Regulation of the PTEN/PI3K/AKT pathway in RCC using the active compounds of natural products in vitro

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Abstract. Since Professor Tu Youyou won the 2015 Nobel Prize in Physiology and Medicine for the discovery of artemisinin, which is used to treat malaria, increased attention has been paid to the extracts obtained from plants, in order to analyze their biological activities, particularly with regard to their antitumor activity. Therefore, the present study explored the biochemical properties of seven natural plant extracts on renal cell carcinoma (RCC). 786-O and OS-RC-2 cells were cultured and treated with different concentrations of the extracts. Then, cell viability, the IC50 value and proliferation was determined using a Cell Counting Kit-8 assay. Apoptosis and cell cycle distribution were evaluated via flow cytometry. The expression levels of proteins were assessed using western blotting, and cellular morphology was observed using a light microscope. The results showed that sophoricoside, aucubin, notoginsenoside R1 and ginsenoside Rg1 did not exhibit notable anti-RCC activity, whereas the effect of ginsenoside Re and allicin on RCC was considerably weak. However, naringenin showed potent anti-proliferative, apoptosis inducing and cell cycle arresting activity on RCC cells via regulation of the PTEN/PI3K/AKT signaling pathway.

Introduction

Renal cancer has a high rate of incidence and mortality worldwide (1), and is the second most common cause of deaths amongst urinary system cancers in men and women (2). Renal cell carcinoma (RCC) develops from renal tubules and accounts for ~85% of all renal cancers, and certain studies have demonstrated that the incidence of RCC has increased by 2-4% per year over the past decades worldwide (3). However, unlike other solid tumors, standard cytotoxic chemotherapy is still ineffective for RCC, and eventually, most patients develop resistance after receiving one or more therapeutic agents (4,5). Thus, to decrease the death rate associated with RCC, there is a need to develop novel antitumor drugs, and natural compounds serve as a source of potential active compounds.

Several thousand years ago, natural products were used for their medicinal properties (6), and increasing evidence has shown that natural products from vegetables, fruits and traditional medicines for anti-cancer research remains an important source of potentially novel treatments (7-11). Although there was a shift towards the study of synthetic drugs based on molecular biology and combinatorial chemistry, these synthetic drugs have the disadvantages of occasional intolerable side effects and expensive prices (12,13). By comparison, treatment with natural products have fewer side effects, whilst exhibiting favorable outcomes (13). However, to the best of our knowledge, there are no wide-scale investigations on the therapeutic activities of natural products on RCC. Hence, it is valuable to study the effects of compounds in natural products on RCC.

The aberrant activity of multiple molecular signaling pathways are closely related to the development and maintenance of
cancer (14,15). Amongst these pathways, the PI3K/AKT/mTOR has been identified as important in the regulation of tumor cell proliferation, survival and angiogenesis in cancer (7). However, the role of these pathways is rarely studied with regard to the anti-RCC effect of certain natural products.

In the present study, the ability of several natural products derived from bioactive plants compounds was assessed with regard to their anti-RCC effects, as well as their ability to modulate the PI3K/AKT/mTOR signaling pathways.

Materials and methods

Cell culture and drug treatment. The 786-O cell line was obtained from the American Type Culture Collection and the OS-RC-2 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. All cells were cultured in RPMI-1640 (HyClone; Cytiva) medium containing 10% FBS (Shanghai ExCell Biology, Inc.) at 37°C with 5% CO₂ in a humidified incubator.

In total, seven natural products were purchased from Beijing Solarbio Science & Technology Co., Ltd.: Sophoricoside (cat. no. SS8650); aucubin (cat. no. SA9840); nortog参enside R1 (cat. no. SN8230); ginsenoside Re (cat. no. SG8310); ginsenoside Rg1 (cat. no. SG8330); naringenin (cat. no. SN8020); and allicin (cat. no. SA8720). According to the manufacturer's instructions, sophoricoside, aucubin and nortog参enside R1 were dissolved in DMSO, ginsenoside Re and ginsenoside Rg1 were dissolved in ddH₂O, and naringenin and allicin were dissolved in absolute ethanol. All natural products were stored at 4°C in the dark and added to cells that had been cultured for 24 h.

Cytotoxicity assay. RCC cells (6x10³ cells/well) were seeded in 96-well culture plates for 24 h, and then treated with the indicated compounds at various concentrations for 48 h; see Fig. 1 for details of treatments. According to the manufacturer's instructions, cell cytotoxicity was detected by treating cells with 10 µl Cell Counting Kit-8 (CCK-8; cat. no. CK04; Dojindo Molecular Technologies, Inc.) at 37°C for 2-4 h. Subsequently, the absorbance was measured on a Thermo Multiskan spectrophotometer (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

Subsequently, the RCC cells were treated with different concentrations of naringenin (0, 1, 2, 4, 6, 8, 10, 12, 14 or 16 µm) or allicin (0, 100, 200, 300, 400, 500, 600, 700, 800 or 900 µm) for 48 h, and then, as described above, the cells were incubated with the CCK-8 solution. The IC₅₀ of naringenin and allicin for each cell line was calculated using SPSS version 13.0 (SPSS, Inc.).

Cell proliferation assay. RCC cells were cultured with naringenin (0, 4 or 8 µm) for different periods of time (0, 24, 48, 72 or 96 h), or, after pretreatment with a PTEN inhibitor (SF6170, MedChemExpress/activator (Oroxin B, MedChemExpress) for 24 h in a 37°C CO₂ incubator, cells were treated with 4 µm naringenin for 24 h. Subsequently, cells were incubated with 10 µl CCK-8 at 37°C for 2-4 h. Finally, cell proliferation was determined by measurement of the absorbance at 450 nm on a spectrophotometer.

Cell apoptosis analysis. An Annexin V binding assay was employed to detect apoptosis using an Annexin V-FITC and PI staining kit (cat. no. AP101-30; MultiSciences Biotech Co., Ltd.) according to the manufacturer's protocols. Briefly, the RCC cells were seeded in 6-well cell plates for 24 h and then RCC cells were treated as described above for the cell proliferation assay. The treated cells were stained with 500 µl 1X Binding Buffer containing 5 µl Annexin V-FITC and 10 µl PI for 5 min in the dark. The percentage of apoptotic cells were measured using a CytoFLEX LX flow cytometer (Beckman Coulter, Inc.). Early + late apoptosis rates were assessed using CytExpert version 2.3.0.84 (Beckman Coulter, Inc.).

Cell cycle assays. 786-O and OS-RC-2 cells were treated as described above for the cell proliferation assays. Subsequently, cells (2-10x10³ cells/well) were digested and washed. According to the manufacturer's protocol of the cell cycle detection kit (cat. no. CCS012; MultiSciences Biotech Co., Ltd.), the cells were incubated in 1 ml DNA staining solution containing 10 µl permeabilization solution (from the kit) for 30 min at room temperature. Finally, analysis of cell cycle distribution was performed using a CytoFLEX LX flow cytometer (Beckman Coulter, Inc.) and cell cycle was analyzed using DNA Modeling Software (Modfit LT 3.2, version number. 3.1.0.0).

Morphological examination. The 786-O and OS-RC-2 cells were cultured in 6-well plates. After overnight incubation to allow adhesion, the RCC cells were treated with the designated concentrations (0 and 8 µM naringenin) for 48 h at 37°C in carbon dioxide cell incubator. The features of mitotically arrested cells were bright, rounded and could be easily detached from the bottom of the plate. The cell morphology was detected and images were captured using a fluorescence inverted microscope (magnification, x400; Motic AE31; Motic Incorporation, Ltd.) (16).

Western blotting. As described above, the RCC cells were treated with naringenin in 6-well plates. Protein was extracted using High-efficiency RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Subsequently, equal amounts of protein (20 µg) were resolved on a 10-12% SDS-PAGE gel and subsequently transferred to PVDF membranes at 200 mA for 120 min. Following this, the membrane was blocked with 5% (w/v) non-fat dry milk for 2 h at room temperature. After washing the membranes with TBS with 0.1% Tween-20 (TBST), they were incubated with the following primary antibodies: Anti-minichromosome maintenance complex component 2 (MCM2; cat. no. 12079), anti-PI3K (cat. no. 4249), anti-AKT (cat. no. 4685), anti-p-AKT (cat. no. 13038), anti-cyclin E1 (cat. no. 20808), anti-Bcl-2 (cat. no. 15071), anti-Bax (cat. no. 5023), anti-phosphorylated (p-)mTOR (cat. no. 5536), anti-caspase-9 (cat. no. 9502), anti-caspase-3 (cat. no. 14220), anti-GAPDH (cat. no. 5174) and anti-β-actin (cat. no. 8457) (all from Cell Signaling Technology, Inc.), anti-cyclin B1 (cat. no. AF6168), anti-cyclin A2 (cat. no. AF0142), anti-P21 (cat. no. AF6290), anti-P27 (cat. no. AF6324), anti-mTOR (cat. no. AF7803), anti-PTEN (cat. no. AF6351) (all from Affinity Biosciences, Ltd.), anti-ki67 (cat. no. ab16667), anti-caspase-8 (cat. no. ab25901; all from Abcam) and anti-cyclin D1 (cat. no. PB0403; Wuhan Boster
Biological Technology Ltd.) overnight at 4˚C. All primary antibodies were used at a dilution of 1:1,000. After washing four times with TBST, membranes were incubated with secondary goat anti-mouse (cat. no. BA1050) or anti-rabbit antibodies (cat. no. BA1054; 1:5,000; both purchased from Wuhan Boster Biological Technology, Ltd.) for 2 h at room temperature. Finally, signals were visualized using a K-12045-D10 ECL system (Advansta, Inc.). The semi-quantification analysis was performed by Tanon GIS version 4.1.2 software (Tanon Science and Technology Co., Ltd.).

Statistical analysis. All data are expressed as the mean ± SD of three independent experiments and were analyzed using SPSS version 13.0 (SPSS, Inc.). One-way ANOVA with a post hoc Bonferroni test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of the seven natural plant compounds on RCC cells. To determine the cytotoxic effects of the seven natural plant compounds against two RCC cell lines, 786-O and OS-RC-2, a CCK-8 assay was conducted. The results revealed an association and species-dependent cytotoxic effect of the examined plant compounds ginsenoside Re, naringenin and allicin. However, whilst ginsenoside Re exhibited anti-RCC activity, this effect was weak; the IC₅₀ values of allicin were 469.10±42.35 and 353.40±18.59 µM in 786-O and OS-RC-2 cells, respectively. The IC₅₀ values of 786-O and OS-RC-2 cells treated with naringenin were 8.91±0.33 and 7.78±2.65 µM, respectively. The four other compounds did not show significant cytotoxic effects (Fig. 1). Therefore, it was determined that naringenin had the most potent cytotoxic effect, and was thus studied further.

Naringenin inhibits the proliferation of 786-O and OS-RC-2 cells. Fig. 2A shows the chemical structure of naringenin. The effect of naringenin on proliferation in RCC cells was next assessed. As shown in Fig. 2B and C, naringenin significantly inhibited RCC cell proliferation. The expression of Ki67 and MCM2 were assessed, and the results showed that naringenin inhibited Ki67 expression with little to no effect on MCM2 expression (Fig. 2D). Therefore, it was suggested that naringenin inhibited RCC cell proliferation by decreasing Ki67 expression.

Naringenin induces G₂ phase cell cycle arrest in 786-O and OS-RC-2 cells. Naringenin significantly increased the proportion of cells in the G₂ phase in the two RCC cell lines (Fig. 3A). At the same time, naringenin reduced cyclin A2, cyclin B1 and cyclin D1 protein expression levels, whilst promoting cyclin E1 and P21 protein expression levels, with no effect on P27 protein expression levels (Fig. 3B). Therefore, it was suggested that naringenin blocked cell cycle progression in the G₂ phase, and the means by which it inhibited RCC proliferation was by regulating the expression of cell cycle proteins.

Naringenin and honokiol induce apoptosis of RCC cells. Induction of cytotoxicity is also associated with the ability to induce the apoptosis of cancer cells (17). To further determine whether naringenin induced apoptosis, morphological analysis was performed using a light microscope. As shown in Fig. 4A, naringenin induced cell apoptosis in 786-O and OS-RC-2 cells, and notable changes in cellular morphology in both cell lines were observed. The cell body became rounded, shrunken and blebbed, and some cells became elongated and dissolved. Quantification of apoptosis was performed using an Annexin V-FITC/PI apoptosis detection kit. The proportion of apoptotic RCC cells was significantly increased after treatment with 4 and 8 µM naringenin for 48 h (Fig. 4B). The expression of apoptosis-related proteins was next assessed. In both cell lines, naringenin decreased Bcl-2 and caspase-3 expression, increased caspase-8 and cleaved-caspase-9 (35 kDa) expression, and had little effect on cleaved-caspase-3, caspase-9,
Figure 2. Naringenin inhibits proliferation of renal cell carcinoma cells. (A) Chemical structure of naringenin. (B) 786-O and (C) OS-RC-2 cells were incubated with naringenin at different concentrations (0, 4 or 8 µM) at different time-points (0, 24, 48, 72 and 96 h). Cell proliferation was measured using Cell Counting Kit-8 assays. (D) Western blot analysis of MCM2 and Ki67 expression levels. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. Abs. Abs, absolute ethanol; MCM2, minichromosome maintenance complex component 2.

Figure 3. Naringenin arrests cell cycle progression of renal cell carcinoma cells in the G2 phase. 786-O and OS-RC-2 cells were treated with naringenin (0, 4 or 8 µM) for 48 h. (A) Cell cycle distribution was determined using flow cytometry. (B) Western blot analysis of cyclin E1, cyclin A2, cyclin B1, cyclin D1, P27 and P21 expression levels. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. Abs. Abs, absolute ethanol.
cleaved-caspase-9 (37 kDa) and Bax protein expression (Fig. 4C). These results demonstrated that naringenin significantly increased the apoptosis of the two RCC cell lines by upregulating the expression of apoptosis-related proteins.

Naringenin inhibits the PTEN/PI3K/p-AKT signaling pathway in RCC cells. Next, the molecular mechanisms involved in the naringenin-induced effects were assessed. As shown in Fig. 5, naringenin treatment upregulated the expression of PTEN, downregulated the expression of PI3K and p-AKT protein, and had no notable effects on AKT, mTOR and p-mTOR expression. Naringenin may thus inhibit RCC progression by inhibiting the PTEN/PI3K/AKT axis.

Inhibition of PTEN expression attenuates the effects of naringenin. Cells were pre-treated with a PTEN inhibitor (SF1670), and then treated with 4 µm naringenin for 24 h. Subsequently, cell proliferation, apoptosis and the protein expression levels of PTEN, PI3K, AKT and p-AKT were determined. Following treatment with SF1670, RCC cell proliferation was increased (Fig. 6A) and apoptosis was decreased compared with naringenin alone (Fig. 6B). Additionally, the expression of PTEN was decreased, the expression levels of PI3K and p-AKT were increased, whilst the expression of AKT was not notably altered compared with the cells treated with naringenin alone (Fig. 6C). These data suggested that PTEN inhibition attenuated the growth inhibitory effects of naringenin via activation of the PI3K/AKT signaling pathway.

Activation of the PTEN expression augments the effects of naringenin. RCC cells were next treated with a PTEN activator (Oroxin B), and subsequently treated with naringenin. The results of the CCK-8 (Fig. 7A) and cell apoptosis (Fig. 7B) assays showed that the naringenin-induced decrease in proliferation and increase in apoptosis were slightly strengthened after treatment with Oroxin B, and the expression of PTEN was increased, whereas those of PI3K and p-AKT were decreased, compared with the group treated with naringenin alone (Fig. 7C). These data indicated that activation of PTEN potentiates the cytotoxic effects of naringenin by inhibiting the PI3K/AKT signaling pathway.
Discussion

RCC is one of the primary causes of cancer-associated death, accounting for 3% of all cancers in women and 5% in men (18). In the urinary system, RCC is the third largest malignant cancer (18). There are numerous management options, but it remains incurable. Compared with synthetic drugs, treatment with natural products have fewer side effects, while exhibiting favorable outcomes (13). Although other studies have reported that several natural products, such as Epigallocatechin Gallate (19), quercetin (20), englerin A (21), honokiol (22), curcumin (23) and resveratrol (24) have shown beneficial results in preclinical studies of RCC, the research on the mechanism of the therapeutic activities of natural products on RCC is remains limited. The present study examined the antitumor effects of natural products on RCC.

In the present study, sophoricoside, aucubin, notoginsenoside R1 and ginsenoside Rg1 did not exhibit cytotoxic effects. Through consulting the literature, the primary therapeutic properties of sophoricoside (25,26), aucubin (27) and notoginsenoside R1 (28) were found to be analgesic, anti-inflammatory, anti-viral and anti-oxidative. Up to date findings demonstrate that aucubin possesses hypolipidemic activity based on its notable anti-inflammatory and antioxidant activity, and may thus serve as a novel drug for treatment of non-alcoholic fatty liver disease (NAFLD) (29). Ginsenoside Rg1 is known for its cardioprotective effects and auxiliary antitumor effects (30,31). In the present study, the IC\textsubscript{50} of 786-O and OS-RC-2 cells treated with naringenin was 8.91±0.33 and 7.78±2.65 µM, respectively. It was suggested that naringenin may serve as a promising agent to prevent or restrict tumor growth. Thus, their bioactivities and the underlying mechanisms regulated by each where assessed.

Subsequently, it was shown that naringenin significantly inhibited RCC cell proliferation by decreasing Ki67 expression. Bao et al (47) revealed that naringenin efficiently inhibited SGC-7901 gastric cancer cell proliferation by downregulating the expression of proliferating cell nuclear antigen in a time- and concentration-dependent manner. In addition, naringenin blocked cell cycle progression in the G\textsubscript{2} phase to inhibit RCC cell proliferation by regulating expression of cell cycle proteins.
Figure 6. Regulatory effects of PTEN inhibition on renal cell carcinoma via regulation of the PI3K/AKT signaling pathway. Cells were pretreated with a PTEN inhibitor (SF1670) for 24 h, followed by 4 µm naringenin for 24 h. (A) Proliferation was assessed using a Cell Counting Kit-8 assay. (B) Flow cytometry was used to determine the proportion of apoptotic cells. (C) Expression levels of PTEN, PI3K, AKT and p-AKT were investigated using western blotting. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. Abs; †P<0.05, ‡P<0.01 vs. naringenin. p-, phosphorylated; Abs, absolute ethanol.

in the current study. Similarly, Md et al (48), Arul et al (49) and Yan et al (50) found that naringenin induced cell cycle arrest at the G2 phase in A549 lung cancer cells, human hepatocellular carcinoma cells and during kidney injury, respectively. Additionally, it has also been shown that naringenin exerts an anticancer effect on MDA-MB-231 breast cancer cells through arresting cell cycle progression at the G0/G1 phase (51). Thus, naringenin has been demonstrated to inhibit the proliferation...
of cancer cells by blocking cell cycle progression at different stages in different types of cancer.

Of note, in the present study naringenin significantly increased apoptosis in the 786-O and OS-RC-2 cell lines by decreasing Bcl-2 and caspase-3 expression, increasing caspase-8 expression and altering their cellular morphology. Although, several studies have suggested that naringenin induces apoptosis via stimulation of caspase-3, caspase-9 and Bax activity, whilst inhibiting Bcl-2 activity (46,47,52,53), it has also been shown that caspase-8 mediates the transition between different cell death modes, acting as a molecular switch that regulates apoptotic, necroptotic and pyroptotic cell death pathways (54). Therefore, it is hypothesized that naringenin can induce apoptosis by regulating caspase-8 in RCC.
Recently, Zhou et al (55) demonstrated that naringin suppresses proliferation and induces apoptosis via repressing the PI3K/AKT pathway in thyroid cancer cells. In prostate cancer cells, naringenin induced apoptotic cell death through the PI3K/AKT and MAPK signaling pathways (56). Furthermore, in the present study it was shown that PI3K and p-AKT expression levels were increased and the naringenin-induced reduction in proliferation and increase in apoptosis were attenuated following treatment with a PTEN inhibitor. However, the inhibitory effect of naringenin on proliferation and increase in apoptosis were enhanced following treatment with a PTEN activator, which increased PI3K and p-AKT expression. Therefore, these results suggested that naringenin inhibited proliferation, induced apoptosis and arrested the cell cycle progression at the G2 phase through regulation of the PTEN/PI3K/AKT signaling pathway in RCC cells.

In conclusion, the beneficial effects of naringenin were demonstrated in the present study. The proliferation, apoptosis and cell cycle progression of RCC cells were regulated by naringenin via the modulation of the PTEN/PI3K/AKT signaling pathway. Thus, it is hypothesized that naringenin may serve as a potential anti-cancer treatment, either alone or as an adjuvant therapy. However, additional in vivo studies are required to further assess its therapeutic value.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW contributed to conception and design of the study, revision of important intellectual content and final approval of the version to be published. XW, ZX and ZL performed the experiments. ZX, ZL, YC, SH and YR contributed to acquisition and analysis of data. SZ and GW contributed to conception and design of the study. XW, GW and SZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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