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Original Article

Inositol hexaphosphate suppresses growth and induces apoptosis in HT-29 colorectal cancer cells in culture: PI3K/Akt pathway as a potential target

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Abstract: Background: Inositol hexaphosphate (IP6) is a polyphosphorylated carbohydrate that is present in high amounts in almost all plants and mammalian cells. IP6 induces apoptosis in multiple types of cancer cells, including prostate cancer, breast cancer, skin tumor, liver cancer and colorectal cancer. However, little is known regarding the molecular mechanisms of its anticancer effects. Therefore, this study was conducted to investigate the activity of IP6 against human colorectal cancer cells (HT-29) and to determine whether the IP6 regulates apoptosis in HT-29 cells by inhibiting the PI3K/Akt signaling pathway. Method: A human colorectal cancer cell line (HT-29) was used for the study. HT-29 cells were treated with 0, 50, 100, 200, and 400 μg/mL of IP6. The MTT colorimetric assay was used to observe the proliferation of HT-29 in vitro, and flow cytometry (FCM) was used to analyze the apoptosis of the HT-29 cells. The relative mRNA expression was determined by real-time PCR, and relative protein levels were analyzed by Western blot analysis. Result: The results of MTT showed that HT-29 cells underwent inhibition of proliferation after exposure to IP6 (100-400 μg/mL) for 12 and 48 h, and this inhibition clearly relied on time and dosage. IP6 induced apoptosis in HT-29 cells in a dose-dependent manner. The mRNA and protein expression of PI3K and Akt decreased in the groups treated with IP6, and IP6 inhibited the phosphorylation of Akt (pAkt), whereas increased the expression of its downstream effector, caspase-9. Conclusion: Our results suggested that by targeting PI3K/Akt pathway, IP6 suppresses cell survival and proliferation, but induces death in HT-29 cells.

Keywords: Inositol hexaphosphate, PI3K/Akt pathway, apoptosis, human colorectal cancer cell, HT-29

Introduction

Colorectal cancer (CRC) is one of the common malignant tumors of the gastrointestinal tract, and its incidence has risen gradually in recent years, which may be due to changes in people's lifestyle and dietary habits [1-3]. In order to prevent the development of colorectal cancer, recent efforts have been made to explore the use of naturally occurring plant extracts as cancer chemopreventive agents.

IP6 is a naturally occurring polyphosphorylated carbohydrate mostly present in high-fiber diets (cereals, legumes, nuts, vegetables and fruits) and mammalian cells [4]. It has been proved that consumption of IP6-rich cereals and legumes is associated with reduction in colon and prostate cancers [5-7]. IP6 is absorbed by cells and metabolized into its lower phosphate forms, which regulate various cellular events [8, 9]. Its hydrolysate, inositol, is also a natural constituent that possesses moderate anticancer activity [10, 12]. IP6 has been shown to have an anticancer effect in several types of cancers, including colorectal cancer [10-18]. However, the molecular mechanisms of its anticancer effects have not been studied in detail.

Previous studies have shown that the mechanism for the anti-proliferative action of IP6 on prostate cancer cells may involve the inhibition of PI3K/Akt activation [19] and a decrease in constitutive NF-κB activity [20]. The up-regulation of caspase-3 and -8 expression and the activation of both caspases may also contribute to the apoptotic cell death of human colorectal cancer cells exposed to IP6 [21].

Caspase-8 and caspase-3 are downstream targets of PI3K/Akt, and the PI3K/Akt pathway plays a critical role in the growth and progression of colorectal cancer [22]. Studies on PI3K inhibition in human cancer cells have shown that PI3K inhibitors, such as LY294002 and wortmannin, have radiosensitizing effects and induce cell apoptosis [23, 24]. These promising studies prompted us to investigate whether the colorectal anticancer efficacy of IP6 is mediated via inhibition of the PI3K/Akt pathway. Further exploration is required in order more precisely to define the mechanism of the anticancer action of IP6 related to this pathway.

The effects of PI3K on tumor growth and progression are mediated by Akt, a downstream effector of PI3K [25]. PI3K induces Akt activation via phosphorylation of key amino acid residues, including Thr-308 [26]. Full activation of Akt also requires phosphorylation of Ser-473, which is regulated by other kinases. PDK-1 over-activation leads to increased Akt phosphorylation (pAkt), and inhibition of this protein kinase by small molecules results in the effective inhibition of cancer cell proliferation [27]. pAkt targets a number of downstream substrates, including caspase-9. Caspase-9 is an important downstream target of Akt. It has been shown that Akt targets caspase-9 at Ser196 and the activation of caspase-9 can lead to the activation of downstream caspases [3, 6, 7] and apoptosis [28-31].

Our study was designed to determine whether IP6 might inhibit the growth of human colorectal carcinoma cells via the PI3K/Akt pathway.

Materials and methods

Cell culture

The human colorectal cancer cell line HT-29 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), was grown in RPMI-1640 supplemented with 10% fetal bovine serum and was incubated in humidified 5% CO_2 at 37°C.

Reagents

Inositol hexaphosphate standards (A0169) were purchased from Muster Biological Science Technology Company (Sichuan, China). Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from LiRui Shanghai biological technology company (cas # 298-93-1, sigma). The Annexin V-FITC/PI Apoptosis Detection Kit was purchased from 7Sea Pharmatech Company Limited (A005-3, Shanghai, China). For Western blot analysis, the following antibodies were used: rabbit monoclonal anti-PI3K (#4257) anti-Akt (#4691) and anti-pAkt (#4060) and anti-caspase-9 (#9506) were purchased from Cell Signaling Technology (USA). Peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG, mouse anti-GAPDH monoclonal antibody and peroxidase-conjugated Affinipure goat anti-mouse IgG were purchased from Zhong Shan Gold Bridge-BIO (Beijing, China). Chemiluminescent HRP substrate (P36599) was purchased from Millipore Corporation (USA). The BCA Protein Assay kit (P0012), SDS-PAGE Sample Loading Buffer (P0015) and Prestained Dual Color Protein Molecular Weight Marker (P0069) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). RT-PCR was run using a two-step RT-PCR Quantiscript RT kit (KR103), and Real Master/SYBR Green (FR202) was purchased from Tiangen Biotech Company (Beijing, China). The primers were designed using Primer5 and Oligo6 software and synthesized in biological engineering company (Shanghai, China).

Cell proliferation assay

The MTT assay was performed in order to investigate the time and dose dependent effect of IP6 on the growth of HT-29 cells. Logarithmic phase cells (1×10^4 cells/well) were seeded in 96-well tissue culture plates in 5% CO_2 at 37°C. After 12 h, the medium was replaced with fresh medium containing 0, 50, 100, 200, or 400 μg/mL of IP6, and the cells were incubated in CO_2 at 37°C for differential time intervals (24 and 48 h). The MTT dye (5 mg/mL) was added to the cells, and the plates were incubated at 37°C for 4 h. The solubilization reagent DMSO (150 μL) was added and absorbance at 490 nm was determined using a microplate reader. A decrease in absorbance was considered a loss of cell viability. Each experiment was repeated three times. The optical density was recorded (OD), and the inhibition rate (IR %) was calculated using the following method: IR (%) = (1-mean OD value in treatment group/mean OD value in control group) ×100.

Apoptosis analysis

Apoptotic cells were measured with the Annexin V-FITC/PI Apoptosis Detection Kit according to

Table 1. RT-PCR primer sequences and product sizes

Gene	Primer sequence	Product size (bp)
β-actin	F 5'-CCTGGCACCCAGCACAAT-3'	144
	R 5'-GGGCCGGACTCGTCATAC-3'	
PI3K	F 5'-GGACCCGATGCGGTTAGA-3'	141
	R 5'-GATGATGGTCGTGGAGGC-3'	
Akt	F 5'-ATGGCACCTTCATTGGCTAC-3'	222
	R 5'-GGGCCGGACTCGTCATAC-3'	
caspase-9	F 5'-GAACTAACAGGCAAGCAGCA-3'	136
	R 5'-CCTCCAGAACCAATGTCCACPC-3'	

Table 2. Effect of IP6 on the growth of HT-29 cells is time- and dose-dependent

Group (μg/mL)	OD value	
	24 h	48 h
0	0.878±0.024	0.930±0.033
50	0.840±0.018	0.881±0.012
100	0.789±0.034*	0.712±0.019a
200	0.662±0.017*,#	0.624±0.016*,#
400	0.555±0.008*,##	0.463±0.029*,##
800	0.540±0.026*,##	0.444±0.020*,##

*P < 0.05 compared to the control group; #P < 0.05 compared to the 100 μg/mL group; ##P < 0.05 compared to the 200 μg/mL group.

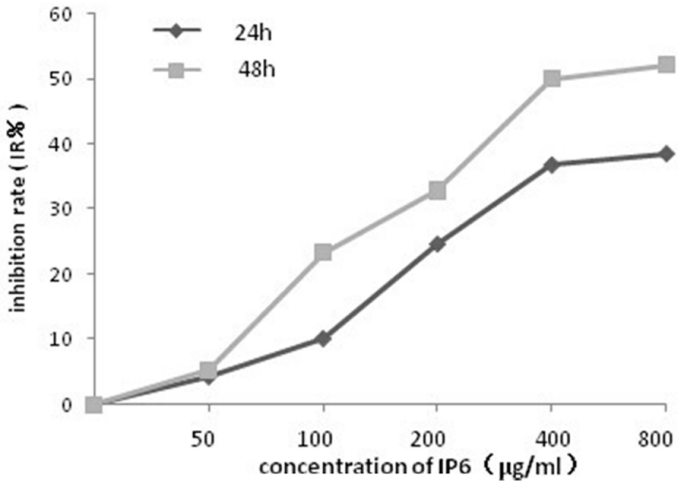

Figure 1. Inhibition of the Cell Growth by IP6 in HT-29 cells. HT-29 cells were treated with IP6 (0, 50, 100, 200, 400 and 800 μg/mL) for 24 and48 h, respectively.

the manufacturer's instructions. Cells were seeded in six-well plates at 2×10^5 cells per well and treated with the different concentration (0, 50, 100, 200, 400 μg/mL) of IP6 for 48 h. The cells were harvested, washed with cold PBS, and resuspended in 400 μL of binding buffer. 5 μL AnnexinV-FITC solution and 10 μL PI were added to these cells, which were incubated for 15 min and 5 min, respectively. Cells were then incubated in the dark for 30 min at room temperature followed by the analysis of the apoptotic cells by flow cytometry.

Western blotting analysis

Cells were cultured in tissue culture flasks overnight and treated with different concentrations (0, 50, 100, 200, 400 μg/mL) of IP6 for 48 h, and total protein was extracted from the cells. The protein concentration was measured using a BCA kit. The cell lysate (30 μg) was separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Equal amounts of total protein extracts (30 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 10% fat-free milk in TBS-T for 2 hours, then incubated with the following primary antibodies at 4°C overnight as follows: anti-PI3K (diluted 1:1000), anti-Akt (diluted 1:1000), anti-phospho-Akt (diluted 1:1000) and GAPDH (diluted 1:2000), followed by incubation with secondary antibody (diluted 1:50000) for 1 h. Protein bands were detected using the Chemiluminescent HRP substrate, and the blot was incubated for 5 min then exposed to a suitable X-ray film for approximately 30 seconds. Bands were identified and quantified using Quantity One (version 4.6.2).

Real-time PCR

Using the Trizol reagent kit, total RNA was extracted from HT-29 cells treated with 0, 50, 100, 200, and 400 μg/mL IP6. The mRNA levels were determined using an eppendorf protein nucleic acid detector. 2 μg of total RNA were reverse transcribed using the Primer Script RT reagent kit followed by reverse transcription with Bio-Rad My Cycler PCR Instruments. The sense and antisense

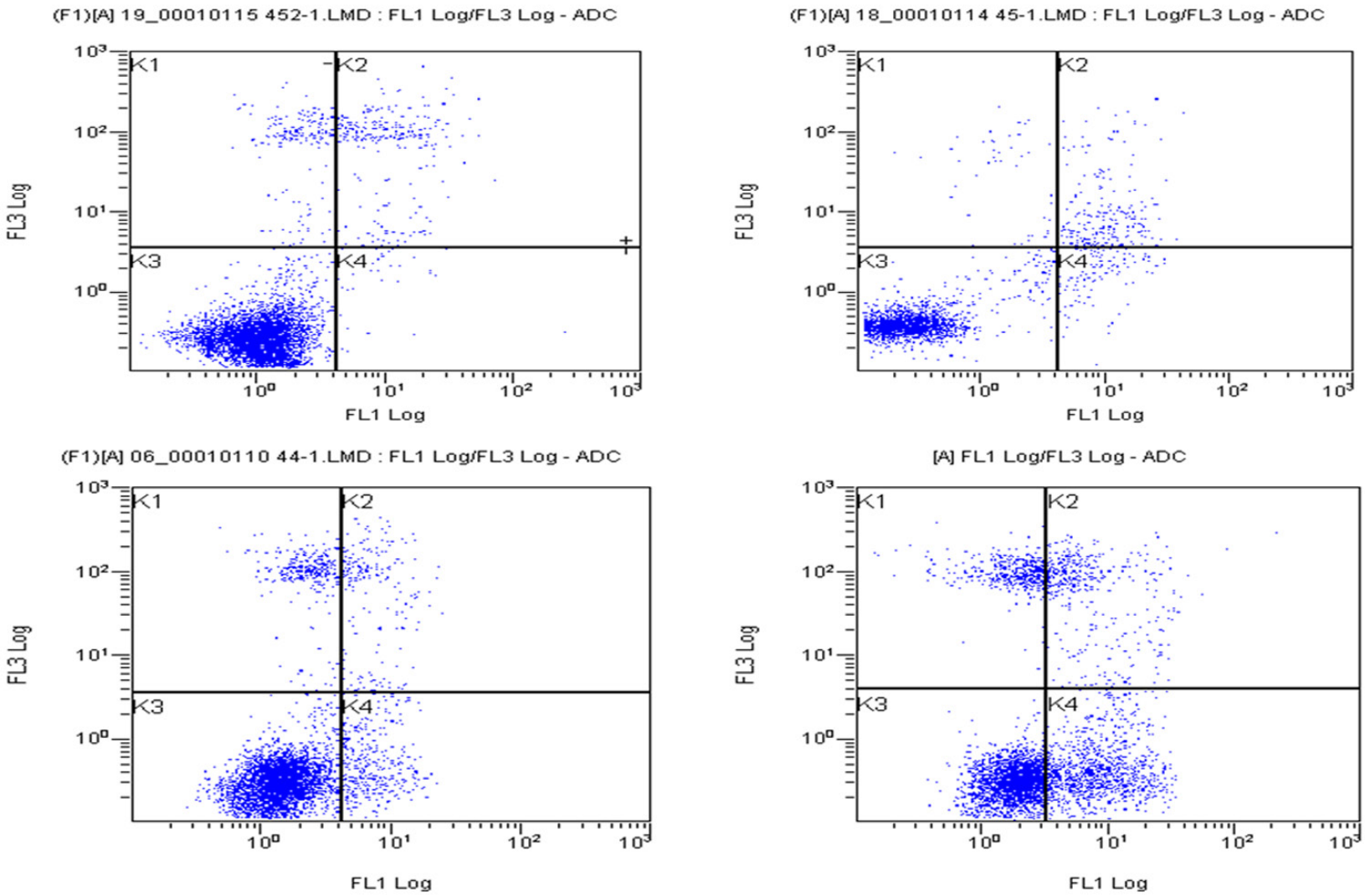

Figure 2. The effect of increasing concentrations of IP6 on cell apoptosis of HT-29 cells in vitro. The HT-29 cells were treated with various concentrations of IP6 (0, 100, 200, and 400 μg/mL) for 48 h. The cells were harvested, stained with PI and analyzed by flow cytometry. The results of apoptosis assessment by the Annexin V/PI single-staining assay. Double negative cells showing the live cell population (lower left), Annexin V positive and PI negative stained cells showing early apoptosis (lower right), Annexin V/PI double stained cells showing late apoptosis (upper right), and PI positive and Annexin V negative stained cells showing apoptotic cells (upper left).

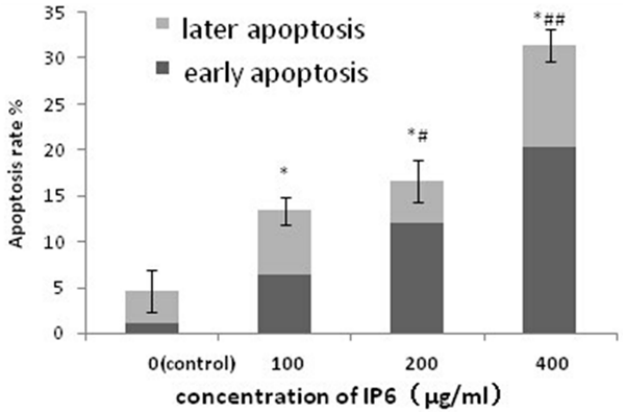

Figure 3. Apoptosis rate of each group after treated with various concentrations of IP6 (0, 100, 200, and 400 μg/mL). IP6 induced a statistically significant increase in the early and late apoptosis in HT-29 cells treated with different concentrations of IP6. The apoptosis cells rate results are expressed as the means ± standard deviation from three independent experiments. *P < 0.05 compared to the control group; #P < 0.05 compared to the 100 ug/mL group; ##P < 0.05 compared to the 200 ug/mL group.

primer sequences and PCR product sizes are shown in **Table 1**. PCR was performed in a 20 μL reaction volume with Eppendorf Mastercycler epgradient according to the manufacturer's instructions. The cycling conditions were as follows: For PI3K:40 cycles of 94°C for 2 min, 94°C for 15 s, and 55°C for 20 s. For Akt: 50 cycles of 94°C for 2 min, 94°C for 15 s, and 58°C for 20 s. For caspase-9: 50 cycles of 94°C for 2 min, 94°C for 15 s, and 57°C for 20 s. The comparative Ct formula $2^{-\Delta\Delta Ct}$ was used to calculate relative gene expression levels.

Statistical analysis

All values are expressed as the mean ± standard deviation. Differences between the groups were assessed with one-way analysis of variance. A value of P < 0.05 was considered statistically significant. All statistical analyses were performed by SPSS software (version 17.0). Each experiment was repeated at least three times.

Results

Cell growth inhibition of IP6 on HT-29 cells

IP6 inhibited cell proliferation in HT-29 cells following treatment with various concentrations of IP6 for 24 h and 48 h, and results of the MTT assay showed that the inhibition rate gradually increased as the concentrations and hours increased. As shown in **Table 2**, the OD values of the higher IP6 treated groups exhibited statistically significant differences compared to the lower IP6 treated group (P < 0.05), but the 50 μg/mL IP6 treated groups exhibited no statistically significant differences (P > 0.05). The inhibition rate of the cells treated with 400 μg/mL and 800 μg/mL IP6 for 48 h was 50.11% and 52.18%, respectively, no statistically significant difference between these groups (as shown in **Figure 1**). It was demonstrated that IP6 is effectively inhibitory within certain concentrations.

Apoptotic cells measured by the Annexin V-FITC/PI method

In **Figure 2**, living cells are grouped in the lower left quadrant, early apoptotic cells are grouped in the lower right quadrant, and late apoptotic cells are grouped in the upper right quadrant. **Figure 3** shows that the concentration of apoptotic cells gradually increased as the concentration of IP6 increased. There were 2.2% apoptotic cells in the control group. In the treatment groups receiving the three higher doses (100, 200, and 400 μg/mL), the proportion of apoptotic cells increased to 11.71%, 20.02.% and 31.43% (including the early apoptosis rate and later apoptosis rate), respectively. The results confirmed the dose-dependent apoptotic effect of IP6 on HT-29 cells.

Effect of IP6 on the expression of PI3K, Akt, pAkt and caspase-9 in HT-29 cells was assessed by western blot analysis

To explore the mechanism of IP6-induced apoptosis of HT-29 cells and to determine whether the PI3K/Akt pathway is involved in this activity, western blot assay was applied. HT-29 cells treated with various concentrations of IP6 (0, 100, 200, 400 μg/mL) for 48 h. The western blot result of the effect of IP6 on PI3K, Akt, pAkt and caspase-9 protein expression were shown in **Figure 4A-D**. The western blot analysis (**Figure 5**) revealed that treatment with IP6 at different concentrations (0, 100, 200, 400 μg/mL) for 48 h significantly reduced the levels of PI3K, Akt and pAkt and increased the levels of caspase-9 in HT-29 cells. Taken together, we can conclude that IP6-induced apoptosis may

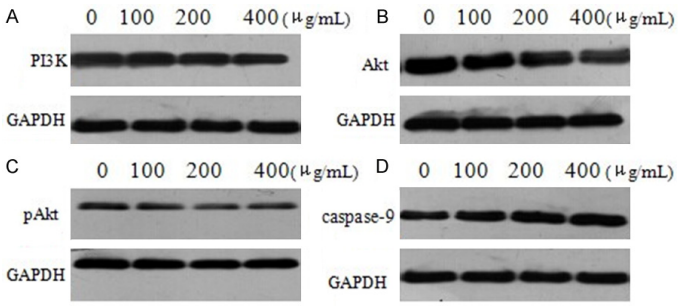

Figure 4. Effect of IP6 on (A) PI3K protein expression, (B) Akt protein expression, (C) pAkt protein expression and (D) caspase-9 protein expression in HT-29 cells treated with various concentrations of IP6 (0, 100, 200, 400 μg/mL) for 48 h. The cells were harvested and analyzed by western blotting. Anti-GAPDH was used as the loading control, bands were identified and quantified using Quantity One.

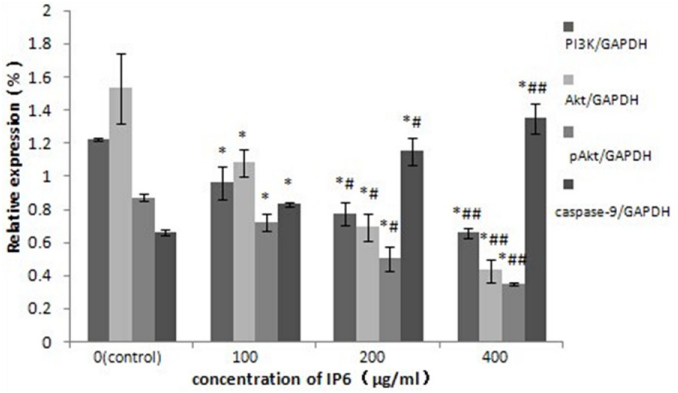

Figure 5. The result of relative expression of related proteins. The results are expressed as the means ± standard deviation from three independent experiments. $^{*}P < 0.05$ compared to the control group; $^{\#}P < 0.05$ compared to the 100 ug/mL group; $^{\#\#}P < 0.05$ compared to the 200 ug/mL group.

works through suppressing the PI3K/Akt pathway.

IP6 decreased the mRNA expression of PI3K and Akt, and it increased the expression of caspase-9 in HT-29 cells

To further verify whether the PI3K/Akt pathway participated in IP6-induced apoptosis, the mRNA expressions of PI3K, Akt and caspase-9 were measured by real time-PCR. The relative gene expression levels were calculated by the comparative Ct formula $2^{-\Delta\Delta Ct}$. The effects of IP6 on the expression levels of target genes and endogenous reference genes Ct values for PI3K, Akt and caspase-9 are shown in **Tables 3-5**. The values are expressed as the means ± standard deviation. The experiments were repeated in triplicate. We found that IP6 inhibits the expression of PI3K and Akt, and compared to the control, their mRNA was gradually inhibited as the dose of IP6 increased. Conversely, caspase-9 mRNA expression increased.

Discussion

Our studies revealed that IP6 has a inhibitory effect on PI3K and its downstream signaling target Akt, whereas it promotes the express of caspase-9. Furthermore, IP6 significantly reduces the expression of pAkt, which might account for the greater biological effect. This means that IP6 has an inhibitory effect on the PI3K/Akt signaling pathway, which plays a key role in the occurrence of cell apoptosis.

IP6 is one of the prime and potential dietary phytochemicals exhibiting broad-spectrum anticancer activities, and its use has been widely described in different tumor types, including breast, pancreas, prostate, lung, liver and colon [8-16]. Several possible mechanisms of its anticancer effects have been studied in many experiments; some of these studies have shown that IP6 inhibits cell proliferation, cell-cycle progression, metastasis, invasion and angiogenesis [32-35]. Further studies of prostate carcinoma have shown that IP6 suppresses cell survival, proliferation and angiogenesis but induces death in PCa cells by targeting the PI3K/Akt pathway [36]. Its apoptotic effect has also been demonstrated in many previous studies, mostly via the regulation of related factors, such as factors in the caspase and Bcl-2 families, like, Bax and Bcl-2 [37]. The expression of these factors might be regulated by Akt, a key factor of the PI3K/Akt pathway [38, 39]. IP6 has been shown to inhibit PI3K activity in JB6 cells as a mechanism of antitumor promotion [40]. Taking this into consideration, together with the evidence that the PI3K/Akt pathway plays a critical role in the growth and progression of colorectal cancer [22], we considered it logical to further investigate the changes of the PI3K/Akt pathway in HT-29 cells after treatment with IP6.

Table 3. IP6 affects the PI3K mRNA expressions of HT-29 cells ($\bar{x}$±S, n=3)

Dose (μg/mL)	β-actin Ct	PI3K Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
0	17.71±0.05	28.24±0.18	10.52±0.13	_	_
100	16.31±0.07	27.48±0.09	11.17±0.01	0.64±0.02	0.64±0.01*
200	17.55±0.07	29.33±0.16	11.78±0.09	1.25±0.09	0.42±0.03*,#
400	15.68±0.23	28.55±0.19	12.87±0.19	2.34±0.19	0.20±0.02*,##

*P < 0.05 compared to the control group; #P < 0.05 compared to the 100 ug/mL group; ##P < 0.05 compared to the 200 ug/mL group.

Table 4. IP6 affects the Akt mRNA expressions of HT-29 cells ($\bar{x}$±S, n=3)

dose (μg/mL)	β-actin Ct	Akt Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
0	17.68±0.06	27.66±0.04	9.98±0.10	0.00±0.10	1.00±0.06
100	16.51±0.08	27.33±0.04	10.82±0.05	0.84±0.05	0.55±0.02*
200	17.49±0.07	28.86±0.12	11.43±0.06	1.45±0.06	0.36±0.01*,#
400	17.60±0.01	29.80±0.14	12.20±0.13	2.22±0.13	0.21±0.02*,##

*P < 0.05 compared to the control group; #P < 0.05 compared to the 100 ug/mL group; ##P < 0.05 compared to the 200 ug/mL group.

Table 5. IP6 affects the caspase-9 mRNA expressions of HT-29 cells ($\bar{x}$±S, n=3)

Dose (μg/mL)	β-actin Ct	caspase-9 Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
0	15.03±0.04	30.92±0.15	15.89±0.15	0.00±0.15	1.00±0.10
100	15.14±0.08	30.49±0.05	15.34±0.11	-0.54±0.11	1.46±0.12*
200	15.36±0.03	29.91±0.02	14.55±0.06	-1.33±0.06	2.52±0.11*,#
400	16.23±0.11	29.65±0.03	13.42±0.13	-2.46±0.13	5.54±0.50*,##

*P < 0.05 compared to the control group; #P < 0.05 compared to the 100 ug/mL group; ##P < 0.05 compared to the 200 ug/mL group.

PI3K/Akt signaling is deregulated through numerous mutations and epigenetic changes, including mutation/amplification of the PI3K and Akt genes [18]. Akt is a downstream target of PI3K, and it is triggered by the lipid second messenger phosphatidylinositol-3, 4, 5-trisphosphate, which is generated by PI3K. Our results show that IP6 reduces the expression of PI3K and that this inhibition was dose-dependent. This inhibitory function in HT-29 cells may be related to the structure of IP6, which is similar to that of D-3-deoxy-3-fluoro-PtdIns, an inhibitor of PI3K [42]. PI3K inhibitors (wortmannin or LY294002) induce cancer cells apoptosis and block cell transformation [23, 24]. In addition, our study showed that IP6 has an inhibitory effect on Akt; this finding is consistent with numerous studies showing the anticancer effect of Akt mRNA expression in different tumor types [43, 44]. Through pyruvate dehydrogenase kinase-1, Akt is phosphorylated to p-Akt. p-Akt in turn inactivates the effector molecules of apoptosis through several mechanisms, promoting the proliferation and metastasis of tumor cells. This is consistent with our result showing a reduction in the expression of pAkt and an increase in the expression of caspase-9. Caspase-9 is an Akt substrate that belongs to the caspase family of proteases, which are generally considered to be initiators of chemotherapy-induced mitochondrial apoptosis [45, 46]. Furthermore, IP6 can be metabolized into its lower phosphate forms (IP1-5); these low-phosphorylated derivatives regulate a variety of cellular events and interfere with the expression of genes [47, 48], that modulate cell signal transduction and the activation of apoptosis.

By investigating the correlation between the PI3K/Akt signaling pathway and the effect of IP6 on HT-29 cells, and by studying the underlying mechanisms of this effect, we concluded that IP6 has an inhibitory effect on HT-29 cells through modulation of the PI3K/Akt pathway; in particular, we found that IP6 has a inhibitory

effect on the express of PI3K, Akt and pAkt, whereas IP6 promotes its downstream signaling target, caspase-9. Above all, the anticancer effect of IP6 was further confirmed in this in vitro study of colorectal cancer. However, further studies are required to determine the downstream signaling that is involved in the inhibition of this signaling pathway by IP6 in HT-29 cells. Furthermore, to delineate the potential of this agent in colorectal cancer treatment, we intend to examine the antitumor effects of IP6 in an animal model.

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Disclosure of conflict of interest

None.

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