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Evaluating Metformin as a Potential Chemosensitizing Agent when Combined with Docetaxel Chemotherapy in Castration-resistant Prostate Cancer Cells

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Abstract. *Background/Aim: Docetaxel, the first-line chemotherapy for metastatic castration-resistant prostate cancer (mCRPC), provides certain survival benefits, but is associated with significant toxicity. A novel therapeutic approach for mCRPC is combining docetaxel with a chemosensitizing agent. We hypothesized that metformin, a potential chemosensitizer, would improve docetaxel efficacy in CRPC cells. Materials and Methods: MTS assays were used to determine the effect of metformin-docetaxel treatment on PC3 and DU145 cell viability. Wound-healing and ATP concentration assays were used to evaluate cell migration and intracellular ATP levels following metformin-docetaxel treatment. Western blotting was used for mechanistic evaluation. Results: Metformin-docetaxel treatment significantly reduced PC3 cell viability. Metformin-docetaxel treatment did not significantly affect cell migration or intracellular ATP levels. Western blotting revealed metformin-docetaxel treatment did not significantly change AMPK or P-AMPK expression patterns. Conclusion: Metformin may be an effective chemosensitizer for certain types of CRPC cells, but further investigation is needed.*

Advanced-stage prostate cancer (PCa) is usually treated with androgen-deprivation therapy (ADT). However, most patients with metastatic disease managed with ADT eventually relapse with castration-resistant prostate cancer (CRPC) and die of the disease (1, 2). The first-line chemotherapy for symptomatic metastatic CRPC is docetaxel, which provides significant survival benefits, but is also associated with significant toxicity and the average survival of metastatic CRPC patients is approximately 19 months (3, 4). Thus, there is still a need to improve the therapeutic options available for advanced-stage PCa patients.

One novel therapeutic approach is combining docetaxel chemotherapy with an agent that enhances its effectiveness, known as a "chemosensitizer". One such potential chemosensitizing agent is metformin, a commonly prescribed and well-tolerated oral biguanide used to treat Type 2 diabetes (5). Evans *et al.* (2005) showed a reduced cancer burden in diabetic patients treated with metformin compared to those treated with other diabetic therapies (6, 7). Since the publication of the Evans paper, metformin has been shown to have anti-neoplastic properties in breast cancer, ovarian cancer, pancreatic cancer, and prostate cancer (8-12).

Metformin inhibits the proliferation of LNCaP, DU145, and PC3 human PCa cells and reduces tumor growth in LNCaP xenografts (8). Metformin has been shown to enhance the tumor-suppressing effect of doxorubicin in four different types of breast cancer cells and prolonged the remission of breast cancer xenograft models (13). Furthermore, metformin combined with a 4-fold lower dose of doxorubicin was shown to be as effective as the standard dose of doxorubicin treatment in breast cancer xenografts (14). Metformin combined with paclitaxel was more effective in reducing tumor growth and increasing apoptosis in breast and lung cancer xenografts than either treatment alone (15). Metformin has also been shown to act synergistically with 5-fluorouracil and oxaliplatin to inhibit cell proliferation and tumor growth of chemo-resistant colorectal cancer cells (16, 17).

Although metformin has been shown to enhance the anti-cancer effect of bicalutamide and simvastatin in advanced stage PCa cells, there is very limited evidence for the use of metformin as a chemosensitizing agent in CRPC (18-20). Given the evidence in the literature demonstrating a

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Key Words: Metformin, docetaxel, chemosensitizing, castration-resistant prostate cancer cells.

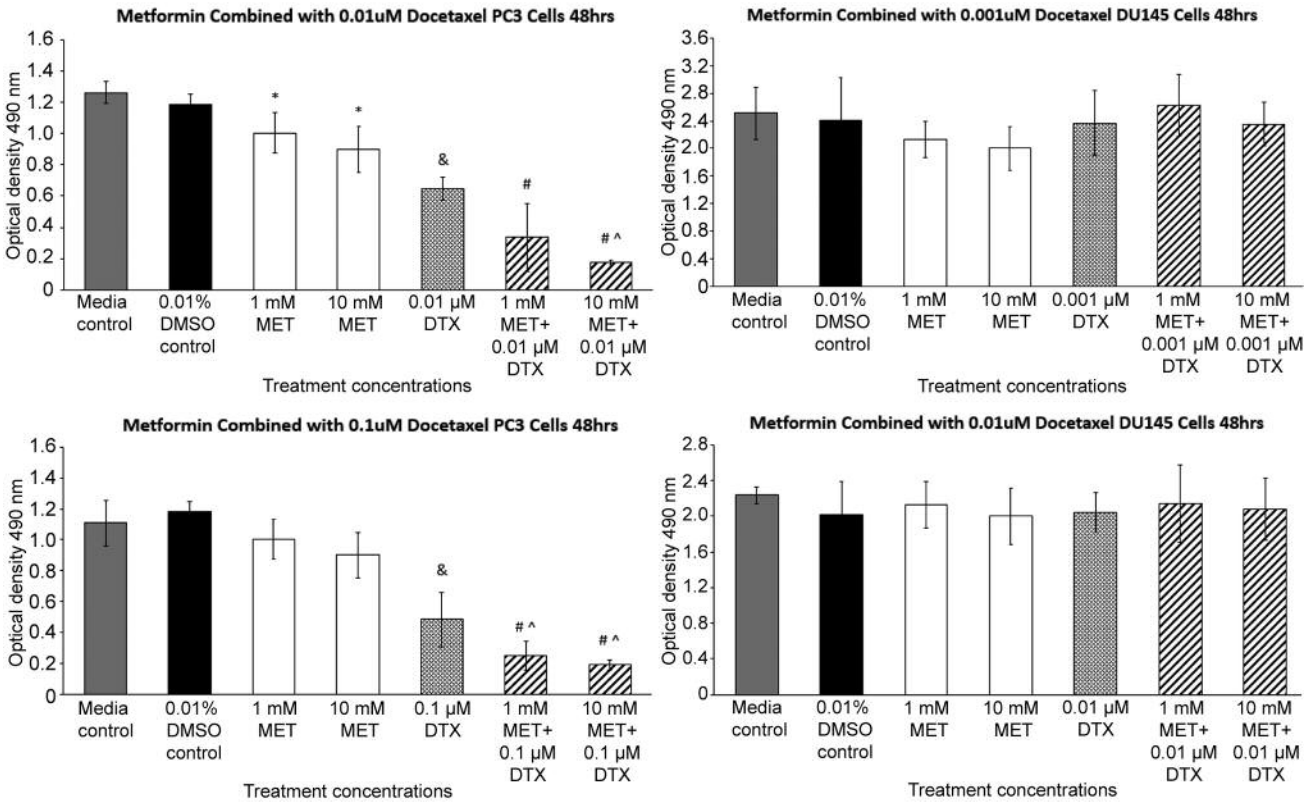

Figure 1. *The effect of 48 hours of combined metformin and docetaxel treatment on the viability of PC3 and DU145 cells. PC3 cells were treated with 1 mM or 10 mM metformin alone, 0.01 μM or 0.1 μM docetaxel alone, or a combination of metformin and docetaxel for 48 h. DU145 cells were treated with 1 mM or 10 mM metformin alone, 0.001 μM or 0.01 μM docetaxel alone, or a combination of metformin and docetaxel for 48 h. The optical density at 490 nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Graphs represent the mean. *p<0.05 compared to media control; &statistically significant compared to 0.01% DMSO control; #statistically significant compared to metformin alone; ^statistically significant compared to docetaxel alone.*

chemosensitizing effect of metformin in other types of solid tumors, but very limited evidence for metformin as a chemosensitizer in CRPC, we hypothesize that metformin will act as a chemosensitizing agent in CRPC cells when combined with docetaxel chemotherapy.

Materials and Methods

Cell culture. Two established human PCa cell lines were used. PC3 and DU145 (androgen-independent) cells were obtained from the American Type Culture Collection (Rockville, MA, USA) and maintained as described previously (18, 21).

Chemicals. Metformin (MET) (Sigma, Oakville, Ontario, Canada) was prepared following the same procedure outlined by Colquhoun et al (2012) (18). Working concentrations (100 mM and 1 mM) were created by dissolving metformin in cell culture medium. Docetaxel (DTX) (Santa Cruz Biotechnology, CA, USA) was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA) and diluted with cell culture medium to a final concentration of 0.01% DMSO as described previously (22).

MTS cell viability assay. The CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay was used to evaluate cell viability (Promega, Madison, WI, USA). Cells (4×10^3/well) were plated in a 96-well plate and left to adhere for 24 h. This was followed by treatment with MET (0.01-100 mM) or DTX (0.001-1 μM of DTX) for 24, 48, or 72 h to establish dose standardization for PC3 and DU145 cells. After determining the effect of MET or DTX alone, the optimal MET and DTX concentrations were chosen for combination treatments. Media and vehicle (0.01% DMSO) controls were used since MET was dissolved in cell culture medium and DTX, previously prepared in DMSO, was dissolved to a final concentration of 0.01% DMSO.

Wound-healing assay. Cell motility was assessed using a wound-healing assay in PC3 cells as described previously (22). Cells (2×10^5) were plated per well in a 24-well plate. Cells were allowed to adhere for 48 h, a scratch was created, and then cells were treated with MET, DTX, or a combination of MET and DTX. A computer-based microscope was used to determine wound-healing at 0 h (baseline) and after 24 h of treatment. This measurement was compared to baseline using Axiovision software (Axiovision Rel 4.6, Carl Zeiss, Gottingen, Germany).

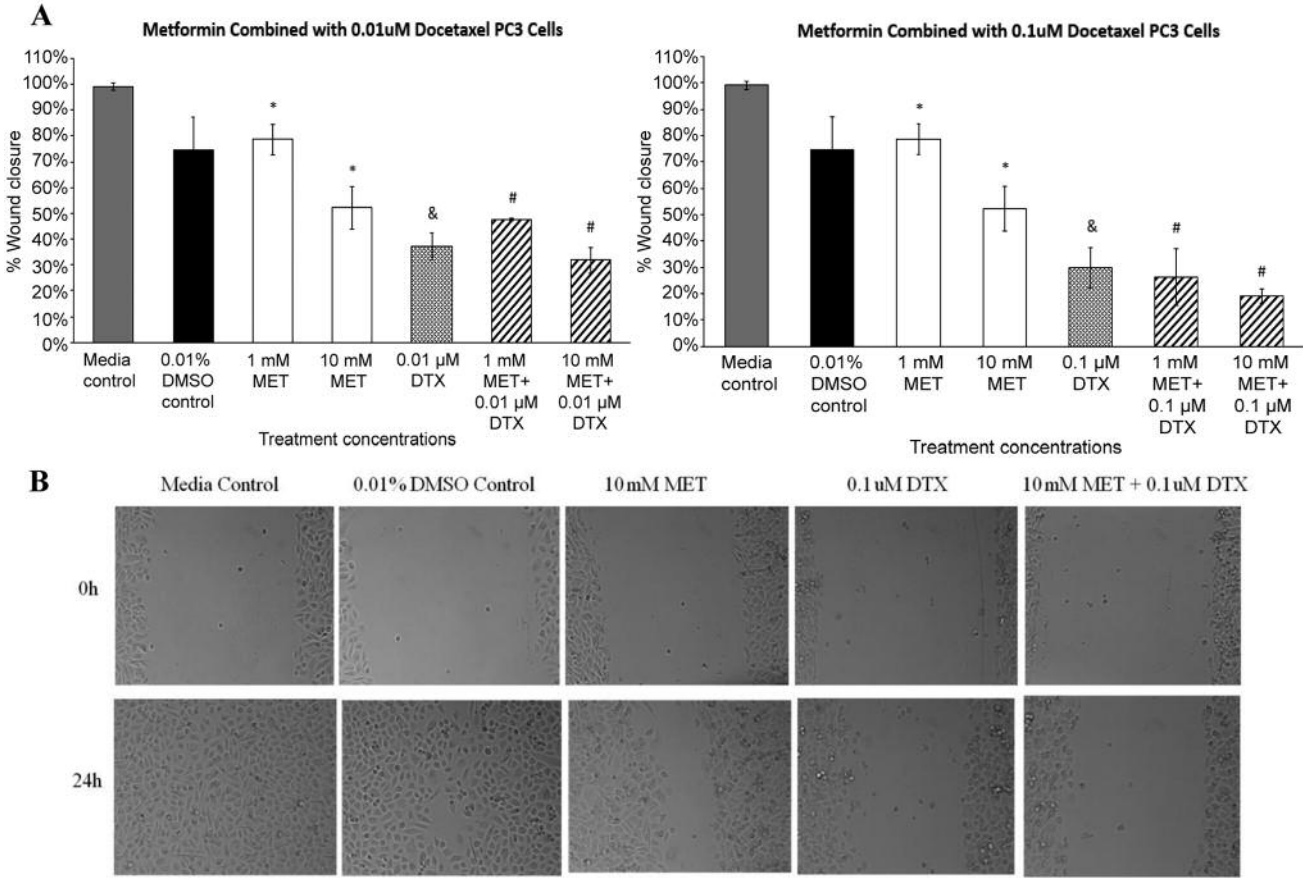

Figure 2. *The effect of 24 h of combined metformin and docetaxel treatment on PC3 cell migration. A. Quantification of wound closure after 24 h of treatment. A lower percent wound closure indicates a greater reduction in cell migration ($p<0.05$ compared to media control; $^{\&}$statistically significant compared to 0.01% DMSO control; $^{\#}$statistically significant compared to metformin alone). B. Representative images used for wound closure quantification. A wound was created in each well of a 24-well plate following PC3 cell plating and adherence. Following wound creation, PC3 cells were treated with 1 mM or 10 mM metformin alone, 0.01 µM or 0.1 µM docetaxel alone, or a combination of metformin and docetaxel for 24 h. Light microscopy was then used to image the wound and the percent wound closure was calculated using measurements obtained from the images. Graphs represent the mean.*

ATP concentration assay. The Luminescent ATP Detection Assay Kit (abcam, Cambridge Science Park, UK) was used to evaluate total levels of cellular ATP in PC3 cells. Cells (4×10^3/well) were plated in a black, opaque 96-well plate for each condition. Cells were allowed to adhere for 48 h and were then treated with 1 mM MET, 0.1 µM DTX, or 1 mM MET + 0.1 µM DTX. All steps involving kit components were completed in subdued lighting to prevent plate phosphorescence which would artificially increase the background signal.

Western blotting. PC3 cells were prepared for lysate collection by plating 1×10^6 cells per 10 cm dish for each control and treatment condition and allowing cells to adhere for 24 h. After 24 h of adherence PC3 cells were treated with 1 mM MET, 0.1 µM DTX or 1 mM MET + 0.1 µM DTX for 48 h. Cell lysis was completed using RIPA buffer with protease and phosphatase inhibitor cocktails. Protein levels were quantified using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Rockford, IL, USA) before loading into a 15-well, 4-12% Bis Tris NuPage gel (Thermo Fischer Scientific, Rockford, IL, USA) to complete electrophoresis. Transfer to a nitrocellulose membrane was completed and protein detection was completed using the following antibodies from Cell Signaling Technology Inc.: AMPK α (Thr 172) and Phospho-AMPK α (Thr172). ImageJ software (US National Institute of Health, Bethesda, MA, USA) was used to complete densiometry for the bands observed. Western blot experiments were completed in duplicate.

Statistical analysis. Experiments were completed in triplicate at least twice. Student's *t*-test was used to analyze between-group differences for all experiments with a significance level of $p<0.05$. The data represented the mean±standard deviation.

Results

Metformin combined with docetaxel significantly reduces cell viability in PC3 cells but not in DU145 cells. The MTS assay was used to evaluate the effect of MET and/or DTX treatment on cell viability. Dose standardization experiments were completed for PC3 and DU145 cells to determine the optimal doses of MET and DTX to be used for combination experiments (data not shown). For PC3 cells, a 48 h time-point

was selected and the MET treatments selected were 1 mM and 10 mM and the DTX treatments selected were 0.01 μM and 0.1 μM. For DU145 cells, a 48 h time-point was also chosen and the MET treatments selected were 1 mM and 10 mM and the DTX treatments selected were 0.001 μM and 0.01 μM.

In PC3 cells after 48 h of treatment, 10 mM MET combined with 0.01 μM DTX significantly reduced cell viability compared to 0.01 μM DTX treatment alone (Figure 1). However, 1 mM MET + 0.01 μM DTX did not have a significant effect compared to 0.01 μM DTX alone (Figure 1). Both 1 mM and 10 mM of MET combined with 0.1 μM DTX significantly reduced cell viability compared to 0.1 μM DTX treatment alone (Figure 1). However, treating DU145 cells with MET, DTX, and multiple combinations of MET and DTX did not have a significant effect on cell viability (Figure 1).

Metformin combined with docetaxel does not significantly reduce cell migration in PC3 cells compared to docetaxel alone. The effect of MET and/or DTX treatment on cell migration was assessed using a wound-healing assay in PC3 cells. 1 mM and 10 mM MET compared to the media control significantly reduced cell migration (Figure 2A). 0.01 μM and 0.1 μM DTX compared to the 0.01% DMSO control significantly reduced cell migration (Figure 2A). The combination of 1 mM or 10 mM MET with either 0.01 μM or 0.1 μM DTX did not have a significant effect on cell migration compared to MET or DTX alone (Figure 2A). A selection of representative images has also been included which were the source of the measurements used for percent wound closure quantification (Figure 2B).

Metformin combined with docetaxel does not significantly reduce ATP concentrations in PC3 cells compared to docetaxel alone. The luminescent ATP detection assay was used to evaluate the effect of MET and/or DTX treatment on total cellular ATP levels since part of the mechanism of action of metformin involves reduced cellular ATP following inhibition of mitochondrial complex 1. In PC3 cells, 48 h of treatment with 1 mM MET or 0.1 μM of DTX alone significantly reduced total cellular ATP levels compared to their respective controls (Figure 3). However, the combination of 1 mM MET with 0.1 μM DTX did not have a significant effect on total ATP levels compared to DTX alone (Figure 3).

Metformin treatment increases phosphorylated AMPK expression in PC3 cells. Western blotting was used to evaluate the effect of MET and/or DTX treatment on AMPK and phosphorylated-AMPK (P-AMPK) expression in PC3 cells. Treatment with 1 mM MET increased AMPK expression slightly but increased P-AMPK expression more significantly compared to the media control (Figure 4). Treatment with 0.1 μM DTX alone decreased AMPK expression compared to the 0.01% DMSO control, but the 0.01% DMSO control and 0.1 μM DTX treatment both increased P-AMPK expression

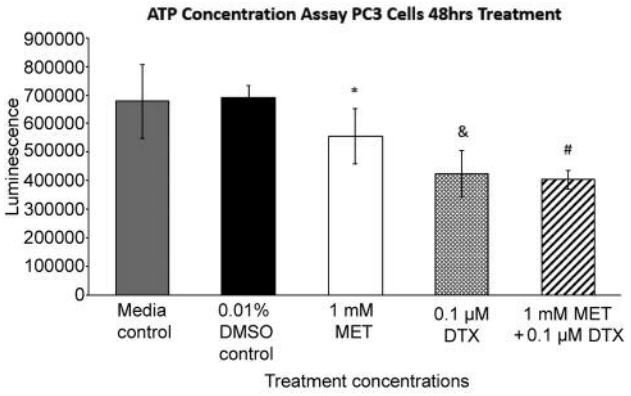

Figure 3. *The effect of 48 h of combined metformin and docetaxel treatment on cellular ATP concentration. The amount of luminescence was measured following the addition of the experimental reagent to determine the concentration of cellular ATP. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Graphs represent the mean. *p<0.05 compared to media control; &statistically significant compared to 0.01% DMSO control; #statistically significant compared to metformin alone. PC3 cells were treated with 1 mM metformin alone, 0.01 μM or 0.1 μM docetaxel alone, or a combination of metformin and docetaxel for 48 h.*

compared to the media control (Figure 4). There was an increase in P-AMPK expression with 0.1 μM DTX treatment compared to the media control. The combination of 1 mM MET and 0.1 μM DTX decreased AMPK expression compared to 1 mM MET treatment and 0.1 μM DTX treatment alone (Figure 4). The combination of 1 mM MET and 0.1 μM DTX also decreased P-AMPK expression compared to 1 mM MET treatment alone and 0.1 μM DTX treatment alone.

Discussion

The current literature indicates an anti-neoplastic effect of MET treatment in multiple types of cancer cells, including PCa cells (8-11). MET has been shown to enhance the effect of bicalutamide and simvastatin in PCa models, and improve the efficacy of chemotherapy in breast cancer, lung cancer, and colon cancer models as discussed in the introduction (13, 15-20). Despite evidence indicating metformin has a chemosensitizing effect in other types of solid tumors, there is extremely limited evidence for using metformin as a chemosensitizing agent in CRPC cells and our study was designed to address this knowledge gap. Collectively, our results revealed that MET or DTX treatment can effectively reduce cell viability and cell migration in PC3 cells, but neither treatment affected DU145 cell viability. Combining MET with DTX significantly reduced PC3 cell viability. Furthermore, MET or DTX treatment could effectively reduce intracellular ATP levels in PC3 cells, but combining MET and DTX had the same efficacy as DTX alone. MET treatment resulted in

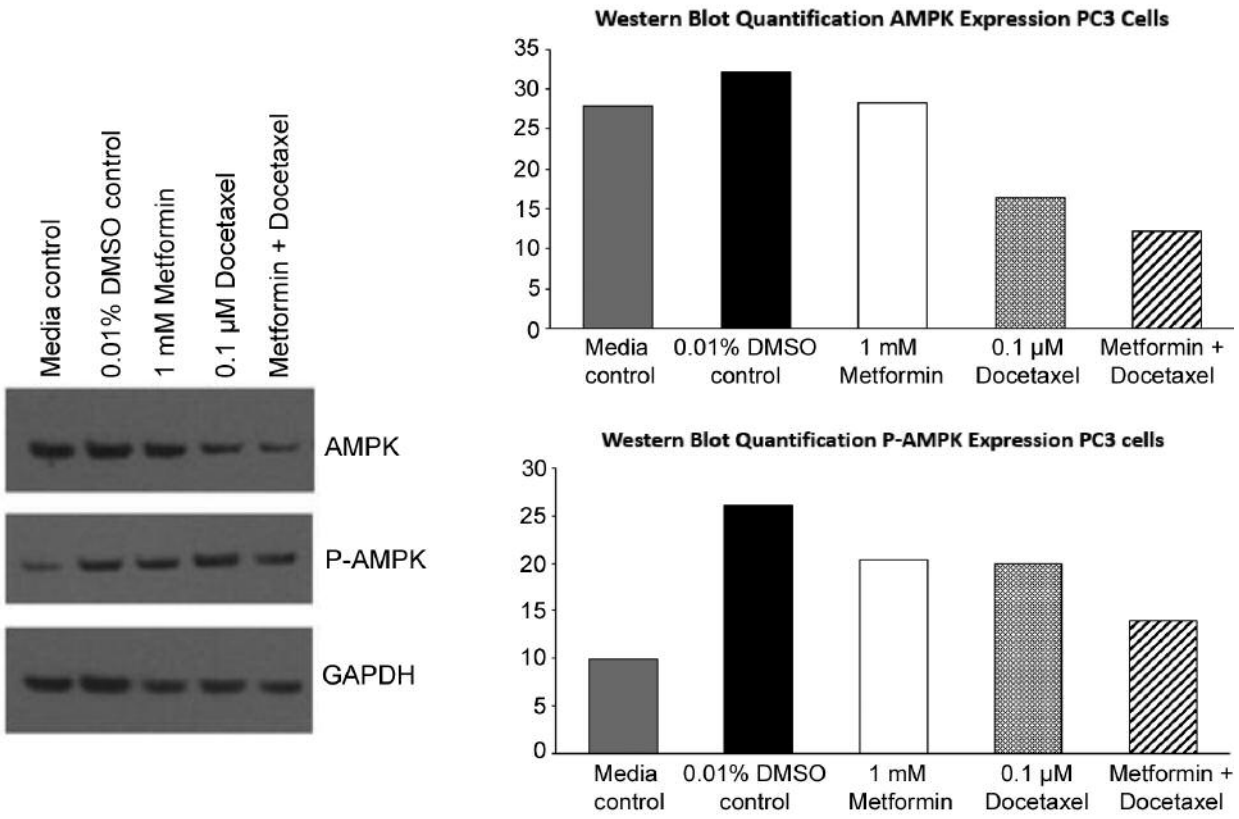

Figure 4. *The effect of metformin combined with docetaxel treatment on the expression of AMPK and phosphorylated AMPK (P-AMPK) in PC3 cells. PC3 cells were treated with 1 mM metformin alone, 0.1 μM docetaxel alone or a combination of 1 mM metformin and 0.1 μM of docetaxel for 48 h. Cell lysates were then harvested and expression of AMPK and P-AMPK was evaluated using Western blotting. GAPDH was used as a loading control. The graphs (right) represent the average quantification of the protein expression of duplicate experiments.*

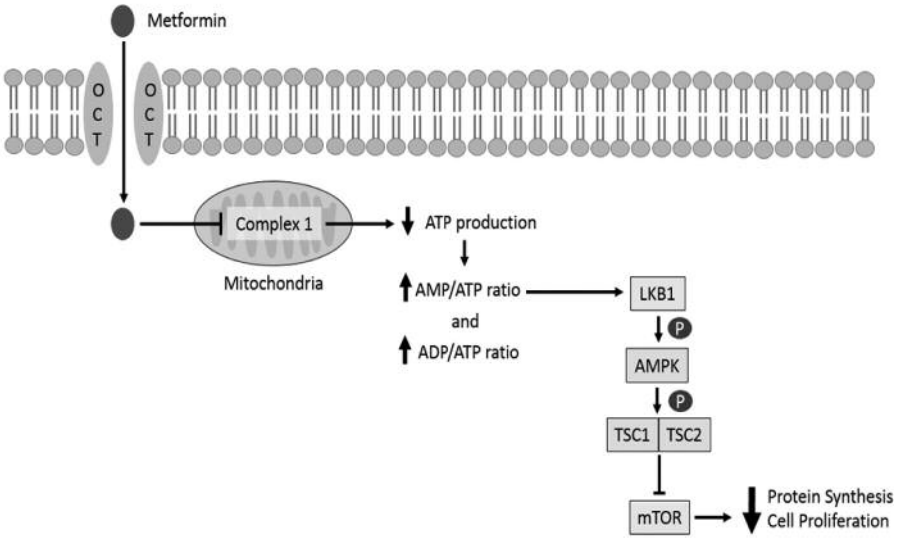

Figure 5. *Proposed mechanism of action of metformin in cancer cells. Metformin is actively transported into the cell by organic cation transporters (OCTs) and then accumulates in the mitochondria due to the mitochondrial membrane potential. Metformin then inhibits mitochondrial complex 1 activity which reduces mitochondrial oxidative phosphorylation and ATP production. A reduction in ATP results in increased ADP/ATP and AMP/ATP ratios. The increased AMP/ATP ratio activates LKB1 and LKB1 then phosphorylates AMPK. Activated AMPK then phosphorylates TSC1 and TSC2 which form an mTOR-inhibition complex which inhibits mTOR signaling and reduces protein synthesis and cell proliferation.*

increased P-AMPK expression, whereas DTX treatment decreased AMPK and P-AMPK expression in PC3 cells.

The mechanism of action of MET is mediated by inhibition of mitochondrial complex 1 which inhibits mitochondrial oxidative phosphorylation and reduces ATP production (23, 24). The decline in cellular ATP increases the AMP/ATP ratio (5). This activates tumor suppressor kinase LKB1 which then phosphorylates AMPK at Thr172 and this activates the catalytic subunit of AMPK, AMPKα (15, 25). P-AMPK inhibits mTOR which disrupts protein synthesis and results in the disruption of tumor cell proliferation (26). A more detailed mechanism of action of metformin is illustrated in Figure 5.

It has been shown that LKB1 is expressed in PC3 cells but not in DU145 cells (27, 28). The presence of LKB1 in PC3 cells and absence of LKB1 in DU145 cells supports the LKB1-dependent phosphorylation mechanism of AMPK since our results show a reduction in cell viability in LKB1-expressing PC3 cells but no change in cell viability in LKB1-null DU145 cells. MET treatment also decreases cell migration in PC3 cells, which is likely mediated through the LKB1-dependent mechanism. The decline in ATP levels following MET treatment in PC3 cells is likely due to its effectiveness in reducing mitochondrial oxidative phosphorylation and thereby reducing intracellular ATP levels. This would then lead to LKB1 activation, AMPK phosphorylation, and AMPK activation, which would inhibit mTOR and therefore reduce cell viability. The decline in ATP levels caused by DTX treatment is likely due to the activation of the DNA damage response which would require large amounts of ATP to repair any damage caused by the chemotherapeutic agent.

The significant increase in P-AMPK in PC3 cells caused by DTX treatment may be due to the phosphorylation of AMPK by the kinase ATM (29). The cytotoxicity of chemotherapeutic agents is mediated by the creation of permanent DNA lesions that trigger ATM activation and DNA damage response signaling (29). PCa cells treated with topoisomerase II inhibitor etoposide showed phosphorylation of ATM and in turn ATM-dependent phosphorylation of AMPK (30). This ATM-dependent phosphorylation of AMPK may be the mechanism through which DTX treatment is causing AMPK phosphorylation in PC3 cells. This can also be linked to the significant reduction in cell viability observed for multiple DTX concentrations in the dose standardization since ATM-dependent AMPK activation would lead to inhibition of mTOR signaling and a reduction in cell viability.

MET combined with DTX does significantly reduce cell viability compared to DTX alone, but this is not the case for cell migration, intracellular ATP concentration, and phosphorylation of AMPK. This may be due to the high efficacy of DTX treatment alone reducing cell migration and intracellular ATP concentration, therefore adding MET may not create an extreme enough energy crisis to enhance the effect of chemotherapy. Another possibility could be related to DTX treatment reducing total AMPK expression. This reduction would lead to a smaller pool of AMPK available for phosphorylation and therefore activation of AMPK by MET treatment would not be as effective, reducing the capacity for MET chemosensitization.

There has been some discussion in the literature regarding the mechanism of action of metformin in PCa cells since it has been shown that MET can act through both AMPK-dependent (27, 31) and AMPK-independent mechanisms (8, 32). The AMPK-independent action of MET has been shown to function in a p53-dependent manner (32). However, PC3 cells are p53-null and DU145 cells have a mutated p53 which results in a lack of p53 function in both cell lines (33). Therefore, MET action may be more dependent on the LKB1-AMPK signaling pathway since the AMPK-independent, p53-dependent mechanism is not available in these cells.

In conclusion, our study is the first to investigate the use of MET as a chemosensitizer in combination with DTX treatment in CRPC cells. Our results indicate that MET may be an effective chemosensitizer for CRPC cells. Further investigation is needed to fully elucidate the specific mechanism by which MET combined with DTX exerts its effects in CRPC cells and provide explanation for the difference in response between cell types. Some possible aspects to investigate include the AMPK signaling pathway, as well as the ATM-dependent AMPK phosphorylation pathway, and expression of p53.

Acknowledgements

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