Co-treatment of breast cancer cells with pharmacologic doses of 2-deoxy-D-glucose and metformin: Starving tumors

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Abstract. A characteristic of tumor cells is the increased aerobic glycolysis for energy production. Thus, inhibition of glycolysis represents a selective therapeutic option. It has been shown that glycolysis inhibitor 2-deoxy-D-glucose (2DG) induces apoptotic cell death in different tumor entities. In addition, the antitumor activity of the anti-diabetic drug metformin has been demonstrated. In the present study, we aimed to ascertain whether the combination of pharmacologic doses of 2DG with metformin increases the antitumor efficacy. Cell viability of MDA-MB-231 and HCC1806 triple-negative breast cancer (TNBC) cells treated without or with 2DG or with metformin alone or with the combination of both agents was measured using Alamar Blue assay. Induction of apoptosis was quantified by measurement of the loss of mitochondrial membrane potential and cleavage of PARP. Treatment of breast cancer cells with glycolysis inhibitor 2DG or with the anti-diabetic drug metformin resulted in a significant decrease in cell viability and an increase in apoptosis. Treatment with 2DG in combination with metformin resulted in significantly reduced viability compared with the single agent treatments. The observed reduction in viability was due to induction of apoptosis. In addition, in regards to apoptosis induction a stronger effect in the case of co-treatment compared with single agent treatments was observed. The glycolytic phenotype of human breast cancer cells can be targeted for therapeutic intervention. Co-treatment with doses of the glycolysis inhibitor 2DG and anti-diabetic drug metformin is tolerable in humans and may be a suitable therapy for human breast cancers.

Introduction

Therapeutic selectivity is one of the most important criteria in the therapy of cancer. In order to achieve effective destruction of cancer tissue without side-effects, it is important to consider the biological differences between normal and cancer cells. One change that occurs in malignant cells is the increase of glycolysis for energy production regardless of the availability of oxygen. This is known as the Warburg effect (1). In addition, in many tumors glucose transporter 1 (GLUT1) is overexpressed resulting in increased glucose uptake (2). The exact molecular mechanisms that lead to these metabolic changes are not yet completely understood, yet increased GLUT1 expression and the central role of glycolysis in tumor cells represent an attractive target of attack for selective tumor therapy (3). Due to the dependence of tumor cells on glycolysis, 2-geoxy-D-glucose (2DG) has been considered as a potential anticancer agent. 2DG is a glucose analog, which leads to inhibition of glycolysis and a decrease in ATP production resulting in induction of apoptosis through activation of caspase-3 in solid tumors (4-8). Combination of chemotherapeutic agents including GnRH receptor-targeted chemotherapy using AEZS-108 (AN-152) and 2DG has been successfully tested (9).

Metformin (1,1-dimethylbiguanide hydrochloride) is a well-established drug used for the treatment of type 2 diabetes. It acts by reducing insulin resistance via different mechanisms, including increased glycogen synthesis, enhanced insulin receptor tyrosine kinase activity, and an increase in glucose transporter 4 (GLUT4) recruitment and activity (10). In addition, metformin affects fasting plasma insulin levels, resulting in the reduction in glucose concentrations in the blood (11). Different preclinical studies have demonstrated the anti-neoplastic effects of metformin in animal models of different tumor entities including cancers of the breast (12-17). The proposed mechanisms of these antitumor effects include an indirect, insulin-dependent pathway by reduction of serum insulin levels, direct modulation of cellular protein synthesis, and direct growth inhibition through effects in the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (PKB and Akt)/AMP-activated protein kinase (AMPK) signaling pathway (18,19).

Increased glycolysis for energy production is necessary for the survival of tumor cells, and thus represents a selective therapeutic target. In the present study, we showed that the glycolytic phenotype of human breast cancer cells can be targeted for therapeutic intervention. We aimed to ascertain whether treatment with well-tolerable doses of 2DG can reduce the viability of triple-negative breast cancer (TNBC) cells *in vitro*. In addition, we tested whether the antitumor

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efficacy of glycolysis inhibition by 2DG can be enhanced by co-treatment with pharmacologic doses of metformin.

Materials and methods

Cell lines and culture conditions. The TNBC cell lines HCC1806 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia, USA). In order to guarantee the identity of the cell lines over the years, the cells were expanded after purchase and aliquots were stored in liquid nitrogen. Every half year a new frozen stock was opened and expanded to carry out the experiments. The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air as previously described (20-22).

Chemicals. 2DG and metformin were purchased from Sigma Chemical Company (Deisenhofen, Germany).

Viability assay. Five hundred cells/well were plated into 96-well plates (Falcon, Heidelberg, Germany) in 100 μl Dulbecco's modified Eagle's medium (DMEM)/5% fetal calf serum (FCS; Biochrom GmbH, Berlin, Germany) without phenol red, 2 mM glutamine, 50 U/ml penicillin/streptomycin, 2.5 μg/ml amphotericin B and 1:100 non-essential amino acids. After cell attachment, 100 μl medium, 100 μl 2DG/medium solution, 100 μl metformin/medium solution or 100 μl solution with 2DG in combination with metformin was added to the wells in six replicates and incubated for 96 h at 37˚C in 5% CO_2. Final concentrations of 2DG, metformin and the combination of both agents are provided in the Results section. Cell number was determined by a colorimetric assay using Alamar Blue (BioSource, Solingen, Germany). The optical density (OD) of the reduced dye was assessed at 570 vs. 630 nm after 4 h at 37˚C.

Mitochondrial membrane potential. Cells were treated for 48 h without or with 2DG, metformin or the combination of both agents. Final concentrations of 2DG, metformin and the combination of both agents are provided in the Results section. Then the cells were washed once with phosphate-buffered saline (PBS) and mitochondrial membrane potential was measured using the JC-1 mitochondrial membrane potential detection kit according the manufacturer's instructions (Biotium, Hayward, CA, USA).

Western blot analysis of cleaved PARP. Cells were treated for 48 h without or with 2DG or metformin or the combination of both. Final concentrations of 2DG, metformin and the combination of both agents are provided in the Results section. Then, the cells were detached with 0.5 g trypsin (Biochrom) and 5 mmol EDTA in 1 l PBS/BSA. The pellets were washed twice with PBS and resuspended with CelLytic™ buffer containing protease inhibitors (both from Sigma). Equal amounts of protein/sample were used and diluted to equal volumes with Laemmli-buffer. The cell lysates were separated on SDS-PAGE (15%, ProSieve® 50 Gel Solution; Cambrex, Verviers, Belgium) under reducing conditions and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare Europe, Munich, Germany). The nitrocellulose membranes were blocked with 5% instant skimmed milk powder, spray-dried (Naturaflor, Töpfer GmbH, Dietmannsried, Germany) in Tris-buffered saline and Tween-20 (TBST) (137 mM NaCl, 2.7 mM KCL, 0.1% Tween-20, 25 mM Tris/Cl, pH 7.4) for 1 h at room temperature (RT), washed with TBST and then incubated at 4˚C overnight with rabbit anti-human active caspase-3 polyclonal antibody (BD Pharmingen, Heidelberg, Germany) in a 1:5,000 dilution in TBST and then, following washings, incubated at RT with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare Europe) at an 1:10,000 dilution in TBST for 1 h. After washings, specifically bound antibody was detected using the SuperSignal™ West Femto Maximum Sensitivity chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA). For quantification, the bands were analyzed using a C-DiGit Blot Scanner (Li-COR, Lincoln, NE, USA).

Statistical analysis. All experiments were repeated at least three times with different passages of the respective cell lines. The data were tested for significant differences by one-way analysis (Figs. 1-4, and 6) of variance or two-way analysis of variance (Fig. 5) followed by Tukey's multiple comparisons test for comparison of individual groups, after a Bartlett test had shown that variances were homogenous using GraphPad Prism 6.01 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Dose-response effects of 2DG treatment on cell viability. Treatment of HCC1806 (Fig. 1A) and MDA-MB-231 (Fig. 1B) human breast cancer cells with increasing concentrations of 2DG (0.1525, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 mM) for 96 h resulted in a significant dose-dependent reduction in viability.

A slight decrease in the number of living HCC1806 cells to 86.4±8.4 (SD)% of the control (C=100%) was observed at 0.1525 mM concentration of 2DG. At a 0.3125 mM concentration of 2DG the decrease in cell number became significant [82.8±8.1% of the control (C=100%; $P<0.05$)]. A concentration of 0.625 mM of 2DG resulted in a significant reduction in living HCC1806 cells to 72.4±8.4% of the control (C=100%; $P<0.001$). At 1.25, 2.5, 5 and 10 mM, as well as at 20 mM concentrations of 2DG a decrease in living HCC1806 cells to 64.5±12.7, 54.4±9.3, 42.8±9.7, 32.8±9.9 and 26.5±8.8% of the control (C=100%; $P<0.0001$ in all concentrations) was observed, respectively.

When the MDA-MB-231 cells were treated with 2DG for 96 h the number of living cells was dose-dependently reduced vs. the control (C=100%) as follows: 0.1525 mM, 81.6±7.9% ($P<0.01$); 0.3125 mM, 85.0±9.9% ($P<0.05$); 0.625 mM, 75.6±7.5% ($P<0.001$); 1.25 mM, 63.0±8.8% ($P<0.0001$); 2.5 mM, 49.4±5.9% ($P<0.0001$); 5 mM, 31.2±8.5% ($P<0.0001$); 10 mM, 20.3±6.1% ($P<0.0001$); 20 mM, 16.1±6.5% ($P<0.0001$).

Dose-response effects of 2DG treatment on mitochondrial membrane potential. Treatment of HCC1806 (Fig. 2A) and MDA-MB-231 (Fig. 2B) human breast cancer cells with 2.5, 5 and 10 mM of 2DG for 48 h resulted in a significant dose-dependent reduction in mitochondrial membrane potential ($\Delta\Psi m$).

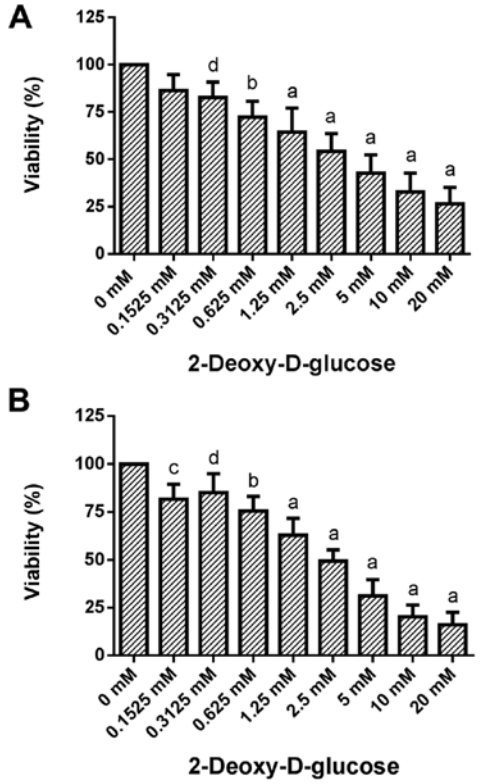

Figure 1. Dose-response experiments on the cell viability of (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells *in vitro*. Cells were incubated for 96 h without (control; 0 mM) or with increasing concentrations of 2DG. Cell number is expressed as a percentage of the control (100%). Columns represent means ± SD of data obtained from five independent experiments in five different passages of the respective cell line; [a]P<0.0001 vs. 0 mM 2DG; [b]P<0.001 vs. 0 mM 2DG; [c]P<0.01 vs. 0 mM 2DG; [d]P<0.05 vs. 0 mM 2DG.

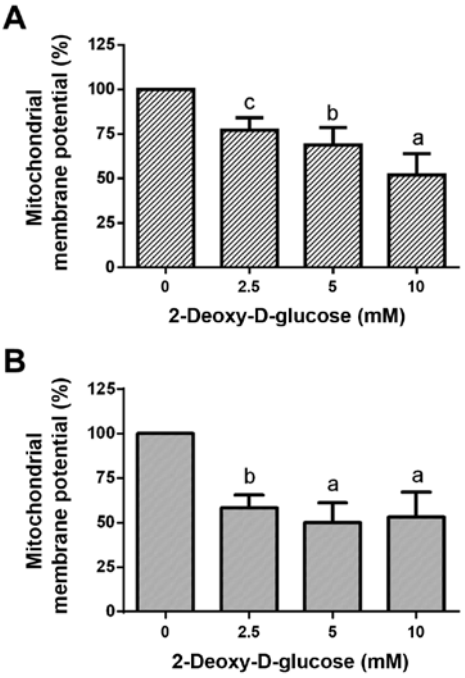

Figure 2. Dose-response effects of the treatment with 2DG on the decrease in mitochondrial membrane potential as a measure of apoptosis. Measurement of mitochondrial membrane potential (ΔΨ) after 48 h of treatment of (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells without (control; 0 mM) or with increasing concentrations of 2DG. Mitochondrial membrane potential is expressed as a percentage of the control (100%). Columns represent means ± SD of data obtained from four independent experiments in four different passages of the respective cell line; [a]P<0.0001 vs. 0 mM 2DG; [b]P<0.001 vs. 0 mM 2DG; [c]P<0.01 vs. 0 mM 2DG.

At a 2.5 mM concentration of 2DG, mitochondrial membrane potential in the HCC1806 cells was significantly decreased to 77.1±7.0% of the control (C=100%; P<0.01). A concentration of 5 mM of 2DG resulted in a significant reduction in mitochondrial membrane potential to 68.9±9.7% of the control (C=100%; P<0.001). At a 10 mM concentration of 2DG, a significant reduction in mitochondrial membrane potential to 51.9±12.0% of the control (C=100%; P<0.0001) was observed.

When MDA-MB-231 cells were treated with 2DG for 48 h, mitochondrial membrane potential was dose-dependently reduced vs. the control (C=100%) as follows: 2.5 mM, 58.3±7.2% (P<0.001); 5 mM, 50.1±11.0% (P<0.0001); 10 mM, 53.2±14.0% (P<0.0001).

Dose-response effects of 2DG treatment on PARP cleavage. Treatment of HCC1806 (Fig. 3A) and MDA-MB-231 (Fig. 3B) human breast cancer cells with 2.5, 5 and 10 mM of 2DG for 48 h resulted in a significant dose-dependent increase in cleaved PARP protein.

Following treatment with a 2.5 mM concentration of 2DG for 48 h, the level of cleaved PARP protein in the HCC1806 cells was increased to 177.3±76.6% of the control (C=100%). A concentration of 5 mM of 2DG resulted in a significant increase in PARP cleavage to 295.8±67.3% of the control (C=100%; P<0.01). At a 10 mM concentration of 2DG, a significant increase in cleaved PARP protein to 339.5±46.5% of the control (C=100%; P<0.001) was observed.

When MDA-MB-231 cells were treated with 2DG for 48 h, cleaved PARP protein was dose-dependently increased vs. the control (C=100%) as follows: 2.5 mM, 129.6±37.3%; 5 mM, 172.1±95.6%; 10 mM, 284.5±119.4% (P<0.05).

Dose-response effects of metformin treatment on cell viability. Treatment of HCC1806 (Fig. 4A) and MDA-MB-231 (Fig. 4B) human breast cancer cells with increasing concentrations of metformin (0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 μM) for 96 h resulted in a significant dose-dependent reduction in viability.

A very slight decrease in the number of living HCC1806 cells to 94.8±7.9% of the control (C=100%) was observed at 0.3125 μM concentration of metformin. A concentration of 0.625 μM of metformin resulted in a slight decrease in cell number to 89.4±6.0% of the control (C=100%). At a 1.25 μM concentration of metformin, the decrease in cell number became significant [84.2±5.9% of the control (C=100%; P<0.05)]. A concentration of 2.5 μM of metformin resulted in a significant reduction in living HCC1806 cells to 76.6±9.5% of the control (C=100%; P<0.001). At 5 and 10 μM, as well as at 20 μM concentrations of metformin a decrease in living HCC1806 cells to 64.3±7.7, 51.6±11.6 and 40.5±8.1% of the control (C=100%; P<0.0001 in all concentrations) was observed, respectively.

When MDA-MB-231 cells were treated with metformin for 96 h the number of living cells was dose-dependently reduced vs. the control (C=100%) as follows: 0.3125 μM, 98.0±4.5%; 0.625 μM, 92.5±9.0%; 1.25 μM, 82.9±9.9% (P<0.01); 2.5 μM,

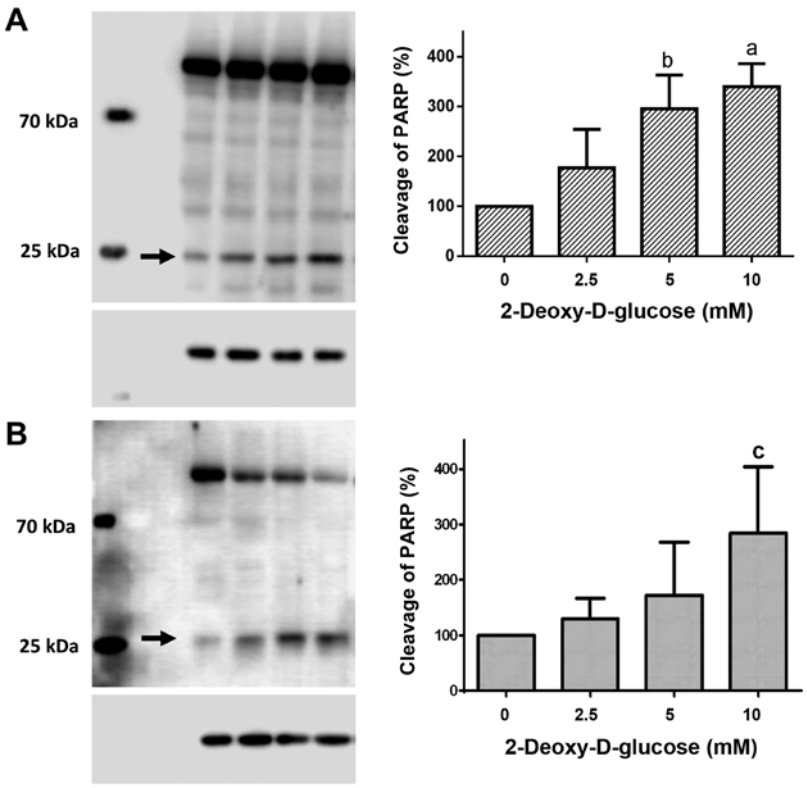

Figure 3. Dose-response effects of the treatment with 2DG on the increase in PARP cleavage as a measure of apoptosis. (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells were treated for 48 h without (control) or with increasing concentrations of 2DG before cleaved PARP protein was measured as described. These are representative data obtained from three independent experiments in three different passages of each cell line; [a]P<0.001 vs. 0 mM 2DG; [b]P<0.01 vs. 0 mM 2DG; [c]P<0.05 vs. 0 mM 2DG.

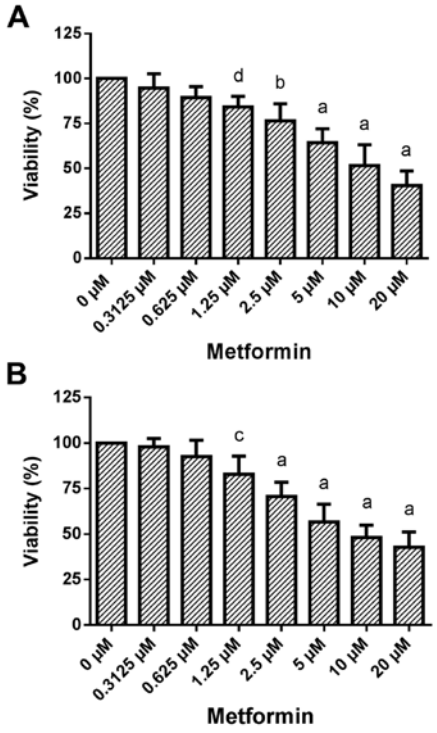

Figure 4. Dose-response experiments on the cell viability of (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells *in vitro*. Cells were incubated for 96 h without (control; 0 mM) or with increasing concentrations of metformin. Cell number is expressed as a percentage of the control (100%). Columns represent means ± SD of data obtained from five independent experiments in five different passages of the respective cell line; [a]P<0.0001 vs. 0 mM metformin; [b]P<0.001 vs. 0 mM metformin; [c]P<0.01 vs. 0 mM metformin; [d]P<0.05 vs. 0 mM metformin.

70.6±7.8% (P<0.0001); 5 μM, 56.7±9.7% (P<0.0001); 10 μM, 48.1±6.8% (P<0.0001); 20 μM, 42.8±8.3% (P<0.0001).

Cell viability after treatment with 2DG alone and in combination with metformin. During treatment of HCC1806 breast cancer cells with 2.5, 5 and 10 mM concentrations of 2DG in combination with 2.5 and 5 μM concentrations of metformin, a classic dose-response effect was not shown (data not shown), indicating that at these concentrations the maximal effects in this cell line were achieved. Therefore, experiments using lower concentrations of 2DG and metformin were performed (Fig. 5). Treatment of HCC1806 human breast cancer cells (Fig. 5A) without or with increasing concentrations (0.1525, 0.3125 and 0.625 mM) of 2DG and without or with increasing concentrations (1.25 and 2.5 μM) of metformin for 96 h resulted in a significant dose-dependent reduction in cell viability. Co-treatment with both agents resulted in a significantly dose-dependent higher reduction of viability than 2DG or metformin alone. At the highest concentrations of 2DG (0.625 mM) and metformin (2.5 μM), viability of the HCC1806 cells was reduced to 28.1±9.7% of the control (C=100%; P<0.0001). Treatment with 0.625 mM 2DG alone resulted in a decrease in viability to 59.5±6.3% of the control (C=100%; P<0.001). After treatment with metformin alone (2.5 μM), viability was reduced to 79.7±16.4% of the control (C=100%).

Treatment of MDA-MB-231 human breast cancer cells (Fig. 5B) without or with increasing concentrations (0.3125, 0.625 and 1.25 mM) of 2DG and without or with

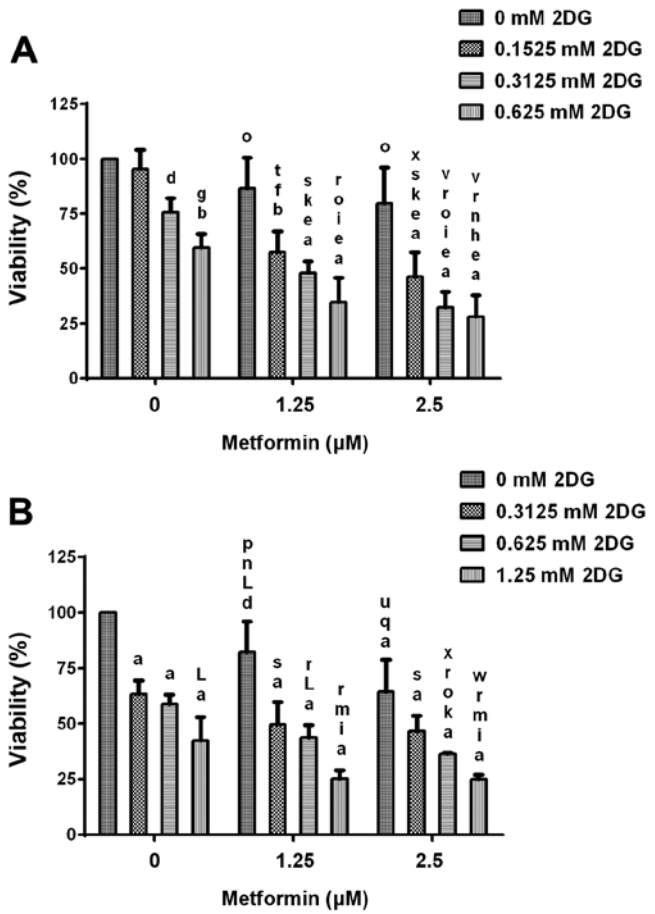

Figure 5. Dose-response experiments on the cell viability of (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells *in vitro*. Cells were incubated for 96 h without (control; 0 mM 2DG, 0 μM metformin) or with increasing concentrations of 2DG (HCC1806, 0.1525, 0.3125 and 0.625 mM; MDA-MB-231, 0.3125, 0.625 and 1.25 mM), metformin (1.25 and 2.5 μM) or both substances. Cell number is expressed as a percentage of the control (100%). Columns represent means ± SD of data obtained from five independent experiments in five different passages of the respective cell line; [a]P<0.0001 vs. 0 mM 2DG/0 μM metformin; [b]P<0.001 vs. 0 mM 2DG/0 μM metformin; [c]P<0.01 vs. 0 mM 2DG/0 μM metformin; [d]P<0.05 vs. 0 mM 2DG/0 μM metformin; [e]P<0.0001 vs. 0.1525 mM 2DG/0 μM metformin; [f]P<0.001 vs. 0.1525 mM 2DG/0 μM metformin; [g]P<0.01 vs. 0.1525 mM 2DG/0 μM metformin; [h]P<0.0001 vs. 0.3125 mM 2DG/0 μM metformin; [i]P<0.001 vs. 0.3125 mM 2DG/0 μM metformin; [k]P<0.01 vs. 0.3125 mM 2DG/0 μM metformin; [L]P<0.05 vs. 0.3125 mM 2DG/0 μM metformin; [m]P<0.001 vs. 0.625 mM 2DG/0 μM metformin; [n]P<0.01 vs. 0.625 mM 2DG/0 μM metformin; [o]P<0.05 vs. 0.625 mM 2DG/0 μM metformin; [p]P<0.0001 vs. 1.25 mM 2DG/0 μM metformin; [q]P<0.05 vs. 1.25 mM 2DG/0 μM metformin; [r]P<0.0001 vs. 0 mM 2DG/1.25 μM metformin; [s]P<0.001 vs. 0 mM 2DG/1.25 μM metformin; [t]P<0.01 vs. 0 mM 2DG/1.25 μM metformin; [u]P<0.05 vs. 0 mM 2DG/1.25 μM metformin; [v]P<0.0001 vs. 0 mM 2DG/2.5 μM metformin; [w]P<0.001 vs. 0 mM 2DG/2.5 μM metformin; [x]P<0.01 vs. 0 mM 2DG/1.25 μM metformin.

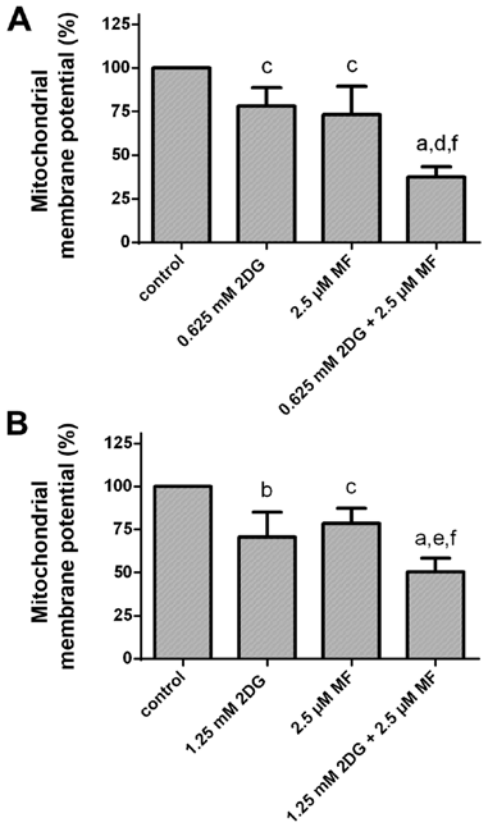

Figure 6. Effects of the treatment with 2DG, metformin or the combination of both substances on the decrease in mitochondrial membrane potential as a measure of apoptosis. Measurement of mitochondrial membrane potential ($\Delta\Psi$m) after 48 h of treatment of (A) HCC1806 and (B) MDA-MB-231 human breast cancer cells without (control; 0 mM 2DG; 0 μM metformin) or with 2DG (HCC1806, 0.625 mM; MDA-MB-231, 1.25 mM), metformin (2.5 μM) or both substances. Mitochondrial membrane potential is expressed as a percentage of the control (100%). Columns represent means ± SD of data obtained from four independent experiments in four different passages of the respective cell line; [a]P<0.0001 vs. control; [b]P<0.01 vs. control; [c]P<0.05 vs. control; [d]P<0.001 vs. 2DG; [e]P<0.05 vs. 2DG; [f]P<0.01 vs. metformin.

increasing concentrations (1.25 and 2.5 μM) of metformin for 96 h resulted in a significant dose-dependent reduction in cell number. Co-treatment with both agents resulted in a significantly higher dose-dependent reduction in cell number than 2DG or metformin alone. At the highest concentrations of 2DG (1.25 mM) and metformin (2.5 μM), the viability of the HCC1806 cells was reduced to 25.1±2.0% of the control (C=100%; P<0.0001). Treatment with 1.25 mM 2DG alone resulted in a decrease in viability to 42.5±10.5% of the control (C=100%; P<0.0001). After treatment with metformin alone (2.5 μM), viability was reduced to 64.5±14.4% of the control (C=100%; P<0.0001).

Effects of 2DG alone and in combination with metformin on mitochondrial membrane potential. Treatment of HCC1806 (Fig. 6A) and MDA-MB-231 (Fig. 6B) human breast cancer cells with 2DG (HCC1806, 0.625 mM; MDA-MB-231, 1.25 mM) or with metformin (2.5 μM) for 48 h resulted in a reduction in mitochondrial membrane potential ($\Delta\Psi$m). Co-treatment with both agents resulted in a significant higher reduction in mitochondrial membrane potential than with 2DG or metformin alone.

After treatment of HCC1806 cells with a 0.625 mM concentration of 2DG for 48 h, the mitochondrial membrane potential was significantly decreased to 78.2±10.6% of the control (C=100%; P<0.05). A concentration of 2.5 μM of metformin resulted in a significant reduction in mitochondrial membrane potential to 73.3±16.1% of the control (C=100%; P<0.05). At 0.625 mM concentration of 2DG in combination with 2.5 μM concentration of metformin, a significant reduction in mitochondrial membrane potential to 37.5±6.0% of

the control (C=100%; P<0.0001 vs. control; P<0.001 vs. 2DG alone; P<0.01 vs. metformin alone) was observed.

When MDA-MB-231 cells were treated with 2DG (1.25 mM) for 48 h, the mitochondrial membrane potential was significantly reduced to 70.6±14.4% of the control (C=100%; P<0.01). A concentration of 2.5 μM of metformin resulted in a significant decrease in mitochondrial membrane potential to 78.5±8.7% of the control (C=100%; P<0.05). At 1.25 mM concentration of 2DG in combination with 2.5 μM concentration of metformin a significant reduction in mitochondrial membrane potential to 50.3±8.0% of the control (C=100%; P<0.0001 vs. control; P<0.05 vs. 2DG alone; P<0.01 vs. metformin alone) was observed.

Discussion

In the present study, we analyzed the combination of 2DG and metformin, two drugs which target two key sources of cell energy and may represent a major advantage over classic chemotherapies alone or in combination with 2DG. Our results showed that inhibition of glycolysis using 2DG significantly reduced the viability of the human breast cancer cells in a dose-dependent manner. In addition, anti-diabetic drug metformin showed significant and dose-dependent antitumor activity in the human breast cancer cells. 2DG in combination with metformin led to a significant higher reduction in viability than 2DG or metformin alone. After co-treatment with 0.625 mM 2DG and 2.5 μM metformin, HCC1806 cell viability was reduced by at least 72%. At 0.625 mM concentration of 2DG and 2.5 μM concentration of metformin, cell viability of the MDA-MB-231 cells was reduced by at least 64%. Even lower concentrations of 2DG and metformin resulted in an impressive decrease in cell viability. These concentrations are below those achieved with doses normally administered in human treatment. In a dose-escalation trial with 2DG orally administered once daily, 63 mg/kg was found to be a clinically well-tolerable dose (23). The median maximum plasma concentration of 2DG at 63 mg/kg was 116 μg/ml (0.7 mM) (23). A typical treatment dose of metformin is 1,000-2,500 mg given twice daily, resulting in steady state plasma concentrations of ~1 μg/ml (7.7 μM) reached within 24-48 h (24,25). During controlled clinical trials, maximum metformin plasma levels did not exceed 5 μg/ml (38.7 μM), even at maximum doses (24). Higher concentrations of 2DG (>1.25 mM) in our co-treatment settings resulted in a further decrease in cell viability *in vitro*. However, these higher concentrations may not be well tolerated in patients.

Treatment of the breast cancer cells with 2DG resulted in a strong induction of apoptosis. Co-treatment with 2DG in combination with metformin induced a significantly higher decrease in viability and a significant higher increase in apoptosis than treatment with the respective single substances. Our observations regarding the antitumor effectiveness of both substances alone and in combination are in agreement with the results of other groups. Zhang and Aft demonstrated the antiproliferative effects of 2DG in cell lines of different tumor entities including breast cancer (26). In addition, our results confirm data shown by Cheong *et al*, indicating that co-treatment with 2DG and metformin is effective against breast cancer cell lines (27). However, Cheong *et al* used concentrations of 2DG (4 mM) and metformin (5 mM), which are higher than well-tolerated concentrations or plasma accessible concentrations in human beings (23-25). In another publication, a combination of 2DG (1 mM) and metformin (1 and 5 mM) was successfully tested in prostate cancer cells using metformin concentrations which are higher than those accessible in blood plasma of human beings (28).

Doses much greater than the pharmacologic concentration of metformin inhibit mitochondrial complex I and induce cell cycle arrest (29-33). Pharmacologic metformin concentrations activate AMP-activated protein kinase (AMPK) and inhibit expression of gluconeogenic genes. In addition, pharmacologic concentrations of metformin inhibit glycerol-3-phosphate dehydrogenase (GPDH) in mitochondria resulting in a decrease in gluconeogenesis (34). The glucose analog 2DG inhibits glycolysis and induces autophagy (35-37). Co-treatment with 2DG and metformin resulted in significant cell death, which is associated with a marked decrease in intracellular ATP concentration, prolonged AMPK activation, and sustained autophagy (27). However, the marked antitumor effects of the combination of 2DG and metformin have the clinical advantage of inducing no negative side-effects. Only lactic acidosis was reported to be a very rare side-effect in metformin-treated patients (38).

2DG as a glycolysis inhibitor represents an attractive option for therapy including combination therapies. Since increased glycolysis occurs in malignant cells, 2DG acts specifically in tumor tissue. Therapy utilizing both specific inhibition of glycolysis using 2DG, and the antitumor activity of metformin appears to be valuable as an effective strategy for the treatment of all inoperable, chemotherapy-resistant or recurring breast cancers and should be further evaluated.

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