 cells

Excisanin A (μM)	ADM (μM)	Fa	CI
1	0.05	0.153	0.538
2	0.1	0.222	0.819
4	0.2	0.444	0.89
8	0.4	0.777	0.753
16	0.8	0.935	0.669

Figure 1. Growth-inhibitory effect of ExcisaninA on Hep3B and MDA-MB-453 cells. **A,** chemical structure of ExcisaninA. **B,** effect of Excisanin A on proliferation of Hep3B and MDA-MB-453 cells. Cells were cultured at 8,000 to 10,000 cells per well in a 96-well plate, exposed to different concentrations of Excisanin A and incubated for 48 and 72 h. *Points,* average of three experiments; *bars,* SD. **C,** synergistic growth inhibition by combination treatment of Excisanin A and other antitumor agents. Cells were cultured at 8,000 to 10,000 cells per well in a 96-well plate, exposed to different concentrations of Excisanin A and 5-FU at a fixed ratio (5:1) in Hep3B cells for 72 h, or exposed to different concentrations of ExcisaninA and ADM at a fixed ratio (20:1) in MDA-MB-453 cells 72 h. The growth inhibition was detected using MTT assay and the combination index were determined by CalcuSyn software. The results are representative of three different experiments.

leupeptin, 100 mmol/L NaF, and 2 mmol/L Na_3VO_4]. The cell lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4°C and the protein concentration was determined using the Bio-Rad protein assay (Bio-Rad labortories). SDS-PAGE sample buffer was added to lysates. Then the lysates were heated to 100°C for 5 min, and 20 to 40 μg of protein was loaded in each well of a 10% to 15% SDS-PAGE gel. Resolved proteins were electrophoretically transferred to PVDF membrane and incubated sequentially with primary antibody and horseradish peroxidase–conjugated second antibody. After washing, the bound antibody complex was detected using an ECL chemiluminescence reagent and XAR film (Kodak) as described by the manufactures (20, 21).

In vivo Antitumor Activity

BALB/c nude mice were obtained from Guangzhou University of Chinese Medicine and were 4- to 6-wk-old. All manipulations were done under sterile conditions. The procedures involving mice and their care were in accordance with the National Institutes of Health Guide for the care and use of Laboratory Animals and with the United Kingdom Coordinating Committee on Cancer Research (1998; ref. 22). Tumor xenografts were established by 2×10^6 Hep3B cells injected s.c. into nude mice. Mice were randomly divided into three groups and each group contained four or five mice. Treatments were initiated on day 6 after inoculation, by which time the volume had reached ~50 mm^3. ExcisaninA (10 mg/kg/d), ExcisaninA (20.0 mg/kg/d), and DMSO control diluent were administered i.p. for 12 d for each group. Tumor volumes and body weight of mice were observed. Tumor volumes were calculated by the formula: $0.5 \times a \times b^2$ in millimeters, where a is the length and b is the width. When all control tumors developed to >2,000 mg, nude mice were killed. After the tumor tissues were excised and weighed, the tissue samples were made into frozen sections with 4 mm width immediately and fixed in cold acetone. Then the frozen sections were stored at −80°C for future studies. Tumor growth inhibition, used to evaluate the tumor response to the drugs, was calculated using the ratio of the average tumor weight of the treated group (T) to the average tumor weight of the control group (C).

Immunofluorescence Staining

Frozen sections of tumor tissue samples were blocked with anti-goat serum albumin for 0.5 h. Then incubate sections with anti–phospho-AKT (Ser473) antibody or anti–phospho-FKHR antibody diluted in blocking buffer (PBS + 0.1% Tween + 1% bovine serum albumin) for 2 h (1:200). After being washed in PBS, sections were incubated with a 1:200 dilution of anti-rabbit Alexa Fluor 488 in the blocking buffer for 1 h. After counterstaining with DAPI (1 μg/mL), frozen sections were observed using confocal microscopy (Olympus). The intensity of immunofluorescence representing the expressions of phospho-AKT and phospho-FKHR proteins was evaluated by repeated staining of the same specimens and by two observers. It was graded as (−) for no immunofluorescence, (±) for weak and indefinitely detectable immunofluorescence, (+) for weak but definitely detectable immunofluorescence, (++) for moderate immunofluorescence, and (+++) for intense immunofluorescence (23).

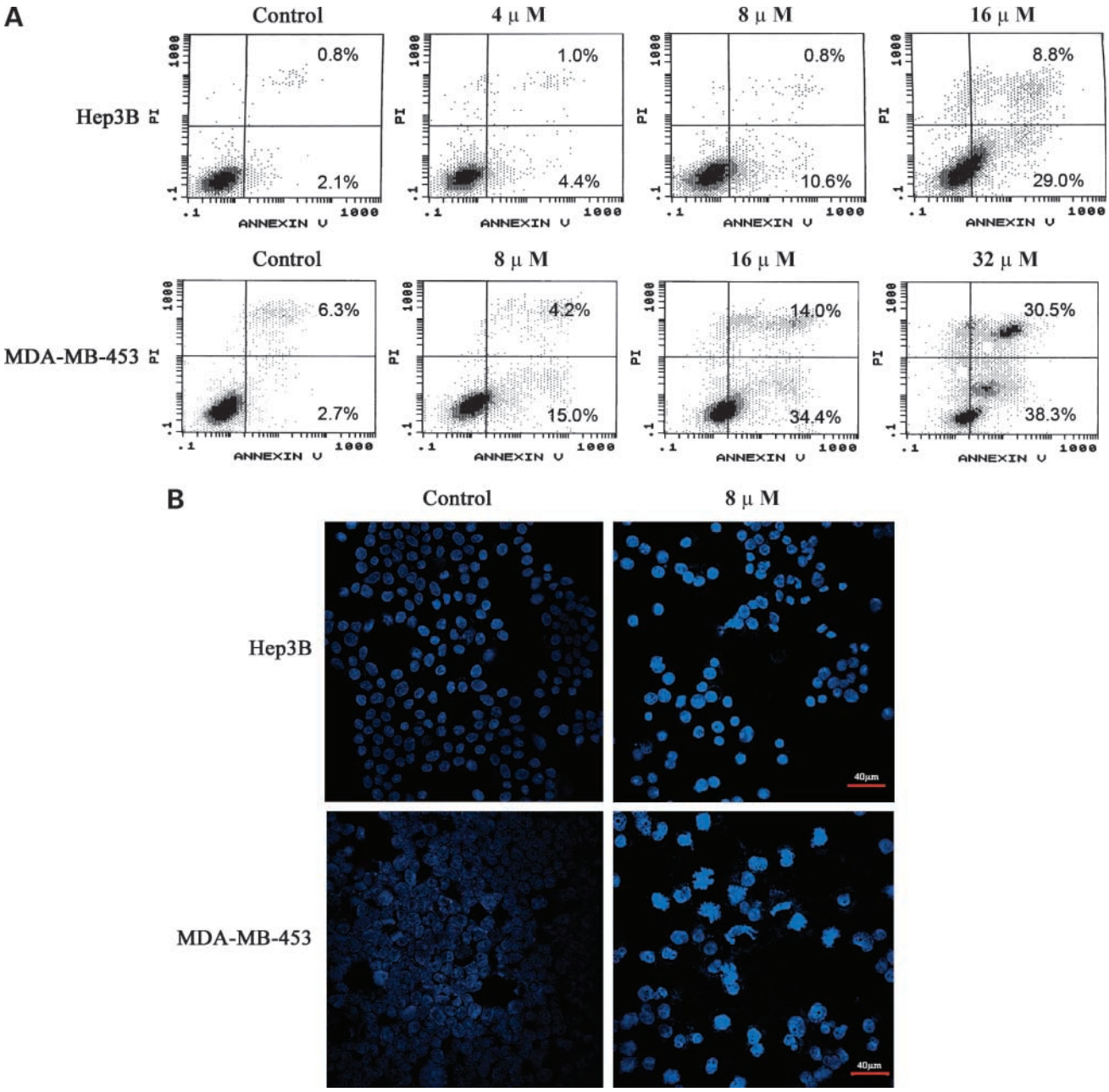


Figure 2. Excisanin A induced apoptosis in Hep3B and MDA-MB453 cells. **A,** AnnexinV analysis of Hep3B cells treated with different concerntrations of Excisanin A for 36 h and MDA-MB-453 cells treated with different concentrations of Excisanin A for 48 h. Excisanin A treatment increased the percentages of AnnexinV$^+$/propidium iodide$^-$(*bottom right quadrant*) and AnnexinV$^+$/propidium iodide$^+$ (*top right quadrant*) cells. **B,** characteristic apoptotic cells were presented in Hep3B cells treated with 8 μmol/L ExcisaninA for 48 h or MDA-MB453 cells treated with 8 μmol/L ExcisaninA for 72 h. Magnification, ×40. The experiments are repeated twice with similar results. Control, 0.1% DMSO.

TUNEL Staining Assay

Frozen tumor sections were fixed in 4% paraform for 20 min and treated with blocking buffer (30%H_2O_2/alcohol methyl, 9:1) for 10 min. Permeabilization involved incubation in 0.1% Triton X-100 for 2 min. After washing twice with PBS, TUNEL reaction was done in a humid chamber for 60 min at 37°C in the dark. After counterstaining with DAPI (1 μg/mL), frozen sections were observed using confocal microscopy (Olympus). TUNEL-positive nuclei were stained green, and all other nuclei were stained blue (24, 25).

Plasmids and Transfection

Hep3B cells were seeded into six-well plate the day before transfection. Transfections of myr-AKT1 (activated) and

empty plasmid were done with Lipofectamine 2000 (Invitrogen) according to the protocol suggested by the manufacture. After 48 h of transfection, the positive clones were selected under G418 (1,000 μg/mL).

Statistical Analysis

The Student's *t* test was used to evaluate the statistical significance of the result at the 95% confidence level, and a *P* value of <0.05 was considered to indicate statistical significance.

Results

ExcisaninA Suppressed Hep3B and MDA-MB-453 Cells Proliferation and Sensitized These Tumor Cells to Other Anticancer Drugs *In vitro*

Using MTT assay, we determined the effect of ExcisaninA on cell proliferation of human solid tumor cell lines. Hep3B and MDA-MB-453 cells were exposed to 0 to 32 μmol/L ExcisaninA for 48 and 72 hours. As shown in Fig. 1B, exposure to ExcisaninA resulted in dose- and time-dependent inhibition of cell viability. At 72 hours, the IC_{50} values showed 6.45 μmol/L in Hep3B cells and 10.3 μmol/L in MDA-MB-453 cells.

Based on the effect of ExcisaninA on Hep3B and MDA-MB-453 cell proliferation alone, the effect of ExcisaninA in combination with other antitumor agents was studied. 5-fluorouracil (5-FU) and ADM are clinically active agents against liver cancers and breast cancers. Thus, we further examined the effects of the combination treatment of ExcisaninA and 5-FU or ADM. As shown in Fig. 1C, 5-FU or ADM in combination with ExcisaninA induced much greater growth inhibition than either 5-FU or ADM alone. The combination of ExcisaninA (from 1–4 μmol/L) and 5-FU (from 0.2–0.8 μmol/L) produced a combination index value of <1 in Hep3B cells. The combination of ExcisaninA (from 1–16 μmol/L) and ADM (from 0.01–0.8 μmol/L) produced a combination index value of <1 in MDA-MB-453 cells. These data indicated that ExcisaninA could sensitize Hep3B and MDA-MB-453 cells to 5-FU or ADM *in vitro*.

ExcisaninA Induced Apoptosis in Hep3B and MDA-MB-453 cells

Phosphatidyl serine translocation to the cell surface is an indicator of early apoptotic cells. To confirm whether the growth inhibition of ExcisaninA was caused by apoptosis *in vitro*, Annexin V-FITC/propidium iodide double staining assay was used to detect the apoptotic cells. In Fig. 2A, the percentage of Annexin V–positive cells increased from 2.9% to 37.8% in Hep3B cells treated with 16 μmol/L ExcisaninA for 36 hours. When MDA-MB-453 cells treated with 16 or 32 μmol/L ExcisaninA for 48 hours, the percentage of Annexin V–positive cells were 48.4% and 68.6%, respectively. Furthermore, cell morphology stained with DAPI was observed to identify the apoptotic cell population. Figure 2B showed that treatment with 0.1% DMSO did not appreciably induce apoptosis in cells, but typical morphologic changes associated with apoptosis-chromatin condensation, apoptotic body formation, and DNA fragmentation were prevalently observed in ExcisaninA-treated Hep3B and MDA-MB-453 cells.

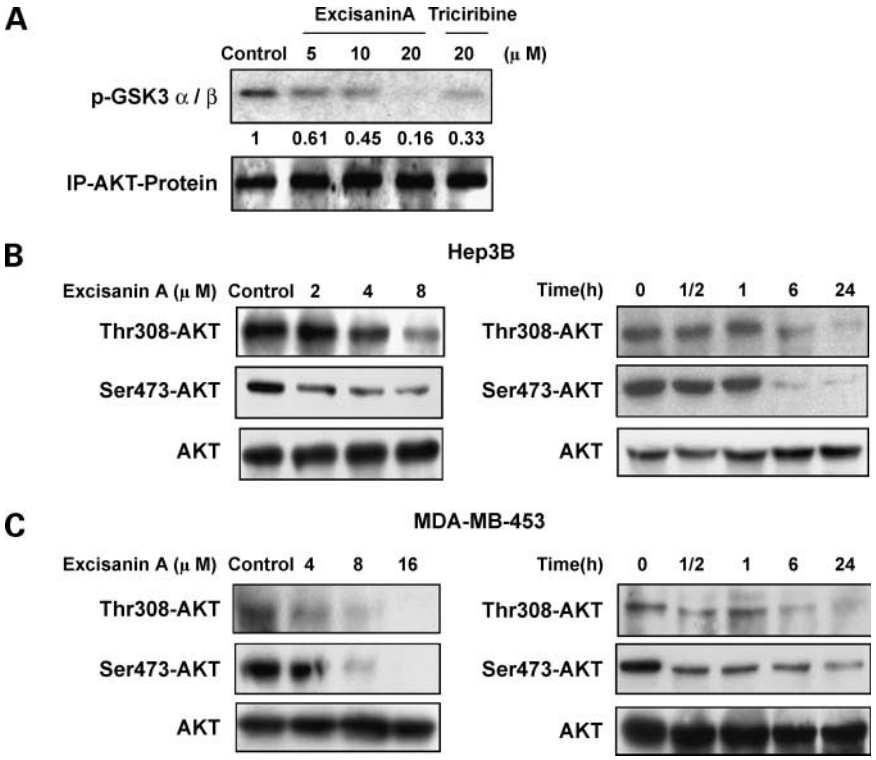


Figure 3. Effect of ExcisaninA on AKT activity and phosphorylation. **A,** cell lysates were used to immunoprecipitate endogenous AKT protein with anti-AKT antibody. Then AKT kinase activity was determined by adding different concentrations of ExcisaninA and 1 μg GSK-3 substrate fusion protein in the presence of ATP for 30 min as described in "Materials and Methods." Triciribine was used as the positive control. Phosphorylation of GSK-3 is measured by Western blot assay using a phospho-GSK-3α/β antibody. **B,** Hep3B cells were treated with different concentrations of Excisanin A for 2 h (*left*); *right,* Hep3B cells were treated with 4 μmol/L Excisanin A for different times. **C,** MDA-MB-453 cells were treated with different concentrations of Excisanin A for 2 h (*left*); *right,* MDA-MB-453 cells were treated with 16 μmol/L Excisanin A for different times. Then the cells were collected and lysed. Western blot was conducted and probed with anti–phospho-AKT and anti-AKT. Data represent three independent experiments. *Control,* 0.1% DMSO.

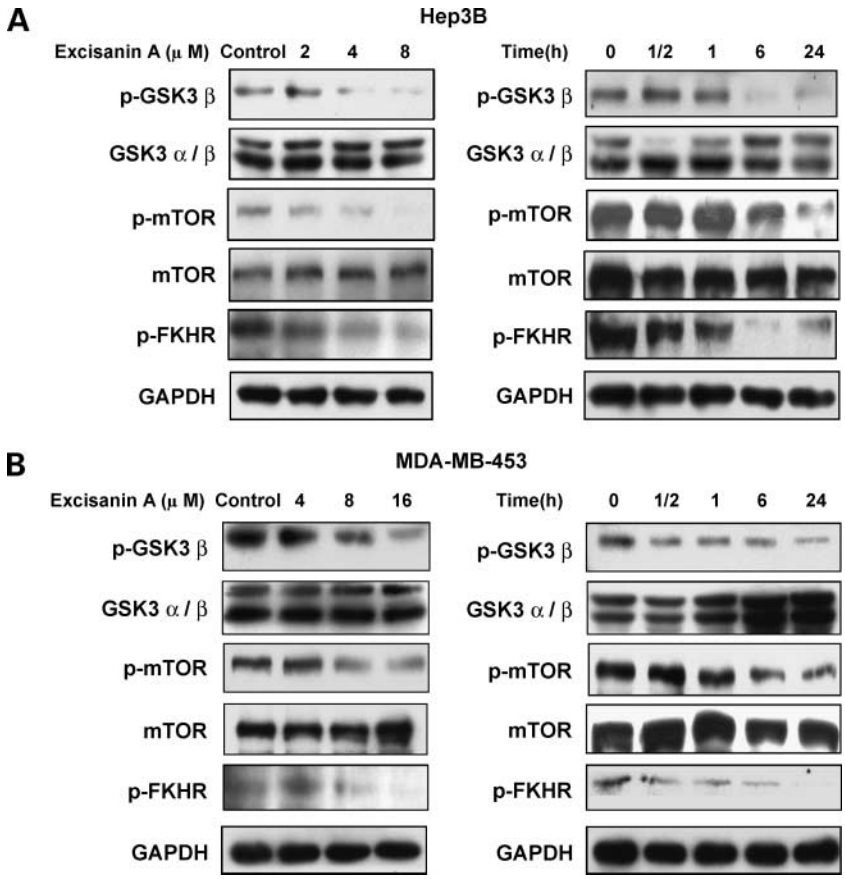


Figure 4. ExcisaninA inhibited phosphorylation of AKT downstream targets. **A,** Hep3B cells were treated with different concentrations of Excisanin A for 2 h; *right,* Hep3B cells were treated with 4 μmol/L ExcisaninA for different times. **B,** MDA-MB-453 cells were treated with different concentrations of Excisanin A for 2 h (*left*); *right,* MDA-MB-453 cells were treated with 16 μmol/L ExcisaninA for different times. Then the cells were collected and lyzed. Total protein isolated was separated by SDS-PAGE and analyzed by immunoblotting with indicated antibodies. Results are representative of three different experiments.

ExcisaninA Inhibited AKT Kinase Activity and Phosphorylation of AKT *In vitro*

It is evident that AKT pathway is one of the most frequently hyperactivated signaling pathways in a variety of human cancers, including human breast cancer and human hepatocellular carcinoma (26–29). High levels of phosphorylated AKT in Hep3B and MDA-MB-453 cells were also observed in our previous work, which indicated that AKT kinase and its down signaling pathway might be aberrantly activated in these two cell lines. Thus, to explore the molecular mechanisms of ExcisaninA-induced apoptosis, we used biochemistry method (AKT Kinase Assay kit) to evaluate the effect of ExcisaninA on the AKT kinase activity in cell-free system. In this experiment, GSK-3 fusion protein was used as a substrate; thus, the changes of GSK-3 phosphorylation can directly reflect the AKT kinase activity (30). Triciribine (AKT Inhibitor V) was a selective small molecule inhibitor of AKT signaling, and used as a positive control (31). As shown in Fig. 3A, ExcisaninA could concentration-dependently suppress phosphorylation of GSK-3α/β. At the concentration of 20 μmol/L ExcisaninA, the level of GSK-3α/β phosphorylation decreased 84% but was only 67% in the positive control (20 μmol/L triciribine). As the AKT kinase activity is regulated by phosphorylation on two regulatory sites, threonine 308 in the activation loop of the catalytic domain and serine 473 in the COOH-terminal regulatory domain, we next assessed the effect of ExcisaninA on the phosphorylation status of AKT on Ser473 and Thr308 in cancer cells. The results showed that ExcisaninA down-regulated phosphorylation of Ser473-AKT and Thr308-AKT in a dose- and time-dependent manner, without affecting the amount of AKT (Fig. 3B and C). Actually, exposure of Hep3B to 4 μmol/L ExcisaninA for 2 hours or exposure of MDA-MB-453 cells to 16 μmol/L ExcisaninA for only 30 minutes caused virtual loss of phosphorylation of AKT on Thr308 and Ser473.

ExcisaninA Inhibited Phosphorylation of Downstream Targets of AKT

It has been stated that AKT exerts its cellular effects through phosphorylation of a number of proteins. More than 20 proteins have been identified as AKT substrates, including the members of Forkhead protein family (FKHR, FKHRL1, and AFX), GSK-3β, mammalian target of rapamycin, p21, p27, MDM2, Bad, ASK, tuberlin/TSC2, and IKKα, etc. (6). As inhibition of substrate phosphorylation can really reflect inhibition of AKT activity, we examined whether ExcisaninA could inhibit phosphorylation of downstream targets of AKT. Figure 4 showed that the phosphrylation levels of FKHR, GSK-3β, or mammalian target of rapamycin were all partially attenuated by ExcisaninA dose dependently and time dependently without affecting the amount of total proteins in Hep3B cells and MDA-453 cells. More

precisely, the reduction of phosphorylation of these proteins occurred within 2 hours after exposure to 4 μmol/L ExcisaninA in Hep3B cells, or within 30 minutes after exposure to 16 μmol/L ExcisaninA in MDA-MB-453 cells. These data indicated that ExcisaninA could induce cell apoptosis by blocking AKT signaling pathway in Hep3B cells and MDA-453 cells.

Effect of ExcisaninA on the Viability of Hep3B/myr-AKT1 Cells

To further confirm ExcisaninA could target AKT kinase, we transfected the Hep3B cells with myr-AKT1 plasmids and selected the positive clone using G418. In comparison with the control cells (Hep3B cells transfected with the vector plasmid), the exogenous AKT and phospho-AKT expression significantly increased in Hep3B/myr-AKT1 cells (Fig. 5A). Although treatment with ExcisaninA obviously reduced the survival rate in Hep3B/myr-AKT1 cells more than that in control cells ($P < 0.05$; Fig. 5B). Thus, this result further confirmed that AKT kinase was indeed the target of ExcisaninA treatment.

Antitumor Activity of ExcisaninA *In vivo*

To detect antitumor activity of ExcisaninA *in vivo*, we established Hep3B xenografts and ExcisaninA administration was initiated on day 6 after implantation. ExcisaninA was alone given by i.p. injection every day at the dose of 10 or 20 mg/kg for 12 days. We could see that treatment with ExcisaninA at 10 mg/kg/d had no obviously effect on tumor growth, and the tumor growth inhibition was ~10.3%. However, ExcisaninA at 20 mg/kg/d could obviously suppress the tumor growth and the tumor growth inhibition was ~46.4% (Fig. 6A). No obvious toxicity was observed in mice receiving the dosage treatment.

To determine whether the growth inhibition of ExcisaninA was caused by apoptosis *in vivo*, tumor frozen sections from Hep3B-bearing nude mice were stained with TUNEL to identify the apoptotic cell population. As shown in Fig. 6B, treatment with 4%DMSO (control) did not appreciably induce apoptosis, whereas ExcisaninA at 20 mg/kg/d stimulated a substantially increased number of TUNEL-positive cells in Hep3B tumors, as indicated by green-nuclear cells (*arrowheads*). Then we detected effect of ExcisaninA on the AKT signaling pathway in tumor samples. Western blot analysis showed that the phosphorylation of AKT (Ser473) and its downstream substrate FKHR dramatically decreased in mice treated with 20 mg/kg/d of ExcisaninA (Fig. 6C). These results were confirmed by immunofluorescence staining in tumor frozen sections. Figure 6D showed that the intensity of fluorescence representing the protein levels of phospho-AKT and phospho-FKHR was (++) to (+++) in the control group and the group with ExcisaninA at 10 mg/kg/d, whereas in the group treated with 20 mg/kg/d of ExcisaninA, the intensity of fluorescence was (±) to (+).

Discussion

Natural products have long been recognized as an important source of therapeutically effective drugs, and their importance in the prevention and treatment of cancer is becoming increasingly evident (32). ExcisaninA is an ent-kaurane diterpenoid compound with varied biological activity for antibacteria, antitumor, and anti-inflammation. It can be identified from many plants of genus *Isodon* that have been widely used in Chinese popular folk to treat cancer and inflammatory diseases. In the present study, we found that ExcisaninA, isolated from *Isodon MacrocalyxinD*, possessed an antiproliferative effect on Hep3B and MDA-MB-453 cells and suppressed Hep3B xenografts tumor growth by inhibiting AKT kinase activity and blocking its signal pathway.

Because of owing α-amethylenecyclopentanone system that is an essential active center for the antitumor activity of diterpenoids (14), ExcisaninA has been reported to exhibit cytotoxic effect in cancer cells. For example, ExcisaninA extracted from *Rabdosia excisa* and its semisynthetic analogues shows significant cytotoxic activity against P388 murine leukemia cells (33, 34). We have also shown that ExcisaninA isolated from *Isodon MacrocalyxinD* could inhibit human colon cancer SW620 cells proliferation (35). In the present study, ExcisaninA not only showed its significantly inhibitory effect on Hep3B and MDA-MB-453 cells proliferation alone but also produced synergistic growth inhibition

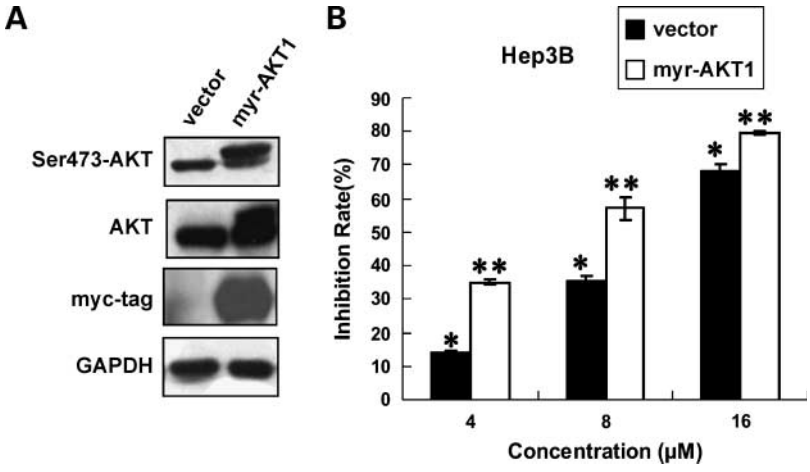


Figure 5. Effect of ExcisaninA on cell viability in Hep3B/myr-AKT1 cells. **A,** the positive clone stably expressed myr-AKT1 was analyzed by Western blotting with indicated antibodies. **B,** effect of ExcisaninA on the viability of Hep3B/myr-AKT1 cells. Cells tranfected with vector or cells stably expressed myr-AKT1 were cultured at 8,000 to 10,000 cells per well in a 96-well plate, exposed to different concentrations of ExcisaninA for 60 h. Cell viability was assayed by MTT. *Columns,* mean of triplicate determinations (** <0.05 versus *); *bars,* SD. Similar results were obtained in another independent experiment.

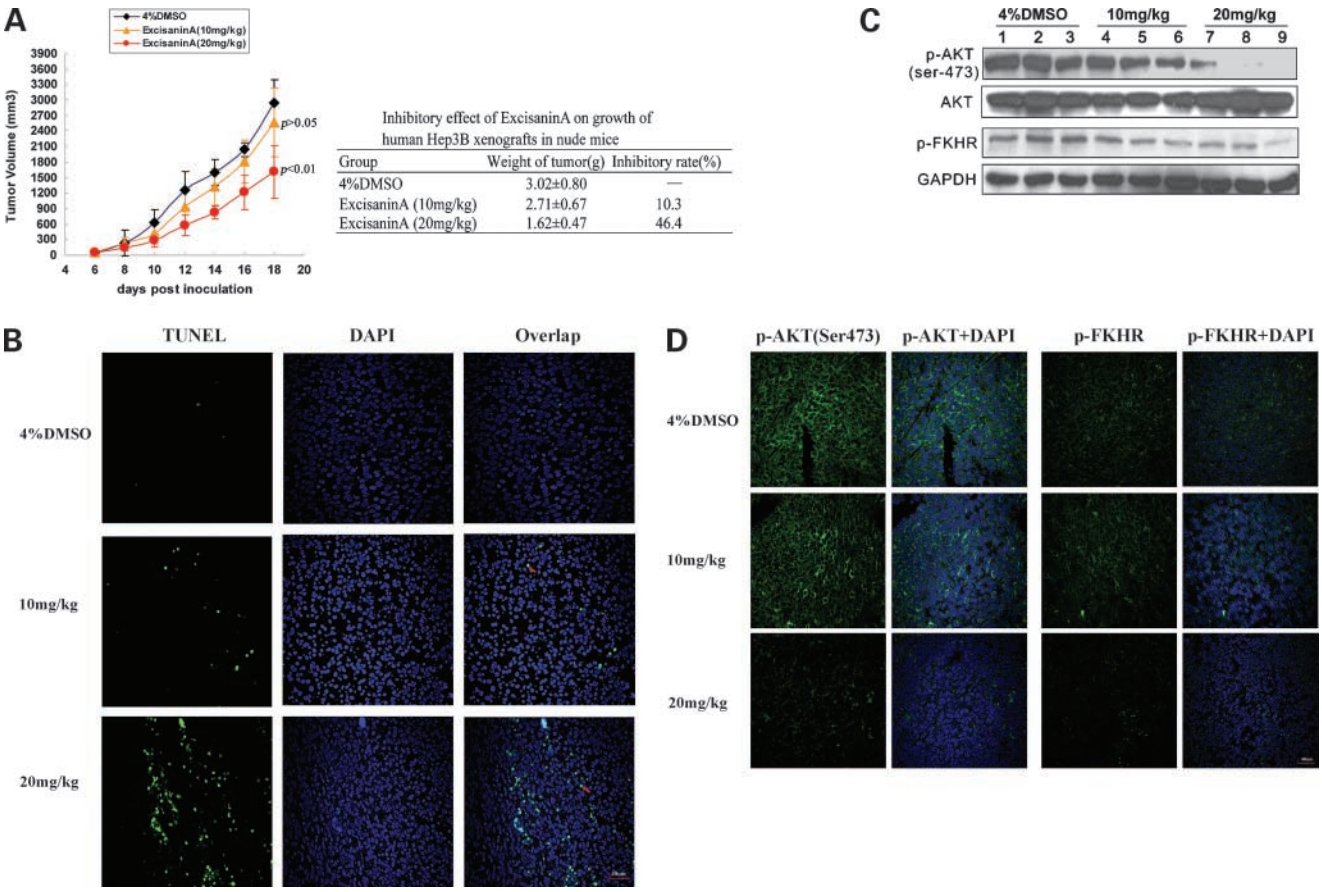


Figure 6. Effects of ExcisaninA on tumor growth of human Hep3B xenografts in nude mice. **A,** tumor xenografts were established by 2 × 10^6 Hep3B cells injected s.c. into nude mice. The treatments began on day 6 after inoculation, including 4% DMSO (n = 4), 10 mg/kg/d ExcisaninA (n = 5), and 20 mg/kg/d ExcisaninA (n = 5) for 12 d. *Left,* injection of ExcisaninA (20 mg/kg/d) could obviously decrease the tumor size in nude mice model of Hep3B xenograft tumor. *Points,* mean of tumors; *bars,* SD. *, P values for treated groups were obtained by comparison with the control group. *Right,* tumor growth inhibition was calculated. **B,** representative images of TUNEL staining of Hep3B xenograft frozen tumor sections after treatment. The apoptotic cells with DNA fragmentation were stained positively as green nuclei (*arrowheads*). Magnification, ×40. **C,** tumor samples of each group were lysed. Western blot analysis was conducted with phospho-AKT (Ser473) and phospho-FKHR antibodies. *Lane 1, 2,* and *3,* group with 4%DMSO treatment; *lane 4, 5,* and *6,* represented the group with ExcisaninA treatment at 10 mg/kg/d; *Lane 7, 8, 9,* group with ExcisaninA treatment at 20 mg/kg/d. This experiment is representative of three experiments. **D,** representative images of immunofluorescence staning of Hep3B xenograft frozen tumor sections for phospho-AKT and phospho-FKHR proteins. Magnification, ×40.

effect with other anticancer drugs *in vitro* (Fig. 1). And ExcisaninA at 20 mg/kg/d could potently suppress Hep3B xenografts tumor growth (Fig. 6A). Further study confirmed that ExcisaninA decreased cell viability and suppressed tumor growth mainly due to the induction of apoptosis, as shown by increasing Annexin V–positive cells and characteristic morphologic changes of apoptosis in the nuleus, as well as increasing TUNEL-positive cells in tumor samples (Figs. 2 and 6B). More importantly, we found that ExcisaninA inhibited AKT kinase activity and blocked its signal pathway *in vitro* and *in vivo*.

It is now apparent that hyperactivation of AKT kinases is one of the most common molecular perturbations in human malignancy (6). Activated AKT can promote cancer cell survival by inhibiting apoptosis, after the phosphorylation of several downstream targets, which include downstream effectors such as Forkhead transcription factors, GSK-3β, mammalian target of rapamycin kinase, Bad, etc. (36). Inactivation of AKT signaling pathway would dephosphorylate these proapoptotic effectors (37, 38). As high levels of phospho-AKT were observed in Hep3B and MDA-MB-453 cells, whether ExcisaninA could target PKB/AKT kinase to induce these two cancer cells apoptosis were investigated. First, we found that ExcisaninA suppressed AKT kinase activity obviously in cell-free system using AKT Kinase Assay kit (Fig. 3A). Then we measured the phosphorylation status of several AKT substrates to evaluate inhibition of AKT kinase activity in cells. As expected, the downstream proapoptotic effectors of AKT including FKHR, GSK-3β, and mammalian target of rapamycin dephosphorylated significantly at very early time without affecting the total amount of proteins after ExcisaninA treatment in Hep3B and MDA-MB-453 cells (Fig. 4). Because reduction in phosphorylation of these downstream effectors occurs early, these effects could not result from induction of apoptosis but instead could conceivably cause apoptosis as a consequence. Third,

the results of Hep3B xenograft models *in vivo* showed ExcisaninA at 20 mg/kg/d obtained good antitumor effect with decreased phosphor-AKT and phospho-FKHR (Fig. 6C and D). Whereas treatment with 10 mg/kg/d of ExcisaninA had little effect on AKT signal pathway, the tumor growth inhibition was only 10.3%. Fourth, ExcisaninA is more sensitive to Hep3B/myr-AKT1 cells. In Hep3B/myr-AKT1 cells, AKT signaling pathway is more hyperactivated, rendering the cells highly dependent on this pathway. Because ExcisaninA could inhibit the AKT activity, the growth inhibition was much higher in Hep3B stably expressed constitutively activated AKT1 cells than that in control cells (Fig. 5). This result is concurrent with another selective AKT inhibitor-API-2, which much more potently inhibits cell growth in AKT-overexpressing/activating cells compared with those with low levels of AKT (31). Furthermore, we observed that AKT phosphorylation decreased after ExcisaninA treatment (Fig. 3B and C). It is possible that the binding of ExcisaninA to AKT may alter its conformation so that the relevant amino acid residues are not available for phosphorylation. Another possibility is that ExcisaninA may decrease the plasma membrane localization of AKT, just like perifosine that inhibits the AKT phosphorylation by altering translocation of AKT to the plasma membrane (39). It is also possible that ExcisaninA may inhibit the activity of upstream molecules of AKT (i.e., phosphoinositide 3-kinase). These possibilities remain to be elucidated with further study. Taken together, all these data suggest that ExcisaninA can inhibit AKT kinase activity and phosphorylation of its target effectors, block the AKT signaling pathway, and subsequently initiate apoptotic events.

AKT kinase is an attractive target for small molecular drug discovery. To date, researchers have developed some AKT inhibitors targeting the ATP binding site, PH domain, and protein substrate binding site, respectively (40). But most of them cannot yet enter into clinical trials due to high toxicity or poor bioavailability. Only several of them, such as GSK690693 and perifosine, are currently in phase I to II trials alone or in combination to treat multiple forms of cancer (41, 42). Although many diterpenoids isolated from genus *Isodon* have been shown to possess strong antitumor activities with low toxicity (14). For example, oridonin and ponicidin have been formally used in clinic to treat cancer in China. In the present study, our data provide evidence of a sustained antitumor effect and a promising development of ExcisaninA. However, how ExcisaninA interacts with AKT kinase remains elusive. Elucidating molecular basis for ExcisaninA and AKT kinase interaction in the further study, will be useful for ExcisaninA as a leading compound to design, synthesize more potent and selective compounds that inhibit the AKT signal pathway.

In summary, ExcisaninA is a potent inhibitor of AKT signaling pathway in tumor cells. Through inhibition of AKT kinase and blockade of its signal pathway, ExcisaninA induces tumor cells apoptosis *in vitro* and inhibits tumor growth *in vivo*. These data provide validation for the development of ExcisaninA or its semisynthetic analogues to treat cancers displaying elevated levels of AKT. Further investigation is required to evaluate whether ExcisaninA or its semisynthetic analogues are clinically useful in this setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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