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Original Research Article

Cardamonin suppresses glycolysis and induces oxidative stress by inhibiting PI3K/AKT/mTOR pathway in bladder cancer cells

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Abstract

Purpose: *To evaluate the effect and underlying mechanisms of action of cardamine on the progression of bladder cancer (BC).*
Methods: *Human bladder epithelium immortalized cell line (SV-HUC-1) and human bladder cancer (BC) cell lines (T24 and UM-UC-3) were used in this investigation. They were treated with cardamine at concentrations of 0, 15, 30, 60 or 120 µmol/L. Cell viability was determined using cell counting kit 8 (CCK-8) assay while 5-ethynyl-2'-deoxyuridine (Edu) assay was used to assess cell proliferation. Cell apoptosis as well as reactive oxygen species (ROS) accumulation were determined by flow cytometry whereas glucose uptake, adenosine triphosphate (ATP) level and lactate production were determined using their respective assay kits. Furthermore, the expression levels of nuclear factor level (erythroid-derived 2)-like 2 (Nrf2), NAD(P)H, quinone oxidoreductase 1 (NQO1), protein kinase B (AKT), phosphorylated-AKT (p-AKT), phosphatidylinositol 3-kinase (PI3K), p-PI3K, mechanistic target of rapamycin kinase (mTOR) and p-mTOR were evaluated by western blot analysis.*
Results: *Cardamine significantly reduced cell viability and inhibited cell proliferation in BC cells in a dose-dependent manner, but did not affect human normal cells. In addition, treatment with the compound induced apoptosis in BC cells; the higher the concentration, the higher the apoptosis level. Besides, cardamine administration suppressed aerobic glycolysis, and decreased the nuclear factor level (Nrf2) level, thereby increasing ROS production in a concentration-dependent manner. Furthermore, it blocked the activation of PI3K/AKT/mTOR signal cascade.*
Conclusion: *Cardamine inhibits glycolysis and PI3K/AKT/mTOR pathway, and also promotes apoptosis as well as oxidative stress in BC cells. Thus, the compound is a potential therapeutic reagent for BC.*

Keywords: *Bladder cancer, Cardamine, Glycolysis, Reactive oxygen species, PI3K/AKT/mTOR pathway*

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INTRODUCTION

Bladder cancer (BC) is identified as one of the most common cancers of the urinary system, with a high recurrence rate and mortality [1]. Although a variety of methods have been used for the treatment of BC, prognosis in many patients, especially those with muscle-invasive

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bladder cancer, is still poor [2]. Therefore, the study of compounds that may inhibit the progression of BC is crucial for its effective treatment.

A vital hallmark of tumorigenesis is cellular metabolic reprogramming [3]. Even under oxygen-rich conditions, tumor cells prefer to consume glucose to produce lactate, which is known as the “Warburg effect” or “aerobic glycolysis” [4]. It has been reported that phosphatidylinositide-3-kinase (PI3K) and AKT are abnormally activated in multiple biological activities [5]. Additionally, emerging findings also indicate that PI3K/AKT signal participates in BC progression [6]. However, the regulatory mechanism of PI3K/AKT involved in the development of BC needs to be further studied.

Cardamine, a cardamom-derived chalcone compound (Figure 1 A), plays a vital role in the treatment of diverse pathological processes, including the targeting of cancer cells [7] and suppression of inflammation [8]. In addition, Wang *et al* discovered that cardamine inhibits the progression of esophageal cancer by inhibiting PI3K/AKT signal [9]. Jin *et al* reported that it reduces glucose uptake and lactate production, and inhibits nuclear factor (erythroid-derived 2)-like 2 (NrF2)-dependent reactive oxygen species (ROS) scavenging system by suppressing HIF-1α-mediated cell metabolism [7]. Nevertheless, the therapeutic effect and mechanism of cardamine on bladder cancer are still unclear.

This study was designed to reveal the role of cardamine in the progress of BC cells and primarily explored the molecular mechanism.

EXPERIMENTAL

Cell culture

Human bladder epithelium immortalized cell line (SV-HUC-1, IM-H068), human BC cell lines T24 (IM-H069) and UM-UC-3 (IM-H070) were acquired from Xiamen Immocell Biotechnology Co., Ltd (China). In addition, SV-HUC-1 cells were maintained in F12K basal medium (IMC-209, Xiamen Immocell Biotechnology Co. Ltd, China). T24 cells were cultured in McCoy's 5A complete medium (IMC-307, Xiamen Immocell Biotechnology Co. Ltd, China) while UM-UC-3 was maintained in DMEM high sugar complete medium (IMC-301, Xiamen Immocell Biotechnology Co. Ltd, China). All media were mixed with 10 % fetal bovine serum (FBS, IMC-101, Xiamen Immocell Biotechnology Co. Ltd, China), and the cells were placed in 37 ℃ atmosphere containing 5 % CO_2.

Cell treatment

Cardamine was purchased from Nanjing Guangrun Biological Products Co. Ltd (batch no. 18956-16-6), and dissolved in dimethyl sulfoxide (DMSO, X11263, XYbio, China) to the desired concentrations. An equivalent volume of DMSO was used as the blank group.

Cell viability assay

Cell viability was assessed using a Cell counting kit-8 (CCK-8) kit (X11934, XYbio, China), following the kit manufacturer’s instruction. Thereafter, 2 × 10^4 SV-HUC-1, T24 or UM-UC-3 cells were cultured in a 96-well plate. After 24 h, the cells were treated with cardamine (0, 15, 30, 60 or 120 μmol/L) for 24 h. Next, 10 μl CCK-8 reagent was supplemented into each well for another 4 h. Finally, the absorbance were recorded in a microplate analyzer (JC-1181, ELx808, USA) at 450 nm.

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

T24 or UM-UC-3 cells were cultured in 96-well plates overnight. Then, the cells were treated with cardamine (0, 15, 30, 60 or 120 μmol/L) for 24 h, followed by treatment with EdU Cell Proliferation Kit with DAB (C0085S, Beyotime, China) based on the kit manufacturer’s protocol. Next, the cell nuclei was stained with 4',6-diamidino-2-phenylindole (DAPI) dye, and the images of the positive cells were obtained using a fluorescence microscope (MF52-N, Guangzhou Mingmei Photoelectric Technology Co. Ltd, China).

Determination of cell apoptosis

Annexin V-FITC/PI apoptosis assay kit (G65873, Shanghai Jingkang Biological Engineering Co. Ltd, China) was used to measure cell apoptotic level following the kit's protocol. T24 or UM-UC-3 cells treated with a specific concentration of cardamine were collected in a 500 μL binding buffer, and stained with 5 μL Annexin V-FITC reagent as well as 5 μL PI reagent. Apoptotic cells were determined using a flow cytometry (CytoFLEX, Beckman Coulter, USA).

Determination of glucose uptake, lactate production and ATP level

The uptake of glucose was determined using a glucose uptake colorimetric detection kit (MAK083, Sigma-Aldrich, USA) in line with the kit's instruction, while production of lactate was measured with a lactic acid (LA) content assay kit (AKAC001C, Boxbio, China) according to the

kit's instruction. The ATP level was determined using an ATP assay kit (MAK190, Sigma-Aldrich, USA), following the kit manufacturer's instruction.

Western blot analysis

The entire cell proteins were harvested employing total protein extraction cell lysis buffer (R0278, Sigma-Aldrich, USA). A BCA protein quantification kit (20201ES76, YESEN, China) was introduced to assay the concentration of proteins. The total protein was isolated with SDS-PAGE, followed by shifting onto polyvinylidene fluoride (PVDF) membranes (36124ES10, YESEN, China). The membranes were treated with 3 % bovine serum albumin (BSA, 36101ES25, YESEN, China) for 40 min and then incubated with a primary antibody, anti-Nrf2 (16396-1-AP, Proteintech, USA), anti-NQO1 (11451-1-AP, Proteintech, USA), anti-phospho-AKT (ab38449, Abcam, UK), anti-AKT (10176-2-AP, Proteintech, USA), and anti-phospho-PI3K (AF3241, Affinity Biosciences, USA)They were also treated with anti-PI3K (ABS234, MilliporeSigma, USA), anti-phospho-mTOR (ab137133, Abcam, UK), anti-mTOR (ab134903, Abcam, UK) or anti-GAPDH (60004-1-Ig, Proteintech, USA) at 4 ℃ overnight, followed by incubation with their corresponding secondary antibodies for 2 h. Finally, the bands of protein were observed using Enhanced ECL Chemiluminescent Substrate Kit (36222ES60, YESEN, China) and quantified by employing imageJ software (National Institutes of Health, USA) [10].

Assessment of ROS

The ROS content was determined using a reactive oxygen species assay kit (S0033S, Beyotime, China) based on the kit manufacturer's protocol. T24 or UM-UC-3 cells treated with the indicated concentration of cardamine was incubated with 10 μM 2', 7' dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min and the ROS level in BC cells recorded flow cytometry (CytoFLEX, Beckman Coulter, USA) [11].

Statistical analysis

All data were analyzed using GraphPad 8.0 (GraphPad software, USA). Significant differences were analyzed using Student's t test (between two groups) or two-way ANOVA (for multiple groups). The data were presented as mean ± standard deviation (SD). All experiments were repeated 3 times. ^^^P < 0.001, ^^p < 0.01 or ^p < 0.05 were considered statistically significant as appropriate.

RESULTS

Cardamine inhibits the proliferation of BC cells

To investigate the effect of cardamine, its toxicity in human normal cells (SV-HUC-1) was investigated. The results showed that cardamine has very low cytotoxicity in SV-HUC-1 cells (Figure 1 B). However, 15 - 120 μmol/L of cardamine notably decreased cell viability in human BC cell lines (T24 and UM-UC-3, Figure 1 C). Besides, cardamine suppressed proliferation of T24 and UM-UC-3 cells in a dose-dependent manner (Figure 1 D and E). Moreover, flow cytometry indicate that the concentration-dependent up-regulation of the apoptosis in T24 and UM-UC-3 cells was induced by 5-30 μmol/L of Cardamine (Figure 1 F and G). These results demonstrate that cardamine suppressed proliferation and facilitated apoptosis in a dose-dependent manner in BC cells.

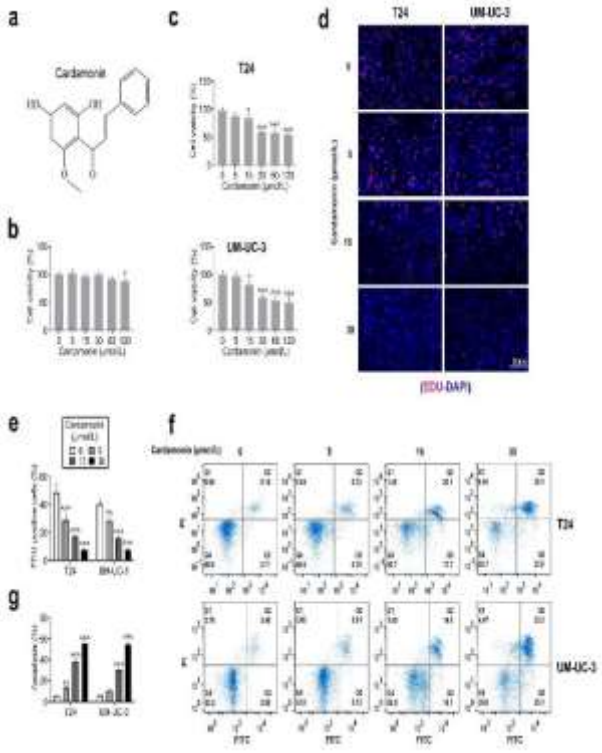

Figure 1: Cardamine inhibits the proliferation of BC cells. (a) Chemical structure of cardamine; (b) Cell viability of SV-HUC-1 cells assessed via CCK-8 assay (^p < 0.05); (c) Cell viability of T24 and UM-UC-3 cells assessed via CCK-8 assay; (d and e) cell proliferation measured via Edu assay (^^^p < 0.001, ^^p < 0.01); (f and g) cell apoptosis. ^^^P < 0.001, ^^p < 0.01). ^^^p < 0.001, ^^p < 0.01 and ^p < 0.05 versus 0 μmol/L group

Cardamine inhibits glycolysis in BC cells

This study sought to determine whether aerobic glycolysis affects cardamine-mediated suppressive role in BC cells. As indicated in Figure 2 A, B and C, 5 - 30 µmol/L of Cardamine remarkably decreased glucose uptake (Figure 2 A), ATP generation (Figure 2 B) and lactate production (Figure 2 C) in a dose-dependent way, in T24 and UM-UC-3 cells. These findings suggest that cardamine dramatically blocked glycolysis in BC cells.

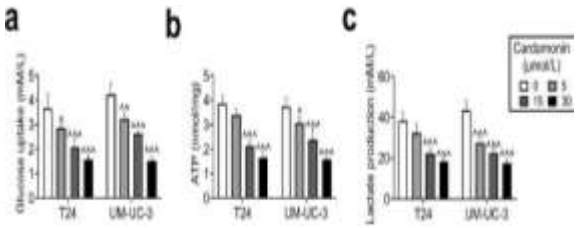

Figure 2: Cardamine inhibits glycolysis in BC cells. (a) Glucose uptake in T24 and UM-UC-3 cells treated with 0 - 30 µmol/L of cardamine (^^^P < 0.001, ^^p < 0.01, ^p < 0.05); (b) ATP generation in T24 and UM-UC-3 cells treated with 0 - 30 µmol/L of cardamine (^^^P < 0.001, ^p < 0.05); (c) Lactate production in T24 and UM-UC-3 cells treated with 0-30 µmol/L of cardamine (^^^p < 0.001). ^^^P < 0.001, ^^p < 0.01 and ^p < 0.05, versus 0 µmol/L group

Cardamine induces oxidative stress in BC cells

Nrf2, a transcription factor strongly associated with metabolic processes and oxidative stress in cancer cells, is capable of regulating the expression of oxidative defense genes, including NQO1. Expression of Nrf2 and NQO1 was measured, and the results revealed that cardamine up-regulated Nrf2 and NQO1 in T24 and UM-UC-3 cells (Figure 3 A). In addition, a previous study proved that the activation of Nrf2-related pathway reduced ROS accumulation in cancer cells. As expected, the treatment with cardamine effectively increased the ROS levels in a concentration-dependent manner (Figure 3 B). Thus, cardamine induced oxidative stress related to ROS accumulation by modulating Nrf2 expression in BC cells.

Cardamine inhibits PI3K/AKT/mTOR pathway in BC cells

It is reported that cardamine was able to inhibit oesophageal cancer development by blocking the PI3K/AKT signal cascade. To further elucidate the molecular mechanism by which cardamine exerts its suppressive effect in BC cells, the modulation of the activation of PI3K/AKT/mTOR pathway by cardamine was investigated. The results showed that cardamine treatment significantly reduced the phosphorylation of PI3K, AKT and mTOR in a dose-dependent way, but cardamine did not affect the total protein levels of PI3K, AKT and mTOR in T24 and UM-UC-3 cells (Figure 4).

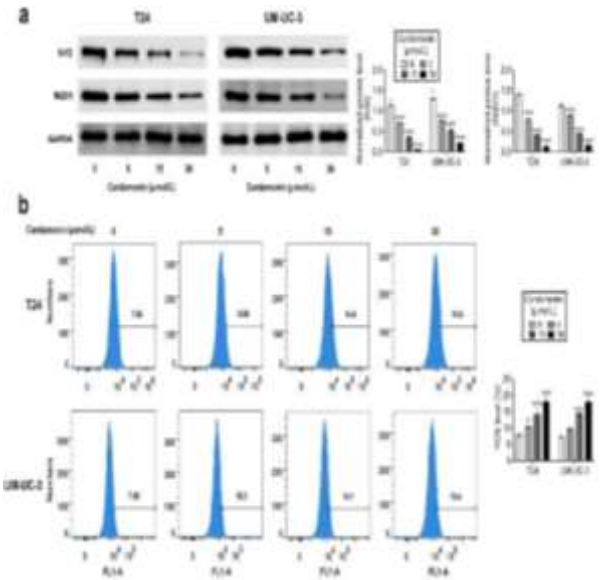

Figure 3: Cardamine induces oxidative stress in BC cells. (a) Expression level of Nrf2 and NQO1 detected via western blot analysis (^^^p < 0.001); (b) ROS accumulation in T24 and UM-UC-3 cells treated with 0-30 µmol/L of cardamine (^^^p < 0.001, ^p < 0.05). ^^^p < 0.001 and ^p < 0.05, versus 0 µmol/L group

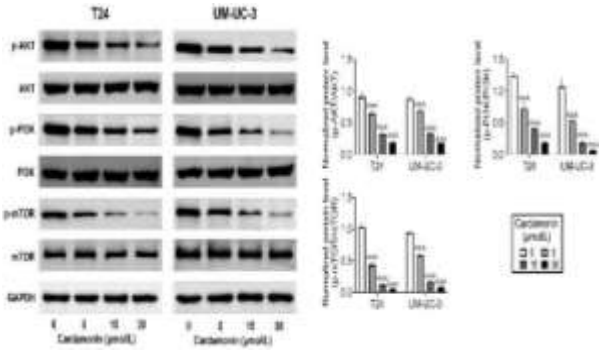

Figure 4: Cardamine inhibits the PI3K/AKT/mTOR pathway in BC cells. Expression level of proteins related to PI3K/AKT/mTOR pathway in T24 and UM-UC-3 cells treated with 0 - 30 µmol/L of cardamine (^^^p < 0.001, ^p < 0.05). ^^^p < 0.001 and ^p < 0.05, versus 0 µmol/L group

DISCUSSION

Cardamine dramatically reduced cell viability in BC cells in a dose-dependent way, while Cardamine treatment did not exhibit cytotoxicity in human normal cells. It also induced BC cells apoptosis and inhibited glycolysis *in vitro*. Moreover, an increase in ROS levels was

observed in BC cells treated with cardamine, which might be caused by the suppression of Nrf2 signal. At the molecular level, cardamine administration inhibited the activation of PI3K//AKT/mTOR signal cascade.

Bladder cancer is considered the third most mutated cancer in the world, behind only lung and skin cancer. Men are about 3 times more likely to develop BC than women, and the five-year survival rate for patients with BC is about 77 %. Nevertheless, the number drops to about 5 % when the cancer has metastasized far away [12]. Recently, certain compounds extracted from plants were found to exhibit good efficacy and low toxicity, hence they are widely used in the treatment of malignancies [13]. For instance, natural drugs such as Qici Sanling decoction [14] have been shown to suppress the occurrence and progression of BC. Cardamine, a common traditional Chinese medicine (TCM), is able to exert inhibitory effects on diverse tumors, including oesophageal cancer [9] and breast cancer [7], by modulating diverse molecular networks. However, the detailed roles of cardamine in BC cells are still unclear. Increasing evidence indicate that TCMs exert certain effects through the inhibition of the proliferation and induction of apoptosis in cancer cells [15]. Consistently, the present study demonstrated that treatment of cardamine notably reduced cell viability and up-regulated the apoptotic rate in BC cells, which indicates that it suppressed the development of BC cells *in vitro*.

Previous investigations have reported that cancer cells presented increased glucose uptake and lactate production, providing sufficient energy for rapid growth [16]. Therefore, regulating glycolysis may be a potential mechanism by which cardamine inhibited the proliferation of BC cells. The present findings show that cardamine treatment significantly reduced glucose uptake, lactate accumulation and ATP level in BC cells in a concentration-dependent way. The results also indicate that cardamine inhibited glycolysis in BC cells.

Additionally, glycolysis is likely to decrease the level of ROS in cancer cells, thereby increasing the survival rate of cancer cells during treatment [17]. Besides, the accumulation of ROS is identified as a vital marker of oxidative stress, which reflects the extent of DNA and protein damage in the cells, and which leads to the occurrence of various cancers [18]. It has been reported that cardamine is associated with ROS accumulation in breast cancer [7]. This study, to the best of our knowledge, is the first to reveal that cardamine accelerates the production of ROS in BC cells in a dose-dependent way, at least partly by reducing the expression of Nrf2 as well as its downstream gene, NOQ1.

PI3K/AKT/mTOR is one of the most widely studied signal cascade in protein kinase family, and the tumorigenesis of multiple cancers is tightly related to the up-regulated activity of AKT. Emerging evidence has revealed that cardamine plays an inhibitory role in esophageal cancer development by inactivating PI3K/AKT pathway [19]. Notably, the present investigation confirms that treatment with cardamine reduced the phosphorylation of PI3K, AKT and mTOR in T24 and UM-UC-3 cells, implying that it suppressed the activity of PI3K/AKT/mTOR pathway in BC cells.

Limitations of the study

Nevertheless, some limitations exist in this study. For example, it was not determined whether cardamine inhibited BC progression *in vivo*. Moreover, further rescue experiments need to be conducted to confirm that cardamine exerts its role in BC cells through PI3K/AKT/mTOR pathway. Furthermore, the clinical effects of cardamine should be determined in subsequent investigations.

CONCLUSION

This work demonstrates that cardamine suppresses proliferation and glycolysis, and induces apoptosis and oxidative stress in BC cells. Furthermore, it inhibits PI3K/AKT/mTOR pathway. Thus, the compound is a novel and promising drug for treating patients with BC.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ping Li and Chaopeng Tang designed the study and carried them out; Ping Li, Chaopeng Tang, Dian Fu and Xiaofeng Xu supervised the data collection, analyzed and interpreted the data; Ping Li, Chaopeng Tang, Jingping Ge and Ruipeng Jia prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript. Ping Li and Chaopeng Tang contributed equally to the work and should be considered co-first authors.

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