Research Article

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Dicholoroacetate exerts anti-cancer activity on human renal cell carcinoma cells

Dikloroasetatın İnsan Böbrek Karsinoma Hücrelerinde Anti-kanser Aktivitesi

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Abstract

Aim: Impaired mitochondrial function is a consequence of HIF1-induced overexpression of pyruvate dehydrogenase kinase (PDK) which phosphorylates and inactivates pyruvate dehydrogenase multi-enzyme complex (PDC), which converts pyruvate to acetyl-CoA for entry into the TCA cycle. Shifting cancer cells from glycolysis to oxidative phosphorylation induces apoptosis, which is a new therapeutic strategy by utilizing PDK inhibitors. In this work, the effect of PDK inhibitor, dichloroacetate (DCA) has been investigated in Human renal carcinoma cell line.

Methods: Adherent epithelium renal cell adenocarcinoma (ACHN) cells were treated with different concentrations of DCA at different time periods. Cell viability was measured by WST assay, cell-cycle profile and apoptosis were assessed by using flow cytometry. Metabolites of the cell extracts were analyzed by LC-MS/MS.

Results: DCA reduced cell viability in a concentration- and time-dependent manner. Treatment with DCA induced G1 arrest and apoptosis in ACHN cells. Additionally, metabolite changes of ACHN cell line upon DCA treatments showed that lactate, citrate, N-acetylaspartate and 5-oxoproline levels, which were high in untreated cells, significantly reduced upon DCA treatment.

Conclusion: Potential anti-carcinogenic effects of DCA, including inhibition of cell proliferation and growth, and induction of apoptosis, as well as the ability of markedly reducing lactate levels make this agent a promising drug candidate in renal adenocarcinomas.

Keywords: Renal adenocarcinoma cell; Dichloroacetate; Cell cycle; Apoptosis; Metabolite; LC-MS/MS.

Özet

Amaç: Bozulmuş mitokondriyal fonksiyonlar; pirüvatı asetil-CoA'ya dönüştürerek TCA çevrimine girmesini sağlayan, pirüvat dehidrogenaz multi-enzim kompleksi (PDC)'ne fosfat grubu ekleyerek inaktive eden, HIF-1 uyarımlı pirüvat dehidrogenaz kinaz (PDK)'ın aşırı ifade edilmesi sonucunda ortaya çıkmaktadır. PDK inhibitörlerinin kullanımı, kanser hücrelerinin glikolizden oksidatif fosforilasyona yönlendirilmesini ve böylelikle apoptoz'un uyarılmasını sağlayan yeni bir tedavi stratejisidir. Bu çalışmanın amacı, insan böbrek karsinoma hücrelerinde bir PDK inhibitörü olan dikloroasetat (DCA)'ın etkilerinin belirlenmesidir.

Metod: ACHN (insan renal adenocarcinoma) hücreleri farklı konsantrasyon ve zaman sürecinde DCA ile muamele edilerek hücre canlılığı, hücre canlılık assayle; hücre döngüsü profilleri ve apoptoz akım sitometresiyle ölçülmüştür. ACHN hücrelerinden elde edilen özütlerdeki metabolit seviyeleri LC-MS/MS ile analiz edilmiştir.

Bulgular: DCA doz ve zamana bağlı olarak ACHN hücre canlılığını azaltırken, 50 mM DCA uygulaması hücre canlılığını önemli derecede düşürmüştür. Bu konsantrasyonda hücrelerin G1 evresinde 24 saat süresince tutuklandığı belirlenmiştir. Bu sonuçlara ilaveten, DCA ACHN hücrelerinde

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konsantrasyon ve zaman bağımlı olarak apoptozu uyarmıştır. LC-MS/MS analizleri bu hücrelerde laktat, sitrat, N-asetilaspartat ve 5-oxoprolin metabolitlerinin yüksek miktarda bulunduğu ve DCA uygulamalarının bu metobolitlerin azalmasına neden olduğunu göstermiştir.

Sonuç: DCA'nın hücre canlılığını azaltması, apoptozu uyarması ve laktat seviyesini düşürmesi gibi potansiyel anti-karsinogenik özellikleri, bu ajanın böbrek adenokarsinoma tedavisinde kullanılabilecek bir ajan olabileceğini işaret etmektedir.

Anahtar Kelimeler: Böbrek adenokarsinoma hücre hattı; dikloroasetat; Hücre döngüsü; Apoptoz; Metabolit; LC-MS/MS.

Introduction

Kidney cancer is the twelfth most common cancer in the world affecting more than 300,000 patients annually. Renal cell carcinoma (RCC) is the most common type of kidney cancer [1, 2]. No curative therapy exists for patients diagnosed with metastatic RCC. It is resistant to treatment such as radiation therapy and chemotherapy [3]. RCC displays impaired mitochondrial function and preferential use of glycolysis despite the presence of high oxygen concentration (known as "aerobic glycolysis" or the "Warburg effect") [4]. Due to mitochondrial suppression, pyruvate produced in glycolysis from glucose is preferentially diverted towards the production of lactate. This acidic (lactic) environment gives an advantage to cancer cells for activation of their proteases for disrupting and killing normal cells [5].

Impairment of mitochondrial function is the consequence of downregulation of pyruvate dehydrogenase complex (PDC) activity, which links glycolysis to TCA cycle by catalyzing conversion of pyruvate to acetyl-CoA. PDC is one of the key regulatory enzymes involved in the control of the carbohydrate metabolism. The activity of PDC is regulated by tissue-specific, up to now known, two phosphatase (PDP) and four kinase (PDK) isoenzymes [6]. PDK phosphorylates and inhibits PDC, whereas PDP removes the phosphate group(s) and thus restores its activity. In the fast growing solid tumors like RCC, hypoxia-inducible factor 1 (HIF1) upregulates expressions of glycolytic enzymes, lactate dehydrogenase as well as PDK isoenzymes [7, 8]. Overexpression of PDK causes marked reduction of PDC activity, thereby severely impairing the link between glycolysis and TCA cycle. Suppression of mitochondrial function by HIF1 via PDK expression also results in higher mitochondrial membrane potential and lower mitochondrial reactive oxygen species (mROS) production, facilitating a state of resistance to mitochondrial-driven apoptosis [4]. Hence, targeting PDKs appears to be a therapeutic strategy to restore mitochondrial function, which in turn causes apoptosis, in rapidly growing cancer cells.

Dichloroacetate (DCA), an inhibitor of PDK, has been effectively used for the treatment congenital forms of lactic acidosis in infants and children, and to enhance glucose oxidation in mitochondrial disorders with low PDC activities [9]. Furthermore, recent studies have indicated that DCA induces apoptosis in lung, breast, and glioblastoma cancer cell lines by shifting metabolism from aerobic glycolysis to glucose oxidation [10–12], indicating that it is also a potential anti-tumor agent in vitro and in vivo. Here we wanted to see the effect of DCA treatment on ACHN cell line by analyzing cell viability, proliferation, apoptosis, as well as metabolite changes.

Materials and methods

Materials

DCA (Sigma-Aldrich) dissolved in dH_2O was used immediately and/or stored at –20°C for further usage. All of the used analytical standards were 98% or more pure according to the manufacturer instructions. HPLC-grade solvents, acetonitrile, methanol, formic acid (Lichrosolv) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Millipore Direct Q3-UV deionized water was used in all studies.

Cell culture

Adherent epithelium renal cell adenocarcinoma (ACHN) cells (CRL-1611) and Human umbilical vein endothelial (HUVEC) cell lines (CRL-1730) were purchased from ATCC that were maintained at Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Germany), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 in the air at 37°C.

Viability assay

Cells (1×10^3) per well were seeded in 96 cell culture plates. After overnight incubation media was replaced with fresh media containing 12.5 mM, 25 mM and 50 mM

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concentrations of DCA. 24, 48 and 72 h incubated cells were washed with 100 µL PBS to remove DCA before addition of assay solution. After addition of WST reagent (Roche, Swiss) plates were incubated in the dark for 1 h. OD was measured using a plate reader (Biotek, Winooski, VT, USA) at 450 nm.

Cell cycle analysis

Cells (3×10^5) per well were placed in six well tissue culture plates and after 24 h incubation, old media replaced with fresh media containing increasing doses of DCA (control, 25 mM and 50 mM) for 24, 48 and 72 h. After washing and harvesting, cells were suspended and incubated in 500 µL staining solution (40 µg/mL RNase A, 33 µg/mL PI, 0.2% NP-40 in PBS) at 37°C for 30 min. Flow cytometry was performed by BD FACS Calibur (BD Bioscience, USA) and data were analyzed using BD-Cell Quest Pro software (USA).

Apoptotic analysis

Cells (3×10^5) per well were spread in six-well tissue culture plates and incubated overnight. Media replaced with fresh media and control, 25 mM and 50 mM DCA was used and compared to control untreated cells. Flow cytometry was performed after 24, 48 and 72 h of incubation. Cells were washed twice with PBS and suspended in Annexin V-FITC Apoptosis Kit (Biovision, USA). Then, the samples were analyzed on BD FACS Calibur flow cytometer (BD Bioscience, USA).

LC-MS/MS studies

The cells were grown as described above and then the cells were seeded onto T-75 plates and culture medium was replaced with serum free medium for 24 h prior to DCA treatment. Then, medium was exchanged with complete medium containing 25 mM and 50 mM concentrations of DCA for 24 h, 48 h and 72 h. Cells were immediately washed three times with ice-cold 0.9% NaCl solution and then the 2 mL cold (−80°C) extraction solutions which contain ACN:MeOH:H_2O:formic acid (40:40:19:1, v/v) was added on to the cells. Cells were scratched from the T-75 plates and transferred into falcon tubes (4°C) and vortexed for 1 min, centrifuged at 3000 rpm and 4°C. The supernatants were stored at −80°C until analysis by LC-MS/MS.

The cell extracts were analyzed by an LC-MS/MS method developed by SEM Laboratories (Istanbul, Turkey). Briefly, LC-MS/MS analyses were conducted using Agilent 6460 triple quadrupole LC/MS system equipped with an Agilent Jet Stream electrospray ionization source and coupled with Agilent 1290 Infinity UHPLC system consisting of an Agilent 1290 Infinity binary pump, high performance autosampler, thermostatted column compartment. MassHunter workstation software was used for data acquisition and analysis. For LC flow: 0.6 mL/min, column temperature was set: 20°C, injection volume: 5 µL. LC-MS/MS (Agilent 6460 QQQ) system was operated MRM (multiple reaction monitoring) mode and optimized source conditions and parameters for organic acids are; ESI, jet stream ionization, gas temp: 150°C, gas flow: 10 L/min, sheath gas temp: 350°C, sheath gas flow: 10 L/min, nebulizer: 40 psi, capillary voltage: 2000 V.

Statistical analysis

Statistical analyses were performed using one-way ANOVA and Tukey's post hoc test using SPSS 20 for Windows. $p < 0.05$ was considered statistically significant.

Results

DCA reduces renal cancer cell viability

To determine the effect of DCA on the viability of ACHN cells, the cell line was exposed to selected concentrations of DCA at selected time periods. In 24 h, compared to untreated control cells, about 10–50% decrease in cell viability with increasing DCA concentration was observed (Figure 1A). Reduction in cell viability was more significant at 48 and 72 h time periods (Figure 1A). Unlike other doses, the rate of decrease of cell viability was linear with 50 mM DCA. For further studies, 25 mM and 50 mM DCA concentrations were used. Additionally, in order to investigate the effect of DCA on non-cancerous cell viability, HUVEC cells were used as a control cell line. In a similar fashion, HUVEC cell viability was decreased in a time- and dose-dependent manner. The cell viability was 35.3 ± 4.5% in 50 mM DCA administered cells for 72 h (Figure 1B). DCA was more effective against ACHN cells compared to HUVEC cells due to the 29.3% of viability difference.

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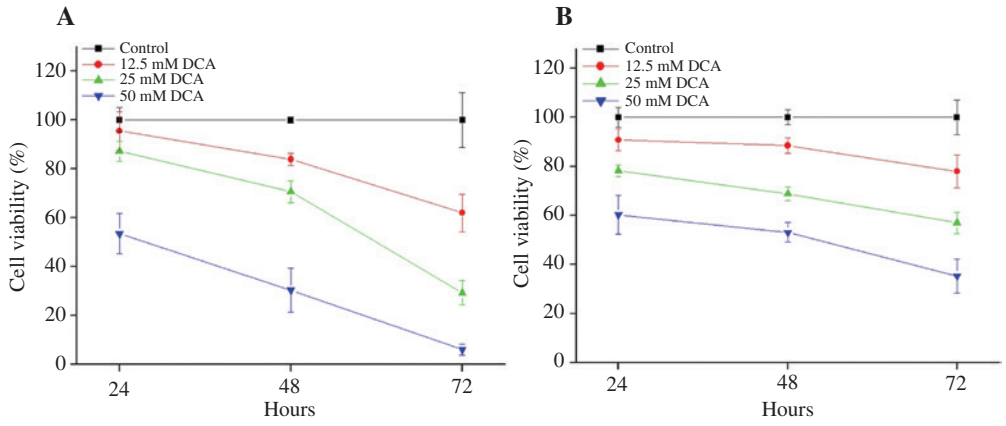


Figure 1: Effect of DCA on cell viability.
Cells were treated with increased doses of DCA or control untreated cells for 0–72 h. The relative number of viable cells was evaluated using WST-1 assay. DCA signifacantly reduced the viability of ACHN cells (A) and HUVEC cells (B). The data normalized to negative control values (no DCA exposure), which were set as 100% cell viability. (B) HUVEC cell line viability.

DCA induces G1 phase arrest in ACHN cell lines

To investigate whether the reduced viability of ACHN cell associated with induction of growth arrest, cells were treated with 25 mM and 50 mM DCA for 24, 48 and 72 h. DNA content was assessed by using PI staining for flow cytometric analysis. DCA treatment changed the cell cycle profile of ACHN cells (Figure 2). Compared to control cells (58.1%), significant amount of ACHN cells (71.1%) were accumulated at G1 phase after 50 mM DCA treatment ($p < 0.005$). Likewise, 50 mM DCA caused accumulation of cells at G1 in 24h, however this effect was insignificant and transient for the HUVEC cells (Supp. Fig. 1).

DCA triggers apoptosis in ACHN cell lines

To examine whether DCA is able to trigger apoptosis in ACHN cells, Annexin-V/PI staining was performed between 0 and 72 h. As shown in Figure 3, Annexin-V positive cells are representing early and late apoptosis at lower right and upper right respectively. Compared to untreated counterpart, the cells treated with 25 mM or greater DCA concentrations went to early (25.5%) and late apoptosis (19%) in 24 h. Moreover, total apoptotic cell death ratio was 21% and 33.9% in 48 h; 27.3% and 28.7% in 72 h at 25 mM and 50 mM DCA treated cells, respectively. In addition, when HUVEC cells also exposed to 25 mM and 50 mM DCA concentrations, 21% and 23.3% of apoptotic cells were observed in 72 h respectively (Supp. Fig. 2). Results indicated that apoptotic ratio of HUVEC cells were lower than ACHN cells in 72 h.

Effect of DCA on the levels of lactate, citrate, N-acetylaspartate, 5-oxoproline in ACHN cells

In order to see the effect of DCA treatment on metabolite changes in ACHN cells, the cell extracts were analyzed by an LC-MS/MS method. Of the 42 organic acids, only four compounds, namely, lactate, citrate, N-acetylaspartate (NAA) and 5-oxoproline (pyroglutamic acid) were quantitatively detected. The others were below detection limits. Figure 4 shows the changes in the levels of these four organic acids in untreated (control) and DCA-treated ACHN cell lines at 24 h, 48 h, and 72 h. As expected, the most abundant metabolite was lactate and its level in control cells was highly increased at 48 h and 72 h, but significantly diminished upon DCA treatment (Figure 4A). Citrate was the second highest metabolite detectable in ACHN cell line. The levels of citrate also markedly increased in control ACHN cell line at 48 and 72 h (Figure 4B). DCA treatment initially caused some increases in citrate levels in 24 h, but later on at 48 and 72 h remarkably reduced its level (Figure 4B). 5-oxoproline, an intracellular product of the γ-glutamyl cycle, was also found to be one of the highly produced metabolites in ACHN cell line. Although it appears that DCA treatment causes some increases in 5-oxoproline levels in 24 h, it causes modest decrease at 48 h, but remarkable one at 72 h (Figure 4C). Surprisingly, NAA, a nervous system-specific metabolite, was another

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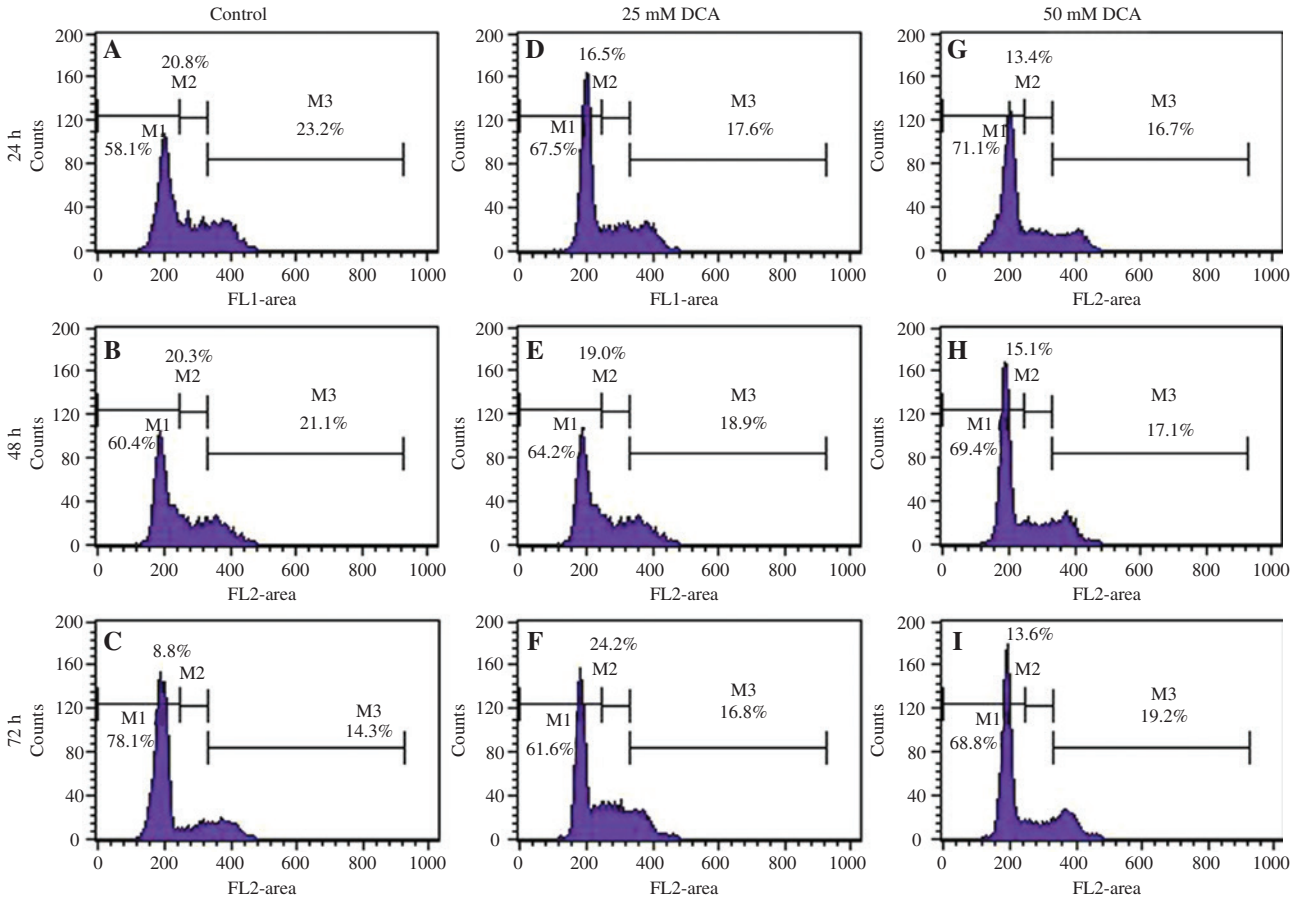


Figure 2: Effect of DCA on cell-cycle profile.
DCA, induces G1 cell-cyle arrest in ACHN cell lines. Cells were treated with 25 and 50 mM doses of DCA or control untreated cells for 24 h (A), 48 h (B) and 72 h (C). Compared to control cells, 50 mM DCA treated cells were blocked in G1 phase in 24 h. M1: G0/G1, M2: S, M3: G2/M.

metabolite which substantially accumulated in ACHN cell line (Figure 4D). DCA treatment also caused marked decrease in NAA levels (Figure 4D).

Under in vivo conditions, drugs diffuse through HUVEC layer before reaching the cancer cells; so HUVEC are selected as the healthy cells. The effects of DCA in HUVEC cell line metabolite levels were also studied by using LC-MS/MS, which only could detect lactic acid and citric acid (Supp. Fig. 3). In 25 mM and 50 mM DCA treatment, lactate levels are slightly decreased, while citrate levels slightly increased. All of the other metabolites were not detected in HUVEC cells.

Discussion

Shifting cancer cells from glycolysis to oxidative phosphorylation is a new therapeutic strategy by utilizing PDK inhibitors such as DCA alone or in combination with conventional drugs [13]. Moreover, DCA has a remarkable property; it is selectively toxic to cancer cells rather than noncancerous cells [12, 14, 15]. In the present study, the effects of DCA were studied in renal cell adenocarcinoma, ACHN cells. Results showed that DCA can reduce viability and induce apoptosis in ACHN cells. Our findings corroborate recent reports where DCA treatments resulted in reduction of viability and apoptosis [14–18]. Moreover, the result of cell cycle analysis revealed that there is a substantial increase of cell proportion in the G1 phase as well as decrease in S phase after DCA treatments. Previously, it was shown that DCA treatment resulted in G1, G2 arrest or no alteration on cell cycle profiles of various cells [14, 16, 19, 20–22]. Dissimilar cell cycle profiles after DCA treatment may indicate that the effects of DCA on cell-cycle is varied depending on cell type and/or physiological status of cells and/or depending on checkpoint mutations that cancer cells possess.

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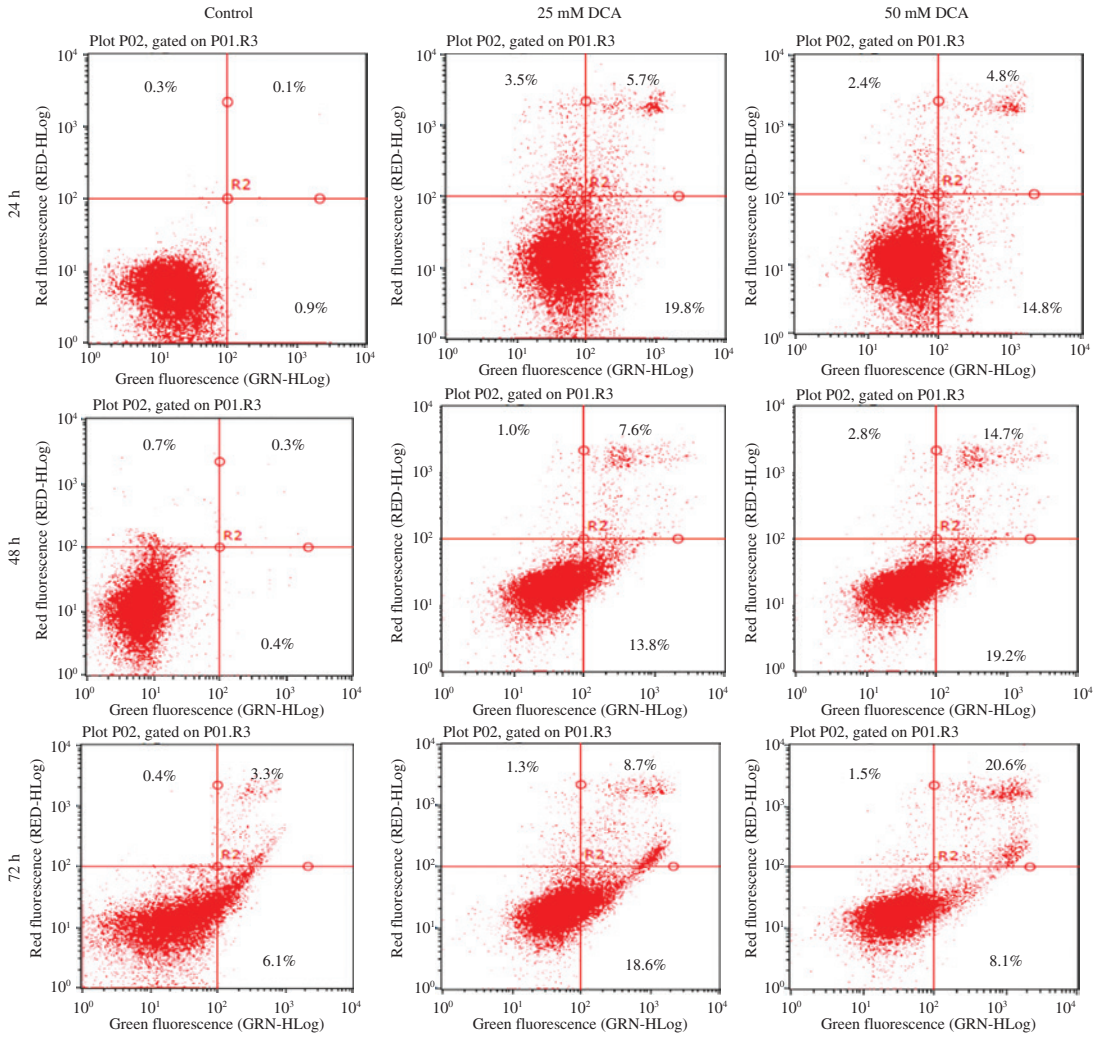


Figure 3: Effect of DCA on apoptosis.
Flow cytometry analysis of ACHN cells treated with 25–50 mM DCA for 24–72 h. DCA treatment induced apoptosis. Lower right and upper right parts show early and late apoptotic cells, respectively.

The apoptotic effect of DCA was shown in endometrial, lung, breast, and glioblastoma, colon, B leukemia, multiple myeloma, melanoma, head and neck cancer cells [10–13, 16–20]. DCA has a great potential as an apoptosis sensitizer and anticancer agent that reverses resistance to mitochondria dependent apoptosis [4, 19]. Moreover, effect of DCA on cancer cells has been found to be varied in that some populations are highly resistant to treatment, while others have high rates of death only at prolonged exposure [15]. Similarly, DCA treatment triggered apoptosis in ACHN cell line.

In this study, we also scrutinized metabolite changes upon DCA treatment in ACHN cell line. The LC-MS/MS analyses revealed that lactate, citrate, N-acetylaspartate and oxoproline levels were high in ACHN cell line. Since it was expected that DCA would inhibit PDK activity and thereby activate PDC, pyruvate produced via glycolysis would be diverted towards the production of acetyl-CoA rather than the production of lactate. Indeed lactate levels significantly reduced upon DCA treatment (Figure 4A), confirming the inhibition of PDK and thus reactivation of PDC. Increased glucose uptake and accumulation of lactate, even under normoxic conditions (aerobic glycolysis), is a common feature of cancer cells. Lactate appears to serve multifunctions in the tumor environment including contribution to the immunologic escape, potential antioxidative effects resulting in radioresistance, and promotion of metastasis and angiogenesis [23]. Besides, aerobic glycolysis in cancer cells plays role in resistance to apoptosis [24]. By decreasing lactate levels DCA appears to make marked contribution to prevent all of these lactate-induced processes in cancer cells.

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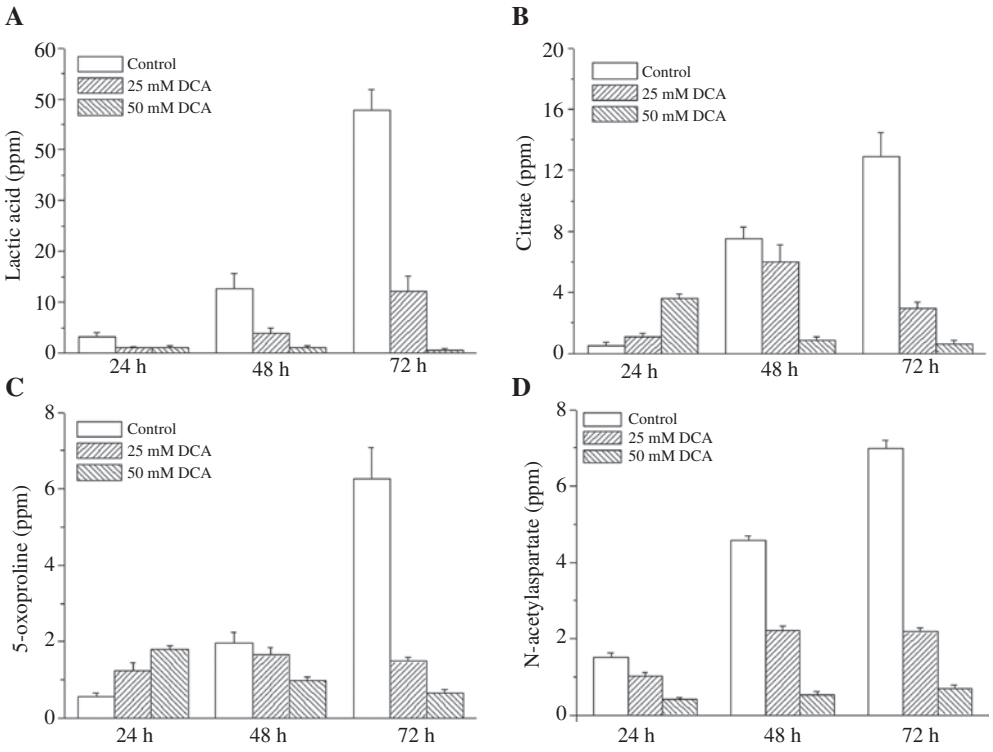


Figure 4: Effect of DCA on metabolite changes.
DCA treatment altered the levels of lactate, citrate, 5-oxoproline and N-acetylaspartate in ACHN cells. The cell extracts of DCA-treated and untreated (control) were analyzed by LC-MS/MS as described in Materials and Methods. The amount of each compound was determined using the quadratic equation given in suppl. Table S1. (A) lactate, (B) citrate, (C) 5-oxoproline, (D) N-acetyl aspartate.

Reactivation of PDC by DCA also led to an increase in citrate levels in 24 h as glucose-derived pyruvate is diverted towards TCA cycle (Figure 4B). Proliferating cancer cells synthesize great amount of lipids and sterols and need high citrate levels for this purpose [25]. Glutaminolysis is the main source of citrate in fast growing cancer cells [25]. In glutaminolysis, glutamine (Gln), one of the main amino acid residues present in high amounts in the blood, is taken by cancer cells and converted into glutamate (Glu) by the action of glutaminase [25]. Glu is then converted into α-ketoglutarate which enters TCA cycle. When glucose-derived pyruvate is diverted towards TCA cycle, increases in TCA cycle intermediates including oxaloacetate, succinate, fumarate and α-ketoglutarate have been reported for clear-cell renal cell carcinoma [4]. Nevertheless, our results show that in the limits of the LC-MS/MS analysis method used here (Suppl. Table S1) these intermediates were not detectable suggesting that these metabolites are not significantly accumulating in ACHN cells. Decrease in the levels of citrate at 48 and 72 h upon DCA treatment suggest that the metabolism of ACHN cells is severely affected. It should be also emphasized that DCA also inhibits synthesis of cholesterol as it is a noncompetitive inhibitor of hydroxymethylglutaryl coenzyme A reductase, the rate-controlling enzyme of cholesterol synthesis [26].

Interestingly, 5-oxoproline and NAA also markedly accumulated in control ACHN cell line (Figure 4C and D). 5-oxoproline is an intermediate of γ-glutamyl cycle and accumulates as a consequence of defects in glutathione synthetase and 5-oxoprolinase activities [27]. Elevated 5-oxoproline and glutathione is observed in various types of tumors, and this makes the neoplastic tissues more resistant to chemotherapy [28]. NAA, on the other hand, is the second most abundant metabolite in the brain and serves as a source of acetate moiety for oligodendrocyte myelination and protein/histone acetylation or a precursor for the synthesis of the neurotransmitter N-acetylaspartylglutamate [29–31]. NAA synthesis which takes place in mitochondria is catalyzed by N-acetyltransferase (NAT) using acetyl-CoA and aspartic acid (Asp) as substrates. Zand et al. reported high levels of tumoral NAA and its biosynthetic enzyme, NAT8L, in the samples of high-grade serous ovarian cancer patients [32]. NAT8L silencing using siRNA significantly reduced tumor growth and survival [32]. High NAT8L gene expression in other cancers (melanoma, renal cell, breast, colon, and uterine cancers) was also reported [32]. It has also

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been demonstrated that high expression of NAT8L occurs in adipose tissues where it notably stimulates glucose incorporation into neutral lipids [33]. It appears that in addition to citrate, NAT8L-driven NAA also provides cytosolic acetyl-CoA for lipid synthesis in fast growing cancer cells. Since glycolysis and TCA cycle are impaired in cancer cells, it is β-oxidation of fatty acids that could mostly provide acetyl-CoA in mitochondria. Inhibition of fatty acid transport into mitochondria was shown to induce apoptosis in hematopoietic cell lines LyD9 and WEHI-231 [34]. The carbon for de novo aspartate synthesis, on the other hand, is supplied by anaplerotic Gln [35]. Aspartate can be produced from α-ketoglutarate via both reductive and oxidative pathways [36, 37]; as mentioned above α-ketoglutarate originates from Gln. Asp is essential for proliferating cells as it is needed for conversion of IMP to AMP in de novo purine synthesis and supplies the carbon backbone for de novo pyrimidine synthesis as well as incorporates in proteins as an amino acid [35]. It seems that besides providing acetyl group for lipid synthesis, NAA also provides Asp for aferementioned processes in the cytosol. Acetylation of aspartate may facilitate its removal from mitochondria as well as enhances glutamine contribution to the replenishment of TCA cycle metabolites. Our study showed that NAA levels decreased at 24 h upon DCA treatment (Figure 4D). When PDC was activated by DCA, β-oxidation and glutaminolysis seemed to be suppressed, and so did NAA synthesis.

LC-MS/MS results showed that only lactic acid and citric acid metabolites could be detected in HUVEC cells when compared to ACHN cells. Lactate and citrate are important for the energy metabolism and inability to detect NAA and 5-oxoproline in HUVEC cells indicates the importance of these metabolites for kidney cancer.

In conclusion, our study shows that DCA is effective in sensitizing renal cell adenocarcinoma cells to apoptosis. This is done by activating PDC and directing pyruvate into TCA cycle rather than lactate, and thereby increasing oxidative phosphorylation and mitochondrial ROS production. It would be interesting to see the effect of inhibition of lactate dehydrogenase A (LDHA) at the same time, which presumably will further increase pyruvate flux into TCA cycle. Future studies will focus on using DCA in combination with a LDHA inhibitor in various cancer cells.

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Conflict of interest statement: There is no conflict of interest.

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