

# Brusatol Suppresses Bladder Cancer by downregulating the expression of Nrf2

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## Research Article

**Keywords:** bladder cancer, brusatol, Akt pathway, Nrf2, EMT

**Posted Date:** September 1st, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-2013818/v1>

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# Abstract

A substance called brunsatol, which is derived from the dried ripe fruit of *Brucea javanica*, has been shown to be active against a variety of human cancers, but not against bladder cancer (BCa). In the present study, we attempted to investigate the effect of brusatol in BCa and verify whether Nrf2 was involved in the mechanism. The effect of brusatol was assessed by CCK8 and colony formation assays. Transwell analysis were performed to detect the migration of bladder cancer cell lines under treatment of brusatol. Apoptosis was determined by flow cytometry. Finally, Western blot was used to explore the mechanism of brusatol regulating the BCa progression and Nrf2. We revealed that brusatol inhibited the growth of BCa cells in vitro and in vivo and decreased their invasion ability. Mechanistic analysis revealed that brusatol decreased the expression of Nrf2 which functions as a tumor promoter in the development of BCa. And Nrf2 overexpression reversed the inhibitory effects induced by brusatol. Finally, brusatol inhibited the proliferation and invasion of BCa cells via Akt signaling-mediated Nrf2 expression. These results suggest that brustasol is a valuable potential antitumor drug available for BCa.

## Introduction

Bladder cancer (BCa) is a malignancy of urinary system that is estimated greater than 17,000 deaths and 83,000 new cases in 2021 in the United States[1]. The male-female ratio of new cases is about 3:1, which is thought to be related to cigarette smoking, occupational exposure, inflammation, radiation and chemotherapy[2]. The most common type of bladder cancer is urothelial cancer. Bladder cancer can be treated in many ways. Guidelines for urology provide detailed recommendations for treatment, such as radical cystectomy, chemotherapy and intravesical immunotherapy with bacillus Calmette Guerin (BCG). BCa patients, however, have a poor overall survival rate. Thus, improved treatment options for BCa is worthy of further study.

Herbal medicines have demonstrated a wide range of pharmacological effects in recent years, including anticancer, anti-inflammatory, and antioxidant properties. Brusatol is an active natural component of *Brucea javanica* and was first evaluated as an experimental treatment for leukemia[3]. Brusatol is a traditional herbal medicine which exhibits potent therapeutic effects, such as proapoptotic, anti-inflammatory, antimetastatic and radiation resistance effects. By inhibiting JAK2/STAT3 signaling, brusatol inhibits laryngeal cancer cell proliferation and metastasis [4]. In another research, Human gastric cancer cells undergo epithelial-mesenchymal transformation induced by lipopolysaccharide and undergo apoptosis induced by brusatol via the PI3K/Akt/NF-kB pathway [5]. These findings suggest that brusatol could be a promising drug for treating cancer.

There have been limited studies on the therapeutic efficacy of brusatol in BCa patients. An interesting study showed that brusatol could inhibit the proliferation of nasopharyngeal carcinoma through Akt/mTOR signaling pathway. And Akt/mTOR signaling pathway is known to regulate the growth of BCa [6, 7]. There is increasing evidence that Nrf2 in tumor cells plays a key role in anticancer therapies and metabolic reprogramming[8]. Here, we sought to identify whether the natural component of *Brucea*

javanica may exert anti-tumor effect. At last, we investigate whether Nrf2 is involved in BCa's response to brusatol.

## **Materials And Methods**

### **Chemicals**

Brusatol (purity,  $\geq 95\%$ ; molecular weight, 520.53; formula, C<sub>26</sub>H<sub>32</sub>O<sub>11</sub>) was obtained from Sigma-Aldrich (Shanghai, China). The stock solution of brusatol was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich (Shanghai, China)) and stored at 20°C. The final concentration of DMSO in the cell culture medium was less than 0.1% in all treatments.

#### **Antibodies and reagents.**

The primary antibodies were all purchased from Proteintech (Wuhan, China): GAPDH, Bax, Bcl2, cleaved caspase 3, Akt, mTOR and Nrf2. Proteintech (Wuhan, China) provided goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies.

#### **Cell lines.**

The human bladder cancer cell lines were purchased by CTCC. 5637 and T24 cells were maintained in RPMI-1640 medium (HyClone, China). In addition to 10% fetal bovine serum (FBS) (Gibco, Australia), these cells also received 1% penicillin G sodium/streptomycin sulfate.

#### **Immunohistochemistry (IHC).**

The method was as previously reported[9].

#### **Western blot.**

The method was as previously reported[9].

#### **CCK-8 based cell viability assay.**

Each well was seeded with 5637 and T24 cells and cultured for 24, 48, 72, or 96 hours. CCK-8 reagent (CK04, Dojindo, Japan) was added at the end of each experiment. Absorption values were measured at 450 nm. The experiments were conducted three times in triplicate.

#### **Tumor cell colony formation assay.**

5637 and T24 cells were seeded in 6-well plates and grown for about 10 days. Following fixation in 4% paraformaldehyde (PFA) followed by staining in 0.1% crystal violet, visible colonies were counted three times.

#### **Transwell migration assay.**

Cells were suspended in serum-free medium in the upper chamber of a 24-well transwell chamber system. And 20% FBS medium was added to the lower chamber. Transwell chambers were collected 48 hours after establishing transwell permeability, and cells on the other side of the membrane were fixed and stained with 0.1% crystal violet. Photographs were taken of the stained chambers after 48 hours. The experiments were conducted three times in triplicate.

### **Cell apoptosis.**

An Annexin-V FITC stain was used to stain 5637 and T24 cells, then they were analyzed by flow cytometry. The experiment was repeated three times.

### **TUNEL assay.**

The TUNEL assay was performed by using an apoptosis detection kit. An optical fluorescence microscope (Olympus) was used to observe the result. Positive cells were identified, counted, and analyzed.

### **Xenograft mouse model.**

The 4 weeks old specific pathogen-free (SPF) male nude mice were used for xenograft mouse model. Subcutaneous injections of  $10^6$  T24 cells suspended in 150  $\mu$ l of PBS were performed on two randomly selected group mice. The two groups were treated with normal saline and brusatol (2 mg/kg) respectively, every other day. Approximately four weeks after sacrifice, the tumors were dissected and stained with IHC. Researchers at Renmin Hospital of Wuhan University followed institutional guidelines for animal care and used animals in this study in accordance with the ethics committee's approval.

## **Statistical analysis.**

The 22.0 SPSS software package was used for all statistical analyses. A two-sided P value < 0.05 was considered statistically significant.

## **Results**

### **Brusatol suppressed the growth of BCa cells.**

First, in CCK-8 assay the cell viability of the 5637 and T24 cell lines decreased as the drug concentration increased (Fig. 1A). The results suggested that brusatol inhibited BCa cell growth concentration-dependently. In the following experiments, we used 20 nM brusatol. We found that brusatol suppressed BCa cell growth in a time-dependent manner (Fig. 1B). As shown in Figs. 1C and 1D, brusatol-treated cells were less able to form colonies than control cells in a concentration-dependent manner.

### **Brusatol inhibited the migration of BCa cells.**

In this part, we explored the effect of brusatol on BCa migration using a transwell chamber. The migration was also delayed in brusatol treatment cells in a concentration-dependent manner (Fig. 2A and 2B). There were researches reported Epithelial-mesenchymal transition (EMT) is associated with the metastasis of cancer cells[10]. So, In BCa cells, we performed western blotting to determine whether brusatol suppressed EMT-associated markers. After brusatol treatment, E-cadherin levels increased, whereas the expression of vimentin as well as N-cadherin decreased on T24 (Fig. 2C) and 5637 (Fig. 2D). Thus, these revealed brusatol maybe inhibited BCa cell migration by suppressing EMT.

### **Brusatol induced apoptosis in BCa cells.**

For determine the potential mechanism underlying the antitumor effect of brusatol on BCa cells, BCa cells were treated with brusatol for 24 h to determine whether it could induce apoptosis. After treatment, there was a significant increase in apoptotic cells in a concentration dependent manner (Fig. 3A and 3B). We also examined whether brusatol suppresses apoptosis-associated markers in BCa cells via western blotting. Consistent with flow cytometry results. Consistent with the results of flow cytometry, the levels of cleaved caspase3 and Bax increased, whereas the expression of Bcl-2 decreased (Fig. 3C and 3D). These findings all suggested that brusatol promoted apoptosis in BCa cells.

### **Brusatol inhibites aggressive behaviors in BCa cells by decreasing the expression of Nrf2.**

We have previously shown that brusatol regulates BCa cell biological functions. The accumulating research has demonstrated that Nrf2 regulates key signaling pathways in tumor cells that contribute to tumor progression. Therefore, we examined whether Nrf2 contributes to cancer progression by measuring Nrf2 protein levels after brusatol treatment in 5637 and T24 cell lines. Western blotting results showed that BCa cells treated with brusatol showed greatly reduced levels of Nrf2 protein (Fig. 4A and 4B). To further investigation, Nrf2 overexpressed cell lines were constructed (Fig. 4C). The CCK-8 assay, clone formation assay, transwell assays results showed that overexpression of Nrf2 could partially counteract the inhibition of brusatol on BCa cells (Fig. 4D, 4E, 4F). This suggests that brusatol regulates Nrf2 to mediate BCa cells' behavior.

### **Akt pathway is required for the regulation of Nrf2 by brusatol in BCa cells.**

Akt signaling is found participating in tumor cells by regulating Nrf2[11, 12]. Therefore, We first examined the activation of the Akt pathway in 5637 and T24 cell lines with or without brusatol treatment to determine whether it regulates Nrf2 through this pathway. The results showed that the levels of p-Akt and p-mTOR in 5637 and T24 cell lines were significantly decreased in the brusatol treatment group (Fig. 5A, 5B). Subsequently, Western blotting, CCK-8, Transwell assays, clone formation assay were performed after the brusatol treatment group was treated with SC79. The WB results showed that SC79 treatment up-regulated Nrf2 protein levels in brusatol treatment group (Fig. 5C, 5D). Similarly, SC79 treatment significantly reversed the inhibition on the proliferation and invasion of brusatol treatment in 5637 and T24 cell lines (Fig. 5E, 5F, 5G). Altogether, these results confirmed that brusatol inhibits the Akt signaling pathway in BCa cells.

## **Brusatol significantly inhibits the growth of BCa in vivo.**

In vitro, we verified brusatol inhibited the growth of BCa cells. Next, we investigated whether brusatol inhibited the growth of BCa cells in vivo via xenograft mouse model. After each injection, each mouse's tumor size was measured, and when compared with the control group, tumor sizes were smaller in the brusatol-treated mice (Fig. 6A). In brusatol treatment group, the tumor growth rate was noticeably slower than that of the control group (Fig. 6B). Accordingly, the levels of positive TUNEL staining were increased (Fig. 6C), whereas IHC analysis demonstrated that the expression levels of Ki-67 and Nrf2 were reduced in the brusatol treatment (Fig. 6D). These data confirmed that brusatol had an obvious antitumor effect in vivo.

## **Discussion**

Bladder cancer is the most common tumor among adult urogenital system [1]. Despite surgery, radiotherapy, and chemotherapy improving prognoses for BCa patients, recurrence is common because of its high aggressiveness and proliferation rate[13]. Previous studies have demonstrated that brusatol inhibites liver cancer and lung cancer, there is a lack of knowledge regarding its role in BCa, as well as the underlying molecular mechanisms. In the present study, we found brusatol effective in inhibiting BCa cells growth in vitro and in vivo. Consistently, we identified brusatol as an inhibitor of the Nrf2 which played an important role in inducing apoptosis. Brusatol inhibits cell growth and invasion but can be reversed by overexpressing Nrf2. Furthermore, we found brusatol down regulates the Nrf2 protein expression by inhibiting the Akt signaling.

First, we observed that brusatol inhibited BCa cells proliferation and viability in a dose-dependent manner. With regard to the mechanism of the antitumor effect of brusatol, recent studies has shown that maybe connected to inducing apoptosis and cell cycle arrest. Apoptosis is known as a common programmed cell death. Similar to these reports, our results show that brusatol induced apoptosis in BCa cells by flow cytometry analysis. In another experiment, brusatol inhibites the migration of BCa cells. As a growing number of studies suggest that EMT of tumor cells may be the major cause of the tumor distant metastasis [14–16]. And distant metastasis of tumors is the major cause of death in BCa. As epithelial cells undergo EMT, their cell-to-cell connections and apicobasal polarity are lost, and the cells acquire a fibroblast-like morphology, paving the way for metastatic invasion[17, 18]. E-cadherin is the key epithelial marker of EMT[19]. In our study, we found that the migration of brusatol-treatment cells were significantly decreased. Furthermore, brusatol significantly inhibited BCa cells' EMT, thus attenuating the malignant progression of BCa.

Cancer cell survival is mediated by Nrf2, one of the most important transcription factors. Studies have shown that the translocation of this protein into the nucleus induces tumor-protective genes transcriptional activation, which in turn protects cells from apoptosis and ultimately promotes tumor progression[20, 21]. Polycyclic aromatic hydrocarbons stimulate Nrf2 through the aryl hydrocarbon receptor (AhR). Nfe2l2's promoter contains xenobiotic response element-like sequences that AhR

transactivates by binding to AhR nuclear translocator (Arnt). By inducing AhR, xenobiotic ligands activated the Nrf2 pathway [22]. In addition, NFE2L2 is activated by breast cancer susceptibility 1 (BRCA1) in human MCF10A mammary cells[23]. In another research, A new study by Wei demonstrated that tagitinin C induces ferroptosis in colorectal cancer cells and activates PERK-Nrf2-HO-1 signaling through ER stress[24]. Blocking Nrf2 also inhibited esophageal squamous cell carcinoma metastases driven by EMT[25]. This is consistent with our findings. Here, we found Nrf2 was markedly downregulated in brusatol treated BCa samples. Moreover, Nrf2 overexpression significantly reverse the inhibition on BCa cells. Under normal conditions, we hypothesized that brusatol and Nrf2 are related since Nrf2 can be ubiquitinated by E3 ligases and degraded via 26S proteasomes[26]. In the deeply experiment, we found BCa's biological behavior was improved by Nrf2 overexpression, suggesting Nrf2 may serve as an upstream target of brusatol.

As is known, Akt/mTOR pathway is an important pathway in various of cancers. Activated AKT inhibits its downstream molecules thereby increasing the progression and metastasis of tumor cells. One of the major downstream markers is mTOR, which inhibits the downstream targets[27]. Interestingly, Akt signaling participates in many biological behaviors of cancer cells by regulating the expression of protein Nrf2[12, 28]. The activation of Akt phosphorylation had an immediate effect on cell growth and invasion treated with brusatol. Our research confirmed these results by showing that brusatol inhibites the Akt/mTOR pathway and weakens Nrf2 expression. All these results imply the Akt pathway may be a potential pathway via which brusatol plays a role in BCa.

In conclusion, we described brusatol could attenuated the progression of BCa cells by regulating cell growth for the first time. Furthermore, the underline mechanism refers to the Akt signaling pathway regulating the transcription factor Nrf2. This mechanism provides a new perspective on the antitumor effect of brusatol in the treatment of BCa.

## **Declarations**

### **Acknowledgements**

This work was supported by Independent scientific research project of Wuhan University (grant numbers 2042019kf0076) and National Natural Science Foundation of China (No. 81972408 and 82000639),

### **Author contributions**

Xiuheng Liu and Lei Wang conceptualized the study. SL contributed to the data curation. Huaxin Wang conducted the formal analysis. XY investigated the study. Chenglin Ye contributed to the methodology. XY and SL wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

### **Data availability**

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

## Declarations Conflict of interest

All authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this research article.

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## Figures

### Figure 1

Brusatol suppressed the proliferation of T24 and 5637 cells. (A) T24 and 5637 cells were treated with different concentrations of brusatol (0, 20, 40, and 80 nM). (B) T24 and 5637 cells were treated with brusatol (20nM) for different time intervals. Cell viability was determined by CCK-8 assay. A colony-forming growth assay detection of T24 (C) and 5637 (D) cells colony formation ability treated with different concentrations of brusatol (0, 20, 40, and 80 nM). The colonies were counted and captured. Data are presented as the means  $\pm$  SDs for 3 independent experiments. \* $P < 0.05$  compared with the control group.

### Figure 2

Brusatol suppressed the invasion of T24 and 5637 cells using the Transwell invasion assay. T24 (A) and 5637 (B) cells were treated with different concentrations of brusatol (0, 20, 40, and 80 nM) for 24h. Quantitative analysis of the average invasive cell numbers in each group. EMT-associated markers such as E-cadherin was increased, whereas vimentin and N-cadherin was decreased on T24 (C) and 5637 (D)

cells in a concentration dependent manner. Data are presented as the means  $\pm$  SDs for 3 independent experiments. \* $P < 0.05$  vs. the control group.

### Figure 3

Brusatol induced apoptosis in BCa cells through activating caspase 3. T24 (A) and 5637 (B) cells were treated with different concentrations of brusatol (0, 20, 40, and 80 nM) for 24 h. Cell apoptosis was detected by flow cytometry using annexin V/PE staining. Representative flow cytometric images and statistical data are showed. T24 (C) and 5637 (D) cells were treated with different concentrations of brusatol (0, 20, 40, and 80 nM) for 24 h, and then subjected to Western blot analysis. The data are presented as means  $\pm$  SDs for 3 independent experiments. \* $P < 0.05$  compared with the control group.

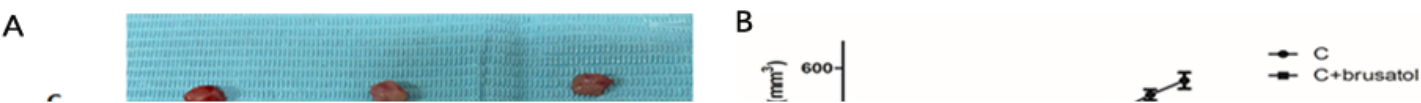
### Figure 4

Brusatol inhibites aggressive behaviors in BCa cells by decreasing the expression of Nrf2. The expression of Nrf2 was decreased in a concentration dependent manner when T24 (A) and 5637 (B) cells were treated with different concentrations of brusatol (0, 20, 40, and 80 nM) for 24 h. (C) The overexpression efficiency of Nrf2 in T24 and 5637 cells. Cell growth was assessed by the Cell Counting Kit-8 assay in cells with Nrf2 overexpression (D). Cell proliferation was assessed by the colony formation ability (E). Transwell assays were used to examine the invasion ability of BCa cells treated as described above (magnification  $\times 200$ ) (F). Results from the quantitative analysis of the percentage of invading cells are shown. Data are presented as the means  $\pm$  SDs for 3 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. the control group

### Figure 5

Akt pathway is required for the regulation of nuclear factor E2-related factor 2 (Nrf2) by brusatol treatment in BCa cells. Levels of Akt/mTOR pathway proteins in brusatol treatment T24 (A) and 5637 (B) cells were detected using Western blotting. (C) Levels Nrf2, p-Akt, and Akt proteins in brusatol treatment T24 (C) and 5637 (D) cells with or without SC79 (2  $\mu$ M, 4 h) were detected using Western blotting. (E) Cell viability was assessed by the CCK8 assay. (F) Cell proliferation was assessed by the colony formation

ability. (G) Transwell assays were used to assess the invasion ability (magnification  $\times 200$ ). Results from the quantitative analysis of the percentage of invading cells are shown. Data are presented as the means  $\pm$  SDs for 3 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. the control group



**Figure 6**

Brusatol significantly inhibits the growth of BCa in vivo. (A) Morphology of the subcutaneous implanted tumor. (B) Mean tumor volume at each time point. (C) A TUNEL assay was performed to detect the apoptotic cells in the tumor tissue. (D) IHC was performed to detect the protein of Ki67 and Nrf2 in the tumor tissue \* $P < 0.05$  vs. the control. All the above data are the mean  $\pm$  SD from an average of three experiments.