Combination of Metformin and Dichloroacetate Inhibits Proliferation and Induce Intrinsic Pathway of Apoptosis in PC-3 Human Prostate Cancer Cells

Metformin ve Dikloroasetat Kombinasyonunun PC-3 Prostat Kanser Hücrelerinde İçsel Apoptotik Yolağı İndükleyerek Proliferasyonu Baskılaması

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ABSTRACT

Objective: Prostate cancer is one of the most common cancers in men and is the second leading cause of male cancer deaths after lung cancer. Activation of apoptosis is an important process to overcome prostate cancer. In this study, we investigated the synergistic anti-proliferative and apoptotic effects of metformin and dichloroacetate (DCA) in human prostate cancer cell line PC-3.
Methods: PC-3 cells were cultured in plate before being exposed to different concentrations of unaccompanied metformin and DCA as well as metformin and DCA combination. Cell proliferation and viability were investigated with WST-1 assay. After the protein isolation from control and treated cells, whole cell lysate was used for determining caspase-3, -8 and -9 activation by western blotting method.
Results: Our results demonstrated that both drugs were found effective for inhibiting cell proliferation. This inhibition effect was markedly enhanced with a low-dose 30mM DCA plus metformin (2.5mM) combination treatment. Our western blot results showed that caspase-3 and -9 were activated after the combination treatment, but caspase-8 was not activated, which suggests that intrinsic apoptosis pathway was activated by DCA and metformin in PC-3 cells.
Conclusion: Metformin and DCA combinations demonstrated growth inhibiting effects on PC-3 prostate cancer cells with inhibition of cell proliferation and increased apoptosis by caspase activation. By the application of combined doses of these drugs, inhibition of viability and proliferation occurred at lower doses in cells. Further research work should be performed in order to further investigate these promising agents as therapeutics and adjuvant substances for prostate cancer.

Key Words: Apoptosis, DCA, metformin, prostate cancer

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ÖZET

Amaç: Prostat kanseri erkeklerde en sık görülen kanserlerden biri olup, akciğer kanserinden sonra erkek kanser ölümlerinde ikinci sıradadır. Apoptozun aktivasyonu prostat kanserinin üstesinden gelmek için önemli bir aşamadır. Bu çalışmada, insan prostat kanseri hücre hattı olan PC-3 hücrelerinde metformin ve dikloroasetatın (DCA) sinerjistik anti-proliferatif ve apoptotik etkileri araştırılmıştır.
Yöntem: PC-3 hücreleri, metformin ve DCA'nın tekli ve kombine farklı konsantrasyonları uygulanmadan önce kültüre edildi. Hücre proliferasyon ve canlılığı WST-1 yöntemi ile analiz edildi. Kontrol ve ilaç uygulanmış hücrelerden protein izolasyonu sonrasında, kaspaz-3, -8 ve -9 aktivasyonuna western blot yöntemi ile bakıldı.
Bulgular: Sonuçlarımız, ilaçların her ikisinin de hücre proliferasyonu inhibisyonunda oldukça etkili olduğunu göstermiştir. Bu inhibisyon etkisi, düşük doz metformin (2.5mM) ve DCA'nın (30mM) birlikte uygulanması ile dikkate değer bir şekilde artmıştır. Western blot sonuçlarımız, bu ilaçların kombinasyonu sonrasında kaspaz-3 ve -9 aktivasyonunun arttığını fakat kaspaz-8 aktivasyonunun olmadığını göstermiştir. Bu sonuç, ilaçların hücrelerde içsel apoptoz yolağını aktive ettiğini işaret etmektedir.
Sonuç: PC-3 prostat kanser hücrelerinde metformin ve DCA kombinasyonunun büyümeyi inhibe edici etkisi, hücre proliferasyonunun inhibisyonu ve kaspaz aktivasyonu aracılı apoptozu artırma yolu ile gözlenmiştir. Bu ilaçların kombine dozlarının uygulanması ile hücrelerin canlılık ve proliferasyonunun inhibisyonu daha düşük dozda elde edilmiştir. Prostat kanseri için terapötik ve adjuvan tedavi olarak bu ajanların kullanılması için daha ileri araştırmaların yapılması gerekmektedir.

Anahtar Sözcükler: Apoptozis, DCA, metformin, prostat kanseri

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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and second leading cause of cancer death amongst men in developed countries. Many patients fail androgen ablation therapy and die of recurrent androgen-independent metastatic prostate cancer (1).

Metformin is a guanidine derivative and commonly used in the treatment of Type 2 diabetes (2). Studies showed a relation between the use of metformin and a lower risk of several cancers, including the prostate cancer (3). The mechanism by which metformin acts is through controlling the protein synthesis and adenosine monophosphate-activated protein kinase (AMPK) / mTOR signaling pathway which is involved in cell proliferation (4). Metformin controls several carcinogenic pathways, however, its tumor inhibitory effect is not yet fully understood (5). Metformin inhibits mitochondrial complex I by direct inhibition of cellular respiration. By this way, it creates a microenvironment similar to caloric restriction in tumor cells which is an undesirable condition. The research work to investigate the effects of metformin on neoplastic pathologies in phase I, II, III clinical trials is ongoing. Metformin stands out as a good candidate for a therapeutic agent due to its low toxicity profile, availability and FDA-approval (6, 7).

Recently, researchers have reported that dichloroacetate (DCA) was also an orally available drug with well-studied pharmacokinetics. It has been tested for the treatment of mitochondrial deficiencies and lactic acidosis which are potential side effects of metformin. Its administration is associated with restoration of apoptotic pathways in cancer cells (8-10). DCA is a known inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK) which phosphorylates pyruvate dehydrogenase (PDH) to make it inactive. In cancer cells, PDK activity is often elevated, acting as a guard to reduce the flux of pyruvate from the cytoplasm into mitochondria metabolism. It is believed that this is an important component of metabolic reprogramming in cancer cells, leading to reduced glucose oxidation and production of lactate (11).

In this study, we examined the anti-proliferative activity of the two metabolism targeting drugs -metformin and DCA- and the synergy between them. Additionally, following unaccompanied or combined treatment with these drugs, the activation of caspase-3, -8 and - 9 were investigated at protein expression level.

METHODS

Cell Culture and Reagents

The prostate cancer cell line PC3 was kindly provided by Prof. Levent Türkeri (Marmara University, Faculty of Medicine, Department of Urology, Istanbul, Turkey). Cells were cultured in RPMI medium with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C with 5% carbon dioxide. Metformin was purchased from Sigma-Aldrich (Darmstadt, Germany) and was dissolved in cell culture medium to prepare stock solution and stored at -20°C. DCA was purchased from Sigma-Aldrich (Darmstadt, Germany) and was dissolved in PBS. The resulting stock solution was stored at -20°C. Both stock solutions were diluted in cell culture medium before treating cells.

Cell Viability Assay

Cells ($5x10^3$ per well) were plated in 96 well culture plate before exposure to different concentrations of unaccompanied metformin, unaccompanied DCA and combinations of the two for 48h. Cell proliferation and viability were assayed with WST-1 (Roche Diagnostics, Germany). Each well was treated by 10µl WST-1 and the cells were incubated for 4h at 37°C. After the incubation, the absorbance of each well was measured spectrophotometrically at 450nm with ELISA reader (Spectramax® M3; Molecular Devices LLC, Sunnyvale, CA, USA).

Protein Isolation and Western Blot Analysis

Cells (2 x 10^6) were plated on 25cm^2 cell culture dishes one day before unaccompanied and combined metformin and DCA treatment. Whole cell lysate was obtained using RIPA buffer (Thermo Fisher Scientific, Waltham, MA USA). Then, 35µg total protein lysate from each sample was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad wet-blot transfer apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blotted with 5% non-fat milk powder at room temperature for 1h and probed with primary antibodies at 4°C overnight. The primary antibodies are caspase-3, caspase-8 (Cell Signaling Technology, Danvers, MA, USA), caspase-9 (Thermo Scientific, Waltham, MA, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA). They were then incubated with the secondary antibody anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA) for 1h to detect the primary antibodies. Proteins were visualized by Kodak Gel Logic 2200 using Lumina Crescendo Western HRP substrate (Millipore, MA, USA).

Statistical analysis

Each data point was measured in three independent experiments. Comparisons between control and drug-treated cell group viability were analyzed using the SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference and the results were expressed as the mean ± standard deviation.

RESULTS

Metformin and DCA synergistically inhibit cell proliferation of PC-3 cells

To determine the effects of exposure to unaccompanied and combined metformin and DCA, PC-3 cells were treated with various concentrations of metformin (0-100mM) and DCA (0-300mM) as well as with their combinations for 48h. The IC_{50} doses, which represent the concentration of a drug that is required for 50% inhibition of metformin (Figure 1A) and DCA (Figure 1B) alone were determined to be 10mM and 50mM, respectively. These drugs were found very effective for inhibiting cell proliferation. This inhibition effect was markedly enhanced with DCA plus low-dose metformin treatment for 48h. After treatment with several combinations, 2.5mM metformin plus 30mM DCA combination equaled IC_{50} values in PC-3 cells (Figure 1C). These results indicated that the two agents synergistically enhanced cell proliferation inhibition.

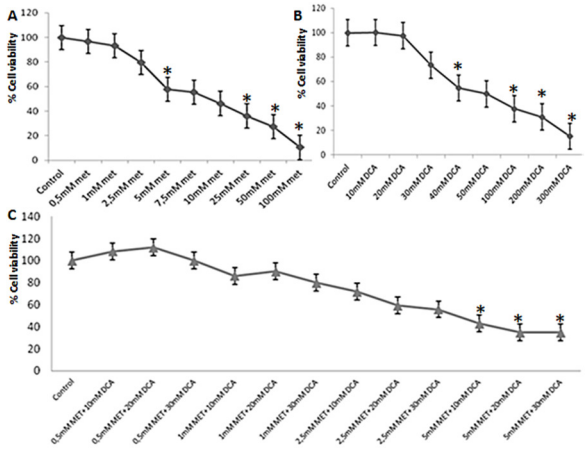

Figure 1. Metformin and DCA synergistically inhibit cell proliferation of PC-3 cells. Effects of these drugs on cell viability were determined using the WST-1 assay. Metformin (**A**) and DCA (**B**) alone effectively inhibited cell proliferation at 48h. Combination treatment (**C**) at 48h however led to the inhibition of cell proliferation at lower doses (2.5mM metformin plus 30mM DCA). The results were reported as means ± standard deviation after three independent experiments. Differences were considered to be significant in all experiments when $p< 0.05$ (*, $p < 0.05$).

Metformin and DCA combination enhances caspase-3 and -9 but not caspase-8 activity

We investigated caspase-3, -8 and -9 activation at protein level by western blotting in PC-3 cell line after treatment with unaccompanied metformin and DCA as well as with combinations of these agents (Figure 2). The cells were treated with 10mM unaccompanied metformin, 50mM unaccompanied DCA and 2.5mM metformin plus 30mM DCA combination for 48h. Metformin-only treatment resulted in no caspase-3 and -8 activation but a slight increase in activation of caspase- 9. Combination treatment drastically inducted caspase-3 and -9 activation in PC-3 cells. We did not observe caspase-8 activation in neither unaccompanied nor combination treatment (Figure 2). We suggest that the cause of the significant increases in caspase-3 and -9 after the combination treatment is the triggering of intrinsic apoptosis by these drugs. Combination treatment was more effective in inducing the apoptotic pathway than treatment with the two unaccompanied agents.

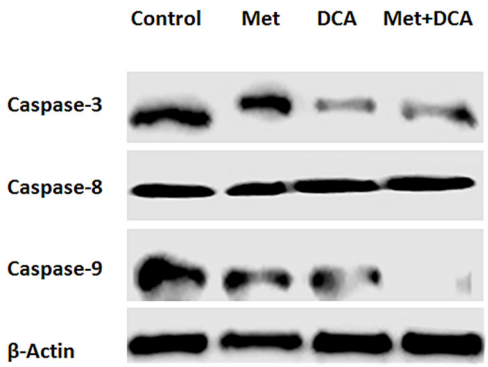

Figure 2. Metformin + DCA combination enhances caspase-3 and -9 activity but not caspase-8 activity. Expression levels of caspase-3, -8 and -9 were determined by western blotting method. After the combination treatment, a remarkably increased activation of caspase-3 and -9 were observed, but no changes were observed in caspase-8 activation after unaccompanied or combination treatments (Met; Metformin, DCA; Dichloroacetate).

DISCUSSION

Metformin is a drug which is used in diabetes mellitus. The results of many studies showed that metformin plays an important role in inhibition of cancer cell growth and proliferation. Anticancer action of metformin is mainly associated with the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) (12). Studies have shown that metformin was able to inhibit proliferation and promote apoptotic cell death of many cancer cells (13-15).

However, researchers discovered that metformin treatment created high lactate levels, which is an untoward outcome. Investigators showed that dichloroacetate (DCA) helped to attenuate the lactate production induced by metformin and enhanced apoptotic effects of metformin (16, 17). DCA is a well-studied drug used for the lactic acidosis treatment. In an *in vitro* study by Bonnet et al., it was reported that DCA played an important role for enhancing apoptosis in cancer cells but not in normal cells (18).

Caspases are a family of intracellular proteases and the primary drivers of apoptotic cell death. They cleave cellular proteins and activate themselves, a process critical for shredding the dying cell. Caspases are initially translated as inactive zymogenic precursors, and activated in response to a variety of cell death stimuli. Activation of caspases requires proteolytic processing of their inactive zymogen into activated small fragments (19). Two major apoptotic pathways have been identified in cells. The first one is membrane bound death receptor-mediated extrinsic signaling pathway to activate caspase-8, while the second one is mitochondria-dependent intrinsic signaling pathway characterized by the activation of caspase-9 by cytochrome C release into the cytosol and subsequent apoptosome formation. These extrinsic and intrinsic apoptotic signaling pathways merge at the level of caspase-3 activation (20).

In this study, we investigated the synergistic anti-proliferative and apoptotic activity of metformin and DCA. Metformin and DCA synergistically enhanced cell proliferation inhibition. In a study by Ben Sahra et al. (21), after unaccompanied metformin treatment, apoptosis was not induced in prostate cancer cells. Our study demonstrated that marked activation of caspases-3 and -9 occurred following combination treatment, however, we did not observe caspase-8 activation after any kind of treatment. This suggests that, the intrinsic mitochondria-dependent apoptotic pathway was activated following combination treatment in PC-3 cells. Increased caspase activation indicates that combined treatment induces cellular apoptosis. The current results corroborate those of previous studies which have evaluated the effects of combination treatment on caspase activation and apoptosis in various other cell types (10, 22).

CONCLUSION

Our results demonstrate that DCA and metformin synergistically induce mitochondrial apoptosis more effectively than unaccompanied metformin and DCA treatment. This combination may offer a new perspective for advanced-prostate cancer research.

Conflict of Interest

No conflict of interest was declared by the authors.

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