Pegylated recombinant human arginase 1 induces autophagy and apoptosis via the ROS-activated AKT/mTOR pathway in bladder cancer cells

Zhuyun Zhao  
Shenzhen Longhua District Central Hospital

Peng Zhang  
Shenzhen Luohu People's Hospital

Wei Li  
Shenzhen Longhua District Central Hospital

Dengchuan Wang  
Shenzhen Longhua District Central Hospital

Changneng Ke  
Shenzhen Longhua District Central Hospital

Yueming Liu  
Shenzhen Longhua District Central Hospital

James Chung-Man Ho  
University of Hong Kong

Paul Ning-Man Cheng  
Bio-cancer Treatment International

Shi Xu  
Shenzhen Longhua District Central Hospital  
https://orcid.org/0000-0002-2178-5666

Research

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Abstract

Background

Bladder cancer is one of the dominant cancers worldwide, especially for male. Currently, the therapeutical regimen of bladder cancer is based on surgery, radiation therapy, chemotherapy and immunotherapy, but the clinical outcome is still needed to improve. Recombinant human arginase (rhArg, BCT-100) is a novel agent to show great anticancer effect on arginine auxotrophic tumor. However, the effect of rhArg on bladder cancer still remains unclear.

Methods

A panel of six bladder cancer cell lines (BIU-87, EJ-1, J82, SCaBER, T24 and 5637) was employed to assess the anticancer effect of BCT-100 \textit{in vitro} by MTT assay. T24 nude mice xenograft models were established to evaluate the anticancer effect of BCT-100 \textit{in vivo}. Protein level (argininosuccinate synthetase 1 (ASS1), ornithine transcarbamylase, cleaved-PARP, PEG, Survivin, p62, Beclin-1, LC3B, p-AKT, p-mTOR) was detected by Western blot. Intracellular, serum and intratumoral arginine concentrations were examined by ELISA. Apoptotic rate, H$_2$O$_2$ and mitochondrial membrane depolarization were tested by flow cytometer. Immunofluorescence on ki67 and TUNEL assay were applied to identify cellular and tumoral apoptotic events.

Results

BCT-100 displayed anticancer effects on bladder cancer cells \textit{in vitro} and \textit{in vivo}. The expression of ASS1 varies in different bladder cancer cell lines, and ornithine transcarbamylase is almost deficient except weakly expressed in SCaBER cell line. Knockdown ASS1 in BIU-87 cells could enhance the cytotoxicity induced by BCT-100. Intracellular arginine level was sharply decreased followed by apoptotic events. Furthermore, BCT-100 induced H$_2$O$_2$ production and mitochondrial membrane depolarization, leading to cytochrome c and smac released from mitochondria to cytosol. The expression of LC3B and Beclin-1 was up-regulated, while p62 was down-regulated in a time dependent manner. Autophagic flux was also observed upon BCT-100 treatment. Besides, the phosphorylation of AKT/mTOR pathway was suppressed in a time dependent fashion in BCT-100-treated T24 cells. N-Acetyl-L-cystein reduced the apoptosis and autophagy induced by BCT-100, while CQ, MK-2206 and rapamycin potentiated the apoptosis triggered by BCT-100.

Conclusions

The present study demonstrated that BCT-100 induced autophagy and apoptosis via ROS mediated AKT/mTOR signaling pathway in bladder cancer cells.
Background

Bladder cancer is one of the most prevalent diseases worldwide, especially for males. It was estimated that bladder cancer accounts for approximately 3.0% of all new cancer diagnoses and 2.1% of cancer deaths (1). Urothelial cell carcinoma is the most common diagnosed subtype in all bladder cancer cases, following with squamous cell carcinomas (2). Similar to lung cancer, using tobacco is the leading risk factor for bladder cancer, however, other risk contributors including arsenic exposure, chlorine and family heredity also matter (3–5). Currently, four options of standard treatments are used for bladder cancer patients, including surgery, radiation therapy, chemotherapy and immunotherapy (6, 7). The first line strategy for bladder cancer is drug combination based on cisplatin and gemcitabine, especially the emerging of immunotherapy, which improves the overall survival rate. However, the nearly half of patients cannot benefit this treatment because of renal insufficiency or cancer cachexia (6, 8). Therefore, it is still urgent to develop novel and safe therapeutic approaches for bladder cancer to improve the clinical outcomes.

Arginine is a semi-essential amino acid for humans, which exerts multifaceted functions in cellular activities, including cell growth, metabolism and survival (9). Arginine is an essential precursor for the synthesis of protein, nitric oxide, polyamines, creatinine and nucleotides (10). In-depth research into various aspects on tumor metabolism reveals metabolic therapy might be a promising option for cancer treatment (11). It has been demonstrated that certain cancer cells (arginine auxotrophy) cannot synthesize arginine independently and are reliant on extracellular arginine for growth (12, 13). Therefore, arginine deprivation might be an Achilles’ Heel for arginine auxotrophic cancers, including hepatic cell carcinoma, prostate cancer, melanoma, small cell lung cancer and leukemia (12–16).

Theoretically, arginine could be metabolized by five enzymatic agents (i.e., nitric oxide synthase, glycine amidinotransferase, arginine decarboxylase, arginine deiminase (ADI) and arginase) to induce arginine depletion. Considering the drug efficacy, immunogenicity, half-time and potential byproducts, only pegylated ADI and arginase were employed to therapy for arginine auxotrophic cancers (17). Accumulating evidences believed that argininosuccinate synthase (ASS1) and ornithine transcarbamylase (OTC) were two important enzymes in urea cycle, which affected the efficacy of arginine depletors greatly (13, 18, 19).

The agent we used in this study is BCT-100, which is a classic recombinant human arginase. Consistent with endogenous arginase, BCT-100 catalyzes arginine to ornithine and urea, leading to arginine depletion (19). It has been demonstrated that BCT-100 showed significant anticancer effects against several arginine auxotrophic cancers and completed clinical trials on HCC with promising outcomes (12, 13, 20). Few studies have emphasized the role of BCT-100 on bladder cancer. Hereby, we explored the underlying mechanisms of arginine deprivation with BCT-100 in bladder cancer as a potential therapeutic approach.

Materials And Methods
Cell lines and culture

A panel of six bladder cell lines (BlU-87, EJ-1, J82, SCaBER, T24 and 5637) and one non-small cell lung cancer cell line (A549) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained on RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco®, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Reagents

BCT-100 was kindly provided by Bio-cancer Treatment International Limited, Hong Kong. N-Acetyl-L-cystein (NAC, Cat No, A7250) and chloroquine (CQ, Cat NO, C6628) were purchased from Sigma. MK-2206 (Cat NO, SF2712-5mg) and Rapamycin (Cat No, S1842-25mg) were bought from Beyotime Biotechnology.

Western blot analysis

Treated cells were harvested, washed and resuspended in NP-40 lysis buffer (Beyotime Biotechnology, Jiangsu, China) with addition of protease inhibitor (1 mM phenylmethylsulphonyl fluoride) for 1hr. A tissue protein extraction reagent kit purchased from Thermo was used to extract protein from xenografts. Supernatants were collected after centrifugation (13000 rpm, 4°C, 30 min). For mitochondrial and cytosolic protein extraction, cell mitochondria isolation kit was purchased from Beyotime Biotechnology. Protein concentration was measured by Bradford Protein Assay Kit (Bio-Rad, Berkeley, CA, USA). 30-60 µg protein was loaded into 8-15% sodium dodecyl sulfate polyacrylamide gel electrophores (SDS–PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked for 1hr at room temperature in PBS containing 5% non-fat dry milk plus 0.1% Tween-20 (PBST) and incubated overnight at 4°C with monoclonal or polyclonal primary antibodies [ASS1, OTC, cleaved PARP (Santa Cruz Biotechnology, CA, USA), PEG (RevMAb, San Francisco, USA), b-Actin (Sigma-Aldrich), cytochrome C, Smac, COX IV, p-AKT, AKT, p-mTOR, mTOR and Survivin (Cell Signaling Technology, MA, USA)]. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, MA, USA) were applied and membranes incubated for 1hr at room temperature. Detection was conducted using an enhanced chemiluminescence (ECL) kit (GE Healthcare). Quantification was performed using GelQuantNET software (Biochem Lab Solutions, CA, USA).

Cell viability assay

SCLC cells were seeded in a 96-well plate, approximately 10^4 per well. After drug exposure, 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) (Sigma–Aldrich) was added to each well for 3hr, followed by triple lysis solution (10% Sodium dodecyl sulfate, 5% isobutanol, 0.012 mol/L HCl in water), 100 µL per well for 2hr. Optical density was measured at 570 nm using a microplate reader Fluo Star Optima (BmgLabtec GmbH, Ortenberg, Germany).

Arginine concentration detection
The arginine level in cells, serum and tumor was measured using an L-arginine ELISA kit purchased from Abcam (Cat NO, ab241028). The procedure was performed according to the manufacturer's instructions. In brief, derivatized samples and standards were incubated with L-arginine antibody overnight at 4°C, followed by incubation with peroxidase conjugate for 1 hr at room temperature, tetramethybenzidine substrate for 10 min in the dark. Wash buffer was needed between every step and stop solution added to halt the reaction. Absorbance (450 nm), using a reference (620 nm), was measured using a microplate reader Fluo Star Optima.

**Short hairpin RNA (shRNA) transfection**

Silencing of ASS1 (shRNA) was using a lentiviral particles kit bought from Santa Cruz. In brief, pretreatment was with polybrene prior to addition of lentiviral particles and incubation for 24 hr. Mixture was then removed to complete medium for a further 48hr incubation. Puromycin dihydrochloride was used to select the stable ASS1-silenced cell line. Corresponding protein was detected by Western blot and cell viability after shRNA was confirmed by MTT assay.

**Autophagic flux analysis**

Autophagic flux in T24 cell was detected by using the mRFP-GFP-LC3 adenovirus (Hanbio, China). After plating the cells in a 6-well plate at a density of 1×10^5 cells/dish and incubating with mRFP-GFP-LC3 adenovirus for 24 hr, the cells were treated with or without BCT-100 (20 mU/mL) for 24 hr. Autophagic flux was observed under an inverted fluorescent microscope (Zeiss, Germany). The yellow puncta indicated autophagosomes, and the red puncta indicated autolysosomes.

**Flow cytometry of Annexin V/PI staining**

Apoptosis was determined by flow cytometry using a FITC-conjugated annexin V/PI kit (Beyotime Biotechnology, Jiangsu, China). Briefly, cells were harvested, washed, and resuspended in binding buffer provided in the kit. After 15 min incubation of annexin V/PI at room temperature, samples were measured using a BD FACSaria II analyzer with FL2/FL4 channels (BD, New Jersey, USA).

**Reactive oxygen species (ROS) measurement**

2’,7’-dichlorodihydro-fluorescein diacetate (H2DCFDA) (Beyotime Biotechnology, Jiangsu, China) were employed to test hydrogen peroxide (H_2O_2). Briefly, treated cells were harvested, washed and incubated with H2DCFDA (1 µM) in medium without FBS for 30 min at 37°C, washing two times following flow cytometry analysis. GSH content was measured by 5-chloro-methylfluorescein diacetate (CMFDA) (Invitrogen). Treated cells were collected, washed and incubated with CMFDA (5 µM) for 30 min at 37°C in FBS-free medium, then changed to complete medium for a further 30 min incubation prior to flow cytometry analysis.

**Tumor suppression effect of BCT-100 using xenograft models**
Xenograft models were established by subcutaneous injection of $10^7$ cells into nude mice (4-5 weeks, 10-14g, female, BALB/cAnN-nu, Laboratory Animal Centre of Shenzhen University, Guangdong, China). When the tumor reached a mean group size of 40–60 mm$^3$, mice were randomized to one of three groups. PBS and BCT-100 (20 and 60 mg/kg) were administered intraperitoneally twice a week. Tumor size was calculated according to the following formula ($\text{Size} = \text{Length} \times \text{Width} \times \text{Depth}/2$) (21). The relative tumor volume (RTV) was calculated by the formula: $\text{RTV} = \frac{V_n}{V_0}$, where $V_n$ was tumor size on day $n$ and $V_0$ was tumor size on the first day of treatment. Mice were sacrificed when tumor volume reached 600 mm$^3$, which was regarded as a humane endpoint. The study protocol was approved by the laboratory animal ethical committee of Guangdong Medical University.

**Terminal deoxynucleotidyl transferase-dUTP Nick End Labeling (TUNEL) assay**

TUNEL assay was conducted using a Click-iT® Plus TUNEL Assay kit (Beyotime Biotechnology, Jiangsu, China) according to the standard protocol provided by the manufacturer. Cell crawling, fixation and permeabilization were required before TUNEL assay. Cells were immersed in terminal deoxynucleotidyl transferase (TdT) reaction buffer, then changed to new TdT reaction buffer that contained EdUTP, TdT and TdT enzyme. Samples were incubated with TUNEL reaction cocktails followed by 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) staining. Images were captured using a Zeiss fluorescence microscope.

**Immunofluorescence staining**

Fixed and permeabilized cell or tumor sections were blocked with 3% bovine serum albumin (BSA) for 1 hr at room temperature, followed by incubation with ki67 antibody overnight at 4°C. After washing with PBST for 30 min, Alexa Fluor anti-rabbit (Life Technologies) antibody was applied followed by incubation for 1 hr protected from light. The slides were mounted with Prolong® Gold anti-fade reagent with DAPI (Life Technologies). Pictures were obtained using a Zeiss fluorescence microscope.

**Statistical analysis**

All data were obtained from at least three independent experiments and are shown as mean ± standard deviation (SD). Statistical analysis of data was performed using Student's two-tailed t-test by Prism5 (GraphPad Software, La Jolla, Southern California, USA).

**Results**

**Sensitivity of bladder cancer cells to BCT-100 correlated with the expression of ASS1 and OTC**

The cell viability of BCT-100 in six bladder cancer cell lines (BIU-87, EJ-1, J82, SCaBER, T24, and 5637) and one lung cancer cell line (A549, positive control) was evaluated by CCK-8 assay. All cell lines were exposed to increasing concentrations of BCT-100 for 3 days. T24 cell line was the most sensitive to BCT-100 treatment. IC$_{50}$ values of these cell lines range from 20 mU/mL (T24) to 200 mU/mL (SCaBER) (Fig.
ASS1 and OTC are considered as two important enzymes for arginine biosynthesis in the urea cycle. The basal expression of ASS1 and OTC protein expression levels were tested in a panel of using Western blot. ASS1 expression was relatively high in A549, BIU-87 and 5637 cells, while OTC showed high expression in SCaBER and A549 cells (Fig. 1B).

In order to explore the role of ASS1 in BCT-100 exposure, we employed shRNA lentiviral package to knockdown ASS1 in BIU-87 cells. Knockdown of ASS1 increased sensitivity to BCT-100 in BIU-87 cells (Fig. 1C), and cleaved poly ADP-ribose polymerase (C-PARP) was up-regulated in the ASS1-silenced group after BCT-100 exposure for 3 days (Fig. 1D).

T24 cell line was selected as model cell line to study the underlying mechanism in the following experiments because it was relatively sensitive to BCT-100 treatment.

**BCT-100 induced cytotoxicity accompanied by arginine decrease in bladder cancer cells**

BCT-100 is one type of recombinant human arginase conjugated to polyethylene glycol (PEG), which was used to indicate the accumulation and location of BCT-100 in vitro and in vivo. PEG was present upon BCT-100 exposure in T24 cells in a dose-dependent manner (Fig. 2A), while intracellular arginine concentration was significantly decreased in a dose dependent manner (Fig. 2B). Meanwhile, cleaved PARP (C-PARP) was also up-regulated to present the cytotoxicity induced by BCT-100 treatment (Fig. 2C). At the same time, we observed an increase in apoptotic cells evidenced by Annexin-V/PI staining (Fig. 2D) and TUNEL assay (Fig. 2E). The apoptotic rate was increased sharply from 4.0±2.4% (control) to 32.0±6.3% (20 mU/mL), 48.0±4.1% (40 mU/mL) in T24 cells.

**Effect of BCT-100 on ROS production, mitochondrial membrane depolarization and mitochondria-dependent apoptosis**

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a probe used to indicate intracellular hydrogen peroxide (H$_2$O$_2$). We found that BCT-100 could significantly induce H$_2$O$_2$ production upon BCT-100 exposure for 24hr (Fig. 3A). The 5-chloromethylfluorescein diacetate (CMFDA) dying was employed to present the GSH level, which was decreased in a dose dependent manner upon BCT-100 treatment for 24hr (Fig. S1). Mitochondrion is the essential organelle for ROS production. JC-1 staining showed that mitochondrial membrane depolarization occurs in T24 cells upon BCT-100 exposure for 3 days (Fig. 3B). Smac and cytochrome c (Cyt-c) are two biomarkers play a crucial role in mitochondrial dependent apoptosis. Smac and Cyt-c were decreased in a dose dependent fashion in mitochondrial extraction, while they presented up-regulation manner in cytosolic protein (Fig. 3C, 3D).

**Autophagy induction and AKT/mTOR pathway activation after BCT-100 exposure**

T24 cells were transfected with mRFP-GFP-LC3 adenovirus to test autophagy flux induced by BCT-100. As shown in Fig. 4A, the number of red and yellow (merged) puncta was remarkably increased in BCT-100 treatment arms. The autophagy related biomarkers Beclin-1 and LC3B were up-regulated in a time-
dependent manner, while p62 was down-regulated upon BCT-100 stimulation (Fig. 4B). It has been considered that AKT-mTOR pathway exert important role in cell proliferation and autophagy. We thus observed that total and phosphorylated AKT and mTOR under BCT-100 treatment. Both phosphorylated AKT (Ser473) and mTOR (Ser2448) were inhibited in a time dependent fashion (Fig. 4C).

**BCT-100-induced ROS initiate cellular apoptosis and autophagy in bladder cancer cells**

ROS is a key regulator in cellular apoptosis and autophagy. And we have observed that BCT-100 indeed induced ROS production upon BCT-100 exposure. To investigate the role of ROS in BCT-100 mediated apoptosis and autophagy, we employed that ROS scavenger (N-Acetyl-L-cystein, NAC) in our study. As shown in Fig. 5A, the cellular ROS level was significantly reduced in combination group compared with BCT-100 alone group (21.5±3.3% Vs 44.2±12.4%), and the apoptotic rates in combination group and BCT-100 arm were 14.1±5.9% and 39.6±7.4%, respectively (Fig. 5B). Moreover, NAC inhibited the expression of C-PARP as well as LC3B, but promoted p62 expression in combination arm (Fig. 5C). The autophagy inhibitor (Chloroquine, CQ) was recruited to clarify the role of autophagy in BCT-100 treatment. CQ combined with BCT-100 could significantly induce cellular apoptosis, since C-PARP was up-regulated and Survivin was decreased in combination group (Fig. 5D). Furthermore, specific AKT and mTOR inhibitors (MK-2206 and Rapamycin) potentiated the cytotoxicity triggered by BCT-100, as C-PARP was increased and Survivin was decreased in combination arm (Fig. 5E, F).

**Suppression of tumor growth by BCT-100 in T24 xenograft models**

In T24 xenograft models of nude mice, BCT-100 (60 mg/kg) significantly blocked tumor growth rate compared with the control group (Fig. 6A). Median survival was prolonged from 25.0 days (control arm) to 26.5 days (20 mg/kg arm), and 31.5 days (60 mg/kg arm) in T24 xenograft models (Fig. 6B). In line with in vitro study, we subsequently examined the accumulation of PEG as well as apoptotic biomarkers in BCT-100 treatment arms in xenograft models, and PEG and C-PARP were increased sharply while Survivin was decreased accordingly (Fig. 6C). Besides, serum arginine concentration in control, 20 mg/kg, and 60 mg/kg arm was 72.7±14.4 µM, 11.8±3.5 µM, 5.6±2.6 µM, respectively (Fig. 6D). Similarly, intratumoral arginine level in T24 xenograft models declined dramatically from 10.8±1.1 µM (control) to 4.7±0.5 µM (20 mg/kg), 1.6±0.3 µM (60 mg/kg) (Fig. 6E). Ki67 is a biomarker associated with cell proliferation. We found that the fluorescence intensity of Ki67 was decreased in a dose dependent manner after BCT-100 treatment by immunofluorescence assay (Fig. 6F).

**Discussion**

Pegylated recombinant human arginase 1 (BCT-100) depleted intracellular and intratumoral arginine sharply and displayed an anti-proliferative effect on tumor growth in bladder cancer cells. The underlying mechanisms were mainly involved in apoptosis and autophagy, which were related to oxidative stress activated AKT/mTOR cell signaling pathway. In bladder cancer xenograft models, high dose of BCT-100 (60 mg/kg) inhibited tumor growth and prolonged median survival remarkably.
Amino acid deprivation has been considered as a promising approach for cancer therapy, because of dysregulation of cellular metabolism in cancer cells, which require more nutrients to support their proliferation. Arginine, asparagine, glutamine, methionine and serine are important amino acids for certain auxotrophic cancers tumor growth (17). One of the most successful examples is L-asparaginase induces asparagine depletion on acute leukemia treatment. Similarly, arginine depletion therapy has been recognized as another encouraging therapeutic strategy for cancer treatment in recent years.

As a targeted and metabolic anticancer therapy, the efficiency of arginine deprivation is related to the expression of ASS1 and OTC, two key enzymes in arginine biosynthesis (22). As reported in previous studies, the ASS1 and/or OTC deficient cell lines with were relatively vulnerable to arginine depletors. For instance, it has been illustrated that silencing ASS1 enhanced the sensitivity to ADI treatment in small cell lung cancer (SCLC) cells (18, 23). Of note, knockdown ASS1 in H69 cells (SCLC) did not show significant differences to BCT-100 treatment, but blocking OTC sensitized H841 cells (SCLC) to BCT-100 exposure (13). In our study, we observed that knockdown ASS1 in BIU-87 cells increased sensitivity to BCT-100 (Fig. 1C, 1D), which was consistent with our hypothesis. Nevertheless knockdown OTC in SCaBER cells did not increase sensitivity to BCT-100 (data not shown). It might be the weak basal expression of OTC in SCaBER cells, and there are non-canonical pathways circumvent OTC to regenerate arginine, but solid evidences are needed to support our view.

The underlying mechanisms of arginine depletors might vary from cancer cell types. ADI, the most well-studied arginine depletory agent, induced mitochondrial dysfunction, ROS production as well as autophagy in prostate cancer (24). The Warburg Effect was involved after ADI treatment in leiomyosarcoma and melanoma (25). Akin to the use of ADI in cancer therapy, the rhArg also demonstrated tumor inhibition effects through autophagy induction, oxidative stress and cell cycle arrest in breast cancer, hepatocellular carcinoma and acute myeloid leukemia (19, 26, 27). Therefore, it is necessary to discuss the mechanism of BCT-100 in bladder cancer cell for better clinical outcomes.

Reactive oxygen species (ROS) homeostasis is critical to steady biological functions of cells. The imbalance of ROS production and metabolism leads to oxidative stress, which is responsible for several pathological processes, such as autophagy, apoptosis, inflammation, DNA damage, aging and neurological diseases (28). Numerous chemotherapeutic drugs exert anti-tumor effects via ROS-dependent cytotoxicity in cancer cells in vitro and in vivo (29–31). In our study, ROS production was boosted and glutathione (GSH) was correspondingly decreased in a dose dependent fashion after BCT-100 stimulation. Besides, the ROS scavenger NAC could eliminate ROS level thus restore the cell viability. Mitochondrion is an important organelle in various biological activities including energy generation, signal transduction, cell differentiation and apoptosis (28). Since mitochondrion is one of the major sources of ROS, we postulated mitochondrial function was affected and BCT-100 induced mitochondrial dependent apoptosis. Consistent with our previous findings, JC-1 staining assay revealed that mitochondrial membrane depolarization (MMD) occurred upon BCT-100 treatment. Concurrently, upon mitochondrial stress, cytochrome C as well as Smac are released from mitochondria to cytosol, indicating
mitochondrial mediated apoptosis initiated. Taken together, excessive ROS production regulated the mitochondrial dependent cytotoxicity by BCT-100 exposure in bladder cancer cells.

Autophagy is an important component in cellular activity in which cytoplasmic materials are directly degraded by lysosomes (32). Cancer cells benefit from exploiting autophagy in stressful conditions such as nutrient deprivation, hypoxia and oxidative stress, but excessive autophagy activities also lead to cell death (33). Due to the two-faced role of autophagy in cancer therapy as a savior or an executioner, it is necessary to determine the function of autophagy in bladder cancer cells after BCT-100 treatment. In this study, our findings revealed that BCT-100 led to up-regulation of Beclin-1 and LC3B, down-regulation of p62 as well as the occurrence of autophagic flux, suggesting autophagy was activated. The autophagy inhibitor CQ potentiated the cytotoxicity triggered by BCT-100, implying the protective role of autophagy in BCT-100-treated T24 cells. Consistent with our findings, the prosurvival role of autophagy of rhArg has been also demonstrated in laryngeal squamous cell carcinoma (34). However, autophagy activated by HuArgI (Co)-PEG5000 leads to autophagic cell death in pancreatic cancer cells (35). AKT-mTOR signaling pathway is known to regulate apoptosis and autophagy, and activation of this pathway provides cells with high growth rate. In the present study, BCT-100 inhibited phosphorylated-AKT and mTOR, without affecting the total protein level, suggesting BCT-100 mediated this signaling pathway. In addition, NAC significantly recover the phosphorylation of AKT thus restore the cell viability. Besides, MK-2206 and rapamycin both enhanced the cytotoxicity induced by BCT-100, implying the crucial role of AKT-mTOR signaling pathway in underlying mechanism.

It is essential to study the anti-cancer effects of BCT-100 in xenograft model, because arginine deprivation offers a systemic effect which requires the tumor microenvironment. BCT-100 suppressed the tumor growth and prolonged the survival time with arginine depletion and tumoral apoptosis. The dosage (20 mg/kg and 60 mg/kg) we used in present study was relatively low in comparison with other malignant tumors models (26, 36), because of the susceptibility to BCT-100 treatment. Furthermore, the body weight of nude mice in medication groups did not markedly decrease contrasted with the control group (data not shown), indicating the side effect of BCT-100 might be acceptable. The most common adverse reaction of cisplatin and gemcitabine in patients with bladder cancer includes vomiting, nephrotoxicity and myelosuppression (37, 38), and it might be an option for BCT-100 combined with chemotherapeutics to diminish the side effect. With nice tolerance in preclinical trials, BCT-100 might potentially serve as a good alternative therapeutic agent in clinical management of bladder cancer.

Many clinical trials of rhArg have initiated in cancer patients including leukemia, melanoma, HCC and prostate cancer. The early phase clinical trial of rhArg has completed in patients with HCC. The patients who received adequate arginine depletion intervention benefit a lot compared with those having less arginase treatment (median progression-free survival, 6.4 vs 1.7 months, 95% CI: 1.67–1.73, p = 0.01) (39). More research findings on ADI clinical trials are available. It has been reported that ADI could prolong progression-free survival in patients with ASS1-deficient mesothelioma (3.2 vs 2.0 months), and the overall survival in ADI and control group was 15.7 and 12.3 months, respectively (40). The side effects of arginine deprivation therapy were relatively slight including liver dysfunction, neutropenia,
fatigue and nausea (20, 40). But formal phase III clinical trial of ADI on advanced HCC did not show an overall survival benefit in second line treatment (41). Further clinical trials of arginine deprivation in cancer patients are still underway.

Nevertheless, there are several limitations in our study. We did not employ NAC, CQ and rapamycin to confirm the corresponding functions in vivo. It would be more reasonable to use PEG as normal control in present study, since BCT-100 is modified PEG, although it is known to be inert to cells (42). Besides, it is necessary to find out non-canonical pathways to regenerate arginine, which provides convincing evidence to support our hypothesis.

**Conclusions**

In conclusion, we demonstrated that recombinant human arginase (BCT-100) displayed good anticancer effects against bladder cancer via apoptosis and autophagy regulated by ROS-activated AKT/mTOR signaling pathway. This provides a scientific ground for future clinical development of recombinant human arginase in the treatment of bladder cancer.

**Abbreviations**

BCT-100, Bio-Cancer Treatment 100; PEG, polyethylene glycol; C-PARP, cleaved poly (ADP-ribose) polymerase; ASS1, argininosuccinate synthetase; OTC, ornithine transcarbamylase; ADI, arginine deiminase; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases.

**Declarations**

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**Authors’ contributions**

Zhuyun Zhao, Peng Zhang, Wei Li and Dengchuan Wang performed the laboratory experiments; Changneng Ke, Yueming Liu, James Chung-Man Ho directed and corrected the study; Paul Ning-Man Cheng provided the needed reagent; and Shi Xu proposed and designed the research as well as drafted the manuscript. All the authors approved the manuscript for publication.

**Ethics approval and consent to participate**
Approval for all the nude mice xenograft experiments was obtained from laboratory animal ethical committee of Guangdong Medical University.

**Consent for publication**

Not applicable.

**Conflicts of interest:** Zhuyun Zhao, Peng Zhang, Wei Li, Dengchuan Wang, Changneng Ke, Yueming Liu, James Chung-Man Ho, Shi Xu report no potential conflict of interest. Paul Ning-Man Cheng is the Chief Executive Officer of Bio-Cancer Treatment International Limited and holds stocks or shares in Bio-Cancer Treatment International Limited.

**References**

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

2. Foth M, Ismail NFB, Kung JSC, Tomlinson D, Knowles MA, Eriksson P, et al. FGFR3 mutation increases bladder tumourigenesis by suppressing acute inflammation. J Pathol. 2018;246(3):331–43.

3. Kiriluk KJ, Prasad SM, Patel AR, Steinberg GD, Smith ND. Bladder cancer risk from occupational and environmental exposures. Urol Oncol. 2012;30(2):199–211.

4. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol. 2013;63(2):234–41.

5. Wang Y, Zou S, Zhao Z, Liu P, Ke C, Xu S. New insights into small-cell lung cancer development and therapy. Cell Biol Int. 2020.

6. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F. Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. Eur Urol. 2017;71(1):96–108.

7. Schneider AK, Chevalier MF, Derre L. The multifaceted immune regulation of bladder cancer. Nat Rev Urol. 2019;16(10):613–30.

8. Katz H, Wassie E, Alshareidi M. Checkpoint inhibitors: the new treatment paradigm for urothelial bladder cancer. Med Oncol. 2017;34(10):170.

9. Fultang L, Vardon A, De Santo C, Mussai F. Molecular basis and current strategies of therapeutic arginine depletion for cancer. Int J Cancer. 2016;139(3):501–9.

10. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37(1):153–68.

11. DeBerardinis RJ, Lum JJ, Hatziavassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab. 2008;7(1):11–20.
12. De Santo C, Booth S, Vardon A, Cousins A, Tubb V, Perry T, et al. The arginine metabolome in acute lymphoblastic leukemia can be targeted by the pegylated-recombinant arginase I BCT-100. Int J Cancer. 2018;142(7):1490–502.

13. Xu S, Lam SK, Cheng PN, Ho JC. Recombinant human arginase induces apoptosis through oxidative stress and cell cycle arrest in small cell lung cancer. Cancer Sci. 2018;109(11):3471–82.

14. Cheng PN, Lam TL, Lam WM, Tsui SM, Cheng AW, Lo WH, et al. Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. Cancer Res. 2007;67(1):309–17.

15. Kim RH, Coates JM, Bowles TL, McNerney GP, Sutcliffe J, Jung JU, et al. Arginase deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Res. 2009;69(2):700–8.

16. Ott PA, Carvajal RD, Pandit-Taskar N, Jungbluth AA, Hoffman EW, Wu BW, et al. Phase I/II study of pegylated arginase deiminase (ADI-PEG 20) in patients with advanced melanoma. Invest New Drugs. 2013;31(2):425–34.

17. Zou S, Wang X, Liu P, Ke C, Xu S. Arginine metabolism and deprivation in cancer therapy. Biomed Pharmacother. 2019;118:109210.

18. Kelly MP, Jungbluth AA, Wu BW, Bomalaski J, Old LJ, Ritter G. Arginase deiminase PEG20 inhibits growth of small cell lung cancers lacking expression of argininosuccinate synthetase. Br J Cancer. 2012;106(2):324–32.

19. Wang Z, Shi X, Li Y, Fan J, Zeng X, Xian Z, et al. Blocking autophagy enhanced cytotoxicity induced by recombinant human arginase in triple-negative breast cancer cells. Cell Death Dis. 2014;5:e1563.

20. Yau T, Cheng PN, Chan P, Chen L, Yuen J, Pang R, et al. Preliminary efficacy, safety, pharmacokinetics, pharmacodynamics and quality of life study of pegylated recombinant human arginase 1 in patients with advanced hepatocellular carcinoma. Invest New Drugs. 2015;33(2):496–504.

21. Epenetos AA, Kousparou C, Filipovic A. Generation of a selectively cytotoxic fusion protein against p53 mutated cancers. Cancer research. 2016;76.

22. Lam TL, Wong GK, Chong HC, Cheng PN, Choi SC, Chow TL, et al. Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest. Cancer letters. 2009;277(1):91–100.

23. Qiu F, Huang J, Sui M. Targeting arginine metabolism pathway to treat arginine-dependent cancers. Cancer letters. 2015;364(1):1–7.

24. Changou CA, Chen YR, Xing L, Yen Y, Chuang FY, Cheng RH, et al. Arginine starvation-associated atypical cellular death involves mitochondrial dysfunction, nuclear DNA leakage, and chromatin autophagy. Proc Natl Acad Sci USA. 2014;111(39):14147–52.

25. Kremer JC, Prudner BC, Lange SES, Bean GR, Schultze MB, Brashears CB, et al. Arginine Deprivation Inhibits the Warburg Effect and Upregulates Glutamine Anaplerosis and Serine Biosynthesis in ASS1-Deficient Cancers. Cell reports. 2017;18(4):991–1004.
26. Chow AK, Ng L, Sing Li H, Cheng CW, Lam CS, Yau TC, et al. Anti-tumor efficacy of a recombinant human arginase in human hepatocellular carcinoma. Curr Cancer Drug Targets. 2012;12(9):1233–43.

27. Tanios R, Bekdash A, Kassab E, Stone E, Georgiou G, Frankel AE, et al. Human recombinant arginase I(Co)-PEG5000 [HuArgI(Co)-PEG5000]-induced arginine depletion is selectively cytotoxic to human acute myeloid leukemia cells. Leuk Res. 2013;37(11):1565–71.

28. Ismail T, Kim Y, Lee H, Lee DS, Lee HS. Interplay Between Mitochondrial Peroxiredoxins and ROS in Cancer Development and Progression. Int J Mol Sci. 2019;20(18).

29. Wangpaichitr M, Wu C, Li YY, Nguyen DJM, Kandemir H, Shah S, et al. Exploiting ROS and metabolic differences to kill cisplatin resistant lung cancer. Oncotarget. 2017.

30. Shin HJ, Kwon HK, Lee JH, Anwar MA, Choi S. Etoposide induced cytotoxicity mediated by ROS and ERK in human kidney proximal tubule cells. Scientific reports. 2016;6:34064.

31. Park C, Cha HJ, Lee H, Hwang-Bo H, Ji SY, Kim MY, et al. Induction of G2/M Cell Cycle Arrest and Apoptosis by Genistein in Human Bladder Cancer T24 Cells through Inhibition of the ROS-Dependent PI3k/Akt Signal Transduction Pathway. Antioxidants (Basel). 2019;8(9).

32. Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. Eur J Pharm Sci. 2019;134:116–37.

33. Liu J, Fan L, Wang H, Sun G. Autophagy, a double-edged sword in anti-angiogenesis therapy. Med Oncol. 2016;33(1):10.

34. Lin C, Wang Z, Li L, He Y, Fan J, Liu Z, et al. The role of autophagy in the cytotoxicity induced by recombinant human arginase in laryngeal squamous cell carcinoma. Appl Microbiol Biotechnol. 2015;99(20):8487–94.

35. Khalil N, Abi-Habib RJ. [HuArgI (co)-PEG5000]-induced arginine deprivation leads to autophagy dependent cell death in pancreatic cancer cells. Invest New Drugs. 2019.

36. Lam TL, Wong GK, Chow HY, Chong HC, Chow TL, Kwok SY, et al. Recombinant human arginase inhibits the in vitro and in vivo proliferation of human melanoma by inducing cell cycle arrest and apoptosis. Pigment cell melanoma research. 2011;24(2):366–76.

37. Ranganath P, Einhorn L, Albany C. Management of Chemotherapy Induced Nausea and Vomiting in Patients on Multiday Cisplatin Based Combination Chemotherapy. Biomed Res Int. 2015;2015:943618.

38. Cao Y, He Y, Chen H, He S, Gu Y, Wang X, et al. Phase I study of gemcitabine-cisplatin versus pemetrexed cisplatin for patients with advanced or metastatic bladder cancer. J BUON. 2018;23(2):475–81.

39. Yau T, Yao TJ, Chan P, Ng K, Fan ST, Poon RT. A new prognostic score system in patients with advanced hepatocellular carcinoma not amendable to locoregional therapy: implication for patient selection in systemic therapy trials. Cancer. 2008;113(10):2742–51.

40. Szlosarek PW, Steele JP, Nolan L, Gilligan D, Taylor P, Spicer J, et al. Arginine Deprivation With Pegylated Arginine Deiminase in Patients With Argininosuccinate Synthetase 1-Deficient Malignant
Pleural Mesothelioma: A Randomized Clinical Trial. JAMA oncology. 2017;3(1):58–66.

41. Abou-Alfa GK, Qin S, Ryoo BY, Lu SN, Yen CJ, Feng YH, et al. Phase III randomized study of second line ADI-PEG 20 plus best supportive care versus placebo plus best supportive care in patients with advanced hepatocellular carcinoma. Ann Oncol. 2018;29(6):1402–8.

42. Ivens IA, Achanzar W, Baumann A, Brandli-Baiocco A, Cavagnaro J, Dempster M, et al. PEGylated Biopharmaceuticals: Current Experience and Considerations for Nonclinical Development. Toxicol Pathol. 2015;43(7):959–83.

Supplemental Figure Legend

Figure S1. GSH content after BCT-100 treatment. (A) CMFDA (5 µM) was used to indicate the intracellular GSH level after BCT-100 treatment (20 mU/mL) for 24 hr by flow cytometer. *P<0.05, **P<0.01, as evaluated by Student's T test.

Figures
Figure 1

Sensitivity of bladder cancer cells to BCT-100 correlated with the expression of ASS1 and OTC. (A) MTT assay was used to determine the cell growth inhibitory effect of BCT-100 on bladder cancer cell lines (BIU-87, EJ-1, J82, ScaBER, T24, and 5637) and A549 for 72 hr. (B) Basal expressions of ASS1 and OTC in bladder cancer cells were evaluated by Western blot. (C) The cell survival rate of ASS1 silenced BIU-87 cells (72 hr) was tested by MTT assay. (D) C-PARP and ASS1 were examined upon BCT-100 treatment for 72 hr in ASS1 knockdown BIU-87 cells. β-Actin was used as a loading control.
Figure 2

BCT-100 induced cytotoxicity accompanied by arginine decrease in bladder cancer cells. (A), (C) T24 cells was exposed to BTC-100 (20 mU/mL) for 72 hr and intracellular PEG and C-PARP were tested by Western blot. (B) Intracellular arginine level was determined BCT-100 treatment for 72 hr. (D) Apoptotic rate was determined by annexin-V and PI staining and analyzed by flow cytometer, cells were treated with BCT-100 (20 mU/mL) for 72 hr. (E) TUNEL assay indicated the apoptotic cells in the same conditions. Data are represented as mean ± S.D. of three independent experiments.*P<0.05, **P<0.01, ***P<0.001, as evaluated by Student's t-test.
Figure 3

The apoptosis induced by BCT-100 was mitochondrial-dependent and accompanied by ROS production. (A) Elevation of H2O2 was observed after BCT-100 treatment (20 mU/mL) for 24 hr by flow cytometer. (B) JC-1 staining was used to show mitochondrial membrane depolarization in T24 cells upon BCT-100 (20 mU/mL) exposure for 24 hr. (C), (D) Cells were treated with 20 mU/mL BCT-100 for 72 hr, and Cytochrome C and Smac in cytosol and mitochondrion were assessed by Western blot. COX IV and β-Actin were used as loading control in mitochondrial and cytosolic fraction respectively. Data are represented as mean ± S.D. of three independent experiments.*P<0.05, **P<0.01, ***P<0.001, as evaluated by Student’s T test.
Figure 4

Autophagy induction and AKT/mTOR pathway activation after BCT-100 exposure. (A) T24 cells were transfected with the mRFP-GFP-LC3 vector for 24 hr and treated with BCT-100 (20 mU/ml) for 24 hr. Representative pictures of fluorescent LC3 puncta were captured by an inverted fluorescent microscope. (B), (C) BCT-100 -treated T24 cells were harvested at different time points. LC3B, p62, Beclin-1, p-AKT, AKT, p-mTOR, mTOR and β-Actin were tested by Western blot. All data are mean ± S.D. of three independent experiments.*P<0.05, **P<0.01, ***P<0.001, as evaluated by Student’s T test.
Figure 5

BCT-100-induced ROS initiate cellular apoptosis and autophagy in bladder cancer cells. (A) T24 cells were pretreated with NAC (5 mM, 1 hr) before incubation with BCT-100 (20 mU/mL) for 24 hr and H2DCFDA (1 µM) was used to determine the ROS level by flow cytometer. (B) T24 cells were pretreated with NAC (5 mM, 1 hr) before incubation with BCT-100 (20 mU/mL) for 72 hr. Annexin V/PI kit was used to determine the apoptotic rate by flow cytometer. (C) AKT, p-AKT, C-PARP, p62, LC3B and β-Actin were evaluated by Western blot. (D) T24 cells were treated with BCT-100 (20 mU/mL) and CQ (10 µM) for 3 days, and Western blot was applied to test the expression of C-PARP, Survivin, LC3B and β-Actin. (E), (F) MK-2206 (2 µM) and Rapamycin (100 nM) were used to combine with or without BCT-100 for 3 days, related protein levels were tested by Western blot. β-Actin was used as a loading control. All data are shown as mean ± S.D. of three independent assays. *p < 0.05 versus control, #p < 0.05 versus BCT-100, as evaluated by Student’s T test.
Figure 6
The tumor suppression effect of BCT-100 in vivo. (A) The relative tumor volume and (B) median survival in control and BCT-100 treatment arms (20 mg/kg and 60 mg/kg). The serum (C) and intratumoral (D) arginine level were detected by L-arginine kit. (F) Immunofluorescence staining of Ki67 was conducted in control and BCT-100 treatment arms. Bar, 30 µm. All data are mean ± S.D. of three independent experiments.*P<0.05, **P<0.01, ***P<0.001, as evaluated by Student’s T test.

**Supplementary Files**

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- Fig.S1.tif