Effects of Licochalcone A on Cell-cycle Distribution and Glycolysis in Human Bladder Cancer T24 Cells

Yan Wang¹,²,#, Jichun Han¹,#, Xiaoyu Chen¹, Penglong Wang¹,³, Wenjin Hao¹, Jun Ma¹, Bo Wang¹,², Xingjie Zhang¹, Fanqing Meng¹, Xiajuan Zhang¹, Dan Wang¹, Qiusheng Zheng¹,²,*

¹Binzhou Medical University, Yantai 264003, China
²Pharmacy School of Shihezi University, Key Laboratory of Xinjiang Endemic Phytomedicine Resources, Ministry of Education, School of Pharmacy, Shihezi, 832002, China
³Ninth Division of hospital of Xinjiang bingtuan, Tacheng, 834700, China

#Yan Wang and Jichun Han contributed equally to the work.
*Corresponding author: zqsyt@sohu.com

Abstract This study investigates the effects of LCA on cell-cycle distribution and glycolysis in human bladder cancer T24 cells. The mRNA expression of HIF-1α, vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA) and Fructose-2, 6-Biphosphatase 3 (PFKFB3) were assessed using RT-PCR. LCA elicited an anti-proliferative effect on human bladder cancer cells line T24 cells in a concentration-dependent manner; LCA induced a G2/M-phase arrest, as well as a down-regulation of the mRNA level of CDK1 and Cyclin B1; after treated with LCA, the activity of CDK1 was down regulated and the p21 was upregulated. LCA inhibited the ATP production, decreased the glucose uptake and the release of lactic acid. The activities of LDH, HK, and PK were significantly down-regulation. LCA could reduce the level of HIF-1α in T24 cells and significantly reduce the expression of its target genes: VEGF, GLUT1, LDHA and PFKFB3. This study demonstrated that LCA treatment caused a significant decrease in the proliferation and a cell cycle arrest of G2/M phase in T24 cells; the mechanism by which LCA inhibits T24 proliferation may be associated with the inhibition of glycolysis as well as cause G2/M phase arrested.

Keywords: licochalcone A, T24, cell-cycle distribution, glycolysis


1. Introduction

It has long been known that energetic metabolism mainly relies upon the mitochondrial oxidative phosphorylation in normal cells. In contrast, due to the hypoxia microenvironment and mitochondrial gene mutations, cancer cells have developed altered metabolism that predominantly produce energy by glycolysis, even in the presence of oxygen, this is known as the “Warburg Effect” [1]. The glycolysis pathway is a series of metabolic reactions catalyzed by multiple enzymes or enzyme complexes. Most enzymes’ activities in the pathway are controlled by two factors including c-myc and HIF-1α [2]. HIF-1α mediates the adaptation of cancer cells to the hypoxic environment by controlling the expression of hundreds of genes, including VEGF, glycolytic enzymes, and glucose transporters [3,4]. In addition to the induction of angiogenesis and glycolysis, hypoxia also inhibits cell cycle progression by blocking the G1-S transition, even though the underlying mechanisms may vary due to disparate experimental conditions [5]. It has been shown that hypoxic inhibition of cell cycle progression requires the up-regulation of the cyclin-dependent kinase inhibitor genes CDKN1A (encoding p21<sup>Cip1</sup>) and/or CDKN1B (encoding p27<sup>Kip1</sup>) [6]. Therefore, developing novel glycolysis inhibitors is an important direction in current cancer research, some glycolysis inhibitors such as 2-deoxy-glucose and 3-bromo-pyruvate have already been approved for clinical trials [7].

Traditional Chinese Medicine (TCM) is particularly appreciated for cancer therapy in China, Chinese herbal medicine provides a fast track and important source for drug discovery and is becoming more and more acceptable around the world [8]. Liquorice is one of the most widely used herbal medicines, its active components have a wide range of pharmacological activities. Licochalcone A (LCA, Figure 1) is a flavonoid extracted from licorice root and has antiparasitic, antibacterial, and antitumor properties [9,10]. LCA reportedly inhibits cell proliferation in gastric [11], colon [10], ovarian [12] and prostatic [9]. Licochalcone A has been reported to induce human gastric cancer BGC-823 cells apoptosis by regulating ROS-mediated MAPKs and PI3K/AKT signaling pathways [13], to induce human bladder cancer T24 cells apoptosis through mitochondria dysfunction and endoplasmic reticulum stress [14], and to inhibit proliferation by inducing reactive oxygen species production [15].
Bladder cancer, a kind of malignant tumor, is one of the most common cancers worldwide, with the highest incidence in industrialized countries [16]. In the present study, we want to investigate the effects of LCA on cell-cycle distribution and glycolysis in human bladder cancer T24 cells.

2. Materials and Methods

2.1. Reagents

LCA (purity ≥98%) was purchased from Tianjin Zhongxin Pharmaceutical Group Co., Ltd. (Tianjin, China). Culture medium (RPMI 1640) was obtained from GIBCO (Invitrogen Corporation). Dimethylsulfoxide (DMSO), sulforhodamine B (SRB), trypan blue, propidium iodide and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bradford protein quantitative kit and RNA extraction kit were purchased from Shanghai Sangong CO., Ltd., (Shanghai, China). First strand cDNA synthesis Kit and cDNA amplification Kit were purchased from Formentas CO., Ltd., (Vilnius, Lithuania). Penicillin and streptomycin were obtained from Shandong Sunrise Pharmaceutical Co., Ltd. (Zibo, China). All other chemicals are of analytical grade and commercially available.

2.2. Cell Line and Cell Culture

Human bladder cancer T24 cells were purchased from China Center for Type Culture Collection (CCTCC, Shanghai, China). The cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2. The cells were split every 3 days and were diluted every day before each experiment.

2.3. Cell Viability Assay

T24 cells were trypsinized and seeded into 96-well plates at 8×10^4 cells/ml and then incubated for 24 h before treatment. Thereafter, the cells were exposed to various concentrations of LCA (0, 20, 40, 60, 80, 100 μmol/l) as indicated for 24 h. The effect of LCA-induced cytotoxicity was evaluated using the SRB assay. Absorbance (A) at 490 nm was determined using a microplate reader (Thermo Varioskan Flash 3001, USA). The experiment was repeated three times under the same conditions.

2.4. Trypan Blue Exclusion Test

After treatment with LCA 24 h, cells were collected into centrifuge tubes, and then cells were stained with 0.4% trypan blue and counted in triplicate with an optic microscope with the aim to estimate the number of live and dead cells. Cell viability was expressed as a percentage of treated cells with respect to appropriate vehicle-treated controls, and toxicity as a percentage of dead cells with respect to the total number of cells.

2.5. Morphological Observation

The cells were placed on a six-well plate at a density of 4×10^4 cells/slide, morphological changes in T24 cells were observed under a phase contrast microscope equipped with a digital camera (Axio Observer, Zeiss, Germany) after 24 h incubation with different LCA concentrations as indicated. Digital images were obtained during observation under an inverted microscope at a 200 × magnification.

2.6. Cell Cycle Analysis

Cells (8×10^4 cells/ml) were treated with LCA (0, 30, 50, 70 μmol/l) for 24 h. Afterwards, the cells were harvested and fixed in 70% ethanol for 30 min on ice. After washing with PBS, the cells were labeled with propidium iodide (PI, 0.05 mg/ml)in the presence of RNaseA (0.5 mg/ml) and incubated at room-temperature in the dark for 30 min. DNA contents were analyzed using the flow cytometer (BD, NJ, USA) equipped with Cell Quest Pro Software. ModFit LT cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

2.7. CDK1 Kinase Activity Assay

Cell extracts were prepared as previously described, kinase activities of CDK1 were performed as previously described [17].

2.8. ELISA Assay For p21 Protein

Protein level of p21 was measured by an ELISA test using Titer Zyme ELISA Kits from Assay Designs.

2.9. Determination Of ATP Production

The ATP assay was performed according to the manufacturer’s instruction. Briefly, harvested cultured cells were lysed with a lysis buffer, followed by centrifugation at 10000×g for 2 min, at 4°C. Finally, in 6-well plates, the level of ATP was determined by mixing 20 μl of the supernatant with 100 μl of luciferase reagent, which catalyzed the light production from ATP and luciferin. Absorbance at 636 nm was determined using a fluorescence plate reader (Thermo Varioskan Flash 3001, USA). Total ATP levels were expressed as μmol/gprotein.

2.10. Glucose Uptake Assay

After treatment, media were collected and diluted 1:4000 in water. The amount of glucose in the media was then detected using the Amplex Red Glucose Assay Kit (APLYGEN, Beijing, China) according to the manufacturer’s instructions. Glucose uptake was determined by subtracting the amount of glucose in each sample from the total amount of glucose in the media (without cells). The detection was performed by spectrophotometer (Thermo Varioskan Flash 3001, USA) at 500 nm.
2.11. Lactic Acid Production Assay

To measure lactic acid production, cells were treated with LCA (0, 30, 50, 70 μmol/l) for 24 h, and media were collected and assayed following the manufacturer’s instructions of the lactic acid production detection kit (KeyGen, Nanjing, China).

2.12. Hexokinase (HK) Determination

The principle of the HK assay kit was that the reaction of glucose and ATP catalyzed by HK generated glucose-6-phosphate and ADP, and the reaction of glucose-6-phosphate and NADP catalyzed by glucose-6-phosphate dehydrogenase generated 6-phosphogluconolactone (G6PDH) and NADPH; with adequate NADP and G6PDH in the coupling reaction of G6PDH, the content of NADPH was proportional to the content of glucose-6-phosphate, and the absorbance at a wavelength of 340 nm of NADPH was proportional to the activity of HK in the sample.

2.13. Pyruvate Kinase (PK) Determination

Pyruvate kinase activity was determined in cell lysate spectrophotometrically using pyruvate kinase assay kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the method described previously. Pyruvate kinase catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP, yielding one molecule of pyruvate and one molecule of ATP.

2.14. Lactate Dehydrogenase (LDH) Determination

Intracellular LDH was determined in the medium and cell lysate, respectively, using lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the method of Dito. It depends on that LDH specifically catalyzes the reduction of pyruvate to lactate with subsequent oxidation of NADH to NAD+. The initial rate of decrease NADH is directly proportional to the rate of the reaction of glucose and ATP catalyzed by HK generated glucose-6-phosphate and ADP, and the reaction of glucose-6-phosphate and NADP catalyzed by glucose-6-phosphate dehydrogenase generated 6-phosphogluconolactone (G6PDH) and NADPH; with adequate NADP and G6PDH in the coupling reaction of G6PDH, the content of NADPH was proportional to the content of glucose-6-phosphate, and the absorbance at a wavelength of 340 nm of NADPH was proportional to the activity of HK in the sample.

2.15. Semiquantitative Reverse Transcription-polymerase Chain Reaction

Cells at a density of 1×10^6 cells/ml were incubated with LCA (0, 30, 50, 70 μmol/l) for 24 h. The total cellular RNA was isolated using a commercial kit (Tiangen Biotech Co., LTD., Beijing, China), 3 μl cDNA template, and 1 μl of each primer. The RT-PCR products were quantified by GelPro analysis software. The primers sequences were as Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tr>
<td>GAPDH</td>
<td>F: 5'GACATCAAGAAAGGTGGTAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'GGATCCACCCCTGCTGTAAGT -3'</td>
</tr>
<tr>
<td>CDK1</td>
<td>F: 5'CCTGAGGGGACGGATT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'GGGACCGGGAATGTTAGGA-3'</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>F: 5'GGTGGTCAGTGGCTGCTTCTC3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'AGGGGCCGAATCATCACGC-3'</td>
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<tr>
<td>VEGF</td>
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</tr>
<tr>
<td></td>
<td>R: 5'CCTGCTCATCAAGAGCACTT-3'</td>
</tr>
<tr>
<td>GLUT1</td>
<td>F: 5'GGGGGAGCGGGATTT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'TGAAGATGGCCACAGCTATG-3'</td>
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<tr>
<td>LDHA</td>
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<td></td>
<td>R: 5'GGATCCACCCCTGCTGTAAGT -3'</td>
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<td>PFKFB3</td>
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</tr>
<tr>
<td></td>
<td>R: 5'GGGAGGGAATGTTAGGA-3'</td>
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2.16. Western Blot Analyses

Cells at a density of 1×10^6 cells/ml were incubated with LCA (0, 30, 50, 70 μmol/l) for 24 h. Soluble lysates (15 μmol/l per lane) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes (Amersham Biosciences, New Jersey, USA), and blocked with 5% nonfat milk in Tris-buffered saline with Tween (TBST) for 2 h at room temperature. Membranes were incubated with anti-HIF-1α (1:500) and anti-β-actin (1:1000) (Amersham Biosciences) in 5% milk/TBST at 4°C overnight. After washing five times with TBST, the membranes were incubated with horseradish peroxidase-conjugated antibody for 1 h at room temperature. Western blots were developed using enhanced chemiluminescence (Thermo, NY, USA) and were exposed to Kodak radiographic film.

2.17. Statistical Analysis

The data were presented as means ± SD from at least three independent experiments and evaluated through the analysis of variance (ANOVA) followed by student’s t-test. P< 0.05 was considered statistically significant. The analyses were performed by using the Origin 8.0 software (Origin Lab Corporation, Northampton, MA, USA).

3. Results

3.1. LCA Inhibited the Proliferation of T24 Cells

To investigate the effect of LCA on the proliferation of T24 Cells, T24 cells were treated with various concentrations of LCA (0, 20, 40, 60, 80 and 100 μmol/l) for 24 h. As shown in Figure 2, a significant inhibition in cell proliferation was observed in a concentration and time-dependent manner. The IC50 value was 55 μmol/l after 24 h of LCA treatment.
Figure 2. Effect of LCA on cells proliferation in human bladder cancer T24 cells. Exponentially growing cells were treated with the indicated concentrations of LCA for 24 h. Cell inhibition rate and lethal rate were assessed by SRB assay or trypan blue exclusion test respectively. The values represented the means ± SD of at least three independent **P< 0.01 as compared with the LCA-untreated control group.

To evaluate the toxicity of LCA, a trypan blue exclusion test was carried out on T24 cells. The results showed that no significant cytotoxicity was observed compared with that of control.

3.2. LCA Induced Morphological Changes in T24 Cells

T24 cells analysis by digital images (Figure 3) showed a concentration-dependent decrease of cellular density. Moreover, a morphological change was detected in cells treated with LCA, the treated cells were loose and the adhesion ability was lost compared to rounded untreated cells.

3.3. LCA Induced G2/M-phase Arrest in T24 Cells

To explore the mechanism of LCA-induced proliferation inhibition, the effect of LCA on the cell cycle was investigated. The results cell cycle distribution analysis by flow cytometry (Figure 4) showed marked and consistent changes in the cell cycle of T24 cells at 24 h. With increased concentrations, the percentage of G2/M phase in LCA-treated T24 cells increased from 6% to 16%.

Figure 3. Morphological alterations of T24 cells treated with determined concentration LCA for 24 h

Figure 4. Effect of LCA on cell cycle stages. After treatment with different concentrations of LCA for 24 h, T24 cells were stained with PI and analyzed for DNA content by flow cytometry.
3.4. Changes of G2/M-related Regulators in mRNA Levels

To investigate the mechanisms involved in the regulation of G2/M-arrest in T24 cells, expression of related regulators were followed by real-time RT-PCR at the mRNA level. Figure 5 showed that after treatment with LCA for 24 h, the mRNA expression of Cyclin B1 and CDK1 remarkably reduced in a concentration-dependent manner.

3.5. LCA Inhibited CDK1 Kinase Activity and Increased p21 Levels In T24 Cells

The entry of cells into mitosis is controlled by the activation of CDK1. As shown in Figure 6, the CDK1 kinase activity markedly decreased (Figure 6A), and p21 level significantly increased (Figure 6B) in response to treatment with LCA for 24 h on a concentration-dependent.

![Figure 5. Effects of LCA on the mRNA expression of CDK1 and Cyclin B1. Transcript levels for CDK1 and Cyclin B1 were monitored by RT-PCR analysis and relative intensities were normalized by levels of GAPDH. LCA-untreated group level was accepted to be “1.0”. Data are presented as means ± SD from three independent experiments. * P<0.05, ** P<0.01 as compared with the LCA-untreated control group.](image1)

![Figure 6. Effect of LCA on CDK1 kinase activity and p21 level. The effect of LCA on the activity of CDK1 was determined by spectrometry, LCA-untreated group level was accepted to be “1.0” (A). The effect of LCA on the levels of p21 in T24 cells protein changes were determined by ELISA in T24 cells (B). Data are presented as means ± SD from three independent experiments. ** P<0.01 compared to the control group.](image2)

3.6. LCA Inhibited the Level of Glycolysis

After T24 cells were treated with LCA (0, 30, 50, 70 μmol/l) for 24 h, the results showed that LCA treatment markedly reduced ATP production (Figure 7A), glucose uptake (Figure 7B) and the extracellular lactic acid production (Figure 7C), indicating that LCA has a potential inhibitive effect on glycolysis in T24 cells.

3.7. LCA Treatment Decreased HK, PK and LDH Activities

When incubated for 24 h, the activities of hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Figure 8) in the LCA-treatment groups significantly decreased, respectively.

3.8. LCA Treatment Decreased the Level of HIF-1α in T24 Cells

In order to find out the possible mechanisms of the significant glycolysis inhibition in T24 cells induced by LCA, we investigated the effects of LCA on the levels of HIF-1α for treatment 24 h. As illustrated in Figure 9, the mRNA expression (A) and the protein expression of factor HIF-1α (B) decreased in LCA-treated groups, LCA exerted an inhibitory effect on HIF-1α in T24 cells.

3.9. Effect of LCA on Levels of HIF-1α-downstream-related Factors in T24 Cells

The levels of HIF-1α-downstream-related factors (VEGF, LDHA, GLUTI, PFKFB3) were investigated after
T24 cells exposed to LCA. As illustrated in Figure 10, RT-PCR showed that the mRNA expression of VEGF, LDHA, GLUT1, PFKFB3 decreased significantly (P< 0.01 or P< 0.05).

Figure 7. Effects of LCA on the level of glycolysis. The content of ATP was determined by the ATP content test kit after 24 h of incubation (A). The content of glucose in medium was detected by glucose oxidase method after 24 h of incubation (B). The content of lactic acid in medium was detected by a lactic acid assay kit after 24 h of incubation (C). Values represent mean values ± S.D., n= 3, **P< 0.01

Figure 8. Effect of LCA on the activities of HK, PK and LDH. The activity of HK, PK and LDH of T24 cells were detected by the related assay kits after 24 h of incubation. LCA-untreated group level was accepted to be “1.0”. Data are presented as means ± SD from three independent experiments. *P< 0.05, **P< 0.01
4. Discussion

Bladder cancer is extremely insensitive to chemotherapy. Hypoxia is a major cause of tumor resistance to radiotherapy and chemotherapy. Hypoxic microenvironments are frequent in solid tumors. Rapid cell proliferation associated with deficient vascularization leads to areas of hypoxia [18]. Hypoxia is a major cause of tumor resistance to radiotherapy and chemotherapy [19]. Tumor hypoxia has direct consequences on clinical and prognostic parameters and is a potential therapeutic target. The hypoxic response depends critically on hypoxia inducible factor-1α (HIF-1α) in pathological as well as physiological processes [20]. HIF-1α overexpression induces angiogenesis in hypoxic tissues and it can lead to increased oxygenation of the organ [21]. HIF-1α mediates the adaptation of cancer cells to the hypoxic environment by controlling the expression of hundreds of genes,
including VEGF, glycolytic enzymes, and glucose transporters [3,4]. HIF-1α, formed by the assembly of HIF-1α and HIF-1β, binds HRE in the promoters of the above genes [22]. HIF-1α is degraded rapidly during normoxia but activated during hypoxia by oxygen-sensing signaling processes [23]. Normoxic basal levels of HIF-1α are sufficient to confer increased target gene expression as well as increased resistance to chemotherapy [24]. Therefore, we hypothesize that inhibition on HIF-1α expression would be a possible approach for anti-bladder cancer strategy. Targeting on hypoxia and glycolytic related genes has become an important strategy for cancer therapy and drug development.

Through long-history clinical application, TCM has well been accepted by pharmacologists and oncologists as a valuable database to screen bioactive compounds for novel drug discovery. In TCM system, liquorice is one of the most widely used herbal medicines, its bioactive components is believed to modulate tumor hypoxia microenvironment and has long been recommended for cancer therapy. Licochalcone A (LCA) is an avonoid extracted from licorice root and has antiparasitic, antibacterial, and antitumor properties. However, the anti-cancer mechanisms of LCA still remain unclear.

The present experimental results show that (1) LCA significantly inhibited the proliferation of T24 cells; (2) LCA treatment markedly reduced glucose uptake the lactic acid production and ATP production; (3) LCA exhibited an inhibitory effect on HIF-1α in T24 cells in vitro. The HIF-1α-mediated genes, VEGF, LDHA, GLUT1 and PFKFB3, were also downregulated gradually along with the increasing doses of LCA concentration in our research, which was consistent with previous studies; (4) LCA significantly decreased the activities of hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH). All results indicate that LCA has a potential inhibitive effect on glycolysis in T24 cells.

In addition to the induction of glycolysis, hypoxia also inhibits cell cycle progression, it has been shown that hypoxic inhibition of cell cycle progression requires the upregulation of the cyclin-dependent kinase inhibitor genes CDKN1A (encoding p21Cip1). In our present study, cell cycle distribution analysis by flow cytometry showed marked and consistent changes in the cell cycle, significant cell cycle arrest at the G2/M phase with LCA treatment was observed in T24 cells. Real-time RT-PCR helped elucidate the underlying mechanism of the G2/M phase arrest by detecting changes in the expression of cell cycle-regulating genes of T24. The cell cycle is tightly mediated by a complex network of positive and negative cell cycle regulatory molecules, including CDKs, CKIs and cyclins [25,26]. In particular, cyclin B and CDK1 proteins participate in the regulation of the progression of G2/M phase [27]. We found mRNA of CDK2 and cyclin B were significantly reduced in T24 cells after 24 h of exposure to LCA; the negative effect of p21Cip1 on DNA synthesis relies primarily on its ability to inhibit CDK–cyclin-dependent pathways. Figures 7 show that the protein levels of p21 were clearly upregulated in T24 cells. In conclusion, LCA induced G2/M-phase arrest of T24 cell.

In our present study, we demonstrated that LCA treatment caused a significant decrease in the proliferation rate, LCA inhibit HIF-1α and HIF-1α-downstream-related factors expression, LCA decrease T24 cells uptaking of glucose by reduced expression of Glucose transporter-1 (GLUT1) and activating ATP generation reaction by down-regulation of pyruvate kinase (PK), causes T24 cells G2/M-phase arrest via glycolysis inhibition.

In sum, LCA can inhibit the proliferation in vitro, had a potential inhibitive effect on glycolysis and induced G2/M-phase arrest of human bladder cancer T24 cells.

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Statement of Competing Interests

The authors have no competing interests.

References


