Submitted: 15 March, 2024 Accepted: 25 April, 2024 Published: 30 May, 2024 DOI:10.22514/jomh.2024.078

ORIGINAL RESEARCH

JOMH Journal of Men's Health

# Jolkinolide B induces reactive oxygen species accumulation and endoplasmic reticulum stress and inhibits MAPK and AKT signaling activation in renal cancer cells

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## Abstract

To investigate the impact of Jolkinolide B (JB) on renal cell carcinoma (RCC), we treated RCC cell lines with various concentrations of JB (0, 5, 25, 50 and 100 $\mu$M) for 24 hours and assessed cell viability using the cell counting kit-8 (CCK-8) analysis. Then, we examined JB's effects on proliferation, migration, apoptosis, reactive oxygen species (ROS) accumulation, and endoplasmic reticulum (ER) stress through 5-ethynyl-2′-deoxyuridine (EdU) incorporation, transwell migration assays, flow cytometry, ROS level determination, and western blot assays. Furthermore, we investigated the potential mechanism using western blot. Our results showed that JB dose-dependently reduced cell viability in both 786-O and Caki1 cells. Additionally, JB at concentrations of 5, 25 and 50 $\mu$M decreased the number of EdU-positive and migrating cells in both cell lines. Additionally, these concentrations of JB increased apoptosis rates, relative protein expressions of cleaved caspase-3 and cleaved caspase-9, ROS levels, and relative protein expressions of C/EBP-homologous protein (CHOP) and activated transcription factor 4 (ATF4) in both 786-O and Caki1 cells. Mechanistically, treatment with all three concentrations of JB significantly downregulated phosphorylated p38 (p-p38)/p38, phosphorylated protein kinase B (p-AKT)/AKT and phosphorylated phosphatidylinositol-3-kinase (p-PI3K)/PI3K in a dose-dependent manner. In summary, JB inhibited proliferation and migration while promoting apoptosis, ROS accumulation, and ER stress in RCC cells, potentially through the inactivation of mitogen-activated protein kinase (MAPK) and AKT signaling pathways.

## Keywords

Renal cancer; Jolkinolide B; ROS; Endoplasmic reticulum stress; MAPK; AKT

## 1. Introduction

Renal cell carcinoma (RCC), originating from the epithelial cells of renal tubules, is a prevalent type of tumor globally. According to 2020 GLOBOCAN statistics, RCC accounted for 2.2% of new cancer cases and approximately 1.8% of cancer-related deaths, ranking it as the 14th most common cancer worldwide [1]. Notably, the incidence of RCC has not only increased over the past decade but is also projected to continue increasing in the future, with an estimated 666,000 new cases and around 301,000 deaths anticipated globally by 2040 [1]. Given the lack of distinct clinical symptoms and effective diagnostic methods, most patients present with distant metastases at diagnosis. Therefore, strategies such as surgical resection, targeted therapy, and immunotherapy have been integrated into RCC treatment protocols to impede disease progression. However, the survival outcomes for RCC patients remain suboptimal, particularly for those with metastatic disease [2]. Thus, there exists an urgent imperative to explore potential pharmaceutical interventions for RCC treatment.

Natural products are increasingly recognized as valuable resources for cancer treatment. Jolkinolide B (JB), a diterpenoid compound ($C_{20}H_{26}O_4$) weighing 330.42 Da, can be retrieved from the root of Euphorbia [3] and has demonstrated a range of pharmacological properties, notably significant inhibitory effects on various solid cancers [4–8]. However, its impact on RCC remains unexplored.

This study investigates the impact of JB on RCC, including its effects on proliferation, migration, and apoptosis in RCC cells. In addition, its influence on reactive oxygen species (ROS) accumulation and endoplasmic reticulum (ER) stress was assessed *in vitro*. Additionally, the potential molecular mechanism underlying JB's actions was explored by examining the expression of proteins involved in the MAPK and AKT pathway.


  

## 2. Materials and methods

### 2.1 Cell treatment

Human renal cortex proximal convoluted tubule epithelial cells human kidney-2 (HK-2, CL-0109) and human RCC cell lines, including 786-O (CL-0010) and Caki1 (CL-0052), were obtained from Procell (Wuhan, China). HK-2 cells were cultured in minimum essential medium (MEM, supplemented with non-essential amino acids (NEAA), PM150410, Procell), 786-O cells in Roswell Park Memorial Institute-1640 (RPMI-1640, PM150110, Procell), and Caki1 cells in McCoy's 5A (PM150710, Procell), all supplemented with 10% fetal bovine serum (FBS, 164210-50, Procell) and 1% penicillin-streptomycin solution (PB180120, Procell), at 37 ℃ with 5% carbon dioxide ($CO_2$). The cells were then treated with various concentrations of JB (0, 5, 25, 50 and 100 $\mu$M) (catalog number: HY-N0732, CAS No.: 37905-08-1, purity: $\geq$99.71%, MedChemExpress, Monmouth Junction, NJ, USA) for 24 hours [7] before being harvested for subsequent assays. Treatment with 0 $\mu$M JB served as the control, with cells treated with phosphate-buffered saline (PBS, P1020, Solarbio, Beijing, China).

### 2.2 Cell viability examination

All three cell lines (HK-2, 786-O and Caki1 cells) were seeded into 96-well plates at a density of $4 \times 10^4$ cells per well and incubated at 37 °C with 5% $CO_2$. The cells were then treated with varying concentrations of JB (0, 5, 25, 50 and 100 $\mu$M) for 24 hours and then incubated with cell counting kit-8 (CCK-8) reagents (CA1210, Solarbio, Beijing, China) at 37 °C for 2 hours. The optical density (OD) was measured at 450 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA), and cell viability was determined accordingly.

### 2.3 The 5-ethynyl-2′-deoxyuridine (EdU) incorporation analysis

786-O and Caki1 cells (density: $6 \times 10^5$ cells per well) were seeded into 6-well plates and incubated at 37 °C with 5% $CO_2$. After treatment with JB for 24 hours, cell proliferation was assessed using the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 647 (C0081L, Beyotime, Shanghai, China), following the manufacturer's instructions. Briefly, 1 mL of EdU working solution (20 $\mu$M) was added to each well and incubated at 37 °C for 2 hours. Subsequently, the cells were fixed with immunostaining fix solution (P0098, Beyotime, Shanghai, China), permeabilized with 0.3% Triton X-100 (P0097, Beyotime, Shanghai, China), and subjected to anti-EdU Click reaction solution treatment for 30 minutes in the absence of light. The cells were then counterstained with Hoechst 33342 (5 $\mu$g/mL, C1025, Beyotime, Shanghai, China). Fluorescence images of the stained cells were captured using a fluorescence microscope (IX71, Olympus, Tokyo, Japan), and the percentage of EdU-positive cells was analyzed based on five randomly selected fields.

### 2.4 Transwell assay

The migration ability of 786-O and Caki1 cells was evaluated using the transwell assay. After treatment with JB for 24 hours, 200 $\mu$L of cell suspension was added to the upper chamber of 24-well transwell plates with an 8-$\mu$m pore size (3422, Corning Company, New York, NY, USA), while 600 $\mu$L of media (RPMI-1640 for 786-O cells and McCoy's 5A for Caki1 cells) containing 10% FBS was added to the lower chamber. After incubation at 37 °C for 24 hours, the cells were fixed with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) and stained with 0.1% crystal violet (G1063, Solarbio, Beijing, China) for 30 minutes. Subsequently, images were taken using an inverted microscope (CKX53, Olympus, Tokyo, Japan), and the number of migrated cells was quantified based on five randomly selected fields.

### 2.5 Flow cytometry

Apoptosis in 786-O and Caki1 cells was assessed using the flow cytometry assay with the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (CA1020, Solarbio, Beijing, China). Following treatment with JB for 24 hours, the cells were washed with pre-cooled PBS and resuspended in 1 mL of binding buffer. Subsequently, the cells were stained with propidium iodide (PI) and FITC-Annexin V for 5 minutes in the dark. Apoptotic cells were then analyzed using a flow cytometer (FACScan, BD Biosciences, Franklin Lake, NJ, USA) and the data were analyzed using BD CellQuest Pro software (version 5.1, BD Biosciences, Franklin Lake, NJ, USA).

### 2.6 Detection of ROS level

After different treatments, the 786-O and Caki1 cells were incubated with 5-(and-6)-chloromethyl-2',7'-dichlorofluorescin diacetate (DCFH-DA) (D6470, Solarbio, Beijing, China) at 37 °C for 30 minutes in the dark. Then, fluorescence images were captured using a fluorescence microscope, and the relative fluorescence levels were quantitatively analyzed.

### 2.7 Western blot

The 786-O and Caki1 cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (R0010, Solarbio, Beijing, China) to extract total proteins, followed by quantification of protein concentrations using the Bicinchoninic Acid Assay (BCA) Protein Assay Kit (PC0020, Solarbio, Beijing, China). Protein samples (20 $\mu$g) were mixed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and separated by SDS-PAGE, then transferred onto a polyvinylidene difluoride (PVDF) membrane (IPVH00010, EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% blocking buffer (SW3015, Solarbio, Beijing, China) for 1 hour at room temperature, followed by overnight incubation at 4 °C with primary antibodies, including anti-cleaved caspase-3 (1:500, ab2302, Abcam, Cambridge, UK), anti-cleaved caspase-9 (1:1000, 9505, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-CHOP (1:1000, ab11419, Abcam), anti-activating transcription factor 4 (ATF4) (1:1000, ab216839, Abcam),

anti-p38 (1:5000, ab170099, Abcam), anti-phosphorylated p38 (p-p38) (1:1000, ab178867, Abcam), anti-AKT (1:500, ab8805, Abcam), anti-p-AKT (1:1000, ab8933, Abcam), anti-PI3K (1:1000, ab191606, Abcam), anti-p-PI3K (1:1000, ab182651, Abcam), and anti-GAPDH (1:2500, ab9485, Abcam). Then, the membranes were washed and incubated with goat anti-rabbit IgG H&L (HRP) (ab6721, 1:10000, Abcam) for 2 hours at 37 °C. Lastly, protein bands were visualized using the enhanced chemiluminescence (ECL) Western Blotting Detection Kit (Goat IgG) (SW2030, Solarbio, Beijing, China).

### 2.8 Statistical analysis

The data are shown as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the *Post hoc* Bonferroni test to compare multiple groups using SPSS 26.0 software (IBM, Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1 JB inhibited the proliferation and migration of RCC cells

To investigate the effects of JB on RCC (Fig. 1A), two RCC cell lines were treated with JB at concentrations of 0, 5, 25, 50 and 100 $\mu$M for 24 hours. As shown in Fig. 1B, the viability of HK-2 cells significantly decreased only upon treatment with 100 $\mu$M JB, while no statistically significant impact on HK-2 cell viability was observed with JB concentrations of 5, 25 and 50 $\mu$M. Conversely, JB dose-dependently reduced the viability of both 786-O and Caki1 cells, with notable decreases observed at concentrations of 25, 50 and 100 $\mu$M. Therefore, JB concentrations of 5, 25 and 50 $\mu$M were selected for subsequent assays. Furthermore, all three concentrations of JB (5, 25 and 50 $\mu$M) significantly reduced the number of EdU-positive cells in both 786-O and Caki1 cell lines (Fig. 1C). Additionally, JB at concentrations of 5, 25 and 50 $\mu$M consistently decreased the migration of both 786-O and Caki1 cells (Fig. 1D). Collectively, these findings indicate that JB suppresses the proliferation and migration of RCC cells.

### 3.2 JB induced apoptosis in RCC cells

To investigate the effect of JB on the apoptosis of RCC, 786-O and Caki1 cells were treated with JB at concentrations of 5, 25 and 50 $\mu$M for 24 hours, and apoptosis rates were measured *via* flow cytometry. As shown in Fig. 2A, all three concentrations of JB prominently increased the apoptosis rate of both 786-O and Caki1 cells. In addition, the relative protein expressions of cleaved caspase-3 and cleaved caspase-9 were significantly increased in both cell lines after treatment with all three concentrations of JB (Fig. 2B), suggesting that JB enhances apoptosis in RCC cells.

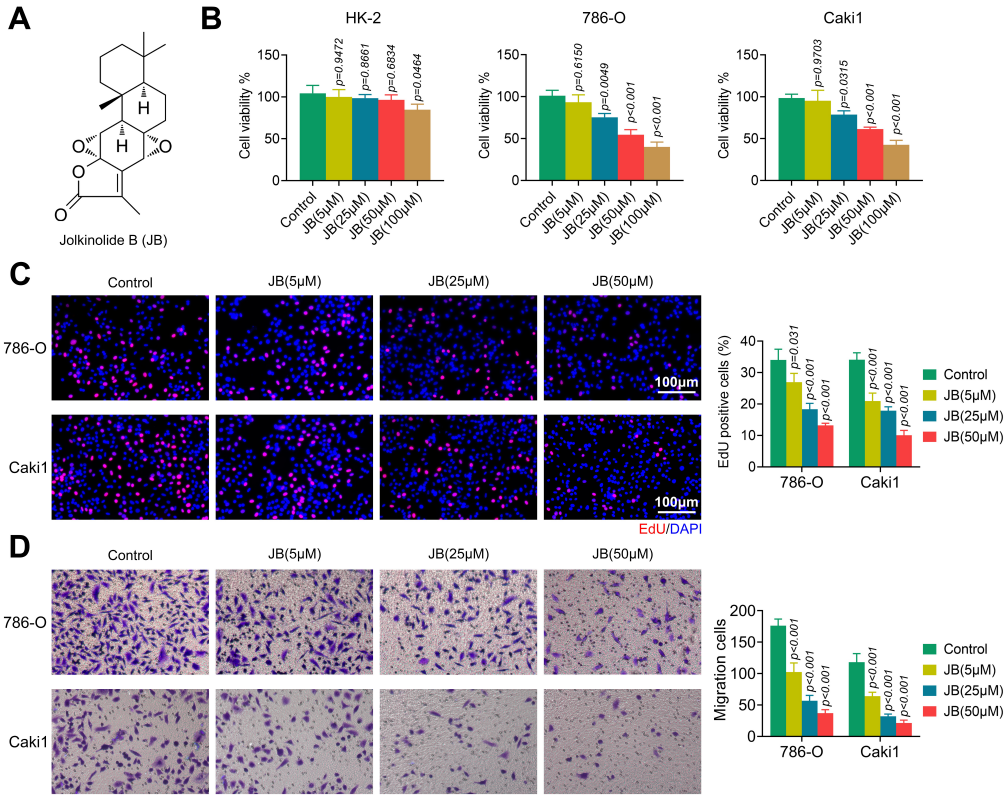


**FIGURE 1. JB suppresses the proliferation and migration of RCC cells**. HK-2 cells and two RCC cell lines were treated with JB (0, 5, 25, 50 and 100 $\mu$M) for 24 h. (A) Chemical structure of JB; (B) Cell viability assessed by CCK-8 assay; (C) Cell proliferation determined by EdU incorporation assays; (D) Migration analyzed by transwell assay. EdU: 5-ethynyl-2′-deoxyuridine; DAPI: 4′,6-diamidino-2-phenylindole; HK-2: human kindey-2.

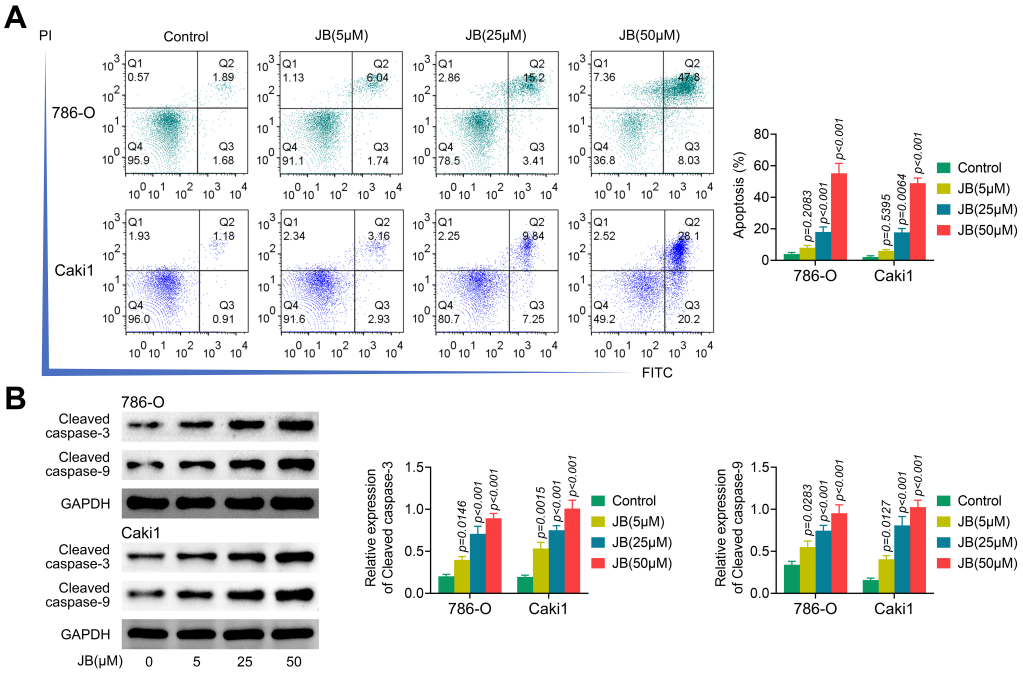


**FIGURE 2. JB induces apoptosis in RCC cells**. 786-O and Caki1 cells were treated with JB (5, 25 and 50 $\mu$M) for 24 h. (A) Apoptosis rate measured by flow cytometry; (B) Relative protein expressions of cleaved caspase-3 and cleaved caspase-9 detected by western blot. The results are normalized to GAPDH. JB: Jolkinolide B; PI: propidium iodide; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

### 3.3 JB elicited ROS accumulation and ER stress in RCC cells

The effect of JB on ROS accumulation and ER stress was investigated after treating 786-O and Caki1 cells with JB at concentrations of 5, 25 and 50 $\mu$M for 24 hours. As illustrated in Fig. 3A, all three concentrations of JB led to a significant increase in relative fluorescence levels when the cells were exposed to DCFH-DA, indicating that JB facilitated ROS accumulation in both 786-O and Caki1 cells. Furthermore, the relative protein expressions of CHOP and ATF4 were significantly enhanced with all three concentrations of JB (Fig. 3B). These findings suggest that JB promotes ROS accumulation and ER stress in RCC cells.

### 3.4 JB suppressed MAPK and AKT signaling in RCC cells

To elucidate the underlying molecular mechanism of JB on RCC, the relative expressions of proteins involved in MAPK and AKT signaling were assessed after treating 786-O and Caki1 cells with JB at concentrations of 5, 25 and 50 $\mu$M for 24 hours. As shown in Fig. 4, the relative levels of p-38/p38, p-AKT/AKT and p-PI3K/PI3K were significantly downregulated with the treatment of all three concentrations of JB in a dose-dependent manner. These results indicate that JB attenuates MAPK and PI3K/AKT signaling in RCC cells.

## 4. Discussion

Renal cell carcinoma (RCC) is a prevalent malignancy often characterized by distant metastases. Despite advancements in its treatment, patients' survival rates remain unsatisfactory [2]. Therefore, research for effective agents against RCC remains crucial. In this regard, JB has demonstrated inhibitory effects on various tumors. Herein, we observed that three concentrations of JB (5, 25 and 50 $\mu$M) reduced cell viability, EdU-positive cells and migration in both 786-O and Caki1 cells. Additionally, JB increased apoptosis rates, as well as the relative protein expressions of cleaved caspase-3 and cleaved caspase-9, ROS levels, and protein expressions of CHOP and ATF4 in both cell lines. Mechanistically, JB downregulated the relative levels of p-38/p38, p-AKT/AKT and p-PI3K/PI3K in a dose-dependent manner. Overall, JB inhibited proliferation and migration while enhancing apoptosis, ROS accumulation, and ER stress in RCC cells, likely through attenuation of MAPK and AKT signaling.

Natural products from various plants are essential candidates in RCC treatment and are known for their suppressive effects on RCC development and progression [9]. In particular, JB has demonstrated efficacy in restraining gastric cancer growth both *in vitro* and *in vivo*, as well as inducing apoptosis and S cell cycle arrest in gastric cancer cells [4]. Similarly, JB has exhibited inhibitory effects on growth and migration while enhancing apoptosis in breast cancer [6]. In this study, JB was found to reduce cell viability, EdU-positive cells and migration while increasing apoptosis rates and the relative protein expressions of cleaved caspase-3 and cleaved caspase-9 in both 786-O and Caki1 cells. Caspase-3, a cysteine-aspartate protease, plays an essential role in apoptosis initiation and is considered a biomarker for apoptosis. Its cleavage initiates DNA fragmentation and activates caspase-6, -7, and -9, leading to an apoptosis cascade. Overall, our findings indicate that JB could inhibit proliferation and migration while promoting apoptosis in RCC cells.

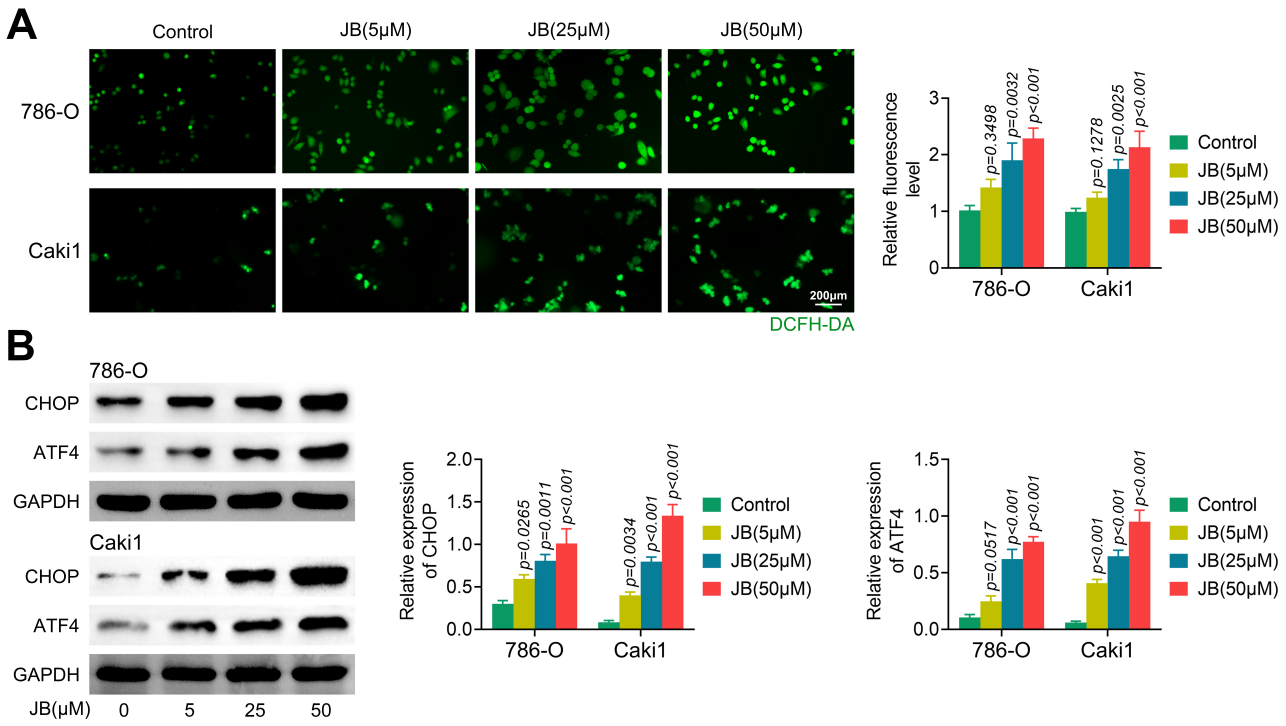


**FIGURE 3. JB promotes ROS accumulation and ER stress in RCC cells.** 786-O and Caki1 cells were treated with JB (5, 25 and 50 $\mu$M) for 24 h. (A) ROS level determined after treatment with DCFH-DA; (B) Relative protein expressions of CHOP and ATF4 examined by western blot. The results are normalized to GAPDH. JB: Jolkinolide B; CHOP: C/EBP-homologous protein; ATF4: activated transcription factor 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

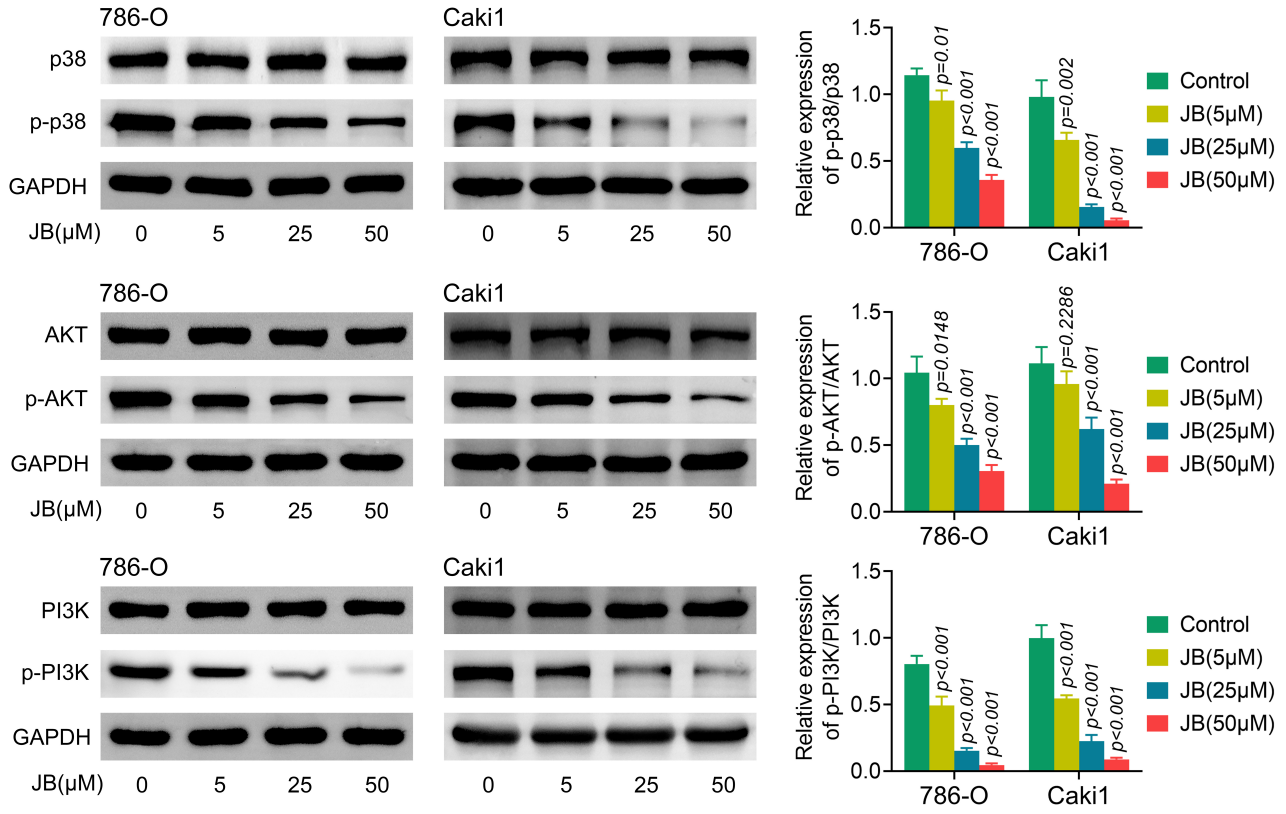


**FIGURE 4. JB inhibits MAPK and PI3K/AKT signaling in RCC cells**. Relative protein expressions of p38, p-38, Akt, p-Akt, PI3K and p-PI3K by western blot. The results are normalized to GAPDH. JB: Jolkinolide B; AKT: protein kinase B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PI3K: phosphatidylinositol-3-kinase.

ROS plays an important role in various normal biochemical functions and pathological processes. In cancer, elevated ROS levels are attributed to dysregulated mitochondria, altered metabolism, oncogenic signaling, and hypoxia, potentially accelerating cancer initiation and development by enhancing genome instability, DNA damage, and pro-tumorigenic signaling [10]. Conversely, increased ROS levels can induce extensive oxidative DNA damage, triggering a DNA damage response (DDR) that impairs cellular repair mechanisms, leading to cell cycle arrest and programmed cell death, thereby impeding cancer progression [11]. Additionally, ROS can directly induce oxidative stress, triggering programmed cell death. However, clinical trials have shown that antioxidants aimed at reducing ROS levels not only fail to prevent cancer progression but also increase cancer risks and promote cancer development [12]. Therefore, elevating ROS levels may serve as an alternative anticancer strategy. In our study, JB increased the relative ROS levels in both 786-O and Caki1 cells, suggesting that JB may promote ROS accumulation in RCC cells, contributing to apoptotic cascade activation. JB has been shown to induce ROS-mediated apoptosis in bladder cancer cells [5], colorectal carcinoma [7] and melanoma [8]. In addition, ROS accumulation induces mitochondrial membrane potential (MMP) depolarization, initiating apoptosis through the classical intrinsic apoptotic pathway [13]. Taken together, our findings indicate that JB promotes ROS accumulation in RCC cells and the impact of JB on mitochondrial function warrants further investigations.

Upon ROS accumulation, oxidative stress increases the levels of misfolded or unfolded proteins, triggering a cellular adaptive program known as ER stress. Thus, targeting ER stress signaling represents a potential approach for cancer treatment [14]. In our study, the relative protein expressions of CHOP and ATF4 were enhanced with all three concentrations of JB. In the ER stress pathway, phosphorylated eukaryotic translation initiator factor (eIF2$\alpha$) activates the transcription of ATF4 and CHOP. Moreover, CHOP serves as a critical marker of ER stress-induced apoptosis and can enhance the ratio of Bax/B-cell lymphoma-2 (BCL-2) and the cleavage of caspase-3. Additionally, ROS accumulation also promotes ER stress in RCC. Therefore, JB may facilitate ER stress to induce apoptosis or trigger apoptosis in a ROS-mediated ER stress manner. JB has been shown to activate ER stress in bladder cancer, leading to apoptosis [15]. Furthermore, JB activated ROS-mediated ER stress, inducing paraptosis in bladder cancer [5]. Overall, JB promotes ER stress in RCC cells.

Increasing evidence are indicating the significant role of the tumor microenvironment in RCC, characterized by notable infiltration of immune cells. This immune-rich environment is intricately connected to metabolic reprogramming within RCC cells, fostering an immunosuppressive environment conducive to tumor progression. Metabolic alterations in RCC can generate immunosuppressive factors, diminish antigen presentation, and modulate immune cell function, ultimately facilitating tumor evasion from immune surveillance [14].

JB's potential to modulate immune cell infiltration and regulate inflammation, known as "immunoflogosis", offers a novel therapeutic avenue. This immunomodulatory effect may occur due to JB's ability to alter the metabolic state of both tumor and immune cells, thus impacting their function within the tumor microenvironment. By influencing key metabolic pathways crucial for immune cell activation and function, JB could potentially enhance the immunogenicity of RCC. Consequently, this could lead to increased immune cell infiltration and decreased levels of immunosuppressive factors, thereby creating a more conducive environment for immune-based therapies [15].

Recent research has shed light on JB's substantial regulatory influence on cellular metabolism, indicating its potential to counteract the metabolic changes observed in RCC. For instance, JB has been demonstrated to affect glycolytic pathways, impacting the energy supply essential for cancer cell proliferation and survival [15]. Through its modulation of glycolysis, JB could decrease the availability of essential metabolic intermediates necessary for the biosynthesis of nucleotides, amino acids, and lipids, ultimately hindering cancer cell growth.

Furthermore, impairments in mitochondrial bioenergetics and OxPhos in RCC contribute to a metabolic environment conducive to cancer progression. JB's reported effects on mitochondrial function, such as its potential to restore mitochondrial bioenergetics and improve OxPhos efficiency, may counteract the metabolic advantages exploited by cancer cells.

MAPK and PI3K/AKT signaling play significant roles in both pathological and physiological processes [16]. Suppression of these pathways to induce apoptosis in RCC has been demonstrated in various plant extracts, such as $\beta$-Elemene [17], eupatilin [18] and gypenosides [19]. Similarly, JB has been shown to inhibit MAPK and AKT signaling, thereby regulating the progression of bladder cancer [15]. Consistent with these findings, the relative levels of p-38/p38, p-AKT/AKT and p-PI3K/PI3K were downregulated with the treatment of all three concentrations of JB in a dose-dependent manner. Thus, JB suppresses MAPK and AKT signaling in RCC cells, suggesting that JB's regulatory effect on RCC development is closely associated with these signaling pathways. However, further studies are required to elucidate their direct role, which should be addressed in subsequent research. Identifying and addressing specific knowledge gaps in our study, as well as those remaining open for future research, could involve a comprehensive evaluation of the current state of RCC research, which could highlight areas where additional studies and innovative approaches are needed to advance the field further.

In future studies, it would be beneficial to explicitly detail any methodological constraints encountered, including but not limited to, the utilization of specific RCC cell lines, experimental conditions employed, and any assumptions made during the analysis. Such clarification would assist readers in understanding the scope of where the findings can be applied. Additionally, future research should aim to discuss the extent to which the results can be generalized to the broader RCC patient population, such as acknowledging the diversity of RCC subtypes and the variability in patient responses to treatment, which may not be fully captured in our study design.

## 5. Conclusions

In summary, JB limited proliferation and migration while promoting apoptosis, ROS accumulation and ER stress in RCC cells, all of which were associated with the attenuation of MAPK and AKT signaling. These findings, combined with preclinical trials, suggest that JB could serve as an alternative therapeutic agent for the treatment of RCC.

### AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

### AUTHOR CONTRIBUTIONS

XW—designed the research study, performed the research, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. SJP—designed the research study, performed the research, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. XJL—designed the research study, performed the research, supervised the data collection, and analyzed and interpreted the data. JL—designed the research study, performed the research, supervised the data collection, and analyzed and interpreted the data. ZGW—designed the research study, performed the research, and supervised the data collection. YHL—designed the research study, and performed the research. QHM—designed the research study, performed the research, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

### ACKNOWLEDGMENT

Not applicable.

### FUNDING

This research received no external funding.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**How to cite this article:** Xuan Wu, Sijing Pan, Xiujuan Li, Jing Liu, Zhigang Wang, Yinghong Lei, *et al*. Jolkinolide B induces reactive oxygen species accumulation and endoplasmic reticulum stress and inhibits MAPK and AKT signaling activation in renal cancer cells. Journal of Men's Health. 2024; 20(5): 119-125. doi: 10.22514/jomh.2024.078.