

Brusatol hinders the advancement of bladder cancer by Chac1/Nrf2/SLC7A11 pathway

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Abstract

Background

Bladder cancer, also known as BCa, is a common tumor that impacts the urinary system. It is marked by a significant fatality rate and an unfavorable outlook. Promising antineoplastic properties are exhibited by brusatol, which is obtained from the dried ripe fruit of *Brucea javanica*. The present study aimed to evaluate the influence of brusatol on the advancement of BCa and uncover the molecular mechanism involved.

Materials and methods

We used Cell Counting Kit-8, colony formation and EdU assays to detect cell activity and degree of apoptosis. We used transwell migration assay to detect cell migration and invasion ability. The mechanism of brusatol inhibition of BCa proliferation was studied by flow cytometry, western blotting.

Results

It was revealed that brusatol could reduce the viability and proliferation of BCa cells. A transwell migration assay revealed that brusatol was able to attenuate the invasion and migration of BCa cells. In addition, treatment with RSL3 or ferrostatin-1 enhanced or reversed the brusatol-induced inhibition of BCa cells. While testing for indicators related to iron death it was determined that treatment with brusatol increased the levels of reactive oxygen species, malondialdehyde and Fe^{2+} in BCa cells. Mechanistically, brusatol induced ferroptosis by upregulating the expression of Chac glutathione-specific gamma-glutamylcyclotransferase (Chac1) in BCa cells. Moreover, treatment with brusatol significantly suppressed the tumor growth in nude mice.

Conclusions

To summarize, the findings of this research demonstrated that brusatol hindered the growth of BCa and triggered ferroptosis by increasing the expression of Chac1. Brusatol has the potential to be considered as a promising candidate for treating BCa.

Introduction

Urinary tract cancer originating from the bladder, known as BCa, is the second most prevalent type of cancer [1]. BCa accounts for over 3% of global cancer diagnoses, with approximately 83,730 new cases and 17,300 deaths related to cancer reported in the United States during 2020 [2]. Around a quarter of BCa cases are labeled as muscle invasive bladder cancer (MIBC), with the rest being categorized as non-

muscle invasive bladder cancer (NMIBC) [3]. Although patients with BCa can be treated by surgery, they often relapse soon after resection and ~ 15% of cases deteriorate to MIBC [3]. In physically fit patients with NMIBC and no contraindications, standard treatment involves chemotherapy utilizing the DNA-damaging agent cisplatin. Nevertheless, the effectiveness of chemotherapy frequently becomes restricted over a period of time as a result of the emergence of treatment resistance. Alternative first-line treatment strategies, including carboplatin-based chemotherapy, immune checkpoint inhibitors, and erdafitinib, have shown potential for achieving long-lasting remission in the presence of acquired therapy resistance [4–6]. Moreover, there has been a growing focus on discovering novel chemical substances that possess enhanced pharmacologifs and pharmacokinetic characteristics.

The Chinese name for brusatol is Ya-Dan-Zi (Fig. 1). The dried mature fruit of *brucea javanica* (L.) was initially assessed as an experimental remedy for leukemia in 1979 [7]. Now, multiple researches have shown the powerful inhibitory impacts of brusatol on the mensplism and growth of cancer cells. For example, research that concentrates on non-small cell lung cancer (NSCLC) has presented proof that brusatol has the potential to be a beneficial addition to current chemotherapy treatments for the control of NSCLC [8]. According to the findings of Lu et al, the utilization of brusatol could hinder the growth and spread of colorectal cancer by impacting the process of epithelial-mesenchymal transition (EMT) through the RhoA/ROCK1 pathway [9]. A previous study showed that brusatol, which inhibits the nuclear factor erythroid 2-related factor 2 (Nrf2), effectively enhanced the antitumor effectiveness of lapatinib against SK-BR-3, SK- OV-3, and AU565 cancer cells, demonstrating a strong synergistic effect [10]. Accordingly, a recent investigation by the authors suggested that brusatol exerted potent tumor suppression in BCa by disrupting redox homeostasis via the Nrf2 pathway (Yu et al, unpublished data).

Previous research has noted that RSL3 and erastin trigger cell demise via a mechanism that is separate from apoptosis. Iron chelators and antioxidants have the ability to impede this form of cellular demise, thereby establishing a correlation between intracellular iron levels and reactive oxygen species (ROS) during this progression. Dixon et al. conducted a study in the year 2012. This type of cell death has been described as different from apoptosis, necrosis, and autophagy due to its unique morphological, biochemical, and genetic characteristics [11]. They named it ferroptosis. The accumulation of iron-dependent reactive oxygen species (ROS) occurs when the lipid repair enzyme glutathione peroxidase 4 (GPX4) is inhibited, thereby triggering ferroptosis. Due to their increased growth needs, cancer cells have a greater requirement for iron in comparison to normal cells. The concept of ferroptosis has opened up new avenues for cancer treatment strategies. Accumulated evidence suggests that ferroptosis is an antitumor factor in several cancers [12–15]. However, the precise molecular mechanism through which brusatol triggers ferroptosis in BCa is still not well comprehended.

Materials and methods

Cell culture and treatment

The T24 and 5637 cell lines were acquired from the Stem Cell Bank of the Chinese Academy of Sciences. T24 cells originate from a patient with urinary bladder cancer in humans. The 5637 cell line is a widely used model for studying high-risk superficial bladder cancer and its interactions with immune cells in systemic mechanisms. T24 and 5637 Cells were grown in RPMI-1640 medium containing 10% FBS obtained from Gibco (Thermo Fisher Scientific, Inc.). The cells were cultured at a temperature of 37°C in a moist environment with a mixture of 5% CO₂ and 95% air. Treatment involved the use of brusatol, acquired from Sigma-Aldrich (Merck KGaA). Cells were cultured until they achieved a confluence of 60–70% before undergoing treatment.

Antibodies

Western-blotting assay was performed with anti-Chac1 (Abcam, ab279365, 1:1000), anti-FTH1 (Cell Signaling Technology, 3998S, 1:1000), anti-SLC7A11 (Cell Signaling Technology, 98051, 1:1000), anti-GPX4 (Cell Signaling Technology, 52455, 1:1000), anti-Nrf2 (Thermo Fisher Scientific, PA5-27882, 1:1000), anti-Gapdh (Thermo Fisher Scientific, #MA5-27912, 1:2000). The secondary antibody was Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab205718, 1:10000). Immunohistochemistry assay was performed with anti-Ki67 (Proteintech, 27309-1-AP, 1:2000), anti-Chac1 (Proteintech, 15207-1-AP, 1:2000), anti-GPX4 (Proteintech, 67763-1-Ig, 1:2000). The secondary antibody was Goat Anti-Rabbit IgG H&L (HRP) (Proteintech, PR30011).

CCK-8 assay

T24 and 5637 cells were seeded in 96-well plates with a density of around 3×10^3 cells per well. Following a 24-hour culture period, the cells were subjected to various concentrations (0, 20, 40, 80 nM) of brusatol. In another trial, T24 and 5637 cells were initially exposed to RSL3 (5 nM) or ferrostatin-1 (50 nM) for a duration of 2 hours at 37°C. Subsequently, this were subjected to a 24-hour treatment of 20 nM brusatol at 37°C. Following each treatment, 10 µl of CCK-8 solution (CK04; Dojindo Laboratories, Inc.) was introduced into every well and left to incubate for a duration of 1 hour. Measurements were taken to determine the absorbance values at a wavelength of 450 nm. The trial was conducted thrice.

Colony formation assay

T24 and 5637 cells were seeded in six-well dishes with around 300 cells per well and cultured for approximately 14 days. After fixing the resulting colonies with 4% PFA, they were stained with 0.1% crystal violet for a duration of 4 hours at room temperature. Next, the colonies were enumerated using a microscope with low magnification. The trial was conducted thrice.

5-Ethynyl-2'-deoxyuridine (EdU) assay

brusatol was added to 96-well plates at a density of 2×10^4 cells per well, and the cells were incubated for 24 hours with different concentrations (0, 20, 40, 80 nM). Following this, the cells were exposed to 50 µM EdU (from Guangzhou RiboBio Co., Ltd.) for a duration of 2 hours at a temperature of 37°C. After rinsing with PBS, the cells were then treated with 4% PFA and left at room temperature for 30 minutes to be fixed. Afterwards, glycine was introduced, and the cells underwent treatment with 0.5% Triton X-100 for a

duration of 10 minutes. Afterwards, the cells were treated with 1X Apollo@ (Guangzhou RiboBio Co.,Ltd.) for a duration of 30 minutes at ambient temperature, while the nuclei were stained with Hoechst (Guangzhou RiboBio Co.,Ltd.) for the same duration at room temperature. Finally, images were acquired using a fluorescence microscope, and the proportion of EdU-labeled cells was determined to assess cell proliferation.

transwell migration assay

For the migration assay, a transwell chamber system from Corning, Inc. was utilized. Matrigel from BD Biosciences was applied to the upper chamber, which was incubated at a temperature of 37°C for a duration of 6 hours. Next, a total of 50,000 cells were introduced into the upper chamber, which was filled with RPMI- 1640 medium without serum. Simultaneously, the lower chamber was filled with medium containing 20% FBS. Following a 48-hour incubation period at a temperature of 37°C, the cells that had migrated to the opposite side of the membrane were immobilized using 4% PFA for 15 minutes at ambient temperature and then subjected to staining with 0.1% crystal violet for 1 hour at room temperature. The number of migrated cells in the lower chamber was determined by observing them under a light microscope. The trial was conducted thrice.

Wound healing assay

The cells were seeded onto 6-well dishes and permitted to proliferate until they established a dense layer. To generate a scratch in the cell layer, a 200 µL plastic pipette tip that was sterile was utilized, followed by two washes of the wells with phosphate-buffered saline (PBS) to eliminate any cells that had become detached. Afterward, the cells were cultured for 48 hours in a medium that did not contain fetal bovine serum (FBS). Photographs of the scrape were taken at various intervals using a microscope that emits light. This experiment was performed three times to ensure reproducibility.

Determination of reduced glutathione (GSH) and malondialdehyde (MDA) levels

T24 and 5637 cells were placed in six-well dishes with a concentration of 2×10^5 cells per well and incubated for 12 hours at 37°C. Following this, cellular extracts were obtained from the cells that were subjected to different concentrations (0, 20, 40, 80 nM) of the medications for a duration of 24 hours at a temperature of 37°C. Furthermore, cellular extracts were additionally obtained from cells that had been previously exposed to RSL3 (5 nM) or ferrostatin-1 (50 nM) for a duration of 2 hours at a temperature of 37°C. Subsequently, these cells were subjected to treatment with brusatol at a concentration of 20 nM for a duration of 24 hours at a temperature of 37°C. The cellular extracts were made in accordance with the guidelines given by the producer. MDA levels were quantified using a lipid peroxidation assay kit (Bio Version Inc, CAT#MDA31-H100), with absorbance values measured at 532 nm. GSH (glutathione) levels were determined by employing a GSH assay kit (Bio Version Inc, CAT#GSM39-K01) and measuring the absorbance values at 412 nm.

ROS analysis

The levels of ROS were assessed by utilizing the ROS detection kit (Beyotime Institute of Biotechnology, CA4 #S0033M) in accordance with the guidelines provided by the manufacturer. T24 and 5637 cells were placed in six-well dishes with a cell density of 6×10^5 cells per well. Following a 24-hour exposure to various levels of brusatol (0, 20, 40, 80 nM), the cells were gathered to detect ROS. In the same manner, the cells were exposed to RSL3 (5 nM) or ferrostatin-1 (50 nM) at a temperature of 37°C for a duration of 2 hours, and then treated with brusatol (20 nM) for 24 hours at 37°C. In order to identify ROS, the cells were exposed to 10 μM of 2,7-dichlorofluorescein diacetate (H2DCF-DA) and incubated at 37°C for one hour. Following two rounds of washing with PBS, the cells were suspended again in 200 μl of PBS. Flow cytometry (BD Biosciences) and Flowjo 7.6 software were utilized to examine the levels of ROS. For every condition, at least 10,000 cells were examined.

Iron assay

We determined the intracellular iron levels in different samples by utilizing an iron assay kit provided by Abcam (CAT #ab83366). To begin with 96-well plates were filled with cell lysates and standards. Assay buffer was also added to each well. The samples were then incubated at 37°C for 30 minutes. Following this, the samples were supplemented with iron probes and incubated at a temperature of 37°C for a further 60 minutes. At a wavelength of 593 nm, the colorimetric microplate reader was used to measure the OD value ultimately.

Transmission electron microscopy

T24 and 5637 cells were placed in six-well plates with a density of 2×10^5 cells per well and incubated for 12 hours at 37°C. Various levels of brusatol (0, 20, 40, 80 nM) were introduced into the culture dishes, and the cells were incubated for an extra 24 hours at a temperature of 37°C. In the same manner, the cells were exposed to RSL3 (5 nM) or ferrostatin-1 (50 nM) at a temperature of 37°C for a duration of 2 hours, and then treated with brusatol (20 nM) for 24 hours at 37°C. Following the procedures, the cells were collected and immobilized in a solution of 2% glutaraldehyde for a duration of 1 hour at ambient temperature. Afterwards, the specimens were treated with 2% uranyl acetate in a dimly lit environment for a duration of 10 minutes at ambient temperature. Subsequently, they were rinsed for a period of 20 seconds. Afterwards, the specimens were treated with lead citrate for a duration of 10 minutes at ambient temperature, followed by a subsequent rinse lasting 20 seconds. Ultimately, the specimens were identified within the electron microscopy facility at Renmin Hospital affiliated with Wuhan University.

Western blot analysis

The RIPA buffer solution (Beyotime Institute of Biotechnology, CAT #P0013B) was utilized to extract the entire cellular protein. To facilitate cell lysis, the samples underwent intermittent ultrasonic dispersion. Following centrifugation at a speed of 10,000 times the force of gravity for a duration of 15 minutes at a temperature of 4°C the liquid remaining after sedimentation was gathered for additional examination. The BCA assay was utilized to determine the protein concentration in the samples. Subsequently, the samples were combined with loading buffer and subjected to denaturation for a duration of 10 minutes. For electrophoresis, a 12% SDS-PAGE gel was used to load a total of 60 μg of protein. After the proteins

were separated, they were subsequently moved onto NC membranes (Millipore Sigma). To prevent nonspecific binding, the membranes were obstructed using 5% nonfat milk for 40 minutes at room temperature. Following that the membranes were then exposed to primary antibodies and incubated overnight at 4°C. After being washed the following day, the membranes were incubated with secondary antibodies at room temperature for a duration of 1 hour. Ultimately, the protein bands were observed by means of chemiluminescence detection on an Odyssey Laser Imaging System (LI COR Inc, USA).

Reverse transcription (RT)-PCR and Quantitative (q)PCR

The RNAiso Plus kit from TaKaRa Biotechnology (9108Q) was used to extract total RNA from T24 and 5637 cells. Next, the isolated RNA underwent reverse transcription utilizing the SuperScript First-strand Synthesis System provided by Invitrogen (K1621) in order to produce cDNA. The synthesized cDNA was stored at -20°C for future use. Real-time PCR reactions were performed using the Novo Start qPCR Super Mix Plus kit from novoprotein (#E096-01A). The levels of expression for the target genes were standardized using the internal control gene GAPDH. Pre-denaturation at 95°C for 30s; then enter the PCR reaction stage, the conditions are 95°C 5s to 60°C 30s, this stage cycle 40 times, and finally enter the lysis stage. The expression of genes was measuring the fold change compared to the control using the 2- $\Delta\Delta C_T$ method. Primers used for qPCR were listed as follows:

Gapdh: 5— *GTCTCTCTGAC* T *C* ∇ *CAGCG* — 3

Chac1: 5— *GTGGTGACGCTC* T *G* ∇ *GATC* — 3

Transfection

To perform siRNA transfection, T24 and 5637 cells were transfected with Chac1-siRNA that targets particular genes or with non-targeting siRNA as a negative control. This transfection was carried out for a duration of 48 hours using Lipofectamine 3000 reagent manufactured by Invitrogen (L3000001). The utilized siRNA sequence was 5'-CCAAGAUGCUCUGACCAACC-3'. The growth medium was replaced with basic medium once the cell density reached 70–80%. The siRNA was combined with Lipofectamine 3000 reagent according to the guidelines provided by the manufacturer, and then introduced into the cells. Afterward, the cells were placed in an incubator set at a temperature of 37°C for a duration of 6 hours. Following the incubation period, the medium was substituted with a full medium. Follow-up interventions and experimental testing were conducted after 72 hours.

Immunohistochemistry (IHC)

IHC is a technique used to visualize specific proteins in tissue samples. After being fixed, the tissue was embedded in paraffin and then sectioned to a thickness of 4 μ m. Dewaxing and rehydration were performed on the sections. Boiling was used to perform antigen retrieval on the sections. Endogenous peroxidase/phosphatase activity was blocked. Primary and secondary antibodies were added and incubated. Next, the sections underwent treatment with DAB chromogen followed by counterstaining using hematoxylin.

Xenograft mouse model

The experiment involved the utilization of ten male BALB/c-nude mice, all of which were 4 weeks old. The mice were kept in conditions that were free from specific pathogens (SPF). The temperature in the room was kept at a range of 26–28°C, while the humidity level was maintained between 40% and 60%. A light/dark cycle of 10 hours of light and 14 hours of darkness was maintained on a daily basis for the mice. Feed should normally be kept in special containers and placed in a ventilated dry place. Sterilized water should be changed 2–3 times a week to ensure uninterrupted drinking. Firstly, the BCa cell line T24 is more malignant compared with 5637 cells. Secondly, Yu et al (unpublished data) found that the BCa cell line, T24, had a higher rate of subcutaneous tumorization than 5637. The T24 cell line was selected for the in vivo test because it is appropriate as a BCa cell line, ensuring the reliability of the results obtained from this cell line. T24 cells were injected subcutaneously into mice at a concentration of 1×10^6 cells per mouse. Three mice were randomly allocated into two groups, consisting of nude mice. The experimental groups were administered intraperitoneal injections of brusatol at a dosage of 2 mg/kg every 48 hours, whereas the control groups were given injections of normal saline. Following a treatment duration of 5 weeks, the mice were administered anesthesia and subsequently euthanized to facilitate additional analysis. Sodium pentobarbital (30 mg/kg) was administered as the anesthetic drug. The euthanasia used for the mice is carbon dioxide induction. Initially airflow should be constant at a carbon dioxide concentration of 30–70%V/min for roughly 1 min. Also the airflow should be maintained for at least 1 min after clinical death to avoid reversal. In accordance with regulations on the welfare of laboratory animals, the humane endpoint for completing these experiments was that tumors do not exceed $2,000 \text{ mm}^3$ in volume or 20 mm in diameter in any one dimension in mice. The mice were all dissected to remove the tumors. The weight and size of each tumor were measured. Afterwards, the tumors were immobilized in 4% PFA and underwent IHC staining to facilitate subsequent analysis.

Statistical analysis

The information is presented as the average plus or minus the standard deviation of duplicate samples. The tests were conducted on three occasions. Statistical analyses were conducted using the IBM Corp. SPSS software package version 22.0.

Results

T24 and 5637 cells are less viable and more proliferative after treatment with brusatol

After being exposed to various concentrations (0, 20, 40, 80 nM) of brusatol for a duration of 24 hours, the CCK-8 test was employed to evaluate the survival rates of T24 and 5637 cell lines derived from human bladder cancer. Both cell lines exhibited a reduction in cell viability that was dependent on the dose, in comparison to the control cells (Fig. 2A). For the time-dependent assay, a concentration of 20 nM was employed. As expected, the T24 and 5637 cell lines derived from human bladder cancer exhibited a

reduction in viability over time upon exposure to brusatol. The decrease in viability exhibited a notable distinction when compared to the control cells (Fig. 2B). Treatment with brusatol resulted in a dose-dependent decrease in colony formation in T24 and 5637 cells (Fig. 2C). According to the EdU assay, brusatol significantly inhibited the proliferation of BCa cells (Fig. 2D). In vitro, the viability and proliferation of BCa cells were effectively decreased by brusatol treatment in a manner that depended on the dose and time.

The migration and invasion of T24 and 5637 cell are inhibited by brusatol

In order to examine the effects of brusatol on the progression of BCa cells, T24 and 5637 cells are exposed to varying doses of brusatol. Figure 3A showed that brusatol treatment resulted in a dose-dependent decrease in invasion in both cell lines, as observed in transwell assays. Furthermore, the brusatol treatment resulted in a notable decrease in cell migration as observed in the wound healing assay, once again in a manner that depended on the dosage (Fig. 3B). Taken together, these results suggest that brusatol efficiently inhibits the migratory and invasive capacities of BCa cells.

The cells T24 and 5637 are susceptible to ferroptosis when exposed to brusatol.

Ferroptosis is a phenomenon marked by the buildup of reactive oxygen species (ROS) caused by lipid peroxidation and the dysfunction of GPX4 enzyme [16]. The experiment demonstrated that the administration of brusatol led to a gradual rise in ROS/Fe²⁺/MDA concentrations and a decline in GSH levels in both T24 and 5637 cells (Fig. 4A-D). To explore the role of ferroptosis in the suppression of cell survival caused by brusatol, T24 and 5637 cells were exposed to the ferroptosis-inducing agent RSL3 or the ferroptosis-blocking compound ferrostatin-1 for a duration of 24 hours at a temperature of 37°C. The findings demonstrated that RSL3 mitigated the reduction in cell viability caused by brusatol, whereas ferrostatin-1 restored the cell viability (Fig. 4E). Moreover, the utilization of flow cytometry demonstrated that the administration of brusatol notably enhanced the buildup of reactive oxygen species (ROS) in T24 and 5637 cells (Fig. 4F). Treatment with brusatol additionally resulted in elevated levels of intracellular Fe²⁺ (Fig. 4G) MDA (Fig. 4H), indicating lipid peroxidation. Furthermore, brusatol markedly decreased the levels of GSH in T24 and 5637 cells (Fig. 4I). In conclusion, the impact of brusatol on mitochondrial structure was investigated using transmission electron microscopy. The findings showed that brusatol significantly caused the vanishing of mitochondrial cristae (Fig. 4J). Collectively, these findings indicated that brusatol induced BCa cell ferroptosis in vitro.

In vitro, brusatol triggers the Chacl/Nrf2/SLC7A11 pathway specific to glutathione and activates the ChaC gamma-glutamylcyclotransferase. The ferroptosis of BCa cells induced by brusatol can be reversed by suppressing Chacl.

GPX4, an enzyme that converts reduced G to its oxidized form called GSSG [17]. Neutralizes ROS (reactive oxygen species). Western blot analysis was conducted to examine brusatol on the protein expression

associated with ferroptosis. In both T24 and 5637 cells, the findings indicated that the administration of brusatol led to a notable reduction in the levels of FTH1, SLC7A11, and GPX4 expression. Moreover, the impact of brusatol treatment [18, 19] extended to Chac1 and Nrf2 levels, recognized as indicators of ferroptotic cell demise. In line with the attributes of ferroptosis, the findings from this investigation demonstrated that brusatol markedly enhanced the levels of Chac1 (both protein and mRNA) while decreasing the levels of Nrf2 in T24 and 5637 cells (Fig. 5A). The expression of Chac1 was subsequently reduced in T24 and 5637 cells (Fig. 5B). Knocked down expression of Chac1 significantly rescued the brusatol induced reduction in cell viability (Fig. 5C) illustrates a reduction in the levels of ROS/Fe²⁺/MDA in T24 and 5637 cells (5D-F). Meanwhile, GSH levels were increased (Fig. 5G). Mitochondrial morphology was examined by transmission electron microscopy. The findings indicated that reduced Chac1 expression resulted in a decline in the vanishing of mitochondrial cristae (Fig. 5H). The downregulation of Chac1 led to a significant increase in the expression of FTH1, SLC7A11, GPX4, and Nrf2 (Fig. 5I). Taken together, these results indicate that brusatol enhances the build-up of reactive oxygen species (ROS) in BCa cells and triggers ferroptosis by increasing the expression of Chac1. The signaling pathway Chac1/Nrf2/SLC7A11 has been demonstrated to have a vital function in controlling ferroptosis mediated by ROS.

In vivo, the growth of xenografts is inhibited by brusatol.

After observing the suppressive impact of brusatol on the viability of BCa cells, we proceeded to examine the effect of brusatol on T24 cell xenografts in BALB/c nude mice. According to the illustration in Fig. 6A and B, the group of mice treated with brusatol showed a notable reduction in tumor size. Cell proliferation in the tumor tissues was evaluated by performing IHC staining for Ki67. Upon treatment with brusatol, the findings revealed a significant suppression of BCa cell growth in the tumor tissues (Fig. 6C). Moreover, the group of mice treated with brusatol exhibited an upregulation of Chac1 expression and a downregulation of GPX4 expression (Fig. 6D and E). Collectively, the aforementioned findings indicated that brusatol markedly inhibited tumor growth in vivo.

Discussion

At the time of the first diagnosis, BCa, the second most prevalent malignancy in the urinary system is predominantly identified as NMIBC. The use of tobacco is a major factor that contributes significantly, along with exposure to hydrocarbons in the workplace, genetic vulnerability, and the consumption of drinking water contaminated with arsenic for NMIBC, the majority of patients experience recurrence but progression to MIBC is rare. As a result, TURBT is widely regarded as the primary therapy for NMIBC, with the potential addition of adjuvant bladder instillation therapy [20]. Conversely, individuals identified with MIBC experience a reduced five-year survival rate and diminished quality of life in contrast to those diagnosed with NMIBC[3]. Chemotherapy is a crucial component in the management of BCa. A research conducted by Liu and colleagues. The study showed that the use of immune checkpoint inhibitors, antibody-drug conjugates, and VEGF inhibitors in combination with chemotherapy drugs has the ability to improve both the rate of response and the overall survival of patients with BCa[21]. However, tumor cells

can develop drug resistance due to drug stimulation and reduce the efficacy. Preoperative chemotherapy, also referred to as neoadjuvant chemotherapy (NAC), has the potential to eradicate micrometastases, lower the TNM stage of the tumor, enhance the likelihood of bladder-sparing surgery, boost patient survival rates, and decrease the risk of mortality[22]. Nevertheless, NAC may result in a delay in surgery for BCa patients who do not respond favorably to NAC, potentially increasing the risk of mortality. Hence, it is crucial to prioritize the creation of efficient and personalized treatment approaches to enhance the outlook for individuals with BCa.

Dixon et al. initially identified ferroptosis as a unique type of programmed cell death. Furthermore, it has been linked to the development of numerous human illnesses, such as cancers[11]. Ferroptosis is often characterized by the presence of excessive iron buildup within cells and the oxidation of lipids, which are widely acknowledged as significant aspects. In terms of morphology, cells undergoing ferroptosis display changes in the structure of mitochondria, such as decreased size, heightened density of the mitochondrial membrane, and the breakdown or lack of mitochondrial cristae. In terms of biochemistry, ferroptosis is distinguished by the buildup of reactive oxygen species (ROS), increased amounts of lipid peroxides and Fe^{2+} , and the suppression of GPX4 and the cystine/glutamate antiporter system (xCT) [23, 24]. GPX4, belonging to the glutathione peroxidase group, is a protein associated with ferroptosis and can be suppressed by RSL3, an inducer of ferroptosis. By catalyzing the conversion of GSH to its oxidized form, GSSG, GPX4 prevents ferroptosis by inhibiting lipid peroxidation and transforming dangerous lipid peroxides into harmless lipid alcohols, as well as converting H_2O_2 to H_2O [25]. SLC7A11, a component of the xCT system, is also closely associated with ferroptosis. Multiple studies in BCa have shown that the dysregulation of GPX4 and SLC7A11 plays a role in triggering ferroptosis, as evidenced by previous research[26–28]. The distinguishing factors for ferroptosis, in comparison to other forms of regulated cell death, are these characteristic features.

Extensive research has been conducted on brusatol, a natural herbal medicine extract for the treatment of different types of tumors such as non-small cell lung cancer (NSCLC), glioblastoma, and pituitary adenomas[29–31]. brusatol demonstrates strong antineoplastic properties in these studies, either by directly impacting cancer cells or augmenting the efficacy of chemotherapy drugs. In NSCLC, a study identified 793 brusatol-binding potential proteins and found that brusatol could directly bind with Skp1. Consequently, brusatol-Skp1 hindered the functioning of the Skp2-SCF E3 ligase and β -TRCP-SCF E3 ligase by interfering with the interaction between Skp1 and the F box protein Skp2[29]. Moreover, the suppressive effect on cell growth and ability to spread to other parts of the body by brusatol has been linked to the buildup of p27 and E-cadherin. A different investigation indicated that brusatol inhibited the growth of glioblastoma cells by negatively controlling the expression of EMC1. Additionally, reducing EMC1 further increased the suppressive impact of brusatol[30]. Brusatol has demonstrated the ability to hinder the proliferation of pituitary adenoma cells and trigger apoptosis in both laboratory and living organisms when considering pituitary adenomas. The modulation of the mTORC1 signaling pathway and the buildup of ROS [31] mediate these impacts. Moreover, the pairing of brusatol and cabergoline has demonstrated an augmented antitumor impact in both pituitary tumor cells and nude mice. Brusatol has

shown the capacity to hinder cell growth in laryngeal cancer by triggering apoptosis and halting the cell cycle in the S phase. Moreover, brusatol has been found to inhibit the process of EMT by suppressing the JAK2/STAT3 signaling pathway. [32]. Additionally, brusatol functions as a crucial Nrf2 inhibitor in the process of ferroptosis[33]. Nevertheless, the complete impact and fundamental mechanism of brusatol in BCa remain unclear and necessitate additional exploration. The study observed that brusatol caused a decrease in viability of T24 and 5637 BCa cell lines, which was dependent on its concentration. Furthermore, the use of brusatol effectively suppressed the growth and invasiveness of these cells. The results indicate that brusatol may have the capability to efficiently suppress the growth and spread of BCa cells. In order to examine the possible triggering of ferroptosis by brusatol, different concentrations of brusatol were administered to T24 and 5637 cells for investigation. The results demonstrated a dose-dependent induction of ferroptosis by brusatol. In particular, the administration of brusatol resulted in: 6 significant reduction in the cellular expression of GPX4, causing the buildup of reactive oxygen species (ROS), iron ions (Fe^{2+}), and malondialdehyde (MDA), whereas the concentration of glutathione (GSH) noticeably declined. Moreover, the combination of brusatol with RSL3 intensified the effects, while the use of ferrostatin-1 rescued cell viability that was compromised by brusatol treatment. Furthermore, the findings demonstrated that brusatol caused a gradual reduction in mitochondrial cristae, which was dependent on the dosage. Taken together, these findings indicate that brusatol is capable of inducing ferroptosis in BCa cells in vitro.

Chac1 belongs to the gamma-glutamyl cyclotransferase family, which specifically breaks down reduced GSH into cysteinylglycine and 5-oxoproline, while not affecting GSSG. Chac1 possesses r-glutamyl cyclotransferase activity and serves as an antioxidant molecule by eliminating GSH[34]. Therefore, Chac1 can affect ferroptosis in humans. Chac1 plays an important physiological role in individual development. Furthermore, elevated expression of the Chac1 gene was detected in various types of tumors and showed a positive association with unfavorable prognosis[19, 35]. Wang et al. discovered that artesunate has the potential to act as a potent growth inhibitor and induce ferroptosis in human Burkitt's lymphoma this effect is mediated through the ATF4-CHOP-Chac1 pathway[36]. Moreover, the activation of the ATF4-Chac1 pathway was observed in glioma cells when exposed to sevoflurane[35]. Dihydroartemisinin was found to trigger cell demise by inducing ferroptosis in primary liver cancer, and the treatment directly boosted the Chac1 promoter's effectiveness [37]. These researches suggested that Chac1 also has an important pathophysiological role. The study found that brusatol had a significant impact on increasing the expression of Chac1 in BCa cells. According to the authors' understanding, this discovery is being reported here for the first time. Subsequently, an experimental cell line with reduced Chac1 expression was employed to investigate the exact mechanism. Knocking down the expression of Chac1 caused a notable rise in the expression of GPX4 and SLC7A11. This rescue of cell viability decline caused by brusatol also resulted in elevated ROS levels. This implies that Chac1 plays a role in controlling ferroptosis. Moreover, it is widely recognized that Nrf2, a regulatory protein, has a vital function in the mechanism of ferroptosis. Several research studies have indicated that brusatol has the ability to function as a suppressor of Nrf2. However, the relationship between brusatol and Nrf2 in BCa has yet to be explored[38, 39]. Therefore, the expression level of Nrf2 was examined after treatment with brusatol to verify this hypothesis. The

obtained results showed a decrease in the expression of Nrf2 after brusatol treatment. The reduction in Nrf2 expression was accompanied by a simultaneous decrease in the level of Chac1 expression. Furthermore, Nrf2 has been documented to control the manifestation of SLC7A11. Therefore, it appears likely that brusatol activated the Chac1/Nrf2/SLC7A11 pathway to trigger ferroptosis

In order to validate the findings of the current research, experiments were conducted on BALB/c nude mouse xenografts to ascertain the impact of brusatol. The study findings revealed that the brusatol-treated group exhibited notably reduced tumor volume in comparison to the control group. This suggests that brusatol has a significant inhibitory effect on tumor growth in vivo. In order to evaluate cell proliferation in various groups, an IHC analysis was conducted, which demonstrated that brusatol significantly inhibited the proliferation of BCa cells in vivo. After treatment with brusatol, the expression of Chac1 was observed to have increased, whereas the level of GPX4 decreased in contrast to the control group. These findings further support the hypothesis that brusatol can induce ferroptosis in vivo.

Conclusion

The findings suggest that brusatol demonstrates an anti-cancer impact on BCa cells by promoting ferroptosis in both laboratory and animal experiments. The research results indicate that brusatol triggers ferroptosis by controlling the Chac1/Nrf2/SLC7A11 pathway. Hence, brusatol holds promise as a potential therapeutic option for treating BCa in clinical environments.

Declarations

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Not applicable.

Author contributions

Xi Yu and Huaxin Wang: Substantial contributions to the conception or design of the work; Xi Yu: Substantial contributions to the acquisition, analysis, and interpretation of data for the work; Ziqi He and Qinghua Wang: Substantial contributions to drafting the work and revising it critically for important intellectual content; Shuai Ke: Substantial contributions to final approval of the version to be published; Shenglan Li and Zhong Wang: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Shenglan Li and Zhong Wang confirm the authenticity of all the raw data.

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Data availability

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

Ethics approval and consent to participate

The animal study was reviewed and approved by the Ethics Committee of Renmin Hospital of Wuhan University (Ethics No. 20220703A).

Consent for publication

All listed authors have actively participated in the study and have read and approved the submitted manuscript.

Competing interests

The authors declare that they have no competing interests.

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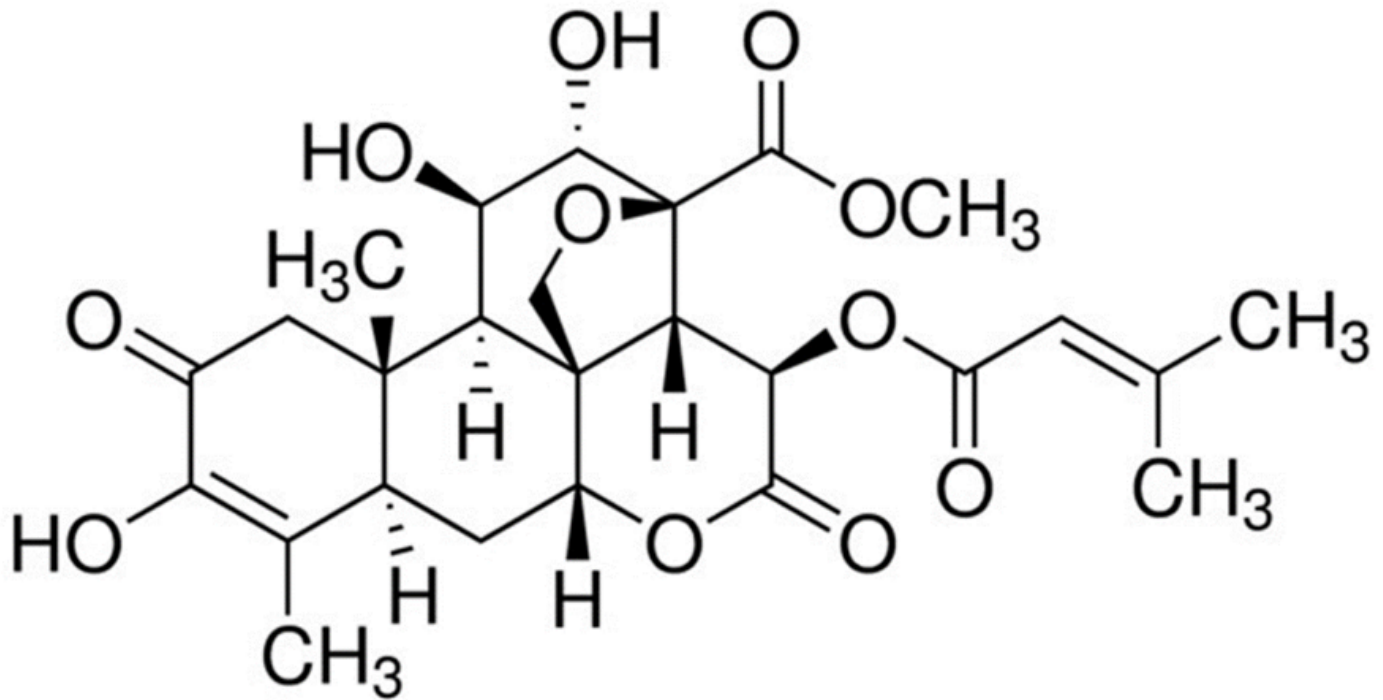
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- ## Figures



Chemical formula of brusatol.

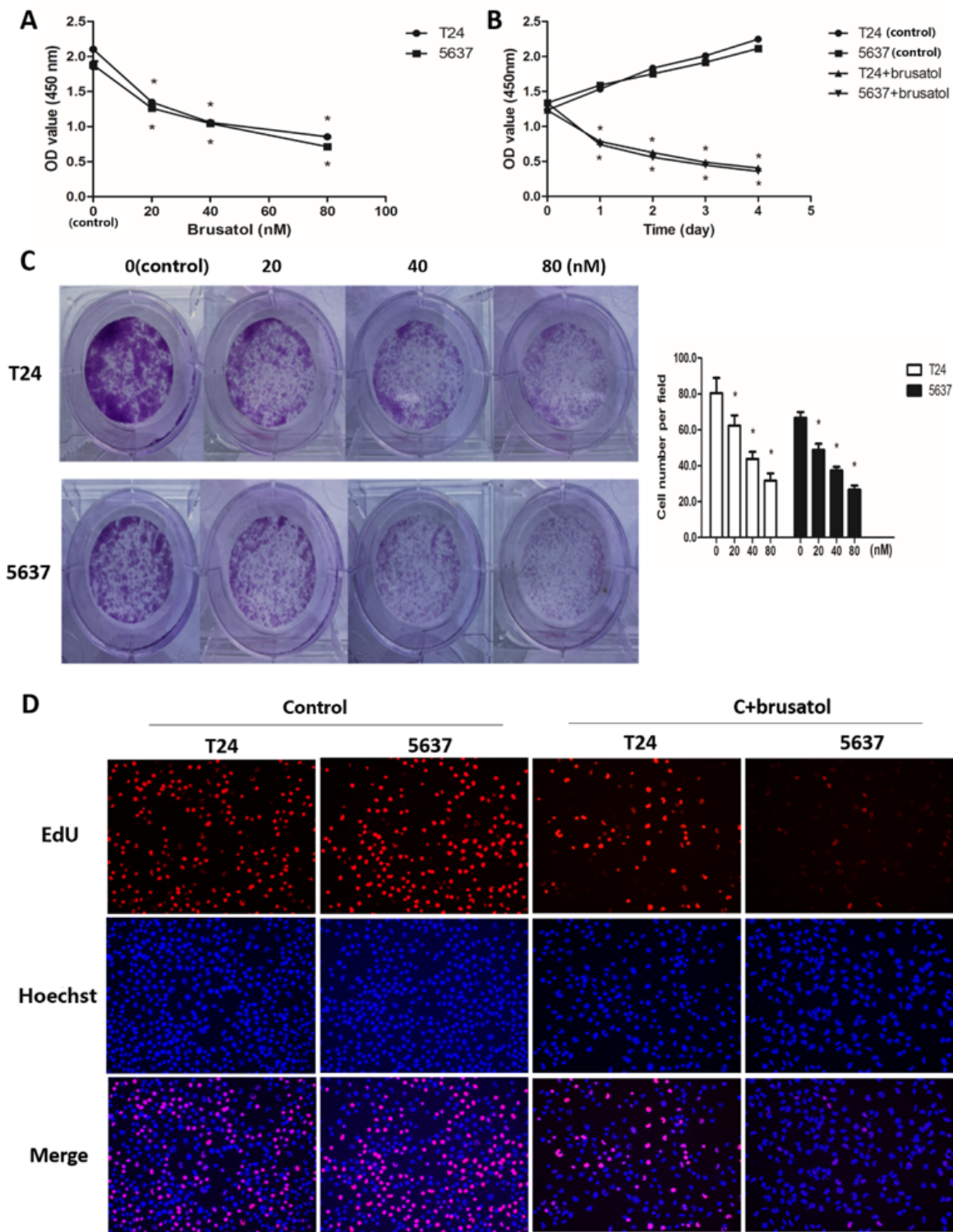


Figure 2

In vitro, brusatol controls the survival and growth of bladder cancer cells. (A) Treatment with brusatol resulted in a dose-dependent decrease in the proliferation of T24 and 5637 cells, as evidenced by CCK-8 assays. (B) Similarly, the CCK-8 assays indicated that the growth of T24 and 5637 cells was reduced by brusatol in a time-dependent manner. (C) Additionally, the colony formation assay demonstrated a significant decrease in the average number of colonies formed by T24 and 5637 cells due to brusatol. (D)

Moreover, the proliferation of T24 and 5637 cells was significantly inhibited by brusatol, as observed in the EdU assay under a fluorescence microscope (magnification, x200). The data is displayed as the average±standard deviations of three separate trials. Significant difference ($P<0.05$) compared to the control group. CCK-8, also known as Cell Counting Kit-8, and EdU, which stands for 5-ethynyl-2'-deoxyuridine.

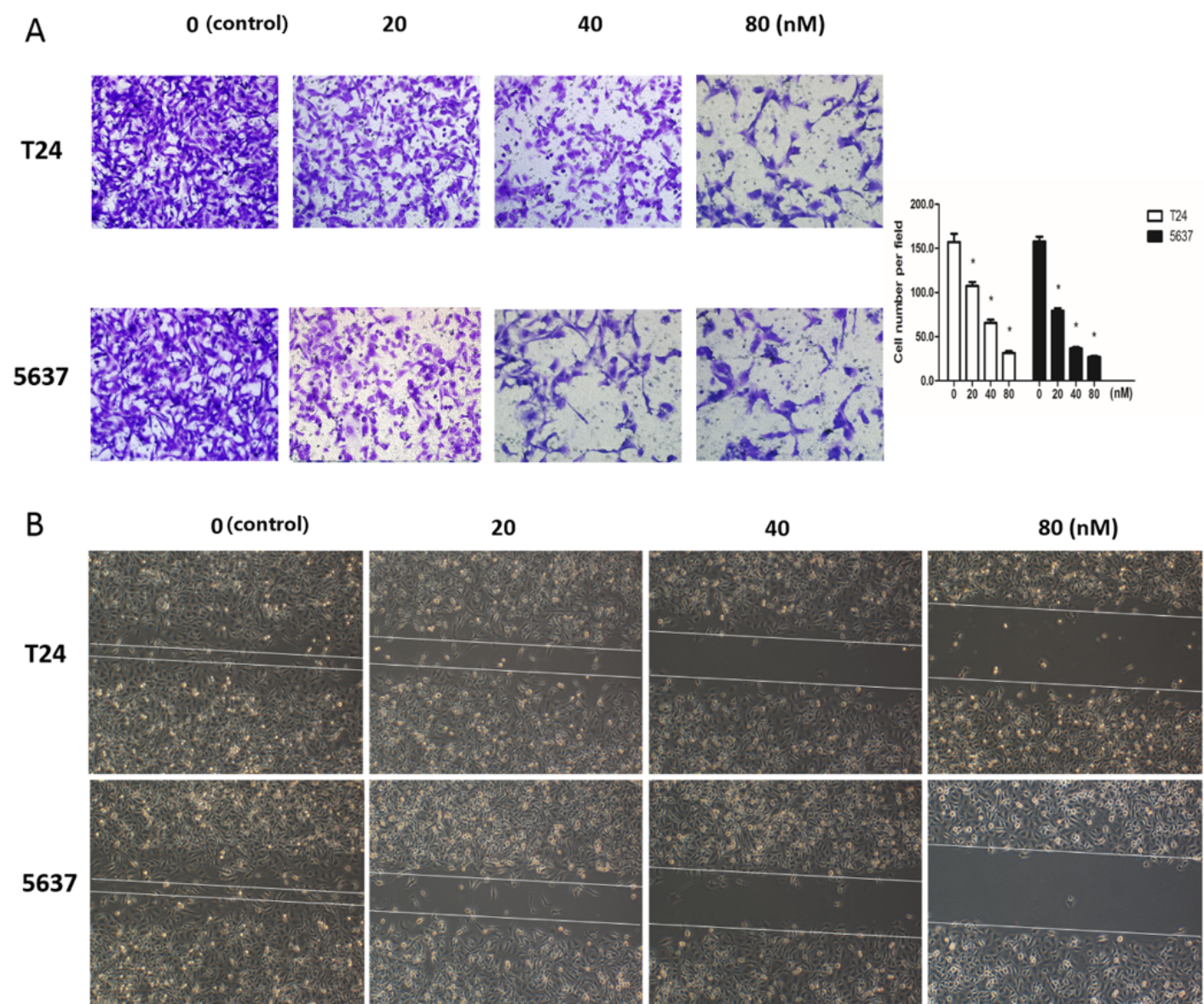


Figure 3

In vitro, brusatol controls the movement and infiltration of cells associated with bladder cancer. (A) Treatment with brusatol significantly reduced the invasion of T24 and 5637 cells, as shown by the transwell assay (magnification, x200). (B) The wound healing experiments demonstrated that the migration of T24 and 5637 cells was reduced in a dose-dependent manner with brusatol treatment (magnification, x40). The data is displayed as the average±standard deviations of three separate trials. Significant difference ($P<0.05$) compared to the control group.

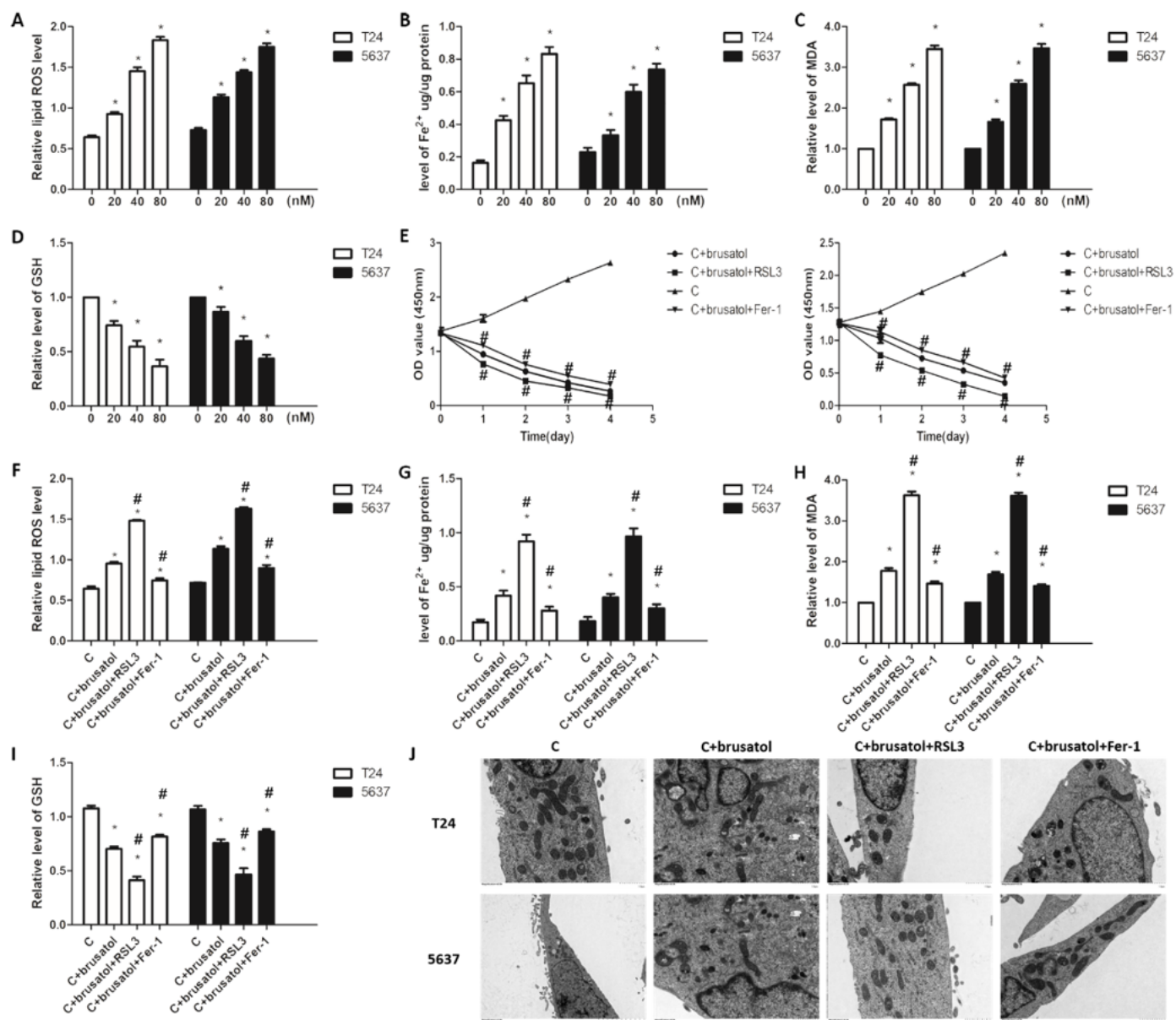


Figure 4

The use of brusatol triggers ferroptosis in cells of bladder cancer. (A) Flow cytometry analysis showed that the concentrations of reactive oxygen species (ROS) in T24 and 5637 cell treated with brusatol were elevated in a manner dependent on the dosage, when compared to the control group. Compared to the control group, the levels of Fe^{2+} (B) and malondialdehyde (MDA) (C) in brusatol treated cells showed a dose-dependent increase. Cells treated with brusatol showed a dose-dependent reduction in the level of GSH (D). RSL3 (5 nM) or ferrostatin-1 (50 nM) and 20 nM brusatol were administered to the T24 and 5637 cells. (E) The control group consisted of the 0-nm group. Cell viability was evaluated by performing Cell Counting Kit-8 experiments. The c group was the label given to the control group. (F) Flow cytometry was used to assess the levels of reactive oxygen species (ROS) in T24 and 5637 cells. The c group was the label given to the control group. The Iron Assay Kit was used to analyze the concentrations of Fe^{2+} (G). The control group was designated the c group. The MDA levels were examined using a kit designed

for MDA analysis (H). The control group was designated as the c group. (I) The GSH levels were examined utilizing a GSH Assay Kit. The control group was designated as the c group. (J) Under a transmission electron microscope, the presenge of mitochondrial cristae was eliminated by the administration of brusatol. The control group was designated as the c group. The data is displayed as the average±standard deviations of three separate trials. The control group is compared to the C+brusatol group with a significance level of $P<0.05$. Additionally, the C+brusatol+RSL3 and C+brusatol+Fer-1 groups are also compared to the C+brusatol group with a significance level of $P<0.05$. ROS refers to reactive oxygen species, MDA stands for malondialdehyde, GSH represents glutathione, and C denotes control.

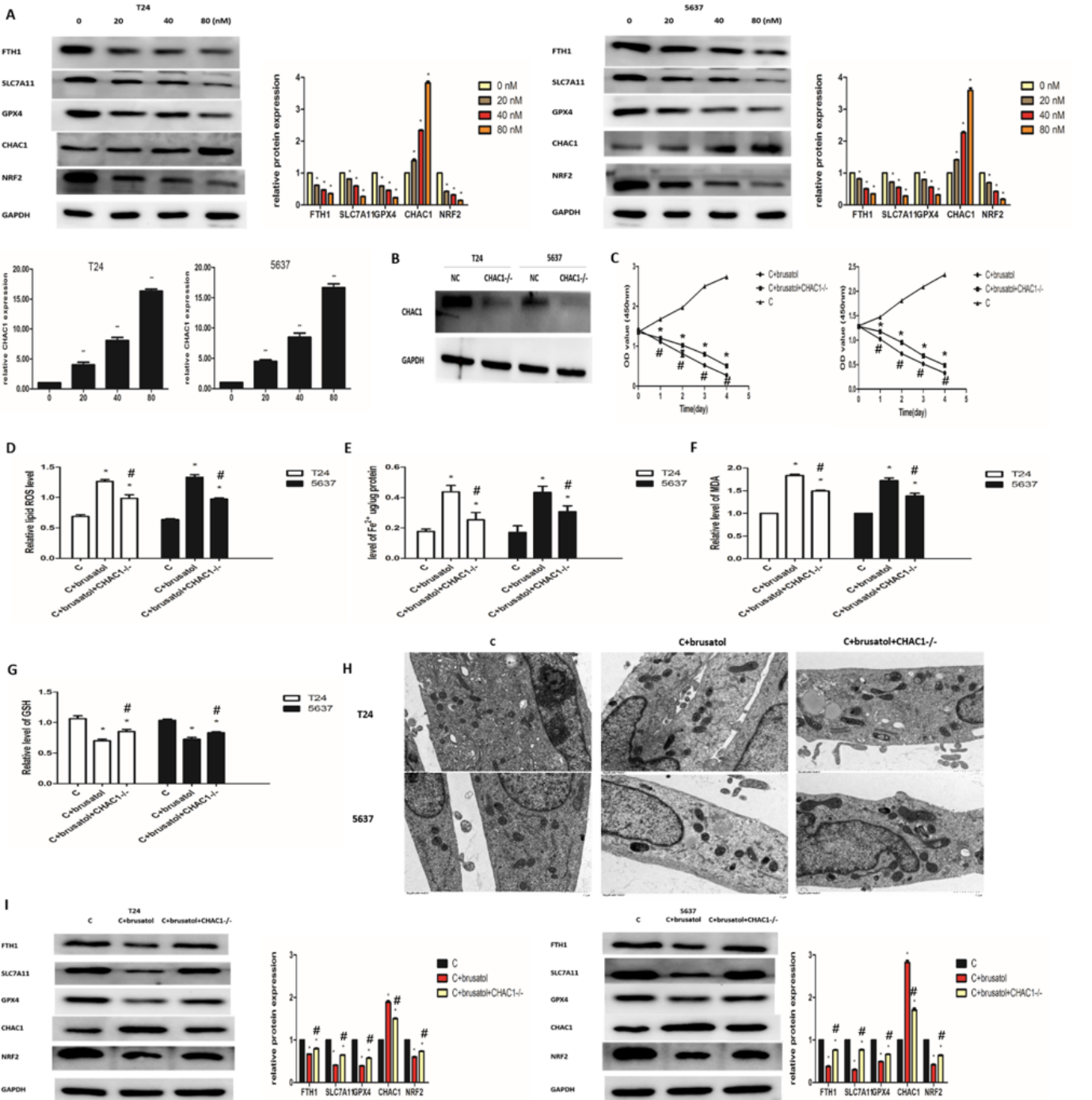


Figure 5

In bladder cancer cells, the upregulation of Chac1 by brusatol leads to the induction of ferroptosis. (A) Western blot analysis was used to evaluate the expression of FTH1, SLC7A11 and GPX4, Chac1, and Nrf2 in T24 and 5637 cells. Furthermore, the qPCR analysis was conducted to evaluate the mRNA expression of Chac1. (B) The Western blot analysis confirmed the reduction of Chac1 expression in T24 and 5637 cells. (C) Cell viability was terminated by performing Cell Counting Kit-8 experiments. (D) Flow cytometric analysis was used to measure the levels of reactive oxygen species (ROS) in T24 and 5637 cells. (E) The Iron Assay Kit was used to analyze the concentrations of Fe^{2+} . (F) The MDA levels were examined utilizing a Kit designed for MDA Assay. (G) The GSH levels were examined by utilizing a GSH Assay Kit. (H) Mitochondrial cristae were observed under a transmission electron microscope. (I) Western blot analysis in T24 and 5637 cells showed that the decrease in Chac1 expression led to an upregulation of FTH1, SLC7A11, GPX4, and Nrf2 expression. The data is displayed as the average $\pm$ standard deviations of three separate trials. Significant difference ($P < 0.05$) compared to the control group. The C+brusatol group is compared to the C+brusatol+CHAC1-/-group, with a significance level of $P < 0.05$. Chac1 and Chac2 are enzymes that specifically transfer the gamma-glutamyl group to glutathione; GPX4 is an enzyme that catalyzes the reduction of hydrogen peroxide using glutathione as a cofactor; Nrf2 is a transcription factor that is related to erythroid 2 and regulates gene expression; MDA refers to malondialdehyde; GSH stands for glutathione; C represents the control group.

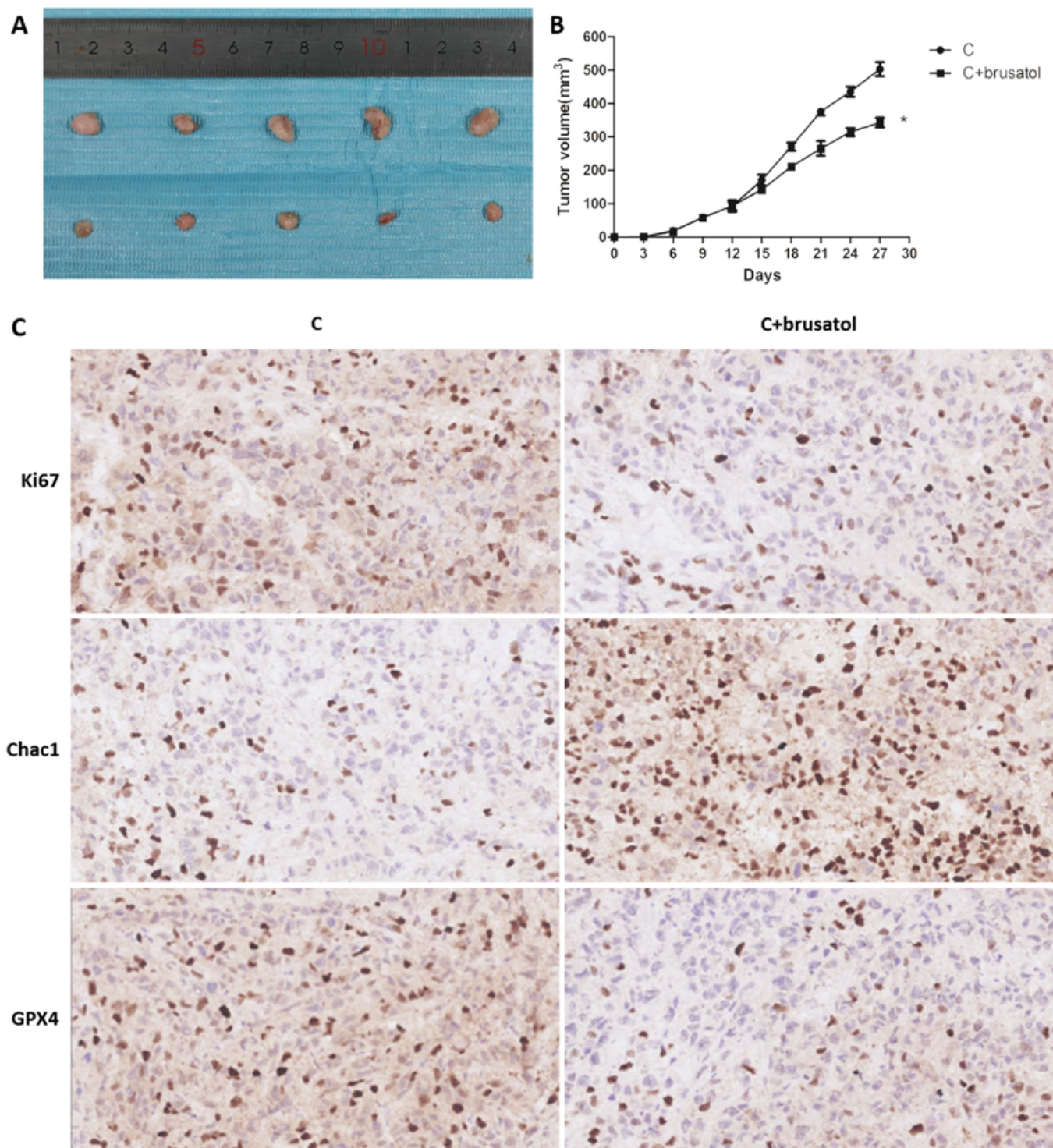


Figure 6

In vivo, the growth of bladder cancer is suppressed by brusatol. (A) The structure of the tumor implanted under the skin was analyzed. (B) Tumor volume was measured at every time point to determine the average. (C) Protein expression of Ki67, Chac1, and GPX4 in the tumor tissue was detected using immunohistochemistry. The control group was compared to determine statistical significance, with a $*P < 0.05$ threshold. The data is displayed as the average $\pm$ standard deviation from three experiments.

Chac1, ChaC glutathione-specific gamma-glutamylcyclotransferase; GPX4, glutathione peroxidase 4; C, control.

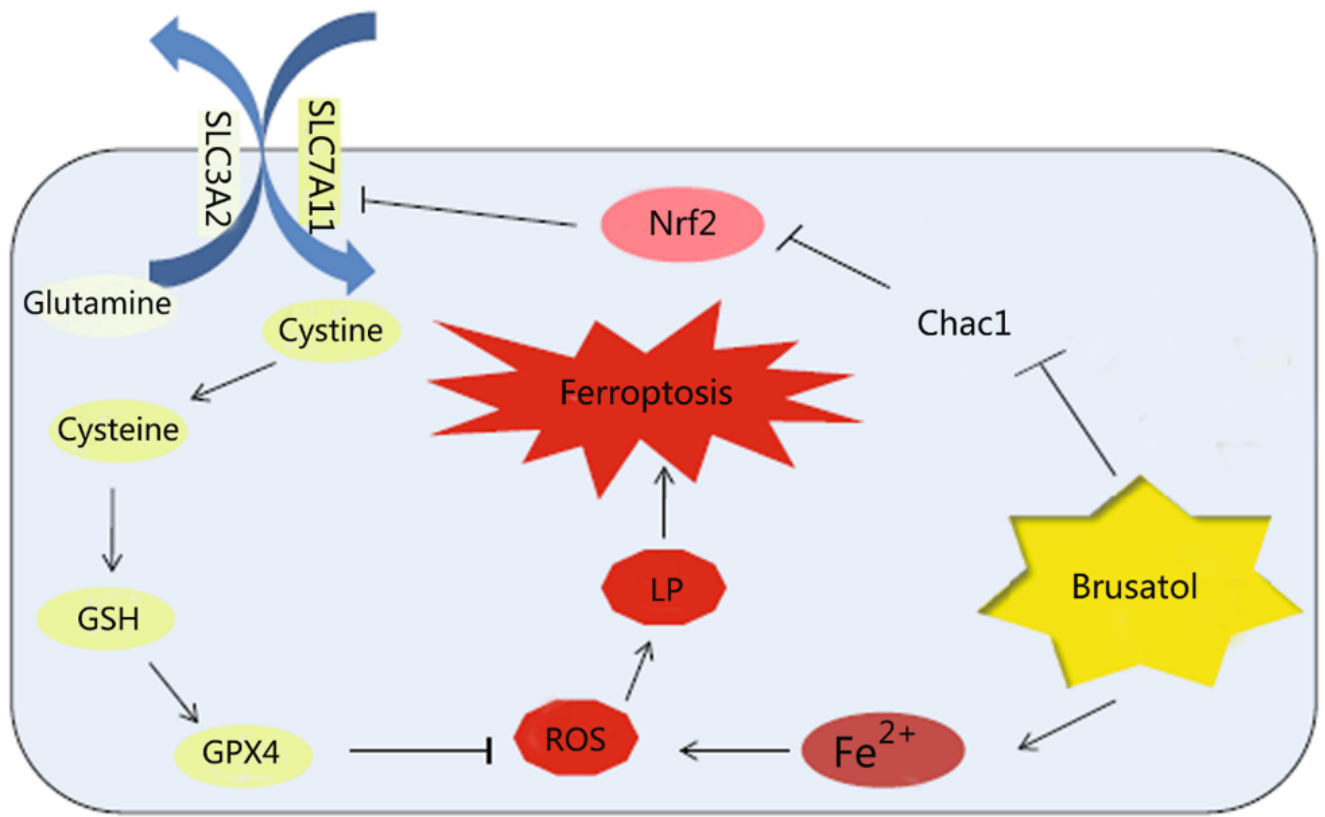


Figure 7

Schematic diagram displays potential mechanism of brusatol-induced ferroptosis in bladder cancer cells.

Nrf2, nuclear factor erythroid 2-related factor 2; Chac1, ChaC glutathione-specific gamma-glutamylcyclotransferase; GSH, glutathione; LP; lipid peroxidation; GPX4, glutathione peroxidase 4; ROS, reactive oxygen species.