RESEARCH ARTICLE

Lack of Metformin Effects on Different Molecular Subtypes of Breast Cancer under Normoglycemic Conditions: An *in vitro* Study

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Abstract

__Background__: In the past few years, a considerable number of preclinical studies have been proposed metformin as a potential anticancer agent, but some of these studies suffer from a number of methodological limitations such as assessment of cytotoxicity in the presence of supraphysiological glucose concentrations or applying suprapharmacological levels of the drug. These objections have limited the translation of published preclinical data to the clinical setting. The present study aimed to investigate direct anticancer effects of metformin on different molecular subtypes of breast cancer with pharmacological concentrations and under normoglycemic conditions *in vitro*. __Materials and Methods__: Breast cancer cell lines from luminal A, luminal B, ErbB2 and triple-negative molecular subtypes were treated with a pharmacological concentration of metformin (2mM) at a glucose concentration of 5.5mM. Time-dependant cell viability was assessed by dye exclusion assay. MTT-based cytotoxicity assays were also performed with metformin alone or in combination with paclitaxel. __Results__: Metformin did not show any growth inhibitory effects or time-dependant cytotoxicity on breast cancer cell lines in the presence of normal glucose concentrations at the therapeutic plasma level. No augmentation of the anti-neoplastic properties of paclitaxel was apparent under the tested conditions. __Conclusions__: Metformin is probably unable to exert cytotoxic or cytostatic effects on breast cancer subtypes at pharmacological concentrations and normal plasma glucose levels. These results highlight the importance of establishing a higher steady-state plasma concentration of metformin in the clinical setting for assessment of anticancer effects in normoglycemic patients.

__Keywords:__ Breast cancer - metformin - cytotoxicity - *in vitro* - normoglycemia

Introduction

Dysregulation of carbohydrate and fatty acid metabolism is a major public health problem with a growing incidence throughout the world. The total number of peoples with diabetes mellitus is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004). Diabetes is most often associated with obesity. The combination of diabetes and obesity increases breast cancer risk up to 20% (Wolf et al., 2005). Furthermore, diabetic women with breast cancer have worse disease-related clinical outcomes than their non-diabetic counterparts (Lipscombe et al., 2008; Peairs et al., 2011).

Metformin is a biguanide oral hypoglycemic agent that has been used for over half a century to treat type 2 diabetes as the first line drug. This drug is currently used by at least 120 million people worldwide (Viollet et al., 2012). Metformin is a safe drug, its most common side effect being gastrointestinal discomfort (Scarpello and Howlett, 2008). Recently, cumulative epidemiological evidences indicate that metformin may prevent or delay the cancer (Evans et al., 2005). Metformin decreases breast cancer risk and improves survival rate in both patients with and without diabetes (Bowker et al., 2006).

Preclinical research has attributed a dual mechanism of action for metformin against cancer (Del Barco et al., 2011). This drug may act directly on neoplastic cells, largely by inhibiting complex-I of the respiratory chain in the mitochondria, leading to increased ratio of adenosine monophosphate (AMP) to adenosine triphosphate (ATP) and liver kinase B1 (LKB1)-mediated activation of AMP kinase (AMPK) resulting in inhibition of mammalian target of rapamycin (mTOR) via phosphorylation of tuberous sclerosis complex 2. These events result in decreased protein translation, inhibition of cell proliferation and increased apoptosis (Goodwin et al., 2009). Metformin may also act through an indirect,

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insulin mediated mechanism. This mechanism involves AMPK-mediated inhibition of hepatic gluconeogenesis, with resulting lowering in insulin plasma concentration, leading to decreased binding of insulin to insulin/insulin-like growth factor-1 receptors (IR/IGF-1R) present on neoplastic cells, reducing signaling through the Ras and phosphatidylinositol-3-kinase (PI3K) pathways leading to decreased proliferation and increased apoptosis (Goodwin et al., 2011).

Despite in the past few years a considerable number of preclinical studies have been proposed the metformin as a potential anticancer agent, but some of these studies suffer from a number of methodological limitations. Much of these works, particularly in the *in vitro* setting, have been assessed the cytotoxicity of metformin in the presence of supraphysiological glucose concentrations. Also in more studies, metformin has been used with suprapharmacological concentrations (Goodwin and Stambolic, 2011). These objections limit the translation of published preclinical data to the clinical setting.

In the present study, we aimed to assess the direct anticancer effect of metformin on cell lines representing different molecular subtypes of breast cancer including luminal A (estrogen receptor [ER] positive, HER-2 negative), luminal B (ER positive, HER-2 positive), ErbB2 (HER-2 positive, ER negative) and triple-negative (TN; ER negative, progesterone receptor [PR] negative, HER2 negative) in pharmacological concentration and normoglycemic condition.

Materials and Methods

Materials and cell lines

Low glucose Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25% trypsin, 1mM EDTA), amphotericin B and penicillin-streptomycin solutions were obtained from Invitrogen (Carlsbad, CA, USA). All cell culture vessels were purchased from BD Biosciences (Franklin Lake, NJ, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) glycine and trypan blue were obtained from Sigma-Aldrich (Steinheim, Germany). Metformin hydrochloride was gifted by Mahban Chemi Company. Paclitaxel was purchased from EBEWE Pharma (Unterach am Attersee, Austria). MCF-7 (luminal A), BT-474 (luminal B) and SK-BR-3 (ErbB2) cell lines were purchased from Iranian Biological Resource Center (Tehran, Iran). MDA-MB-231 (TN) cell line was obtained from National Cell Bank of Iran (Tehran, Iran).

Cell viability assay

For viability assay, cells were seeded at the density of 2×10^4 cells in 24-well plates in $400 \mu l$ low glucose (5.5mM) DMEM medium containing 10% FBS and 2mM L-glutamine in triplicate. The plates were incubated in humidified air containing 5% CO_2 at 37°C. Three days later, 2mM concentration of metformin was added. During exposure phase, cells were harvested every 24 hours up to 72 hour and the cell viability was assessed using a

hemocytometer after staining with trypan blue.

MTT-based cytotoxicity assay

MTT-based cytotoxicity assay was carried out in accordance with the protocol previously described (Plumb et al., 1989). Briefly, the cell lines were conveyed to 96-well microtitration plates with a seeding density of 1,000 cells per well in $200 \mu l$ low glucose DMEM medium containing 10% FBS. Three days later, when the cells were entered to the logarithmic phase of growth, exposure period was started by adding 10nM, 50nM, 100nM, 150nM and 200nM concentrations of paclitaxel alone or in combination with 2mM concentration of metformin. Each treatment was run triplicate. 72 hours later, the drugs were removed from the wells. In order to demonstrate retention of regenerative capacity of the exposed survived cells, a 96 hours recovery period were considered.

During the recovery period, the plates were fed daily with fresh medium. At the end of the recovery period, $50 \mu l$ of MTT (5mg/mL) solution was added to each well and then the plates were further incubated for 4 hours. All remaining supernatant were removed and $200 \mu l$ of DMSO was added to dissolve the formed insoluble formazan crystals. $25 \mu l$ of glycine buffer was added to each well to adjust the final pH. Then, absorbance was immediately recorded at 570nm using microtitration plate reader (BioTek®, USA). The absolute values of the absorbance were converted to surviving fraction data as the percentage of living cells of the control.

Statistical analysis

Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Bonferroni test to adjust for multiple testing. Linear regression analysis was used to find out the concentration-response relationship. Level of significance was set at $p < 0.05$. The statistical analyses were carried out using BioStat 2008 software.

Results

As presented in Table 1, metformin didn't exert significant alteration in cell viability in any exposure time and in any cell line.

There was no any statistically significant difference between the surviving fractions of metformin-treated cells and controls, in any cell line tested.

As shown in Figure 1A, there were seen statistically significant negative correlations between paclitaxel

Table 1. Effect of Metformin on Viability of Breast Cancer Cell Lines in Normoglycemic Conditions

Cell lines	0	24h	48h	72h
MCF-7	94.2±3.1	91.8±4.3	92.6±2.2	89.7±3.9
BT-474	95.2±4.3	92.7±3.6	91.4±6.3	93.1±4.4
SK-BR-3	89.8±5.3	92.1±3.7	90.5±4.9	88.2±3.2
MDA-MB-231	95.7±3.1	93.6±4.2	90.2±5.1	94.8±3.4

*Different molecular subtypes of breast cancer lineages including MCF-7 (luminal A), BT-474 (luminal B), SK-BR-3 (HER2), and MDA-MB-231 (triple negative) were cultured in a medium with 5.5mM glucose concentration and treated with pharmacological concentration of metformin (2mM). Cell viability was assessed every 24 hours using trypan blue. Data are represented as mean±standard error (%)

concentration and surviving fraction of MCF-7 cell line after treatment with paclitaxel alone or in combination with pharmacological concentration of metformin (p=0.001, r=-0.96; p=0.009, r=-0.96; respectively). The IC_{50} of paclitaxel for MCF-7 was calculated as 68.71nM, when used alone. After co-treatment of MCF-7 cell line with paclitaxel and metformin, this value was computed as 69.53nM. There was no any statistically significant difference between surviving fractions of MCF-7 cells treated with paclitaxel alone or co-treated with metformin and commensurate concentrations of paclitaxel.

In BT-474 cell line, surviving fraction significantly correlated with concentration of paclitaxel both in mono-treatment or co-treatment with metformin, as depicted in Figure 1B (p=0.011, r=-0.91; p=0.012, r=-0.91; respectively). In this cell line, the IC_{50} of paclitaxel alone or in combination with metformin was calculated as 66.67nM and 66.20nM, respectively. Metformin didn't change any statistically significant difference in the surviving fraction of paclitaxel-treated cells of luminal-B subtype.

In SK-BR-3 cell line, there were significant negative correlations between paclitaxel concentration and retention of regenerative capacity in both, absence and presence of metformin (p=0.010, r=-0.92; p=0.009, r=-0.92, respectively). In this cell line, the IC50 values for paclitaxel, in the absence and presence of metformin were calculated as 74.68nM and 74.01nM, respectively. There was no observed any significant difference in the surviving fractions of co-treatment group with metformin and paclitaxel compared to mono-treatment group with paclitaxel at commensurate concentrations of this cytotoxic agent (Figure 1C).

Statistically significant negative correlations were observed between paclitaxel concentration and surviving fractions of MDA-MB-231 cell line in the absence or presence of metformin (p=0.021, r=-0.88; p=0.015, r=-0.90; respectively). The IC_{50} of paclitaxel in this cell line was computed 54.92nM in the absence of metformin, and 58.83 pnM in co-treatment with metformin. Co-treatment
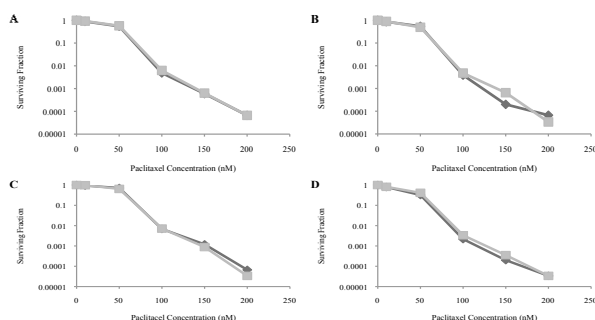

Figure 1. Assessment of Metformin Effect on Paclitaxel Cytotoxicity in Relevant Cell Lines of Breast Cancer Molecular Subtypes Including MCF-7 (Luminal A; A), BT-474 (Luminal B; B), SK-BR-3 (HER2; C), and MDA-MB-231 (Triple Negative; D) in Normoglycemic Conditions By MTT-based Assay. Surviving fractions plotted in Y axis, are calculated as percentage of living cells of the control. Black curves represent survival of cells treated with 10, 50, 100, 150 and 200nM of paclitaxel alone. Grey curves indicate to the survival of cells co-treated with metformin (2mM) and serial concentrations of paclitaxel. As illustrated in this figure, metformin didn't alter the paclitaxel cytotoxicity in normoglycemic status

with metformin didn't alter the surviving fraction of MDA-MB-231 cell line, compared to mono-treatment with paclitaxel (Figure 1D).

Discussion

The present study aimed to investigate the direct anticancer effects of metformin and its probable synergism with paclitaxel on different molecular subtypes of breast cancer in pharmacological concentrations and normoglycemic conditions. Metformin didn't show any growth inhibitory effects on breast cancer cell lines in the presence of normal glucose concentrations at therapeutic plasma levels. Metformin also didn't augment the anti-neoplastic properties of paclitaxel at the mentioned conditions.

Since the initial observation by Evans and colleagues indicating that metformin use in diabetic patients was associated with decreased cancer incidence (Evans et al., 2005), several studies have been performed about the anti-neoplastic effects of this biguanide derivative at preclinical level. Zakikhani et al. (2006) studied the effect of metformin on proliferation of some neoplastic cell lines. They showed the growth inhibition of cancer cells MCF-7 (breast), PC-3 (prostate), SKO3 and OVCAR3 (ovary), but not in HeLa (cervix), at a range of metformin concentrations between 2.5 and 20 mM in high-glucose medium. They attributed this anti-proliferative effect of metformin to decreased mTOR and s6 kinase activation and a general decline in mRNA translation. The lack of response in HeLa cell line was also ascribed to its non-functional LKB1 allele (Zakikhani et al., 2006).

Ryan and colleagues assessed the effect of metformin on protein synthesis in breast cancer cell lines. They expressed that treatment of MCF-7 cells with metformin led to a reduction in mRNA translation with a maximal inhibition of 30% at 5 mM to 20 mM concentrations. But metformin didn't alter protein synthesis in MDA-MB-231 cell line. Since this cell line doesn't express LKB1 mRNA or protein, they accentuated on the role of LKB1 in metformin mechanism of action (Dowling et al., 2007).

Wang et al. (2011) studied the effect of metformin on ER positive MCF-7 and triple negative MDA-MB-231 cell lines. They reported that the growth inhibition rates of MCF-7 cells were higher than MDA-MB-231 at each concentration. According to their results, metformin inhibited the growth of this TN cell line only at 20 mM and 40 mM. They also didn't observe any significant alterations in apoptosis after treatment with metformin (Wang et al., 2011). The results of Wang's study were inconsistent with what Liu and colleagues reported. Liu and coworkers expressed that metformin inhibits cell proliferation in all molecular subtypes of breast cancer with a more prominent cytotoxic effect in TN cell lines. They also reported that metformin induced apoptosis in breast cancer cell lines (Liu et al., 2012).

Cheng and coworkers studied the effect of metformin on SK-BR-3. They observed significant growth inhibitions in drug concentrations above 0.5mM. They also reported an increased apoptosis and decreased HSP90 expression in metformin-treated HER2 positive cell line (Cheng et

al., 2013).

In another study, antiproliferative effect of metformin was examined on MCF-7 and BT-20 lineages as ER-positive and ER-negative breast cancer cell lines. The cell proliferation of BT-20 was significantly inhibited in the presence of 15mM metformin. This drug showed a higher cytotoxic effect on BT-20 compared to the ER-positive cell line (Szewczyk et al., 2012).

Recent studies indicate that metformin may selectively suppress cancer stem cells (Liu et al., 2011; Cufi wt al., 2012). It has been illustrated that metformin overcomes primary resistance to trastuzumab in HER-2 positive human tumor xenograft of JIM-1 cell line by selective killing of CD44 high CD24 low cells (Cufi et al., 2012). This effect has been attributed to the inhibition of HER-2/IGF-1 receptor interactions (Liu et al., 2011). Hirsch's and colleagues' work confirmed the selective killing of cancer stem cells by metformin. They attributed this effect of metformin to inhibition of NF-ϰB and phosphorylation of STAT3 in cancer stem cells. Another finding of their work was that metformin can only inhibit the growth of xenograft tumors of inflammatory cell line which express IL-6 (Hirsch et al., 2013).

With an overview to the published literature on the anticancer effects of metformin, we can find out that some of these works suffer from methodological limitations. Much of these works have evaluated the growth inhibitory effects of metformin in the presence of 25mM glucose, which is 4.5 times higher than normoglycemic values. On the other hand, many studies have achieved the cytotoxic effects of metformin in suprapharmacological concentrations. In the present study, we assessed the effect of pharmacological concentration of metformin on proliferation of different molecular subtypes of breast cancer in normoglycemic conditions. Our study didn't demonstrate any antiproliferative effect of metformin in the test conditions. These results highlight the importance of establishing a higher steady-state plasma concentration of metformin in clinical setting for assessment of anticancer effects of metformin in normoglycemic patients. Further research is needed to illustrate the role of glucose concentration on the antiproliferative effects of metformin.

Acknowledgements

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