

# Targeting lactate dehydrogenase-A promotes docetaxel-induced cytotoxicity predominantly in castration-resistant prostate cancer cells

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**Abstract.** Docetaxel (DOC) is one of the most effective chemotherapeutic agents against castration-resistant prostate cancer (CRPC). Despite an impressive initial clinical response, the majority of patients eventually develop resistance to DOC. In tumor metabolism, where tumors preferentially utilize anaerobic metabolism, lactate dehydrogenase (LDH) serves an important role. LDH controls the conversion of pyruvate to lactate, with LDH-A, one of the predominant isoforms of LDH, controlling this metabolic process. In the present study, the role of LDH-A in drug resistance of human prostate cancer (PC) was examined by analyzing 4 PC cell lines, including castration-providing strains PC3, DU145, LNCaP and LN-CSS (which is a hormone refractory cell line established from LNCaP). Sodium oxamate (SO) was used as a specific LDH-A inhibitor. Changes in the expression level of LDH-A were analyzed by western blotting. Cell growth and survival were evaluated with a WST-1 assay. Cell cycle progression and apoptotic inducibility were evaluated by flow cytometry using propidium iodide and Annexin V staining. LDH expression was strongly associated with DOC sensitivity in PC cells. SO inhibited growth of PC cells, which was considered to be caused by the inhibition of LDH-A expression. Synergistic cytotoxicity was observed by combining DOC and SO in LN-CSS cells, but not in LNCaP cells. This combination treatment induced additive cytotoxic effects in PC-3 and DU145 cells, caused cell cycle arrest in G2-M phase and increased the number of cells in the sub-G1 phase of cell cycle in LN-CSS cells. SO promoted DOC induced apoptosis

in LN-CSS cells, which was partially caused by the inhibition of DOC-induced increase in LDH-A expression. The results strongly indicated that LDH-A serves an important role in DOC resistance in advanced PC cells and inhibition of LDH-A expression promotes susceptibility to DOC, particularly in CRPC cells. The present study may provide valuable information for developing targeted therapies for CRPC in the future.

## Introduction

Prostate cancer (PC) remains the third leading cause of cancer-associated mortalities in males in the developed continents of Oceania, Europe and North America in 2011 (1), with castration-resistant PC (CRPC) being the most lethal stage of this disease (2). Docetaxel (DOC)-based chemotherapy is a first-line cytotoxic treatment, offering symptomatic and survival benefits for patients diagnosed with metastatic CRPC (3,4). However, clinically, DOC therapy only benefits ~50% of patients at the cost of significant toxicity (3). Additionally, patients who respond to this treatment will develop resistance to chemotherapy (2). Therefore, there is an immediate requirement to identify novel therapeutic strategies to overcome resistance to DOC in patients with CRPC (5). The anaerobic glycolytic pathway becomes activated in various advanced cancer types, including PC, and this is commonly known as the Warburg effect (6-8). Lactate dehydrogenase (LDH) is one of the important enzymes in this anaerobic glycolytic pathway. Human LDH is a tetrameric enzyme that is composed of three different monomeric subunits: LDH-A, LDH-B and LDH-C (9). LDH-C has an important role in male fertility and the C subunit is only part of the homotetrameric enzyme (9). The A and B subunits are primarily exhibited in skeletal muscles/liver and heart, respectively (10). LDH-A overexpression has been implicated in tumor development, particularly in disseminated cancer types, including hypoxic carcinoma and metastatic cancer cells, and has been correlated with tumor vitality (11). Additionally, suppression of LDH-A is known to cause oxidative stress in various cancer cell lines and suppress tumor growth (12). It was previously reported that, in PC, elevated serum LDH levels reduced the prognosis

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of patients with CRPC (8). However, there are no reports demonstrating that suppressing LDH-A inhibits the growth of PC cells. In the present study, the effects of LDH-A inhibition in PC cells were analyzed. The influence of LDH-A inhibition on the chemosensitivity of PC cells was also examined, since chemotherapy is the main treatment strategy for advanced PC (13), including CRPC.

## Materials and methods

**Cells and cell culture.** PC cell lines PC-3, DU145 and LNCaP were provided by Dr Hirotsugu Uemura (Department of Urology, Kindai University, Osaka, Japan), which were procured from the American Type Culture Collection (Manassas, VA, USA). PC-3, DU145 and LNCaP cells were cultured at 37°C in RPMI-1640 and supplemented with 10% inactivated fetal bovine serum (cat. no. SH30910.03; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and penicillin-streptomycin solution (cat. no. 15140-22; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hormone-resistant LNCaP derivative cells, termed LN-CSS, were established and maintained at 37°C in RPMI-1640 and supplemented with 10% charcoal stripped fetal bovine serum (cat. no. F6765; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and penicillin-streptomycin solution.

**Cell viability assay.** For each cell line,  $0.5\text{--}1 \times 10^4$  cells/well were plated in 96-well plates. After 24 h, the medium was treated with or without DOC (1.0, 2.5, 5.0, 7.5 and 10.0 nM) or SO (50, 100, 200 and 400 mM), and incubated at 37°C for 48 h. Cells were also treated with DOC in combination with SO at 37°C for 48 h in order to investigate the effect of drug combination. Cell viability was determined with a WST-1 assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) using a 96-well microplate reader (SoftMax Pro 5.X; Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturer's protocol. The cytotoxicity of SO/DOC drug combination in PC cells was evaluated using the Chou-Talalay combination index (CI) method (CalcuSyn Biosoft V2.0; BIO SOFT, Cambridge, UK) (14).

**Cell apoptosis assay and cell cycle analysis assay.** The PC cells were treated with DOC (1 nM) and SO (50 mM) at 37°C for 72 h. Apoptosis was detected by Annexin V staining using a MEBCYTO-Apoptosis kit (MBL International Co., Woburn, MA, USA), according to the manufacturer's protocol. The cells were then analyzed with a flow cytometry (BD FACSCanto II; BD Biosciences; Becton-Dickinson and Company, Franklin Lakes, NJ, USA). The flow cytometry results were analyzed using FlowJo v10 (FlowJo LLC, Ashland, OR, USA).

**Western blotting.** Cells were lysed with sample loading buffer (200 mM Tris-HCL, 12% glycerol, 2% SDS, 1% 2-mercaptoethanol and 0.005% Bromo Phenol Blue; pH 8.4) directly in culture dishes and the proteins extracted from cells (PC3,  $3 \times 10^4$  cells/lane; DU145,  $7 \times 10^4$  cells/lane; LNCaP,  $12 \times 10^4$  cells/lane; and LN-CSS,  $15 \times 10^4$  cells/lane) were separated by electrophoresis using 12.5% Tris-BES gels (Q-PAGE mini; cat. no. 09-155; TEFCO, Tokyo, Japan). Separated proteins were then transferred onto a polyvinylidene fluoride

membrane (Immobilon-P; cat. no. RPN2232; EMD Millipore, Billerica, MA, USA). Following blocking with 5% dried milk and 1% normal goat serum (cat. no. G6767; Sigma-Aldrich; Merck KGaA)-PBS for 1 h at room temperature, the membrane was probed with rabbit monoclonal anti-LDH-A antibody (1:1,000; cat. no. 3582S; Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C. After three washes with TBS with 0.05% Tween-20, membranes were incubated with ImmPRESS Horseradish Peroxidase Polymer Reagents (1:5,000; anti mouse IgG, cat. no. MP-7402; anti-rabbit IgG, cat. no. MP-7451; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature followed by a final wash with PBS. Immune complexes were visualized using a ECL Prime Western Blotting Detection reagent (cat. no. RPN2232; Amersham; GE Healthcare Life Sciences), according to the manufacturer's protocols and signals were digitally captured using an image analyzer (LAS 4000 and Amersham Imager 600; Fuji Photo Film Co., Ltd., Tokyo, Japan). Subsequently, the membrane was treated with stripping buffer (0.1 M glycine-HCl buffer; pH 2.5) to dissociate and strip the primary antibody from the western blot analysis membrane, and then mouse monoclonal anti-b actin antibody (1:1,000; cat. no. A5316; Sigma-Aldrich; Merck KGaA), which was used a reference control, was re-probed overnight at 4°C.

**Transfection of small interfering RNA (siRNA).** LN-CSS cells ( $2 \times 10^5$  cells/well) were plated in 6-well plates. On the following day, cells were transfected with 160 nM LDH-A siRNA (cat. no. 4390824; sense, 5'-CAGUGGAUAUCUUGA CCUAtt-3' and antisense, 5'-UAGGUCAAGAUAUCCACU Gga-3'; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol using Lipofectamine® RNAiMAX (cat. no. 13778030; Thermo Fisher Scientific, Inc.) and incubated at 37°C for 48 h. Scrambled siRNA (cat. no. 4390843; Negative Control #1 siRNA; Thermo Fisher Scientific, Inc.) was used as a negative control.

**Statistical analysis.** The one-way analysis of variance, followed by the least significant difference post-hoc test (Tukey-kramer test), was performed to analyze data using JMP Ver.13.2.1 (SAS Institute, Inc., Cary, NC, USA). All data was represented as mean  $\pm$  standard error.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LDH protein expression and DOC sensitivity in PC cells.** Firstly, the association between LDH expression level and sensitivity to DOC was examined in PC cell lines. The WST-1 assay demonstrated that treatment of PC cells with DOC for 72 h resulted in growth inhibition that was dependent on DOC concentration. The half-maximal inhibitory concentration of DOC was determined to be 7.5 nM in PC3 cells, 2.5 nM in DU145, 2.5 nM in LNCaP and 10 nM in LN-CSS, indicating that LN-CSS and PC3 cells were relatively resistant to DOC. Western blot analysis demonstrated that LNCaP and LN-CSS cells exhibited high expression of LDH-A protein, compared with PC3 and DU145 cells. It was also observed that LN-CSS cells expressed increased expression of LDH-A protein, compared with LNCaP cells (Fig. 1). Collectively, these results

indicated that resistance to DOC may be associated with the expression level of LDH-A protein in PC cells.

*SO causes minimal toxicity in normal cells and inhibits growth in PC cells.* SO is an inhibitor of gluconeogenesis and glycolysis (15). It is a structural analog of pyruvate, which inhibits LDH and disrupts the entire gluconeogenic pathway (16). Due to cancer cells being frequently dependent on glycolysis for ATP production (17), SO has implications as an anticancer compound. Firstly, the inhibitory effect of SO on the survival of PC cells and its effect on normal cells were investigated. A WST-1 assay demonstrated that treatment of PC cells with SO (50 mM) for 72 h resulted in growth inhibition (PC3, ~30%; DU145, ~55%; LNCaP, ~20%; and LN-CSS, ~55%) and this was dependent on SO concentration (Fig. 2A). In contrast, normal lymphocytes exhibited  $\leq 10\%$  suppression of growth at 50 mM SO (Fig. 2B), indicating that they are less susceptible to SO. Subsequently, the suppression of LDH-A expression by SO was confirmed in PC cells. Treatment of PC cells with SO resulted in a time-dependent decrease in LDH-A protein expression (Fig. 2C).

*Cytotoxic effects of combination treatment of DOC and SO.* The combined effect of DOC (1 nM) and SO (50 mM) was examined. The SO treatment concentration is 50 mM due to SO treatment exerting a marginal cytotoxic effect on normal lymphocytes within the concentrations of 50 mM of SO (Fig. 2B) and the minimum cytotoxicity of DOC is 1 nM (Fig. 1A).

As depicted in Fig. 3A, following the combination treatment with DOC (1 nM) and SO (50 mM), synergistic cytotoxicity was observed in LN-CSS cells (CI, 0.5), but not in LNCaP cells (CI, 6.5). The cytotoxic effect of SO/DOC combination in PC cells was evaluated using the Chou-Talalay CI method, which calculates quantitative definitions for additive effect (CI=1), synergism (CI<1) and antagonism (CI>1) in drug combinations (CalcuSyn Biosoft V2.0).

Subsequently, whether the increased cytotoxic effect caused by SO and DOC drug combination is responsible for the decrease in LDH expression by SO was investigated. Therefore, siRNA was used to knockdown the expression of LDH-A in these cells. As depicted in Fig. 3B, western blotting analysis indicated that the expression of LDH-A was downregulated by LDH-A siRNA and cells with decreased expression of LDH-A had increased sensitivity to DOC (Fig. 3C).

Furthermore, the expression of LDH-A during combined treatment was confirmed by western blotting and it was demonstrated that LDH-A protein expression levels were increased by treatment with DOC and SO can block this DOC-induced increase in LDH-A protein expression (Fig. 3D and E).

These results indicated that SO promotes DOC-induced apoptosis by blocking DOC-induced increase of LDH-A protein expression in LN-CSS cells.

*Cell cycle analysis of PC cells following combined treatment with SO and DOC.* The change in cell cycle distribution following treatment with DOC in combination with or without SO in LN-CSS and LNCaP cells was investigated. Cell cycle analysis revealed that the combination of DOC and SO for 72 h resulted in maximum accumulation of cells in the G2-M

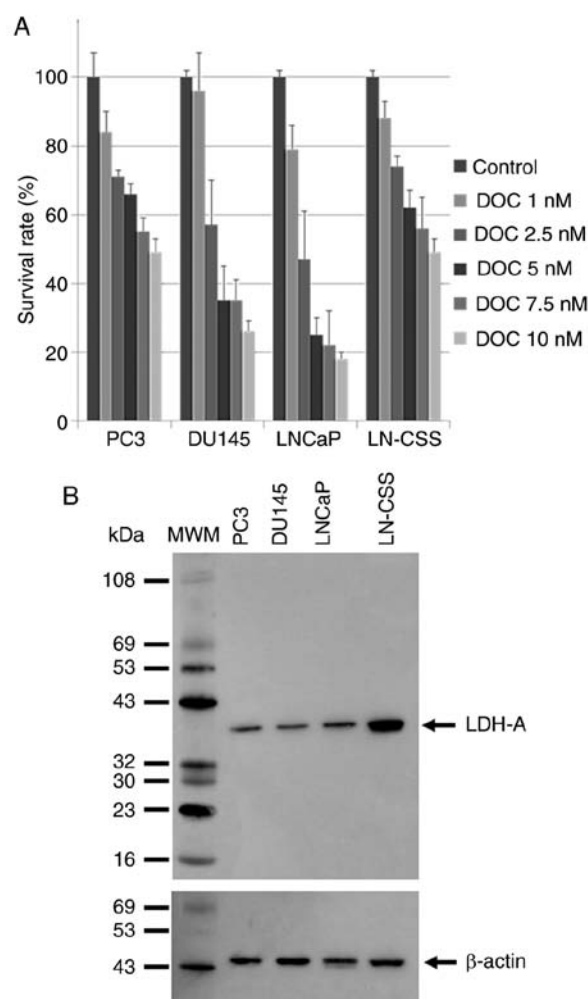


Figure 1. LDH-A (MW, 37 kDa) expression is associated with DOC sensitivity in prostate cancer cells. (A) PC3, DU145, LNCaP and LN-CSS cells were treated with 1.0, 2.5, 5.0, 7.5 and 10.0 nM DOC, followed by the measurement of cell viability. The data in the columns represent the mean of 3 independent experiments and the bars represent the standard deviation. (B) Western blotting was performed to examine the LDH-A expression levels, where  $\beta$ -actin served as a loading control. DOC, docetaxel; LDH-A, lactate dehydrogenase-A; MW, molecular weight. MWM, molecular weight marker.

phase, followed by sub-G1 accumulation in LN-CSS cells, but not in LNCaP cells (Fig. 4A and B).

*SO and DOC-induced apoptosis.* Cell cycle analysis indicated that, following drug treatment, a relatively large number of cells were detected in the sub-G1 phase, in which there was an accumulation of dead cells, indicating the induction of apoptosis by the drug. Therefore, whether this cell death was due to apoptosis was examined.

An Annexin V assay demonstrated that LN-CSS cells treated with DOC alone exhibited decreased apoptosis (16.4%), compared with apoptosis induced by combination treatment (40.4%;  $P<0.001$ ; Fig. 5).

## Discussion

In the present study, the role of LDH-A in human PC cell lines, specifically in CRPC, was investigated. It was determined that LDH protein expression was strongly associated with

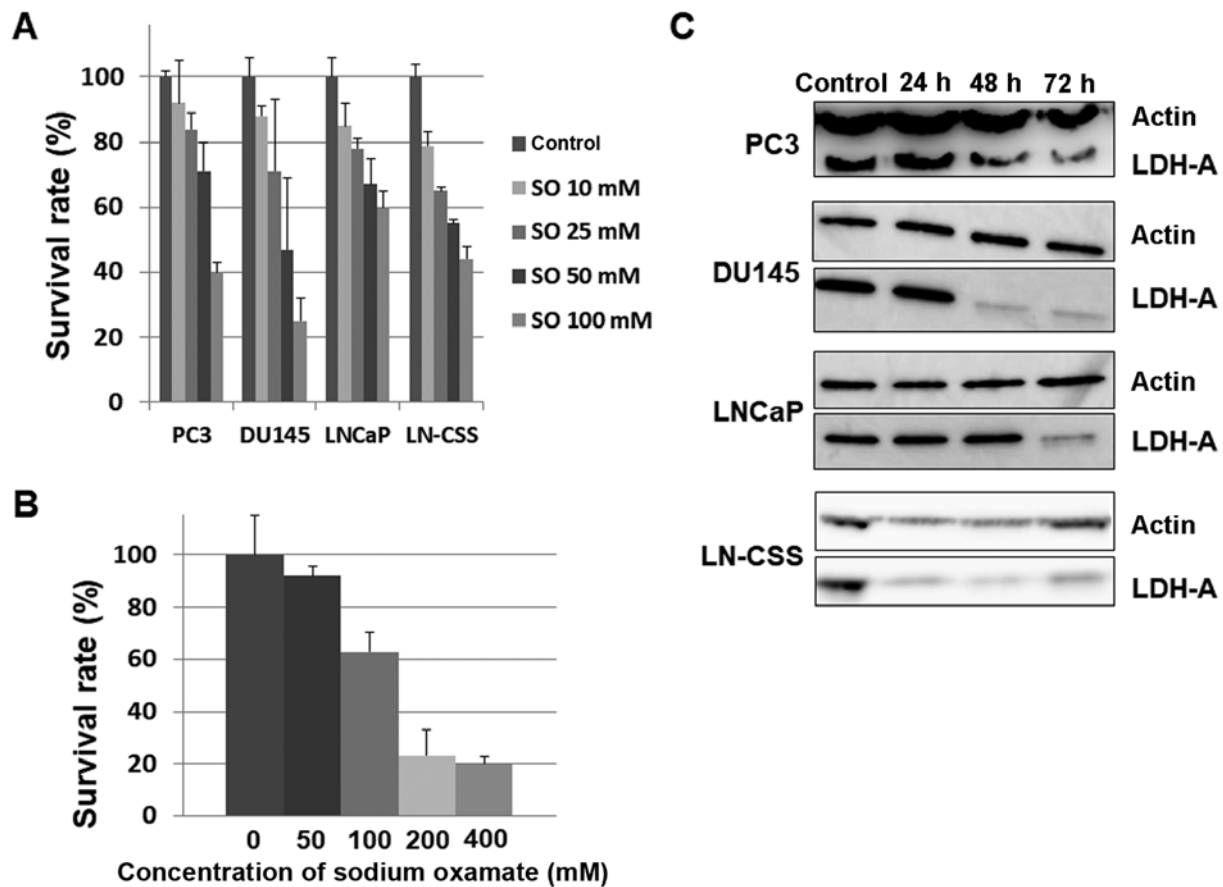


Figure 2. The specific LDH-A inhibitor SO exhibits inhibition of cell growth in prostate cancer cells with minimal toxicity to normal cells. (A) PC3, DU145, LNCaP and LN-CSS cells were treated with 10, 25, 50 and 100 mM SO, followed by measurement of cell viability. (B) Normal lymphocytes were treated with 50, 100, 200 and 400 mM SO, followed by measurement of cell viability. SO treatment exerted marginal cytotoxic effect in normal lymphocytes within the concentrations of 50 mM SO. (C) Western blotting was performed to examine the LDH-A expression levels in PC3, DU145, LNCaP and LN-CSS cells, where  $\beta$ -actin served as a loading control. The data in the columns represent the mean of 3 independent experiments and the bars represent the standard deviation. SO, sodium oxamate; LDH-A, lactate dehydrogenase-A.

DOC sensitivity in PC cells. Compared with castration-naïve LNCaP cells, castration-resistant LN-CSS cells exhibited an increased expression of LDH-A, with SO causing cell inhibition, resulting in increased CRPC cell sensitivity to DOC. Furthermore, compared with DOC or SO monotherapy, a combination therapy of DOC and SO facilitated cell apoptosis and demonstrating a strong effect in suppressing cell growth in LN-CSS cells (hormone-resistant LNCaP cells). The present results indicated that LDH-A is strongly associated with DOC resistance and may result in a novel therapeutic strategy for overcoming DOC resistance, particularly in patients with CRPC. DOC is an anticancer drug that is used not only used for PC treatment, but also various other cancer types, including breast cancer. DOC resistance causes cancer recurrence and metastasis, which can ultimately result in death (18).

Although a number of studies demonstrated resistance to DOC in cancer cells, its specific mechanism remains unknown (19). Cancer cells differ from normal cells in their metabolic properties, with normal cells relying primarily on the process of mitochondrial oxidative phosphorylation, thereby utilizing oxygen and glucose to produce energy (20). In contrast, cancer cells depend primarily on glycolysis, which is the anaerobic breakdown of glucose into ATP, the energy-storing molecule, even in the presence of available

oxygen (21-25). Since metabolic changes can supply sufficient energy and biosynthetic precursors to cancer cells, metabolic enzymes involved in glycolysis are potential therapeutic targets.

LDH-A is one of the main isoforms of LDH frequently exhibited in human cells and tissues (26). It was demonstrated that an increased level of LDH in the serum is associated with poor prognosis in patients with CRPC (8). However, the role of LDH in chemoresistance of CRPC has not been investigated. In the present study, castration-resistant LN-CSS cells were used to compare the expression and activity of LDH-A with other PC cells, in the development of DOC resistance, including the parental castration-naïve LNCaP cells. To the best of our knowledge, this is the first report to provide direct evidence in support of a role for LDH-A in acquired DOC resistance in human PC cells.

It was determined that DOC treatment resulted in increased LDH-A protein expression. It has been demonstrated that DOC induces the expression of LDH-A, which promotes cellular glycolysis and helps cancer cells to survive (17). Previous studies reported that cancer cells suppress apoptosis driven by cytochrome *c* through unregulated glucose metabolism (27,28). Therefore, the DOC induced overexpression and activity of LDH-A detected in DOC-resistant cells may be an adaptation of these cells to DOC treatment, as well as a mechanism to modulate glucose metabolism and glycolysis, thereby avoiding

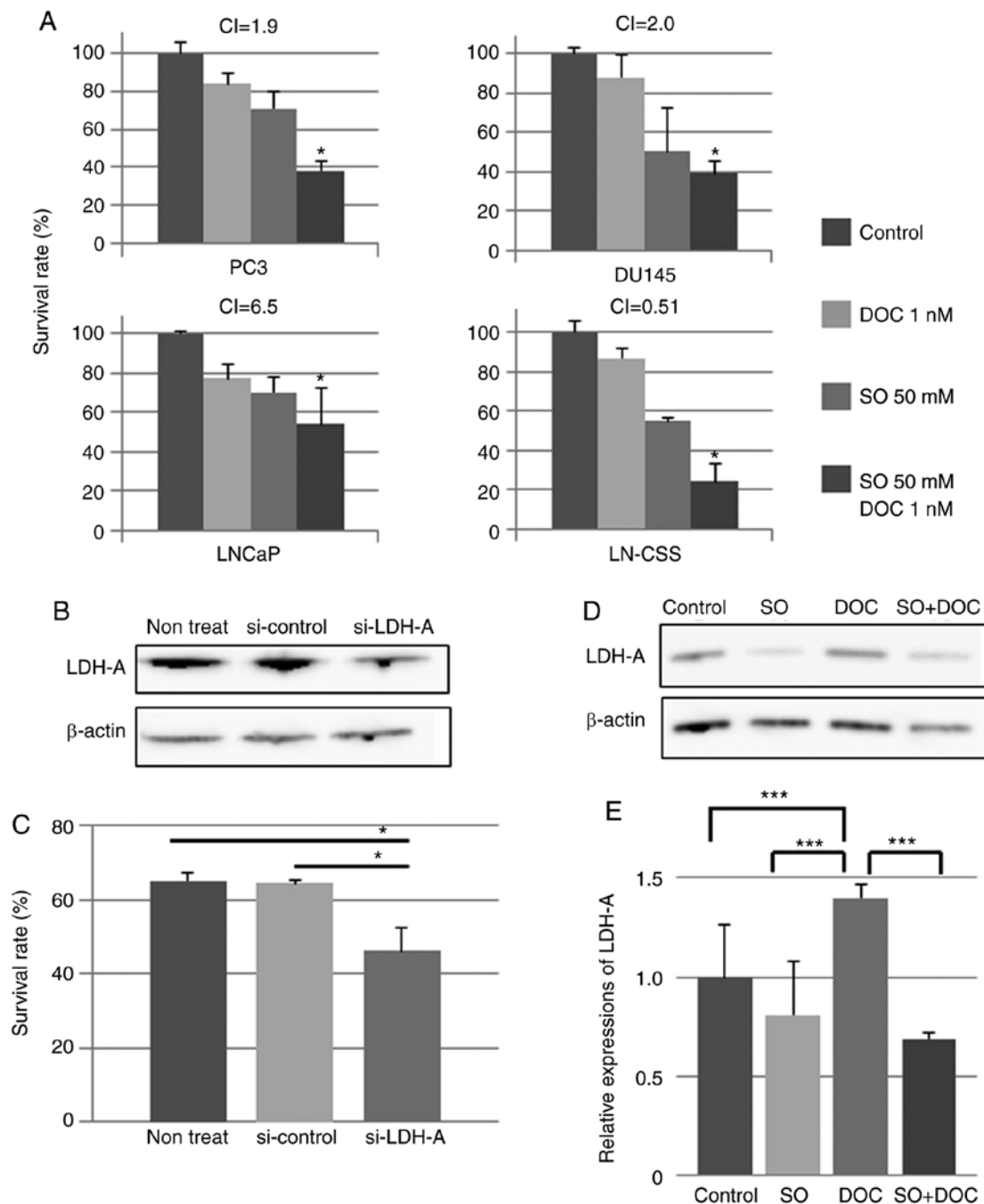


Figure 3. The combination of DOC and SO exhibits synergistic cytotoxic effects on castration-resistant LN-CSS cells. PC3, DU145, LNCaP and LN-CSS cells were treated with and without a combination of 50 mM SO and 1 nM DOC, followed by measurement of cell viability. Synergistic cytotoxicity was observed following the combination treatment with DOC and SO in LN-CSS cells, but not in the other PC cell lines, including LNCaP cells. (A) The cytotoxicity of SO/DOC combination in PC cells was evaluated using the Chou-Talalay CI method, which offers quantitative definitions for additive effect (CI=1), synergism (CI<1) and antagonism (CI>1) in drug combinations. \*P<0.05 vs. control. (B) LN-CSS cells were transfected with scrambled siRNA or LDH-A siRNA for 48 h, and then treated with 1 nM DOC. Western blotting was performed to examine LDH-A expression levels in LN-CSS cells, and β-actin served as a loading control. (C) Knockdown of LDH-A sensitized LN-CSS cells to DOC (1 nM). The data in the columns represent the mean of 3 independent experiments and the bars represent the standard deviation. \*P<0.05. (D) LN-CSS cells were treated with and without a combination of 50 mM SO and 1 nM DOC, and β-actin served as a loading control. (E) Band intensities were quantified to assess the levels of LDH-A. Control bands were samples without treatment. \*\*\*P<0.001. CI, combination index; SO, sodium oxamate; LDH-A, lactate dehydrogenase-A; DOC, docetaxel; PC, prostate cancer; si, small interfering.

apoptosis induced by DOC. Targeting LDH by SO interrupts this feed-forward cycle and re-sensitizes cancer cells to DOC (7). These results indicated that LDH may potentially serve as a target for overcoming DOC resistance in patients with CRPC.

In CRPC, the combination treatment of DOC and SO was demonstrated to be more effective than DOC or SO monotherapy. This combination therapy demonstrated a

synergistic antitumor effect by promoting apoptosis of PC cells. Although SO interferes with the cell cycle from the G2 to M phase (29), the present study indicated that it causes apoptotic cell death, which is associated with the treatment of DOC-resistant CRPC. However, high concentrations of SO may limit its therapeutic potential in clinical practice (12). From a clinical perspective, it is notable that SO exhibits

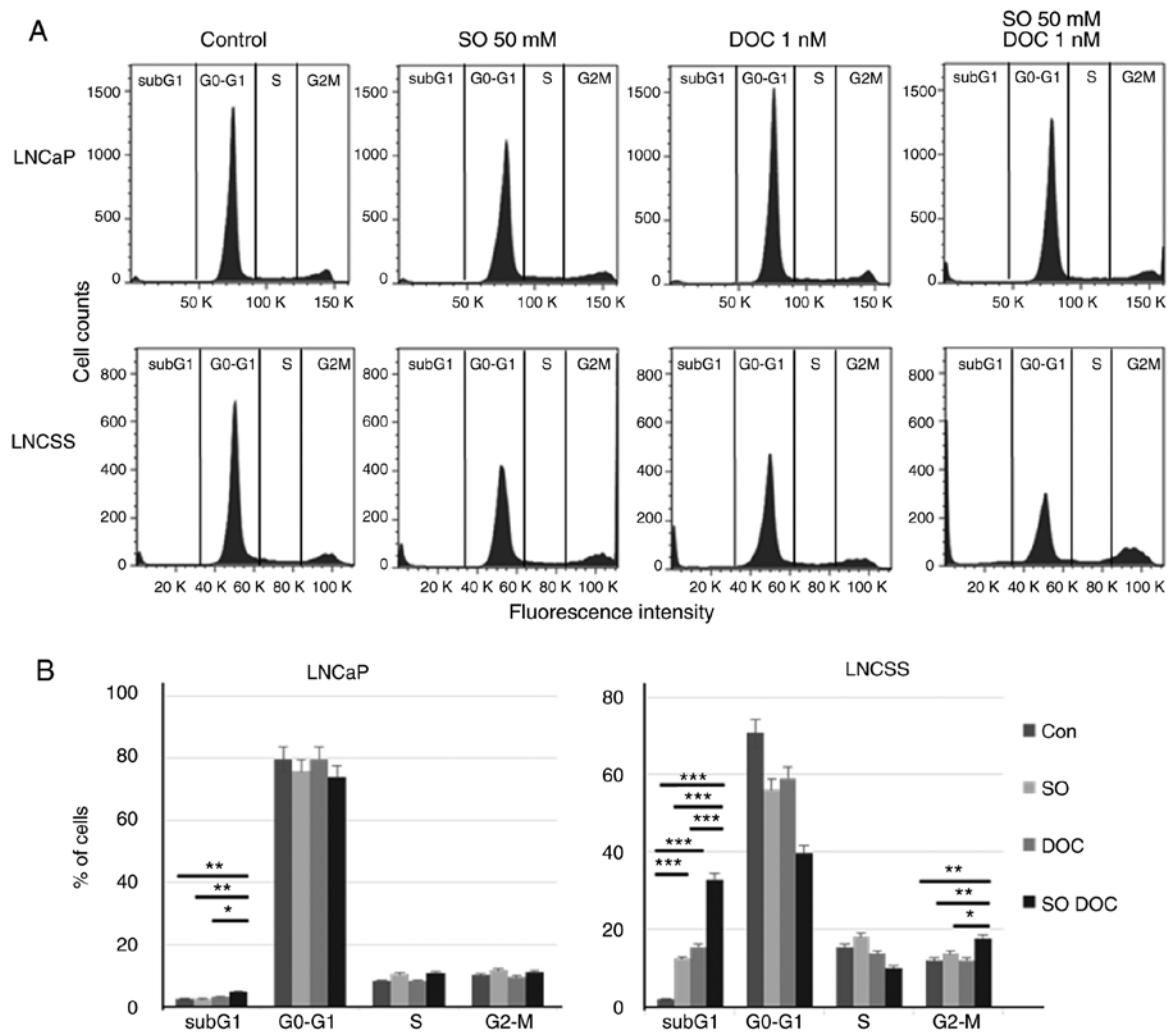


Figure 4. Combination of DOC and SO induces the accumulation of cells in the G2-M phase, followed by sub-G1 accumulation in LN-CSS cells. (A) LN-CSS and LNCaP cells were treated with and without a combination of SO and DOC, and cell cycle distribution was examined by flow cytometry. The figure is one of 3 independent experiments. (B) The data in the columns represent the mean of 3 independent experiments and the bars represent the standard deviation. The results of statistical analysis are depicted for subG1 and G2-M. \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$ . SO, sodium oxamate; DOC, docetaxel.

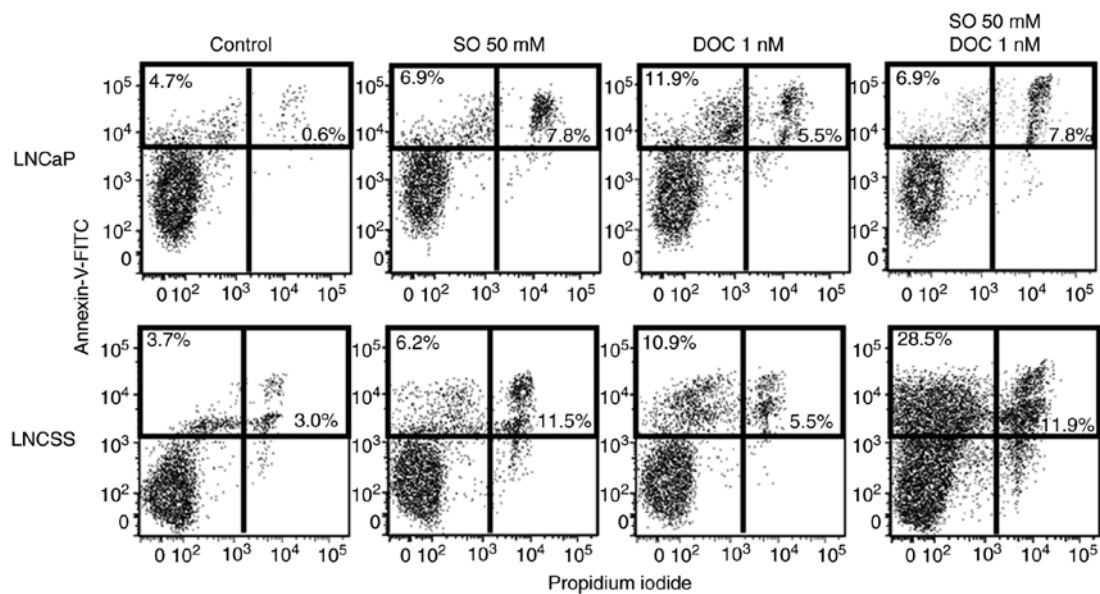


Figure 5. SO promotes DOC-induced apoptosis by blocking DOC-induced increase in LDH-A protein expression in LN-CSS cells. LN-CSS and LNCaP cells were treated with and without a combination of 50 mM SO and 1 nM DOC, and apoptosis was examined by flow cytometry using Annexin V. The figure is a representation of 3 independent experiments. SO, sodium oxamate; DOC, docetaxel; FITC, fluorescein isothiocyanate.



cytotoxic effects only in tumor cells, while normal cells are largely unaffected. In the present study, SO had marginal effect on the growth of normal lymphocyte cells indicating that SO primarily blocks anaerobic glycolysis, which is an important characteristic of tumor cells.

The present results indicated that LDH-A serves an important role in DOC resistance in advanced PC cells. The inhibition of LDH-A promotes DOC sensitivity, particularly in CRPC cells. The present study may provide valuable information for the development of targeted therapies in patients with CRPC in the future.

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

HM, MS, SM, KK, IK, GN, YK, MW, KZ, KK, KN, SS and KY equally conducted in the conception and design of the study, acquisition and interpretation of data, drafting the article and final approval of the version to be published.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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