Effect of electropermeabilization of cell membrane on metformin action on MDA-MB-231 breast cancer cells

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Abstract. *Interest in cancer metabolism opens entirely new area of research in cancer biology. Metabolic alterations in cancer cells enable novel targeted therapeutic approaches. Metformin, an established metabolic drug can be used as anti-cancer treatment and might act against cancer indirectly or systemically. However high discrepancies between achievable physiological concentrations in vivo and the effect of half maximal inhibitory concentration in vitro are not adequately explained. The drug enter cell cytoplasm by organic cation transporter and its effectiveness could limit intracellular concentration of the drug. The aim of our study was to enable direct access of metformin into cytosol by cell membrane permeabilization. We applied external electric pulses that cause transient cell membrane permeabilitation by electroporation and enable free diffusion of metformin into the cell. We tested the effect of electroporation on metformin activity on breast cancer cell line MDA-MB-231 in vitro. Our results indicate that metformin in combination with electroporation does not increases cytotoxicity of metformin in MDA-MB-231 cells.*

1 Introduction

Cancer cells differ from normal cells in several key biological characteristics as described in the renowned paper "The Hallmarks of Cancer" [1]. Recently, metabolic reprogramming in cancer cells has been recognized as a new hallmark of cancer cells. Metabolic alterations in cancer cells were already recognized in 1920s by Otto von Warburg who was first to observe that tumor cells maintain glycolysis even under normoxic conditions – known as Warburg effect. The re-emergence of interest in cancer metabolism opened entirely new area of research in cancer biology and oncology since metabolic alterations in cancer cells offer the possibility for novel therapeutic approaches that selectively target cancer cell metabolism and use of already established metabolic drug for anti-cancer treatment [2].

Epidemiological studies suggest that metformin, one of widely used type 2 diabetes drugs, might reduce the risk and mortality of cancer in type 2 diabetes [3]. On the one hand, metformin might protect against cancer indirectly by its systemic lowering of insulin level and glucose homeostasis. In addition of its systemic effects metformin targets cancer cells directly as an inhibitor of complex I of electron transport chain [4]. Breast cancer is one of most common cancers, among which triple-negative cancers represent most aggressive types. MDA-MB-231 cell line is a model cell line of triple negative human breast cancer cells that is very often used to study different anti-cancer agents.

Direct anti-cancer effects of metformin have been thoroughly examined in cultured MDA-MB-231 cells, but its effectiveness as cytotoxic effects remains questionable due to inconsistent *in vitro* results. Clearly, mechanisms that may link metformin to direct anti-cancer effects require further characterization. Metformin ameliorates systemic glucose homeostasis via at least two mechanisms. One mechanism involves activation of the AMP-activated protein kinase (AMPK) [5]. However, the mechanism of metformin action are not completely understood.

In vitro some studies have shown reduced viability after metformin treatment of MDA-MB-231 cells in concentrations as low as 30-500 μM while others have shown its effects on proliferation and viability only at very high concentrations (10-40 mM). However, in patients with type 2 diabetes peak plasma concentrations of metformin are 10–30 μM. While inconsistent *in vitro* results suggest that differences in experimental design might modulate sensitivity of MDA-MB-231 cells to metformin. Recently we have shown [6] that glucose level and protocol of medium renewal strongly affects metformin action *in vitro*. Still, high discrepancies between achievable physiological concentrations and the effect of half maximal inhibitory concentration (IC50) *in vitro* are not adequately explained. Based on those discrepancies it was hypothsed that due to its charge metformin can be concentrated inside mitochondria thus achieving much higher local concentration do to the electrochemical potential gradient. In addition, the effectiveness also depends on metformin ability to cross the plasma membrane. It is known that metformin needs organic cation transporter OCT1 for active transport across the plasma membrane [4]. Therefore, its cytoplasmic concentration could be increased by cell membrane permeabilization.

So far none of the studies examined the effects of metformin *in vitro* in combination with electroporation. Electroporation is a physical method that uses electric pulses to permeabilise cell membrane. It has been used to transfer small molecules or macromolecules into cells

in vitro and *in vivo* [7]–[9] and is currently used in clinical applications of electrochemotherapy [10], [11].

In this study, we examined the effect of metformin in combination with electroporation on cell survival in comparison with classical treatment with metformin alone. We used MDA-MB-231 cell line cultured *in vitro* and performed preliminary experiments on cell suspension.

2 Materials & Methods

2.1 MDA-MB-231 cells

Triple negative MDA-MB-231 breast cancer cell line was from ATCC (USA). MDA-MB-231 cells were maintained in high-glucose (4.5 g/l) RPMI-1640 medium (Genaxxon bioscience, Germany) without pyruvate, supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Sigma-Aldrich). They were incubated in humidified atmosphere (95% air/5% CO2) at 37˚C. Experiments were performed in RPMI-1640 with 10% FBS and with 1 g/l (5.6 mM) glucose (Sigma-Aldrich). Cell culture medium was renewed every 24 hours.

2.2 Pulse protocols

Electric pulses were generated by electric pulse generator Electro cell B10 HVLV (BetaTech, France). Cells in suspension were prepared with tripsinization. Tripsinized cells were centrifuged at 1000 RPMI for 5 minutes and pelet was resuspended in KPB puffer (10 mM KH_2PO_4/K_2HPO_4 1 mM $MgCl_2$ and 250 mM sucrose) to obtain 10^6 cells/ml. 100 µl of the sample was electroporated in 4 mm Eppendorff cuvettes and 25 vol % of FBS was added to all cells after electroporation. The effect of electroporation in combination with four concentrations of metformin, 30 µM, 300 µM, 1 mM and 5 mM [6] was tested. Pulsing protocols consisted of 4 consecutive square pulses applied at repetition frequency 1 Hz. Three amplitudes of electric fields (E) were used: E = 0.6 kV/cm, 0.8 kV/cm 1, 1.2 kV/cm and 1.4 kV/cm. Pulse durations (t) was 200 µs. Control samples were not electroporated (Control 0 – negative control, E = 0 kV/cm and without metformin). Electric field strength (E) was calculated by the formula $E = U / d$, where U denotes applied voltage and d the electrode distance.

2.3 Determination of MDA-MB-231 cell viability by Hoechst and propidium iodide staining

The effect of metformin and electroporation on cell survival and morphology was inspected visually at inverted microscope (Zeiss Axiovert 200, Germany) under bight field at 10× objective magnification.

The survival of adherent MDA-MB-231 cells was determined 48h after experiment using Hoechst 33342 (Thermo Fisher Scientific) Cells treated in suspension were plated in 24 well plates with the cell density 4×10^4 per well and fluorescence was determined at 350 nm/461 nm (excitation/emission) using Tecan Infinite 200 (Tecan, Männedorf, Switzerland). To lyse cells, 0.03% SDS was added for 30 minutes at room temperature. To stain dsDNA, 100 mM NaCl, 50 mM TRIS-HCl, (pH=8.25) buffer, containing 5 µg/ml Hoechst 33342 was added. Background fluorescence intensity was subtracted and relative cell number in each sample was presented as the percentage of fluorescence intensity of treated samples relative to control sample (untreated cells). All experiments were performed at least in duplicates.

Cell membrane permeability was determined by propidium iodide (PI) staining. PI is cell membrane impermeant stain that can be used to detect cell membrane permeabilization. PI was added to KPB imediately before electric pulse application and cell membrane permeabilization was determined by flow cytometry (Attune NxT, Life Technologies). For each sample 10.000 events were recorded and analyzed.

Effects of metformin on cell energy metabolism was analyzed by measuring oxygen consumption rates (OCR; a measure of oxidative phosphorylation) and extracellular acidification rates (ECAR; a measure of glycolysis) of cells with Seahorse XFp Analyser. Basal and stressed (with 1 µM oligomycin and 1 µM FCCP injection) metabolic phenotypes of MDA-MB-231 cells were determined with Seahorse Energy Phenotype Test according to manufacturer instructions.

3 Results & Discussion

Electroporation experiments were performed to determine the effect of metformin on cell survival after exposure of MDA cells to different electric pulse amplitudes and metformin concentrations. Electroporation transiently permeabilize cell membrane allowing direct acces of metformin to the cytoplasm.
The effect of electroporation that causes cell membrane permeabilization on plated cells is shown in Figure 1. A bright field images of cells treated with different concentrations of metformin (the left column) and cell treated with metformin and permeabilized by electroporation (4×200 µs 1.2 kV/cm, 1 Hz, the right column) are presented.

Our results indicate that electroporation in combination with metformin does not have a significant impact on treated cells as can be observed in selected micrographs (Figure 1). The combined treatment does not affect cell survival quantified spectrofluorometrically (Figure 2). Both electroporation alone and metformin alone reduce cell viability to 80% and 60%. Those effects are consistent with previously published results [6], [7]. The combination of both does not result in further reduction of cell viability.

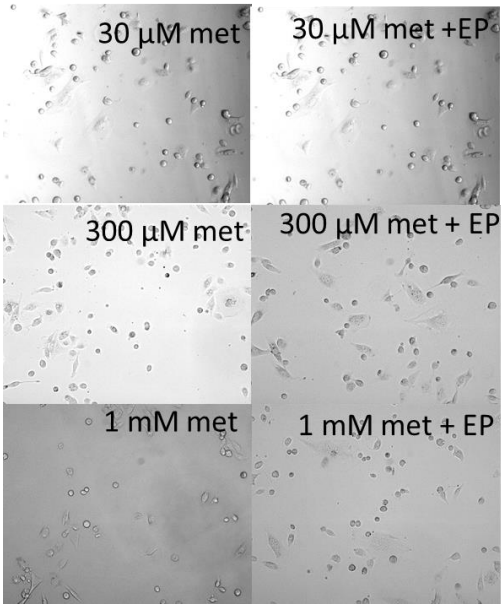

Figure 1: Bright field images of MDA-MB-231 cells treated with different concentrations of metformin alone or in combination with electoporation 48h after treatment. Electric pulse parameters were 4 x 200 µs, 1 Hz, 1.2 kV/cm. Metformine concentrations were 30 µM, 300 µM and 1 mM. Objective magnification 10×.

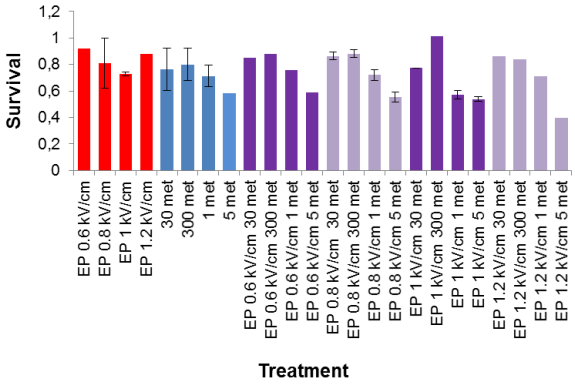

Figure 2: Cells treated in suspension: effect of increasing electric field strength and metformin concentration or combination of electric pulse parameters and concentrations of metformin on survival of MDA-MB-231 cells 48h after treatment

The effect of electric pulses on cell membrane permeability of MDA cell line was detected in a separate experiment and is presented in Figure 3 and Table 1.

The results indicate that almost all population of cells is efficiently permeabilized at 1 kV/cm allowing entrance of small molecules, as determined by PI staining (PI molecular weight 668.39 g/mol). Metformin is a small molecule with molecular weight 129.16 g/mol and can therefore enter the cells permeable to PI. The detected permeabilization of MDA cell line is similar to other cell lines of similar sizes [7].

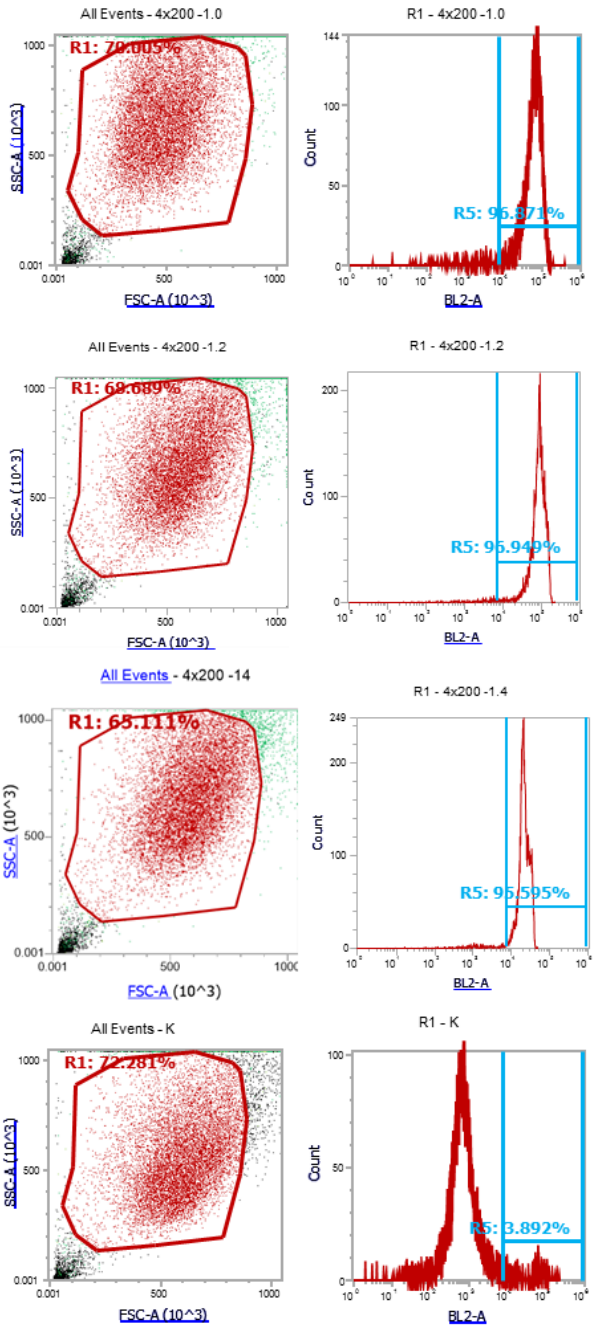

Figure 3: Cell permeabilization of MDA-MB-231 measured with PI uptake. On the left side scater and forward scatter of the samples, viable cells were gated in R1 region. On the right (R5) percentage of PI positive cells in the gated region determined on fluorescence channel BL2. Electric pulse parameters were 4 pulses of 200 µs duration, amplitude of 1, 1.2 and 1.4 kV/cm and repetition frequency 1 HZ. Control cells (at the bottom) were not exposed to electric pulses.

Table 1: The percentage of PI uptake by electroporated MDA-MB-231 cell in suspension. NK, negative control represents cells not exposed to electric pulses. For electroporation we used 4 electric pulses of 200µs duration amplitude of 1 kV/cm, 1.2 kV/cm and 1.4 kV/cm and repetition frequency 1Hz.

	NK	1 kV/cm	1.2 kV/cm	1.4 kV/cm
PI	4 %	97 %	97 %	96 %

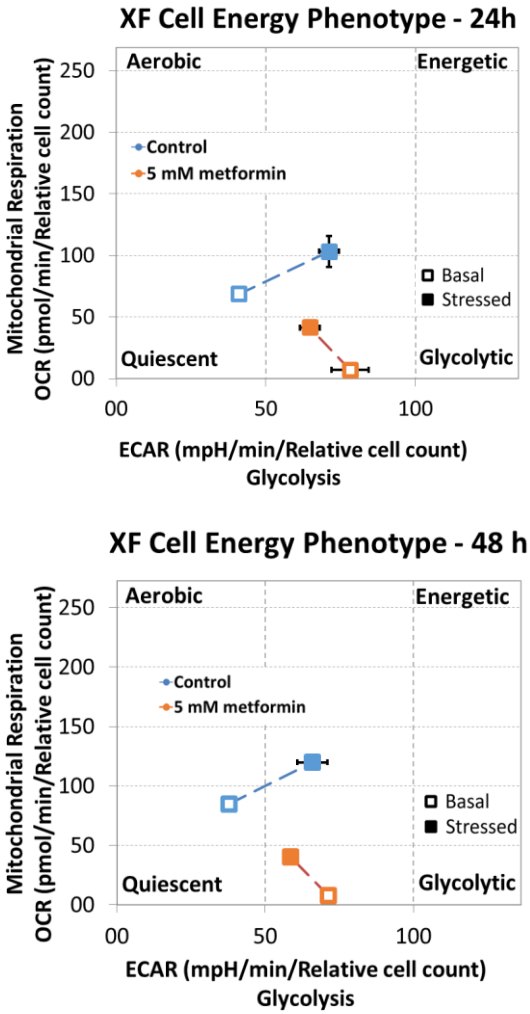

Figure 4: Cell energy metabolism of MDA-MB-231 cells treated with metformin 48h after treatment. Empty squares represent basal, while full squares represent stressed energy phenotype. T-bars indicate standard deviations between the wells. Results were normalized to relative cell counts.

Combination of electroporation and metformin in one treatment (Figure 1 and 2) does not further reduce cell viability indicating that OCT1 transporters [4] responsible for active transport of metformin across cell membrane are sufficient to obtain efficient intracellular concentrations of metformin. Namely, the increased cell membrane permeability caused by electric pulses of different amplitudes (Table 1, Figure 3) does no further increase cytotoxic effect of metformin alone [6].

This was further confirmed by Seahorse experiment, where it can be clearly seen that already after 24h metformin at 5 mM concentration almost completely blocks oxidative phosphorylation both in basal and stressed conditions as determined by decrease in OCR (oxygen consumption rate) consistently with its inhibition of mitochondrial complex I (Figure 4) one of the intracellular metformin targets [4]. Therefore, access of metformin to cell interior in non-electroporated cells is not a limiting factor in cell line MDA-MB-231.

To conclude, electroporation does not increase metformin action on MDA-MB-231 cells and probably its synergistic effects could be obtained in cell lines with low level of OCT1 transporters.

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