Brusatol inhibits the growth of prostate cancer cells and reduces HIF-1α/VEGF expression and glycolysis under hypoxia

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ORIGINAL ARTICLE

Abstract

Prostate cancer (PCa) has a high rate of morbidity and mortality, which urges us to find a unique and effective drug for treatment. Brusatol, a triterpenoid-degraded derivative, possesses antitumor activities. However, the significance of Brusatol in prostate cancer has not yet been completely elucidated. Therefore, this study aimed to explore how Brusatol affected prostate cancer cells. DU145 and PC-3 cell lines were chosen as experimental models. After Brusatol was added to relevant cells in culture, CCK-8 and colony formation experiments were used to assess cell viability; apoptosis rates were calculated using flow cytometry; and transwell assays were utilized to assess cell migration and invasion ability. Vimentin, N-cadherin, E-cadherin, zonula occludens-1 (ZO-1), hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT1), hexokinase 2 (HK2), and lactate dehydrogenase (LDHA) protein expression were evaluated by western blotting, and glucose consumption in cells was assessed using related equipment. In DU145 and PC-3 cells, Brusatol drastically reduced cell proliferation, promoted apoptosis, hindered migration and invasion. Considerably decreased HIF-1α and VEGF protein levels under hypoxia were detected. Furthermore, the expression of GLUT1, HK2, and LDHA was diminished, resulting in decreased glucose consumption in a Brusatol concentration-dependent manner. These findings demonstrate that Brusatol serves as a potent antitumor drug that suppresses DU145 and PC-3 cancer cell growth, metastasis, and glycolysis. This discovery could provide a possible clinical treatment strategy for prostate cancer.

Keywords: Brusatol; hypoxia; HIF-1α; prostate cancer; VEGF; glycolysis

Introduction

Prostate cancer (PCa) is a prevalent malignancy in urinary system disease and it is the primary cause of cancer-related death among males (Bray *et al*., 2018; Siegel, Miller, & Jemal, 2020). Unfortunately, given the current therapeutic advances, prostate cancer burden is rising, and most treatments remain unsatisfactory (Wang *et al*., 2018). Thus, developing an effective treatment for prostate cancer is a huge challenge.

In recent years, natural active components extracted from traditional Chinese herbs have been used in the treatment of cancer in increasing numbers (Li *et al*, 2020; Nishimura *et al*., 2021). Cannabinoids exhibited anticancer properties on ovarian or cervical carcinoma by inducing cell apoptosis (Roncati *et al*., 2021; Yan *et al*., 2015). Modified Ermiao granules have also been reported to reverse cervical intraepithelial neoplasia (Xu *et al*., 2021). Similarly, Brusatol, a form of bioactive natural quassinoid, is obtained from Brucea javanica, a Chinese herbal

medicine (Li *et al*., 2019; Zhang *et al*., 2013). Recent reports indicate that Brusatol induces various biological responses, exerting anti-inflammatory (Ding *et al*., 2021), antiviral (Li *et al*., 2019), and antitumor activities (Bovilla *et al*., 2021; Lu *et al*., 2022; Wang *et al*., 2021). Furthermore, a growing number of studies demonstrate the antitumor activities of Brusatol, especially in colorectal cancer (Lu *et al*., 2022), hepatocellular carcinoma [33341052], breast cancer (Bovilla *et al*., 2021), and non-small cell lung cancer (NSCLC) (Xing *et al*., 2022) cell lines. Specifically, the suppression of protein synthesis is the main mechanism responsible for Brusatol's antitumor action at high micromolar doses (Harder *et al*., 2017; Liao, Kupchan, & Horwitz, 1976). Brusatol has lately been linked to several different protein targets. For example, when used to treat NSCLC, Brusatol inhibited cancer growth and metastasis by binding to Skp1 (Xing *et al*., 2022). It can also boost cell cytotoxicity and chemotherapy efficacy by reducing Nrf2-mediated antioxidant response (Xie *et al*., 2021). Nonetheless, the significance of Brusatol in prostate cancer has not been comprehensively researched and fully understood.

Hypoxia is a prominent and frequent feature of the tumor microenvironment that induces the hypoxia-inducible factors (HIFs), which are abundantly expressed in numerous cancer cells (Vaupel & Mayer, 2007). Activated HIFs enhance tumor angiogenesis, tumor growth, metastasis, and metabolic reprogramming via inducing the transcription of survival-related genes (Oh *et al*., 2017; Ziello *et al*., 2007). Increasing evidence demonstrated that Brusatol suppressed the growth of tumor cells by regulating HIF-related protein activity (Lu *et al*., 2016; Oh *et al*., 2017). Lu *et al*. first identified the inhibitory effect of Brusatol on the HIF-1 signaling cascade in colon cancer cells and found that Brusatol was involved in glycolysis of tumor cells (Lu *et al*., 2016). Further evidence suggests that Brusatol-mediated suppression of c-Myc enhances HIF-1α degradation and leads to cell apoptosis in hypoxia-exposed colorectal cancer cells (Oh *et al*., 2017).

In this study, we established that Brusatol exerts antitumor effects on prostate cancer cells through inhibiting their proliferation, migration, and invasion, downregulating hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression, and suppressing glycolysis. The study implies that Brusatol may serve as a promising therapy for prostate cancer.

Materials and Methods

Chemicals and antibodies

Brusatol (ChemFaces, CFN93119) was obtained from Wuhan Tianzhi Biotechnology Co., Ltd. (Wuhan, China). Dulbecco's Modified Eagle's Medium (DMEM, 12800017), fetal bovine serum (FBS, 10099-141), Penicillin-Streptomycin (15140122), and phosphate-buffered saline solution (PBS, 11965092) were acquired from Gbico. Antibodies against Vimentin (#ab92547, 1:1000), N-cadherin (#ab76011, 1:1000), E-cadherin (#ab40772, 1:1000), ZO-1(#ab276131, 1:1000), HIF-1α (#ab51608, 1:1000), VEGF (#ab32152, 1:1000), GLUT1 (#ab115730, 1:1000), HK2 (#ab209847, 1:1000), LDHA (#ab101562, 1:1000), β-actin (#ab8226, 1:1000) were purchased from Abcam. From Cell Signaling Technology, anti-mouse (#4410, 1:10,000) and anti-rabbit (#4414, 1:10,000) peroxidase-conjugated secondary antibodies were obtained.

Cell lines and culture conditions

DU145 (ATCC®HTB-81) and PC-3 (ATCC®CRL-1435), human prostate cancer cell lines, were supplied by American Type Collection Culture (ATCC, Manassas, VA, USA). DU145 and PC-3 cells were cultured in DMEM (Gibco, 12800017), added with 10% FBS (Gibco, 10099-141), 10,000 U/mL Penicillin-Streptomycin (Gibco, 15140122). All of the cells were maintained at room temperature in a humidity chamber containing 5% CO_2.

Hypoxia experiments

An anaerobic device (Thermo Fisher Scientific, Marietta, OH, USA) was used to conduct experiments on the consequences of hypoxia. CO_2 and N_2 were used in a gas mixture that was used to set oxygen concentrations. Hypoxic parameters were optimized as 0.5% O_2 and 5% CO_2 at 37°C. Normoxia was designated as 20% O_2 and 5% CO_2 at room temperature.

Before each experiment, DU145 and PC-3 cells were cultivated to 70–90% confluence in a conventional O_2 content. Before being placed in normoxic or hypoxic chambers for 4 h, the cell culture solution was withdrawn and reconstituted with an equilibrated medium supplemented or non-supplemented with different concentrations of Brusatol (ChemFaces, CFN93119) for 4 h.

Viability assay

A total of 2500 cells per well were plated in 96-well plates with 100 μL of 10% FBS-supplemented DMEM. After reaching 70% confluency, cells were incubated with Brusatol (ChemFaces, CFN93119) at various concentrations for 48 h. Then, cells were slightly washed twice using PBS after the growth media was discarded. Consequently, the Cell Counting Kit-8 (CCK-8, Abcam, Cambridge, UK) was used to assess cell viability pursuant

to the manufacturer's protocol, and using the microplate reader (Bio-Rad, Hercules, CA, USA) to evaluate cell absorbance in each well at 450 nm. The experiment was repeated in triplicate.

Colony formation assay

Cells (2×10^2/well) in logarithmic growth phase were treated with varying concentrations of Brusatol (ChemFaces, CFN93119) in DMEM (Gibco, 12800017) containing 10% FBS (Gibco, 10099-141), and every 3 days, fresh media plus the drug were added. Two weeks were needed for the cells to colonize. To visualize the colonies, cells were fixed with 4% paraformaldehyde (Xuanya, XY-PCR-1654) at 37°C for 15–20 min, then rinsed with PBS (Gibco,11965092), and stained with 1% crystal violet (Beyotime, China). Ultimately, the ImageJ software (CKsoftware) was utilized to acquire photographs and calculate the data. The experiment was conducted in triplicate.

Flow cytometry assay

The Apoptosis Detection Kit (eBioscience, 88-8005-72) was used to quantify apoptotic cells. In 6-well plates, DU145 or PC-3 Cells (2×10^5/well) were transplanted, then with Brusatol treatment (0, 10, 50, and 100 μM) for 24 h. The cells were washed in PBS, resuspended in binding buffer, and supplemented with Annexin V-FITC for 15 min. Then, added PI in suspension and evaluated by the FACS-Calibur flow cytometer (BD, USA). The experiment was performed in triplicate.

Transwell assay

Matrigel (Corning, USA, 356234) gel was diluted and completely mixed with medium (serum-free DEME, Gibco, 12800017). The mixture was equally distributed across an upper transwell chamber (Boyden chambers, neuroprobe, BY312), and placed at 37°C until congealed. 5×10^4 DU145 and PC-3 cells were seeded onto the transwell upper chamber in serum-free DMEM supplemented with different doses of Brusatol (ChemFaces, CFN93119). The transwell lower chamber was supplied with 500 μL DMEM (with 10% FBS). The cells were incubated for invasion (24 h) or for migration (48 h). Lower chamber cells were fixed with 4% paraformaldehyde (Xuanya, XY-PCR-1654) for 15 min at 37°C. Following, cells were stained with 1% crystal violet (Beyotime, China). Subsequently, randomly selected 5 fields of view from each group were photographed under the microscope. The experiment was conducted in triplicate.

Western blot analysis

Vimentin, N-cadherin, E-cadherin, ZO-1, HIF-1α, VEGF, GLUT1, HK2, LDHA, and β-actin expression in DU145 and PC-3 cells were analyzed by western blotting. Briefly, after being extracted by a RIPA buffer (Beyotime, Shanghai, China), protein debris was eliminated by centrifuging (Thermo Fisher, 75004061) at 12,000 rpm for 10 min at 4°C. Then, protein quantification was determined by using a bicinchoninic acid kit (BCA; Beyotime, Shanghai, China). After denaturing proteins, equivalent quantities (10 μg) of proteins were separated according to molecular weight by electrophoresis and then transferred to PVDF membranes (LMAI Bio, LM1136). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk. Then, membrane was incubated with primary antibodies overnight at 4 °C, washed by TBST, and treated with corresponding secondary antibodies for 1 hour at 37 °C. Afterward, specific protein bands in membranes were observed using an ECL detection system (GE Healthcare, Piscataway, NJ, USA). All the experiments were conducted in triplicate.

Glucose measurement

In all, 5×10^4 cells per well were seeded in 6-well plates and treated with DMEM medium containing different concentrations of Brusatol for 4 h before being exposed to 0.5% O_2 for 4 h. After 24 h, supernatants were collected. Then, glucose consumption was assessed with a Glucose Assay Kit (Applygen Technologies, Beijing, China) according to the manufacturer's instructions. The experiment was repeated thrice.

Statistical analysis

All data were statistically analyzed using SPSS 22.0 (IBM, Armonk, NY, USA) and Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Significant differences were determined using the Student's *t*-test. Three distinct experiment results were provided as the mean ± standard deviation (SD). P values ≤ 0.05 were regarded statistically significant.

Results

Brusatol suppresses the proliferation of prostate cancer cells

To analyze the impact of Brusatol on prostate cancer, we incubated DU145 and PC-3 cells with Brusatol (0–100 μM) for 48 hours before estimating cell viability

via CCK-8 assay. Brusatol impaired the viability of DU145 and PC-3 cells, and at 100 μM concentration Brusatol significantly reduced cell viability by approximately 50% (Figure 1A). Similarly, the colony formation assay revealed a concentration-dependent reduction of colonies in DU145 and PC-3 cells (Figure 1B). Moreover, the number of apoptosis cells in DU145 and PC-3 cells was identified by flow cytometry, indicating the facilitated dose-dependent Brusatol-induced cell apoptosis (Figure 1C). Overall, these findings reveal that Brusatol could reduce prostate cancer cell proliferation and induce cell apoptosis.

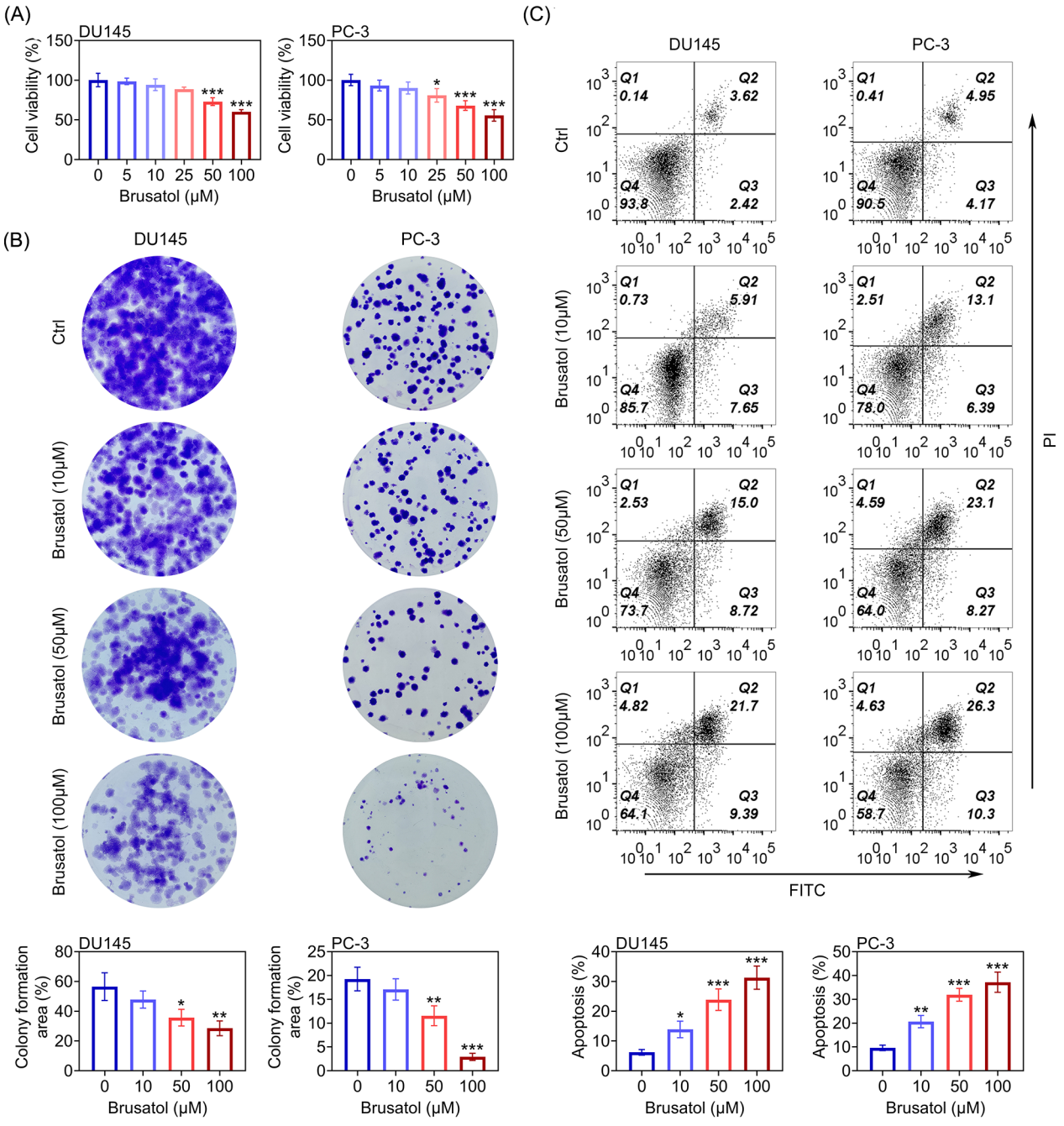

Figure 1. Brusatol inhibits prostate cancer cell growth. (A) Brusatol was added to DU145 and PC-3 cells in various concentrations (0, 5, 10, 25, 50, and 100 μM) for 48 hours. CCK8 experiments were performed to assess the cell viability. *$P<0.05$, ***$P<0.001$. (B) Colony formation of DU145 and PC-3 cells with Brusatol treatment (0, 10, 50, and 100 μM). *$P<0.05$, **$P<0.01$, ***$P<0.001$. (C) DU145 and PC-3 cells were incubated with Brusatol at different concentrations (0, 10, 50, and 100 μM) for 24 h. The cell apoptosis rate was examined using flow cytometry. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Brusatol impairs the invasion and migration ability of prostate cancer cells

Furthermore, we intend to evaluate whether Brusatol influences the epithelial-mesenchymal transition (EMT) in prostate cancer. Transwell assays were performed to examine the impact of Brusatol on the invasion and migration of prostate cancer cells (DU145 and PC-3 cells). Reduced cell invasion and migration were seen in the presence of increasing concentrations of Brusatol, as opposed to the control treatment (Figure 2A). Then, EMT phenotypes expression in Brusatol-exposed prostate cancer cells were measured using western blotting. As shown in Figure 2B, comparing with the control group, Brusatol suppressed the expression of mesenchymal markers N-cadherin and Vimentin while increasing the expression of the epithelial marker E-cadherin and ZO-1 in the DU145 and PC-3 cells. All indicated that Brusatol inhibited the EMT in prostate cancer cells. Specifically, Brusatol could reverse the invasive and metastatic properties of prostate cancer cells.

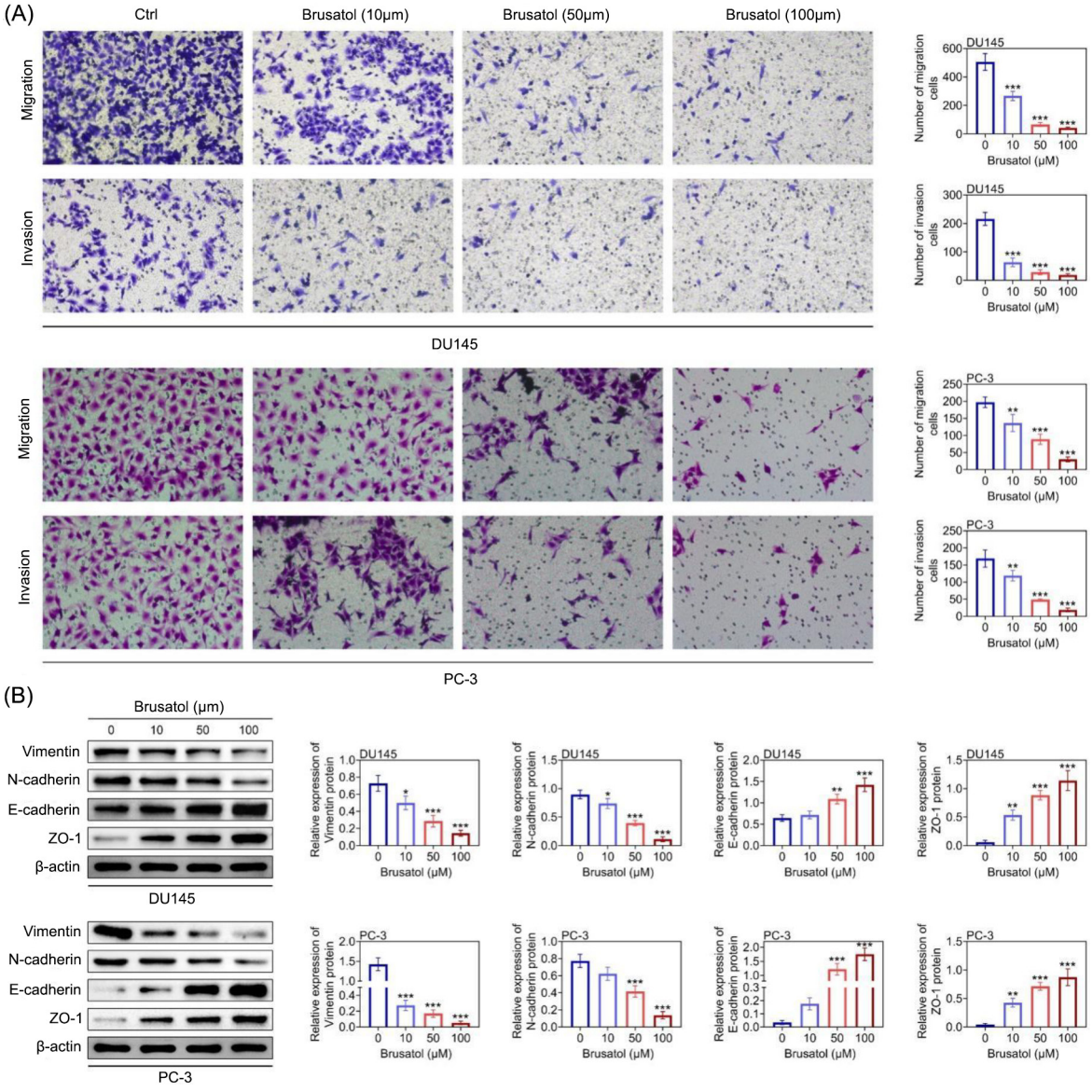

Figure 2. Brusatol weakens the ability of invasion and migration in prostate cancer cells. (A) Transwell assays were performed after Brusatol (0, 10, 50, and 100 μM) treatment. Representative images of cell invasion and migration in the lower chambers were then acquired (magnification, ×200). **$P < 0.01$, ***$P < 0.001$. (B) Whole-cell extract (WCE) from Brusatol-treated (0, 10, 50, and 100 μM) DU145 and PC-3 cells were subjected to western blotting. Histograms show the relative gray value of proteins evaluated by Image Lab. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Brusatol down-regulates the expression of HIF-1α/VEGF in prostate cancer cells under hypoxia

Early research reports that hypoxia is required for maintaining tumorigenic properties (Cowman & Koh, 2022). As the relevant regulators of the hypoxia transcriptional response, HIF-1α and VEGF have been identified to be involved in the tumorigenesis and progression of multiple malignancies (Fang *et al.*, 2021; Mu *et al.*, 2021; Wei *et al.*, 2022). Therefore, to characterize the effect of Brusatol on HIF-1α and VEGF expression patterns in prostate cancer cells subjected to hypoxia, we pretreated DU145 and PC-3 cells with Brusatol and then exposed them to hypoxia, whereas the control group cells were maintained in normoxic condition. As demonstrated in Figure 3, western blotting was performed, confirming that Brusatol reduced concentration-dependently the expression of HIF-1α and VEGF in DU145 and PC-3 cells under hypoxia.

Brusatol inhibits glycolysis in prostate cancer cells under hypoxia

It is widely acknowledged that the glycolytic pathway plays a pivotal role in tumor progression (Leimgruber *et al.*, 2020). We tested the expression of glycolytic pathway-related enzymes, including GLUT1, HK2, and LDHA, in Brusatol-treated prostate cancer cells. Different doses of Brusatol were incubated with DU145 and PC-3 cells, then subjected to hypoxia. As shown in Figure 4A, Brusatol decreased GLUT1, HK2, and LDHA protein expression in a concentration-dependent manner. Furthermore, glucose consumption in DU145 and PC-3 cells decreased significantly after treatment with Brusatol for 4 h as expected, which suggested that Brusatol inhibited glycolysis in prostate cancer cells under hypoxia (Figure 4B).

Discussion

The antitumor bioactivities of Brusatol has been extensively studied in various malignancies, including melanoma (Wang *et al.*, 2018), hepatocellular carcinoma (Lee *et al.*, 2020; Ye *et al.*, 2018), and breast cancers (Yang, Tian, Guo, & Ren, 2020), which exert their effects through mediating cell proliferation, apoptosis, migration, and invasion. Yang *et al.* demonstrated that Brusatol elicited an antitumor response in breast cancer via inhibiting cell growth and contributing to an increased expression of apoptosis-related protein (Yang *et al.*, 2020). Brusatol increased ROS-induced cell cycle arrest and cellular apoptosis via suppressing the AKT-Nrf2 signaling pathway in melanoma (Wang *et al.*, 2018). The comprehensive antitumor activity of Brusatol in hepatocellular carcinoma is mediated by inhibiting cell viability and promoting apoptosis through inhibition of the PI3K/Akt/mTOR pathway (Ye *et al.*, 2018). Similarly, our findings demonstrated that Brusatol reduced cell proliferation, induced apoptosis, and suppressed migration and invasion in DU145 and PC-3 cells. These data suggested that Brusatol might have an antitumor effect on prostate cancer treatment. However, additional research is required to identify the underlying mechanisms of Brusatol's effects on prostate cancer.

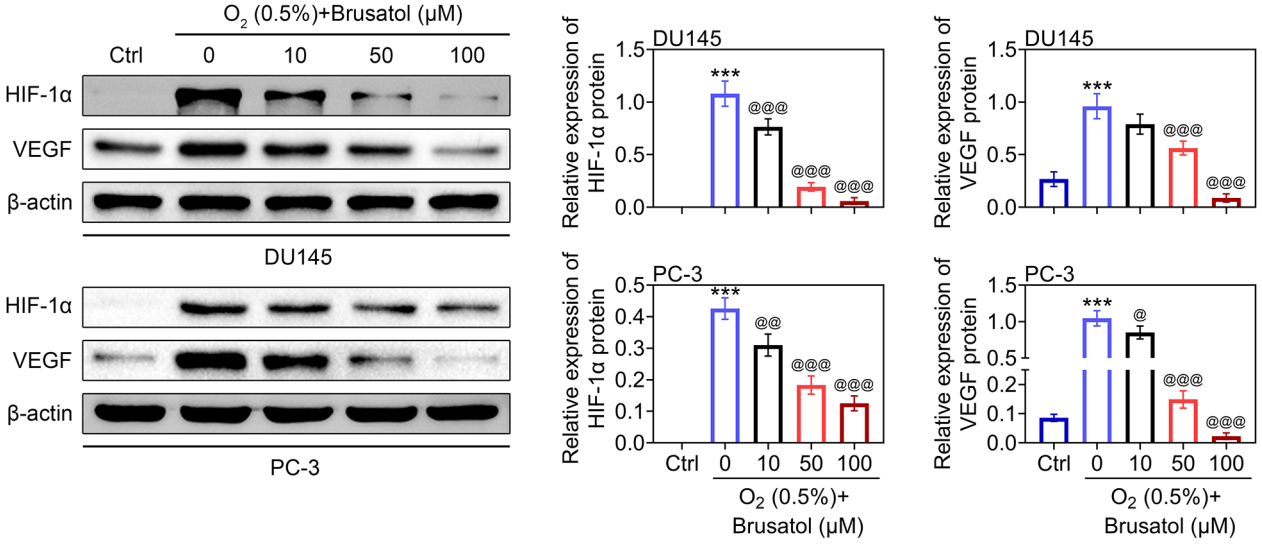

Figure 3. Brusatol reduces the expression of HIF-1α and VEGF in prostate cancer cells under hypoxia. DU145 and PC-3 cells were treated for 4 h with Brusatol (0, 10, 50, and 100 μM), then forced to hypoxia for 4 h, and HIF-1α and VEGF protein expression was evaluated by western blotting. ******P* **< 0.001 versus the control group. @@*P* < 0.01, @@@*P* < 0.001 versus other Brusatol-treated groups.**

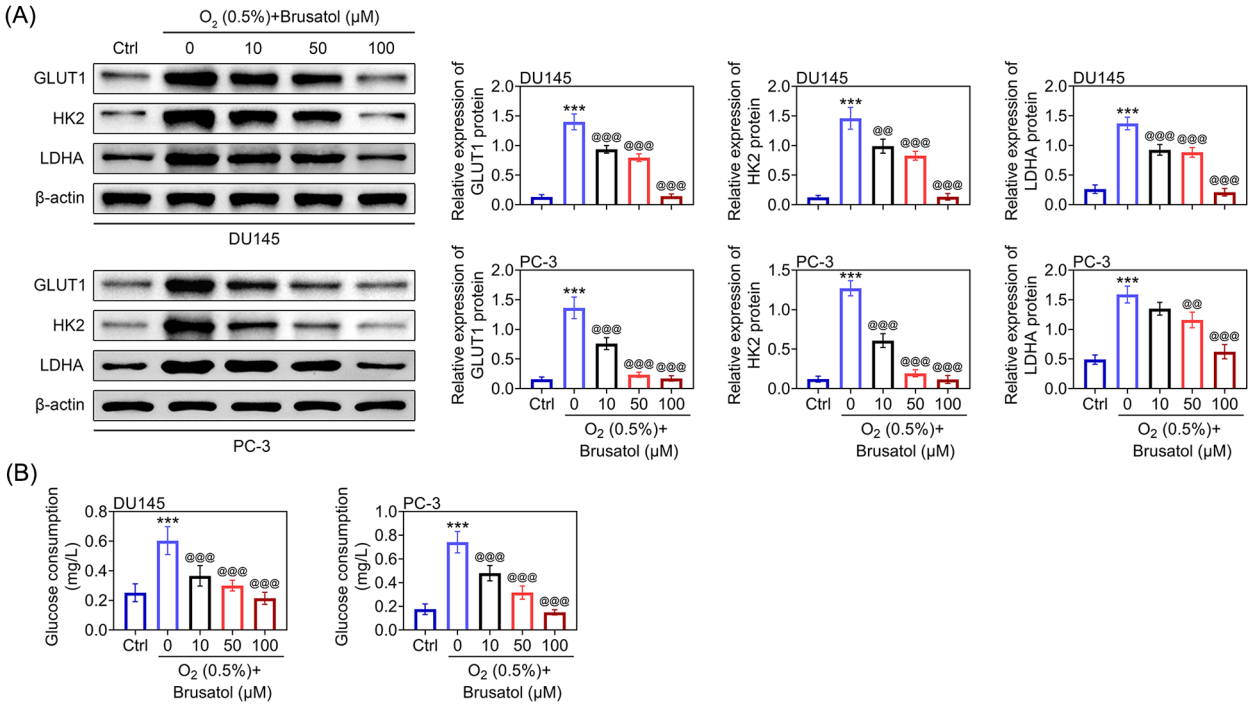

Figure 4. Brusatol inhibited glycolysis in prostate cancer cells under hypoxia. (A) DU145 and PC-3 cells were treated with Brusatol (0, 10, 50, and 100 μM) for 4 h, then cultured under hypoxia (0.5% O2) for 4 h. The control group (Brusatol-free) was cultured in normoxic for 8 h. The protein levels of GLUT1, HK2, and LDHA were detected by western blotting and normalized with β-actin protein levels. ***$P<0.001$ versus the control group. @@$P<0.01$, @@@$P<0.001$ versus other Brusatol treatment groups. (B) The glucose consumption was measured with Glucose Assay Kit. ***$P<0.001$ versus the control group. @@@$P<0.001$ versus other Brusatol-treated groups.

Cells proliferate at a rate that outpaces the generation of new blood vessels, resulting in insufficient oxygen in tumor tissues. The hypoxic microenvironment, in turn, is critical for tumor progression since it is essential for sustained growth and metastasis, conferring a poor prognostic phenotype (Moon *et al*., 2021). Under hypoxia, HIF-1α is stabilized and activated by translocating to the nucleus (Palazon *et al*., 2014). As a transcription factor, HIF-1α promotes the transcription of VEGF and then cooperatively stimulates various cancer cells to acquire invasion and metastasis as well as drives tumor progression (Lu *et al*., 2002; Semenza, 2003). For instance, activated HIF-1α and VEGF play a part in facilitating metastasis and angiogenesis, relevant to the poor prognosis of gastric cancer (Luo *et al*., 2021; Mu *et al*., 2021). Therapeutically targeting HIF-1α reduces and inhibits growth and metastasis of relapsed anaplastic Wilms tumor (Liu *et al*., 2021). Furthermore, various researches have proven that HIF-1α and VEGF might act as potential therapeutic targets. Through the reduction of HIF-1α and VEGF expression, Genipin hinders the invasion and migration of colon cancer cells (Lee *et al*., 2018). Salinomycin suppresses tumor angiogenesis by targeting HIF-1α/VEGF, delaying or preventing breast cancer progression (Dewangan *et al*., 2019). Confirming this, in our study, we discovered that Brusatol dose-dependently suppressed the accumulation of HIF-1α and VEGF in prostate cancer cells under hypoxia.

Interestingly, under hypoxia, cancer cells are compelled to upregulate the expression of genes (GLUT1, HK2, LDHA) implicated in the glycolytic pathway (Vander Heiden *et al*., 2009). Importantly, numerous studies have revealed that these glycolysis-related genes promote tumor growth in various malignancies, implying that targeting these genes could substantially inhibit tumor progression (Fox *et al*., 2018; Guo *et al*., 2021; Liu *et al*., 2021; Zhang *et al*., 2021). Glycosylated poly (amido amine)/celastrol (PAMAM/Cel) complex, for example, were found to suppress tumor growth and metastasis via binding to GLUT1 (Wei *et al*., 2022). Tanshinone IIA exerts a tumor-inhibiting action by blocking the expression of the aerobic glycolysis HK2 gene in oral squamous cell carcinoma (Li *et al*., 2020). Additionally, by targeting LDHA, Dimethyl itaconate inhibits the development of thymic carcinoma (Hayashi *et al*., 2021). Similarly, we found that GLUT1, HK2, and LDHA protein expression were deregulated, and the glucose consumption was decreased correspondently with Brusatol in a concentration-dependent way.

Conclusions

These findings imply that Brusatol dramatically reduced prostate cancer cell proliferation, migration, and invasion; induced apoptosis; and de-regulated HIF, VEGF, and glycolysis-related protein (GLUT1, HK2, and LDHA) expression. The study revealed that Brusatol has an antitumor effect on prostate cancer. Further studies are needed to explore the underling effects of Brusatol on prostate cancer.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

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.

Contribution of authors

All authors contributed to the study conception and design. Material preparation and the experiments were performed by Mi Wang, Liyang Dai and Wei Yan. Data collection and analysis were performed by Ying Chen and Yakun Wang. The first draft of the manuscript was written by Mi Wang and Liyang Dai and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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