Inhibitory effects of petasin on human colon carcinoma cells mediated by inactivation of Akt/mTOR pathway

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Abstract

Background: Colorectal cancer is the third most common cancer worldwide and still lack of effective therapy so far. Petasin, a natural product found in plants of the genus Petasites, has been reported to possess anticancer activity. The present study aimed to investigate the anticancer activity of petasin both in vitro and in vivo. The molecular mechanism of petasin was also further explored.

Methods: Caco-2, LoVo, SW-620, and HT-29 cell lines were used to detect the inhibitory effect of petasin on colon cancer proliferation. Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cell apoptosis was analyzed by flow cytometry. Hoechst 33258 staining was used to visualize morphological changes. Cell migration was assessed using a wound-healing migration assay, and cell invasion was investigated using Transwell chambers. Western blotting assays were employed to evaluate the expression levels of proteins in the protein kinase B/mammalian target of rapamycin (Akt/mTOR) signaling pathway. Finally, in vivo activity of petasin was evaluated using the SW-620 subcutaneous tumor model established in Balb/c nude mice. Twelve rats were randomly divided into control group and 10 mg/kg petasin group. The tumor volume was calculated every 7 days for 28 days. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to assess the apoptotic effect of petasin. Differences between two groups were assessed by analysis of independent-sample t-tests.

Results: Petasin significantly inhibited the proliferation of human colon carcinoma cell lines, induced apoptosis, and suppressed migration and invasion in SW-620 cells. Western blotting results showed that petasin decreased the phosphorylation of Akt (1.01 ± 0.16 vs. 0.74 ± 0.06, P = 0.042), mTOR (0.71 ± 0.12 vs. 0.32 ± 0.11, P = 0.013), and P70S6K (1.23 ± 0.21 vs. 0.85 ± 0.14, P = 0.008), elevated the expression of caspase-3 (0.41 ± 0.09 vs. 0.74 ± 0.12, P = 0.018) and caspase-9 (1.10 ± 0.27 vs. 1.98 ± 0.22, P = 0.009), decreased the Bcl-2 protein (2.75 ± 0.47 vs. 1.51 ± 0.36, P = 0.008), downregulated the expression of matrix metalloproteinase (MMP)-3 (1.51 ± 0.31 vs. 0.82 ± 0.11, P = 0.021) and MMP-9 (1.56 ± 0.32 vs. 0.94 ± 0.15, P = 0.039) in SW-620 cell. In vivo, 10 mg/kg petasin inhibited tumor growth in Balb/c nude mice (924.18 ± 101.23 mm3 vs. 577.67 ± 75.12 mm3 at day 28, P = 0.001) and induced apoptosis (3.6 ± 0.7% vs. 36.0 ± 4.9%, P = 0.001) in tumor tissues.

Conclusions: Petasin inhibits the proliferation of colon cancer SW-620 cells via inactivating the Akt/mTOR pathway. Our findings suggest petasin as a potential candidate for colon cancer therapy.

Keywords: Petasin; Colon cancer; Apoptosis; Migration; Invasion; Akt/mTOR pathway

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, following only lung and breast cancer. [1] CRC accounts for almost 10% of total cancer cases and ranks as the fourth leading cause of cancer-related deaths. [2] Data show that net healthcare costs in the first year after CRC diagnosis range from $36,000 for stage I to $74,000 for stage IV disease. [3] CRC survivors also experience high out-of-pocket costs and lost productivity. [4] These problems impose a significant health burden globally. With growing and aging populations and an increasingly modernized lifestyle, CRC will present more societal challenges in the future. Despite improvements in screening programs for early detection, reduced prevalence of risk factors, and advances in targeted therapy to reduced morbidity and increase survival, median survival is only about 20 months in patients with metastatic CRC. [5] Although researchers spare no effort on the research and development of new anticancer agents, it is regret to find that only 5% of anticancer agents that have activity in preclinical development are subsequently licensed after
phase III testing at present.\textsuperscript{[6]} Furthermore, in the current treatments of CRC, the 5-year survival for late-stage CRC is $<20\%$.\textsuperscript{[5]} Hence, development of new drugs for the treatment of CRC is urgently needed.

Since ancient times natural products have been a rich source of novel therapeutic agents for many diseases.\textsuperscript{[7,8]} Petasin is a natural product found in plants of the genus Petasites, which is endemic to Europe as well as parts of Asia and North America. Petasin has many beneficial medicinal properties, such as antimigraine and anti-allergy properties, and is used to treat hypertension, tumors, and asthma.\textsuperscript{[9,10]} Wang et al reported that petasin inhibits testosterone production and release of corticosterone from rat zona fasciculata-reticularis cells, and obstructs proliferation of human T24 bladder carcinoma cells.\textsuperscript{[11,12]} These authors also found that petasin induces apoptosis in prostate cancer cells, suggesting that S-petasin and iso-S-petasin could be useful as anticancer agents.\textsuperscript{[13]} However, the activity of petasin against colon cancer cells remains unknown.

This study investigated the antiproliferative properties of petasin using a human colon carcinoma cell line. Target endpoints included cytotoxicity, apoptosis, cell migration, and cell invasion. The effects of petasin on the protein kinase B/mammalian target of rapamycin (Akt/mTOR) signaling pathway involved in colon carcinogenesis were also investigated. Finally, in this study, the anti-proliferative activity of petasin was studied in vivo using Balb/c nude mice bearing tumors of a pre-established subcutaneous SW-620 cell line.

Methods

Ethical approval

All animal protocols were approved by the Institutional Animal Care and Use Committee of Lanzhou University Second Hospital and the research protocol complied with institutional guidelines of the Animal Care and Use Committee at Lanzhou University Second Hospital.

Cell lines and cell culture

Human colon carcinoma cell line Caco-2 was purchased from CoBioer biotechnology Co., Ltd. (Nanjing, China). The LoVo cell line was purchased from SunBio Biotechnology Co., Ltd. (Shanghai, China). SW-620 cell line was obtained from the School of Basic Medical Sciences of Lanzhou University (Lanzhou, China). The HT-29 cell line was obtained from Cell Resource Center in the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL penicillin and 100 IU/mL streptomycin). All cell lines were grown in a humidified atmosphere with 5% CO$_2$ at 37$^\circ$C.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Beyotime Biotechnology, Suzhou, China) assay was implied to detect the proliferation of human colon carcinoma cells. Each cell line was cultured in 96-well plates at a density of $5.0 \times 10^4$ per well. After 24 h of incubation for attachment, the cells were treated for 24, 48, or 72 h with different concentrations of petasin (1, 5, and 25 $\mu$mol/L) or with the same volume of phosphate-buffered saline (PBS). Petasin was purchased from Tianrui Biotech Co., Ltd. (Xi’an, China); the purity of petasin was 98% as determined by high-performance liquid chromatography. Cell proliferation was assessed at each time point. Spent medium was replaced with fresh medium containing 10 $\mu$L MTT. After incubation at 37$^\circ$C for another 4 h, the medium was removed and 100 $\mu$L of DMSO was added to each well, and plates agitated for 10 min. Absorbance was measured at 570 nm. Experiments were performed using triplicate wells and repeated at least three times. Results are presented as a percentage inhibition compared to untreated control.

Cell apoptosis assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining (Nan Jing KeyGen Biotech Co., Ltd, Nanjing, China) was used to assess apoptosis. Briefly, SW-620 cells were seeded in six-well plates at a density of $1.0 \times 10^5$ cells per well and incubated for 24 h. Subsequently, cells were treated with 25 $\mu$mol/L petasin or PBS for another 48 h. Cells were collected and centrifuged at 2000 $\times g$ for 5 min, then washed in cold PBS, resuspended in 500 $\mu$L binding buffer, and incubated with 5 $\mu$L Annexin V-FITC and 5 $\mu$L PI. After 10 min of incubation in the dark at room temperature, cell counts were obtained using a flow cytometer.

Morphological changes to cell nuclei were visualized using Hoechst 33258 (Beyotime Biotechnology) staining. Cells were treated as above. After 48 h treatment with 25 $\mu$mol/L petasin or PBS, cells were incubated with 1 mL of Hoechst 33258 dye at 37$^\circ$C for 20 to 30 min, then washed twice with PBS. Cells were examined using fluorescence microscopy. All experiments were repeated three times.

Wound-healing migration assay

Cell migration was assessed using a wound-healing migration assay.\textsuperscript{[14,15]} Briefly, SW-620 cells were plated onto 12-well plates at a density of $1.0 \times 10^5$ per well. After 24 h for attachment, $in vitro$ scratch wounds were created by scraping cell monolayers with a 10-$\mu$L sterile pipette tip. Suspended cells were washed away, and cells remaining on the plates were treated with 25 $\mu$mol/L petasin or PBS in serum-free media for 24 h. Photomicrographs were taken immediately (time 0 h) and at 24 h after the treatment (time 24 h) with an inverted microscope equipped with a digital camera. Both photomicrographs were taken at the same position. Migration was quantified and analyzed with Image-pro plus version 6.0 software (Media Cybernetics, Silver Spring, MD, USA). All experiments were repeated three times.

Cell invasion assay

Cell invasion was investigated using Transwell chambers (24 wells, 8-mm pore size; Millipore, Billerica, MA,
USA). In brief, 600 μL culture medium was added to the bottom chamber, 1 × 10⁶ SW-620 cells were suspended in serum-free medium and placed in the upper chamber. After treatment with 25 μmol/L petasin or PBS for 24 h, noninvasive cells on the top surface of the membrane were mechanically removed, and cells that traversed and spread on the lower surface of the filter were fixed with 95% ethanol and stained with 0.1% crystal violet. Cells adhering to the bottom surface of the membrane were counted in five randomly selected microscope fields. Each experiment was repeated three times.

**Western blotting analysis**

After treatment with 25 μmol/L petasin for 24 h, total proteins were extracted from each group of the SW-620 cells. Briefly, all cells were lysed in RIPA buffer on ice for 30 min, which contained protease inhibitors and phosphatase inhibitors (100:10:4). Cellular debris was removed by centrifuging at 19,830 × g for 15 min at 4°C. Supernatants were collected as total cellular proteins. Protein concentrations were determined with bicinchoninic acid (BCA) protein assay kits (Beyotime Biotechnology). After denaturation for 5 min at 99°C, equal amounts of protein from each group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore). Membranes were blocked in 5% nonfat milk at room temperature, then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. Primary antibodies detected in the study were: rabbit anti-Akt, rabbit anti-phospho-Akt, rabbit anti-mTOR, rabbit anti-phospho-mTOR, rabbit anti-P70S6K, rabbit anti-phospho-P70S6K (Abcam, Cambridge, UK), rabbit anti-caspase-3, mouse anti-caspase-9, rabbit anti-Bcl-2, rabbit anti-phospho-P70S6K, rabbit anti-MMP-3, rabbit anti-MMP-9, rabbit anti-Bcl-2 (Cell Signaling Technology, Danvers, MA, USA). Proteins were visualized with enhanced chemiluminescence Western-blotting detection reagents and analyzed with Image-pro plus version 6.0 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was incorporated as a loading control.

**Tumorigenicity assay in nude mice**

Balb/c athymic (nu/+ or nu−) male mice (4 weeks old) were used in the experiments. SW-620 cells were suspended in 200 μL growth medium/Matrigel and hypodermically injected into right axillaries of mice. According to the random number table, animals were randomly divided into two groups (n = 6) when diameters of resulting tumors measured about 3 mm. Vehicle or 10 mg/kg petasin were administered intragastrically twice a day for 28 days. The length (L) and width (W) of the tumors were measured with calipers to calculate tumor volumes (V = L × W²/2) every 7 days for 28 days.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay**

To assess DNA fragmentation, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed. Slices of mouse tumor xenograft tissues were washed three times in PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 2 min, and incubated with TUNEL reaction mixture (Roche, Indianapolis, IN, USA) in the dark for 1 h at 37°C. Slices were washed with PBS three times and then examined under fluorescence microscopy.

**Statistical analysis**

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 17.0 software or Microsoft Excel 16.0 (Microsoft, Redmond, WA, USA). Differences between the two groups were assessed by analysis of independent-sample t tests. One-way analysis of variance (ANOVA) was used for intergroup comparisons and multiple comparisons. Post hoc tests between groups were evaluated with Student’s t tests. Data are presented as mean ± standard deviation and α = 0.05 was considered statistically significant.

**Results**

**Petasin inhibits the proliferation of human colon carcinoma cell lines**

Petasin inhibited proliferation of human colon carcinoma cell lines, including SW-620, Caco-2, Lovo, and HT-29. Cell viability was analyzed with the MTT assay in vitro. Results showed that treatment with petasin (1, 5, 25 μmol/L) exerted dose-dependent cytotoxicity on all four cell lines [Figure 1A]. The IC50 (half maximal inhibitory concentration) of petasin on the four cell lines-SW-620, Caco-2, Lovo and HT-29 at 48 h were 30.07, 209.67, 228.59, and 78.08 μmol/L, respectively. Among these cell lines, the SW-620 cell line was the most sensitive to petasin exposure. Percent inhibition of SW-620 cell growth after treatment with 25 μmol/L petasin for 24, 48, and 72 h was 21.16 ± 3.59% (P < 0.01), 38.52 ± 4.55% (P < 0.01), and 47.15 ± 7.63% (P < 0.001), respectively [Figure 1A]. The SW-620 cell line was chosen for further investigation of the mechanism of the anticancer effect of petasin.

**Petasin induces apoptosis in SW-620 cells**

It is generally accepted that activation of apoptosis in cancer cells is an important target for cancer treatment. To determine if petasin induces apoptosis in SW-620 cells, Annexin-V-FITC/PI staining and flow cytometry were employed. Treatment with 25 μmol/L petasin for 48 h significantly induced apoptosis in SW-620 cells. The percentage of apoptotic cells in the control group was only 6.01 ± 1.56%, but the percentage increased to 31.03 ± 3.52% in the petasin-treated group (P < 0.01) [Figure 2A]. Hoechst 33258 staining tests yielded the same results. Treatment with 25 μmol/L petasin for 48 h induced clear changes in cell structure and nuclear condensation, in contrast to nuclei of the control cells, which were round, with sharp edges and uniform staining [Figure 2B].

**Petasin inhibits the migration and invasion of SW-620 cells**

Cell migration was investigated using a wound-healing assay. SW-620 cells in the control group showed strong
healing ability after 24 h. Treatment with petasin significantly decreased the percent migration from 68.1 ± 9.6% to 21.1 ± 2.3% (P < 0.01) [Figure 3A]. Similarly, in cell invasion tests, petasin significantly decreased the number of cells growing on the bottom of the membrane (268 ± 36 vs. 61 ± 11, P < 0.01) [Figure 3B].

**Petasin inactivates Akt/mTOR signaling pathway in SW-620 cells**

In order to gain deeper insights into the mechanism of anti-proliferation effects of petasin, the expression of proteins involved in the Akt/mTOR signaling pathway, apoptosis-related proteins, and MMPs were examined [Figure 4]. Previous studies indicated that blocking Akt/mTOR pathway activity would show promising anti-cancer impacts. Here, the results indicated that treatment with 2.5 µmol/L petasin for 24 h significantly decreased the phosphorylation of Akt (1.01 ± 0.16 vs. 0.74 ± 0.06, P < 0.05), mTOR (0.71 ± 0.12 vs. 0.32 ± 0.11, P < 0.05), and P70S6K (1.23 ± 0.21 vs. 0.85 ± 0.14, P < 0.01) when compared with control cells. This inhibition of the Akt/mTOR pathway could activate an intrinsic apoptotic program. Hence, the expression of caspase-3, caspase-9, and anti-apoptotic protein Bcl-2 was evaluated. Petasin elevated expression of caspase-3 (0.41 ± 0.09 vs. 0.74 ± 0.12, P < 0.05) and caspase-9 (1.10 ± 0.27 vs. 1.98 ± 0.22, P < 0.01), decreased Bcl-2 protein (2.75 ± 0.47 vs. 1.51 ± 0.36, P < 0.01), all consistent with induction of apoptosis SW-620 cells. Additionally, Akt regulates the expression of matrix metalloproteinase (MMP) genes that promote p65- and p52-DNA-binding activities of NF-κB; MMPs are thus vital enzymes for extracellular matrix degradation during tumor invasion and metastasis. In the present study, petasin suppressed the expression of MMP 3 (1.51 ± 0.31 vs. 0.82 ± 0.11, P < 0.05) and MMP 9 (1.56 ± 0.32 vs. 0.94 ± 0.15, P < 0.05) in SW-620 cells. These results suggested that the anticancer effects of petasin may be partly due to the inactivation of the Akt/mTOR signaling pathway.

**The in vivo anticancer effect of petasin**

The in vivo anticancer effect of petasin was investigated using an SW-620 subcutaneous tumor model established in Balb/c athymic (nu+/nu+) male mice. Mouse treated with 10 mg/kg petasin exhibited significant reduction in tumor size compared to untreated mice at days 21 and 28. Tumor size in treated mice was reduced from 488.90 ± 48.60 to 289.22 ± 22.60 mm³ at day 21 (P < 0.05), and 924.18 ± 101.23 to 577.67 ± 75.12 mm³ at day 28 (P < 0.01), respectively [Figure 5A]. Besides, we
Figure 2: Petasin induced apoptosis in SW-620 cells. SW-620 cells were treated with 25 μmol/L petasin for 48 h. (A) Apoptosis was detected with Annexin-V-fluorescein isothiocyanate/propidium iodide staining and flow cytometry. (B) Morphological changes in apoptotic cells were evaluated by Hoechst 33258 staining, original magnification ×200. Differences between the two groups were assessed by analysis of independent sample t tests. n = 3, †P < 0.01 vs. control cells.

Figure 3: Petasin inhibited the migration and invasion abilities of SW-620 cells. SW-620 cells were treated with 25 μmol/L petasin for 24 h. (A) Cell migration was assessed using a wound-healing assay. (B) Cell invasion was assessed using Transwell chambers. Invasive cells were stained with 0.2% crystal violet, original magnification ×200. Differences between the two groups were assessed by analysis of independent sample t tests. n = 3, †P < 0.01 vs. control cells.
also found induction of apoptosis existed in petasin-treated mice. As shown in Figure 5A, the number of TUNEL-positive cells was significantly increased in petasin-treated mice when compared to control (3.6 ± 0.7% vs. 36.0 ± 4.9%, \( P < 0.01 \)).

Discussion

Previous studies reported that petasin possesses anti-proliferative activity on human T24 bladder carcinoma cells and prostate cancer cells, suggesting that petasin could be a useful anticancer agent.\(^{[12,13]}\) But whether it could work on colon cancer remains unknown. In the present study, petasin was shown to significantly inhibit the proliferation of human colon cell lines in a dose-dependent manner. Further, induction of apoptosis by petasin treatment in SW-620 cells was identified by Annexin-V-FITC/PI and Hoechst 33258 staining. In addition, treatment of SW-620 cells with petasin suppressed their migration and invasion ability. Anticancer activity of petasin was further confirmed \textit{in vivo}. In the SW-620 subcutaneous tumor model established in Balb/c athymic (nu/nu*) male mice, treatment with 10 mg/kg petasin delayed the growth of tumors and induced apoptosis in tumor tissues.

The Akt/mTOR pathway plays a vital role in regulating cell survival, growth, and metabolism in normal cells. Hyperactivation of Akt/mTOR pathway is implicated in various oncogenic processes across multiple types of cancer.\(^{[22,23]}\) Hence, blocking Akt/mTOR pathway activity could be a promising target for cancer treatment. Recently, Lv et al.\(^{[24]}\) reported a novel phosphoinositide 3-kinase (PI3K)/mTOR dual inhibitor XH002 that decreased the phosphorylation of PI3K/Akt/mTOR pathway proteins and inhibited tumor growth of epidermal growth factor receptor (EGFR)-tyrosine-kinase-inhibitor (TKI)-resistant NCI-H1975 xenografts and exhibited robust antitumor activity in non-small-cell lung cancer. Kenna et al.\(^{[25]}\) reviewed PI3K-Akt-mTOR pathway inhibitors in breast cancer cohorts and found that a variety of promising agents are currently in development for breast cancer treatment. In addition, it is reported that activation of the PI3K/Akt pathway was closely related with a poor prognosis in stage II colon cancer; phosphorylation of Akt is a prognostic factor for disease-free survival.\(^{[26]}\) Thus, inactivation of the Akt/mTOR pathway may be a useful therapeutic target for different types of cancer. In the present study, phosphorylation of Akt, mTOR, and downstream protein P70S6K were decreased after petasin treatment in SW-620 cells,

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**Figure 4:** Petasin inactivated the Akt/mTOR signaling pathway, upregulated apoptotic proteins, and decreased MMP expression in SW-620 cells. SW-620 cells were treated with 25 \( \mu \)mol/L petasin for 24 h. Western blotting analysis was performed to determine the expression of Akt/mTOR/P70S6K, caspase-3, caspase-9, Bcl-2, MMP-3, and MMP-9. (A) Representative images of immunoblots. (B) Quantification of protein expression levels in experimental groups. GAPDH was used as the loading control. Differences between two groups were assessed by analysis of independent sample t tests. \( n = 3, \ P < 0.05, \ * P < 0.01 \) vs. control cells. mTOR: Mammalian target of rapamycin; MMP: Matrix metalloproteinase; p-mTOR: Phosphorylation of mTOR; P70S6K: P70S6 kinase; p-P70S6K: phosphorylation of P70S6K; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
indicating that petasin inhibits the expression of Akt/mTOR pathway proteins.

The Akt/mTOR pathway is also an apoptotic transduction pathway. Decreased phosphorylation of Akt/mTOR pathway could activate an intrinsic apoptotic program. As a family of protease enzymes, caspases play essential roles in apoptotic processes. Once apoptosis is initiated, caspases are activated, which culminate in DNA fragmentation and other apoptosis-related cellular changes. Petasin upregulates expression of caspase-3 and caspase-9, both of which are key members of the caspase family. In addition, the expression of anti-apoptotic protein Bcl-2 was inhibited after exposure to petasin. Results indicate that apoptosis induced by petasin in SW-620 cells might be related to the inactivation of Akt/mTOR pathway. On the other hand, hyperexpression of MMP is also along with the activation of Akt/mTOR pathway, which accelerates tumor migration and invasion. The data obtained in the present study reveal that after petasin treatment, MMP 3 and MMP 9 was remarkably down regulated in parallel to the inactivation of the Akt/mTOR signaling pathway. All these data suggested that inactivation of the Akt/mTOR pathway of petasin eventually induces apoptosis and suppresses migration and invasion in SW-620 cells.

In a recent study, Wang et al reported that petasin could induce apoptosis via the activation of mitochondria-related pathways in prostate cancer cells and finally inhibited the proliferation of prostate cancer cells. They concluded that petasin may influence several cell behaviors, such as cell proliferation, and induction of apoptosis, and ultimately induce morphological changes, which were consistent with our findings in the present study. All of these effects suggested that petasin could be potential anticancer agents. The exact mechanism of the effects of petasin on tumor cells needs to be further revealed in the future.

In conclusion, the antitumor activity of petasin on human colon cancer cells both in vitro and in vivo suggests petasin as a possible candidate for human colon cancer therapy. This activity of petasin might be partially due to the inactivation of the Akt/mTOR pathway.

Confl icts of interest

None.
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