The Induction of Metformin Inhibitory Effects on Tumor Cell Growth in Hypoxic Condition

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ABSTRACT

It is aimed to evaluate the actual anti-cancerous effects of metformin on cancer cells in hypoxic condition.

Non-cancerous cells (HEK293) and cancer cells (MCF-7) were cultured in both hypoxia and normoxia conditions and treated with different concentrations of metformin. The proliferation, apoptosis, and necrosis rate were assessed using MTT test and Annexin V assay. The S6K1 phosphorylation was assessed using western blotting. Zymography was used to measure the activity of metalloproteinase-9 (MMP-9).

Metformin treatment inhibited proliferation of cancer cells in the optimal concentration of 10 mM under hypoxia condition, while it showed no effects on non-cancerous cell viability. The statistical analysis of MTT assay indicated that the pro-apoptotic function of metformin for cancer cells under hypoxia condition compared to normoxia was significant with different metformin concentrations (p<0.01). However, the effect of metformin treatments for non-cancerous cells under hypoxia condition compared to normoxia was not significant. Western-blot analysis indicated a significant decrease in S6K1 phosphorylation in cancer cells under hypoxia condition (p<0.05). Nevertheless, there was no considerable difference between normoxia and hypoxia conditions in non-cancerous cells. MMP-9 zymography analysis revealed that the highest inhibition of MMP-9 activity was observed in hypoxia condition by 20mM of metformin concentration only in cancer cell.

The results indicate that in hypoxia condition metformin exerts its anti-cancerous function by inhibiting proliferation and tumor progression and inducing cell apoptosis more effectively than normoxia condition. In line with cancer cell conditions, most importantly hypoxic condition, metformin can be considered as a potential anti-cancerous drug.

Keywords: Breast cancer; Hypoxia; Metformin
INTRODUCTION

Breast cancer is the major cause of cancer-related deaths and the most common malignancy of women in the world. Invasion and metastasis are considered to be the primary reasons of treatment failures and death in women.\(^1\) Formations of the hypoxic acidic and extracellular environment in tumor are important factors of invasion and metastasis. Besides, metabolic reprogramming in cancerous cells is a recently-recognized hallmark of cancers.\(^2\) Radiation and drug resistance in breast cancer therapies are strongly linked to their self-altered metabolisms.\(^3\) Therefore, Oxygen is one of the crucial factors which is altered in cancer cells. Almost 40% of all breast cancers and nearly half of the locally advanced ones are hypoxic. Moreover, Hypoxia-inducible factor (HIF), a transcription factor, acts in both sensing and responding to changes in cellular oxygen.\(^4\) HIF targets genes encoding insulin-like growth factor 2 (IGF2), Transforming growth factor alpha (TGF-\(\alpha\)), Vascular endothelial growth factor (VEGF) and lysyl oxidase (LOX), which regulate cell survival, proliferation, angiogenesis, invasion and metastasis respectively, play vital roles in changing tumor characteristics.\(^5,6\) HIF-1\(\alpha\) is overexpressed in many human cancers compared to normal tissues.\(^7\) This overexpression is due to the activation of oncogenes involved in phosphoinositide 3-kinase (PI3K) and Mitogen-activated protein kinases (MAPK) signaling cascades, as well as loss of function mutations in tumor suppressor genes (Von Hippel-Lindau, Phosphatase and tensin homolog, and p53).\(^8\) It has also been shown to result in increased expression of HIF-1downstream target genes,\(^9,10\) including matrix metalloproteinase-2 (MMP-2), thus causes tumor progression under hypoxic condition.\(^11,12\) Hence, induction of PI3k receptor by a growth factor, may result in a cascade of protein activation, and finally leads to the phosphorylation of an important protein, known as mammalian target of rapamycin (mTOR).\(^13\) Consequently, mTOR phosphorylation and activation induce mRNA synthesis by phosphorylating The ribosomal protein S6 kinase 1 (S6K1).\(^14,15\) Studies have demonstrated that protein kinase B (AKT) pathway is also involved in the regulation of MMP-9 protein.\(^11\)

Metformin is mostly known as an anti-diabetic drug and activates the AMP-activated protein kinase (AMPK) pathway, an important sensor of the energy levels of the cell and an inhibitor of mTOR catalytic activity which reduces S6K1 phosphorylation.\(^16-18\) In addition, metformin functions as an inhibitor of HIF expression and thus has a role in reduction of cancer morbidity.\(^17\)

According to the recent progresses in metformin related studies, it may be considered as a new agent for cancer related therapies.\(^19\) However, previous studies did not evaluate the effects of metformin on tumor cells in real microenvironments including an adequate amount of oxygen for cell cultures.\(^20\) For this reason, in order to have acceptable data on the efficiency of antitumor drugs, hypoxia condition has been considered in the cell cultures of our experiments.

MATERIALS AND METHODS

Cell Cultures and Reagents

Metformin (1.1-dimethylbiguanide hydrochloride) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye were purchased from Sigma (St. Louis, MO). Human Breast Carcinoma (MCF-7) cell line and human embryonic kidney 293 (HEK293) as a non-cancerous Cell line were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute), and were maintained with low glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, UK) and penicillin (100 units/mL)-streptomycin (100 \(\mu\)g/mL) (Sigma, UK) and incubated at 37°C and 5% CO\(_2\). In this study all cell culture experiments were done in two normoxia and hypoxia (0.1% O\(_2\)) conditions. Hypoxia condition was obtained via a chamber (Inside CO\(_2\) incubator) with inlet and outlet valves. The inlet valve was connected to a gas mixture cylinder (95% nitrogen, 5% CO\(_2\) and 0.1% Oxygen) and outlet valve to CO\(_2\) incubator.

Cell Proliferation Assays

The effects of metformin treatment on cell proliferation were examined by MTT assay according to Feng et al. experiments.\(^21\) Briefly the MCF-7 and HEK293 cells were plated and grown in 96-well plates at a volume of 100 \(\mu\)l of 10000 cells/well and 6000 cells/well respectively for 24 hours. Afterward, cells were treated with four different concentrations of metformin (0, 5, 10 & 20 mM) and incubated under normoxia and hypoxia conditions for 24 and 72 hours. The viable cell densities were determined by metabolic conversion of the soluble MTT. MTT (5 mg/mL) was
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added to the 96 well plates and incubated for 4 hours. The MTT reaction was terminated by adding of 150 μl of MTT solvent after supernatant was discarded. Subsequently, the results of MTT assay were evaluated by measuring absorption at 595 nm. The effect of metformin was calculated as a percentage of control cell growth obtained from untreated cells harvested in the same 96 well plates. Each experiment was performed in triplicate to assess for consistency of results.

Apoptosis Assay
To evaluate the mechanism of growth inhibition by metformin, the cell cycle profile was analyzed after metformin treatment. Both cell lines were plated at 1×10^5 cells/well in 24 well plates in their corresponding media for 24 hours. Subsequently, the cells were treated by different metformin concentrations (0, 10 & 20 mM) for 24 hours in normoxia and hypoxia conditions. Apoptosis assay was done using the Annexin V-FITC assay kit (AbDSerotec, USA) in which the cells were stained with Annexin V-FITC and Propidium Iodide. Fluorochrome FITC-labelled Annexin V is a sensitive protein probe which has a high affinity for phosphatidyl serine (PS). The assay was done according to the manufacturer’s instruction. Briefly, the cells were washed in PBS by gentle shaking or by pipetting up and down. Cells were re-suspended in pre-diluted binding buffer, adjusting to the cell density of 2-5×10^5 cells/ml.

Subsequently 5μl of Annexin V-FITC was added to 195μl of cell suspension and incubated for 10 minutes at room temperature in the dark. Cells were washed in pre-diluted binding buffer and re-suspended in 190μl pre-diluted binding buffer. Finally 10μl of the Propidium Iodide solution was added to the previous mixture. The flow cytometer was properly set such that the Mean Fluorescence Intensity (MFI) of the Annexin V negative population was between 1 and 10. Optimal parameter settings was found using a positive control. For a positive control, cells were incubated with 3% formaldehyde in buffer for 30 minutes on ice. Formaldehyde was washed away and cells were suspended in cold binding buffer at 2-5×10^6 cells/ml. The rest of the staining steps were similar to other cells which were described above. Finally, Cells were analyzed using a Cyflow ML 16 flow cytometer (Partec, Germany). Flowmax software (Partec, Germany) was also used for data analysis.

Western Blot Analysis
Cells were lysed in RIPA Buffer (50 mMTris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5 mM DTT, 2% SDS) with complete Protease Inhibitor Cocktail (Roche, Germany). The cell lysate was centrifuged at 12000rpm for 20 minutes at 4°C. The protein concentration was measured using the Bradford Total Protein Assay. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidenefluoride membranes (Invitrogen, Carlsbad, CA) at 100 V for 2 hours. The transferred membrane was blocked overnight with 3% BSA. Primary hybridization by Rabbit anti S6K1 (phosphor T389) and anti β-actin antibodies, followed by secondary hybridization by Rabbit anti GiatfG-HRP conjugated (Abcam, US) in 1/2000 dilution were exerted each for 2 hours at room temperature and developed by DAB substrate kit (Thermo, US) enable chromogenic detection.

Detection of MMP-9 by Zymography
Zymography was done for semi-quantitative analysis of MMP9 (gelatinase B) levels secreted into the culture medium with minor modification. In brief, samples were mixed with 5μl 4 × SDS sample buffer (2% w/v SDS, 40% urea, 200 Mm Tris-Borate, pH 8.6, and 0.001% bromphenol blue) in the absence of a reducing agent and resolved by electrophoresis at 80 V for 3h on 7% polyacrylamide gel containing 1% SDS and gelatin B at a final concentration of 0.5 mg/ml. Thereafter, gels were washed twice in 2.5% Triton X-100 for 30 min each to remove the SDS and then were incubated for 18 h at 37°C in reaction buffer (0.1 M Tris-HCl, 10 mM CaCl2, and pH 7.4). After staining the gel with 0.2% Coomassie Brilliant Blue R-250, gelatinolytic activity of MMP-9 was visualized as a clear band in the uniformly stained background. The optical density (OD) of the area of each clear band was determined using the ImageJ software (NIH, US).

Statistical Analysis
Every one of the expression analysis assays including proliferation assay, Apoptosis, Zymography and gene expressions were carried out in triplicates and the generated data were presented as the means ± SD. T-test and one-way ANOVA test have been conducted for the data collected from assays. A p value of less than 0.05 was considered as significant difference. All statistical analyses were carried out with SPSS 16 (SPSS Inc.).
RESULTS

Effects of Hypoxia on Anti-proliferative Activity of Metformin

The results of MTT assay data analysis and the light microscopic shots of cells with two different metformin concentrations under hypoxia and normoxia conditions are presented in Figure 1a and 1b, respectively.

According to the data analysis and chart, by increasing metformin treatment concentration, the proliferation rate decreased in MCF-7 cells. Furthermore, with equal metformin concentration in MCF-7 cell lines, hypoxic cells showed lower proliferation rate than normoxic cells and the higher decrease in proliferation rate was observed in hypoxic cells (about 80%) with 20 mM metformin concentration.

In HEK293 cells, under normal condition any considerable change was not observed in proliferation with 0 and 10 mM metformin treatments. However, with 20 mM metformin concentration there was 38% decrease in proliferation rate. HEK293 cells cultured under hypoxia condition showed no changes with 0 and 5 mM metformin concentration whereas showed 26% and 48% decrease in proliferation rate with 10 and 20 mM metformin treatments, respectively (Figure 1a).

As can be seen from the microscopic shots, the number of non-cancerous cells with no metformin treatment was greater in normoxia condition than hypoxia. Furthermore, these cultured cells also showed the greatest decrease in the number of viable cells with 20 mM metformin concentration which had long podia. The longed podia belong to the metformin treated cells with low proliferation rate while the shortened ones were in control cells. However, considering all causes and effects together, in non-cancerous cells the decrease in proliferation rate did not show a significant change between hypoxia and normoxia conditions and overall metformin showed less anti-proliferative effect by 10 mM concentration, especially under normal condition (Figure 1b).

However, in MCF-7 control cells (with no metformin treatment) both cells cultured under hypoxia and normoxia conditions demonstrated a reliable proliferation rate and hypoxic cells showed less number of cell underwent apoptosis which indicated the positive effect of hypoxia on cancer cell proliferation. In addition, by increasing metformin concentration MCF-7 cells revealed a lower proliferation rate and higher apoptotic cells. The difference between normoxia and hypoxia in cancer cells were significant (Figure 1b).

![Figure 1](image.png)

Figure 1. The effects of metformin on cell viability of MCF-7 and HEK293 cells under hypoxia and normoxia condition. (a) Cells were treated with 5, 10 and 20 mM metformin under hypoxia and normoxia conditions. Data were expressed as mean ± SE (n=5). (b) Photomicrographs of MCF-7 cells (b1) versus HEK293 cells (b2) were taken by a light microscope at a magnification of 10X. The cells were treated with 10 mM and 20 mM metformin and cultured under hypoxia and normoxia conditions. Comparing the microscopic shots of treated cell lines under hypoxia and normoxia conditions support the achieved data. Since, optimum anti-growth effect of metformin was observed in MCF-7 cell lines with 10mM metformin concentration under hypoxia condition.
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The highest difference between non-cancerous and cancer cells under hypoxia condition was observed in 10 mM metformin concentration, which indicated the optimum metformin treatment concentration ($p=0.006$).

**Effects of Metformin on Cell Apoptosis and Necrosis under Hypoxia Condition**

A One-way ANOVA test followed by Dunnett’s test has been conducted for the data collected by apoptosis assay. As shown in figure 2, for MCF-7 cell line there is a significant difference in the apoptosis rate between hypoxia and normoxia (Figure 2a). These differences are analytically significant in 20 mM metformin concentration with $p=0.031$ and 10 mM metformin concentration with $p=0.008$. In contrast, HEK293 cells that have been cultured in two hypoxia and normoxia conditions, showed an acceptable difference in the apoptosis rate just in control groups with no metformin treatment with the $p=0.021$, which indicates the toxicity effect of hypoxia on HEK293 cells (Figure 2b).

![Figure 2](image-url)

*Figure 2. Effects of metformin on cell apoptosis and necrosis of MCF-7 and HEK293 cells under hypoxia and normoxia conditions. T-test and one-way ANOVA test followed by a Dunnett’s test have been conducted for the data collected from apoptosis assay. The plots show data as mean ± SD. Plots (a) and (b) show the MCF-7 and HEK293 cell apoptosis rates with 0, 10 and 20 mM metformin concentrations under normoxia condition compared to hypoxic counterparts. Plot (c) shows the data of 10 and 20 mM metformin treatment of each cell line under hypoxia and normoxia conditions compared with control (with no metformin treatment) group. Plot (d) shows the necrosis percentage for MCF-7 and HEK293 cell lines treated with 10 and 20 mM metformin and compared with control groups. The one-way ANOVA test showed no acceptable changes between the aforementioned groups. NS, *, and ** indicate “no significant”, “$p<0.05$” and “$p<0.01$” respectively.*
Figure 3. Effect of optimum concentration of metformin treatment on cell apoptosis and necrosis. (a) and (b) represent the apoptotic patterns under 10mM metformin concentration. The typical dual-staining pattern shows cells in early (bottom right quadrant) and late (top right quadrant) apoptotic states. The viable cells are double negative (bottom left quadrant) patterns observed. Data analysis revealed an optimum metformin concentration on 10mM for cancer cells under hypoxia condition compared to normoxia, while, there is no significant change for non-cancerous cells.

Comparing each treated cell line with its control counterpart indicated significant increases in cell apoptosis of cancer cells by metformin treatment which was greater under hypoxia condition \((p=0.002)\). However, for non-cancerous cells the only statistical significance results was found in the presence of 20 mM metformin concentration under hypoxia condition \((p=0.018, \text{Figure } 2c)\).

The necrosis rate was assessed using apoptosis assay and was shown in Figure 2d. In general, there was no significant change in necrosis by both oxygen supplies and metformin concentrations.

As indicated in biparametric histograms of the cells with 10mM metformin treatment, the staining patterns for cancer cells showed a significant difference under hypoxia condition compared to normoxia while there was no significant change for non-cancerous cells (Figure 3).

Effects of Metformin on S6K1 Phosphorylation

Western blot was carried out to assess S6K1 phosphorylation and \(\beta\)-actin as a control protein in MCF-7 and HEK293 cell lines cultured under hypoxia and normoxia conditions and treated with metformin. The photos of western blotting and statistical analysis are shown in Figure 4.

HEK293 cells were treated with 10 mM metformin concentration (optimum concentration) while cancer cells were treated with 10 and 20 mM metformin concentration. The data indicated that in cancer cells, S6K1 phosphorylation decreased by increasing metformin concentration (Figure 4a). However, in non-cancerous cells there was no major change in phosphorylation rate by metformin treatment (Figure 4b).

As it was recorded in Figure 4, with 10 and 20 mM metformin treatments and comparing with hypoxia and normoxia control pairs, in MCF-7 cells the decrease in S6K1 phosphorylation was statistically significant in both metformin concentrations \((p=0.027 \text{ and } 0.041 \text{ respectively})\), whereas in non-cancerous cells there was no significant change between 10 mM metformin concentration and control counterparts (Figure 4d).

MMP-9 Activation and Regulation by Metformin

For this experiment, MCF-7 cell lines with different concentrations of metformin were applied to gelatin-zymography for the separation of gelatinase and the zymogram. Representative gel presented in Figure 5 indicated that metformin treatment on cancer cells caused a significant reduction in MMP-9 activity. A statistically significant inhibition of MMP-9 activity was observed at 10mM concentration of metformin treatment under hypoxia condition compared to control group \((p=0.038)\). Furthermore, comparing the cells cultured under hypoxia with their normoxia counterparts, the impact of metformin on reducing MMP-9 activity of cancer cells was remarkably higher in hypoxia condition rather than normoxia which resulted to about 50% reduction in MMP-9 activity.
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Figure 4. S6K1 phosphorylation in cancer and non-cancerous cells. (a) Western-blot analysis in cancer cells showed that S6K1 phosphorylation decreased with increased metformin concentration. (b) Western-blot analysis in non-cancerous cells showed no reliable change in S6K1 phosphorylation with metformin treatment. (c) One-way ANOVA test for MCF-7 cells showed that each cells treated with 10 and 20 mM metformin concentrations showed significant difference with control counterparts ($p<0.05$) and t-test analysis revealed that only in 10 mM metformin concentration, the difference between hypoxic cell line and normoxic pair was statistically significant. (d) Data analysis of S6K1 phosphorylation of non-cancerous cells revealed that there was no significant difference between treated and untreated cells and between normoxia and hypoxia pairs. Data expressed as mean±SD. * and NS represent $p<0.05$ and No Significant, respectively.

Figure 5. MMP-9 activity. MCF-7 cell lines with 0 and 10 mM concentrations of metformin were applied to gelatin-zymography. Data analysis (t-test) was conducted to compare treated cells with control pairs. The inhibition of MMP-9 activity was observed to be significant in hypoxia condition by 10 mM of metformin concentration ($p<0.05$). Data were expressed as mean±SD (n=3).
Some studies have reported the effects of metformin, commonly prescribed as an anti-diabetic drug, on the prevention and treatment of various types of malignancies such as lung, breast, colorectal, and prostate cancers. Some probable reasons for the anti-tumorigenic implications of metformin are considered as its well-established clinical safety, preferential activity against tumor cells, and capability of combination with conventional cancer therapies. The most important function of metformin is inhibition of mTOR, followed by HIF activation which is one of the most essential factors in hypoxia condition, thus has a crucial role in cell growth and survival.

The microenvironment of cancer cells is different from normal cells due to their high proliferation rate and lower energy supplements by nutrient circulation. Therefore, cancer cells change their metabolic pathways to survive. Moreover, cancer cells need a large amount of energy for their quick growth and multiplication; hence, consume a great deal of glucose. In vivo cancer cells endure a lack of oxygen due to their rapid growth. The previous studies have not considered hypoxia in their in vitro experiments, and cultured the experimental cell lines by supplying adequate oxygen level.

In this study it was investigated the treatment effects of metformin on breast cancer cell proliferation and apoptosis, cell morphology changes, S6K1 phosphorylation, and MMP-9 activity to evaluate the anti-tumor effect of metformin treatment. However, in order to collect reliable data, we cultured our cancer and non-cancerous cell lines under hypoxia and normoxia conditions to assess the actual inhibitory effects of metformin for cancer cells. The MTT assay and light microscopy of the cultured cell lines revealed significant reduction in cell viability of cancer cells treated by metformin which confirmed the anti-proliferative effect of metformin. Moreover, cancer cell treatment with metformin treatment under hypoxia condition resulted in a more significant enhancement in cell death than cells under normoxia condition. In contrast, in non-cancerous cells, 10 mM and less metformin treatment did not have a significant effect on cell viability.

Normal cells in vivo are under normal conditions; however, cancer cells endure hypoxia condition. Thus, the best comparison would be between HEK293 cells under normal conditions versus MCF-7 cells under hypoxia condition. Therefore, we observed that the best concentration of metformin treatment is 10 mM since, the difference between two cell lines in their viability and apoptosis was in the highest. Nevertheless, in high concentration (20 mM) the difference became lower which was due to the toxicity characteristics of high concentration metformin for non-cancerous cells.

In line with MTT test, apoptosis test showed a significant difference between the above mentioned groups. While HEK293 cell lines cultured under normoxia condition and treated with 10 mM metformin concentration showed 26% apoptosis, hypoxic MCF-7 cells lines treated with the same metformin concentration underwent 84% apoptosis (p<0.01).

S6K1 phosphorylation may considered to be an indicator of mTOR pathway activation. Here it was demonstrated that metformin inhibits mTOR pathway by reducing S6K1 phosphorylation. Western blotting showed that metformin inhibits S6K1 phosphorylation and reduces its protein activity. The cellular pathways that affect S6K1 phosphorylation are vital pathways in cancer cell proliferation and growth. Like that previous reports, the data analysis also revealed that there is a significant change in S6K1 phosphorylation in cancer cells between hypoxia and normoxia condition, which indicates the additional effects of hypoxia to previously proved inhibitory effect of metformin on S6K1 phosphorylation (p<0.05). Together, it could be concluded that 10 mM metformin concentration is the optimum concentration for decreasing S6K1 phosphorylation due to its low effects to non-cancerous cells under hypoxia condition and highly anti-tumor effects on cancer cells under hypoxia condition by inhibiting S6K1 phosphorylation.

Metalloproteinases (MMPs) are mainly responsible for destroying the basement membrane and synthesizing zymogenes. Generally they are capable of degrading the whole extracellular matrix (ECM), so representing a critical factor in metastasis. Along with MMP-2 are the main proteinases that are able to degrade ECM, therefore representing a new target of cancer metastasis prevention studies. In the current study, we showed higher inhibitor effect of metformin on MMP-9 in hypoxia rather than normoxia. Also, it can affect cancer cells more efficiently in hypoxia condition compared to normoxia, which is a common characteristic of cancer cells.

In conclusion, metformin causes a higher reduction
in cell growth and proliferation and increases apoptosis in cancer cells and affects metabolism and growth pathways by direct and indirect effects. It also inhibits S6k1 activation, which is an important factor to maintain cell survival and growth in hypoxia condition. Moreover, metformin holds the potential to act as an anti-metastasis drug by suppressing MMP-9 activation, since MMP-9 is one of the important factors required for cell metastasis. However, further investigations need to be done accordingly.

Collectively, the aforementioned results indicate that metformin exerts its anti-tumor effect more efficiently under hypoxia condition rather than normoxia, which increase its selectivity and specificity of cancerous cells and reduce its side effects and harms on non-cancerous cells. To sum up, having characteristics such as specific toxicity against tumor cells along with having better performance under hypoxia condition, low side effects and costs, high stability at room temperature, metformin keeps the potential of acting as a drug in cancer therapeutics. However further studies required to clarify the mechanisms of observed effects.

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