



Targeting Cancer Cell Metabolism with Metformin, Dichloroacetate and Memantine in Glioblastoma (GBM)

Gulsah ALBAYRAK¹, Ece KONAC¹, Umit Akin DERE², Hakan EMMEZ²

¹Gazi University, Faculty of Medicine, Department of Medical Biology and Genetics, Ankara, Turkey

²Gazi University, Faculty of Medicine, Department of Neurosurgery, Ankara, Turkey

Corresponding author: Ece KONAC ✉ ecemercanoglu@yahoo.com

ABSTRACT

AIM: To investigate the effects of metformin, dichloroacetate (DCA), and memantine on T98G and U87-MG human glioblastoma (GBM) cells to target tumor cell metabolism in a multi-directional manner.

MATERIAL and METHODS: IC50 levels for metformin, DCA, metformin+DCA and memantine were determined by MTT assay in T98G and U87-MG cells in vitro. Casp3, Bcl-2, Bax, c-Myc and GSK-3B protein expressions were investigated post treatments. Fifteen GBM+ tumor tissues were assessed for Casp-3, Bcl-2, Bad, Bax for apoptotic protein expression patterns.

RESULTS: Cancer cell metabolism targeting drugs metformin, DCA, metformin+DCA and memantine induced cytotoxicity in a dose-dependent manner in T98G and U87-MG cells. IC50 for memantine is found as 0.5 mM ($p < 0.01$) which is nearly 10 times lower concentration than that of metformin. Fifteen GBM+ tumor tissues had differential apoptotic protein expressions.

CONCLUSION: Memantine exerted anti-cancer mechanism of action in T98G and U87-MG cells, however, such a mechanism requires deeper investigation for GBM treatment.

KEYWORDS: Glioblastoma, Cancer cell, Metabolism, Metformin, Dichloroacetate, Memantine

INTRODUCTION

Glioblastoma (GBM) is one of the most aggressive tumors of the central nervous system, and represents approximately 50% of all glial tumor types (12,27). The median survival rate for GBM patients does not change significantly using the current standard of care treatments, which involve tumor resection followed by radiotherapy and Temozolomide treatment. The median survival is approximately 12-14 months despite the combined use of surgery, radiotherapy and chemotherapy (7,23).

GBM tumors have a wide range of genetic variations leading to different therapeutic responses (5,9,21). Intratumor heterogeneity might be the key to identify the cause of treatment failure (22). Chemotherapy drugs such as Temozolamide increases the mutational load within the cancer genome when compared to the untreated GBM cells (17). Alternative therapy approaches, therefore, are urgently needed in GBM treatment.

Metformin is a common anti-diabetic drug used in Type 2 diabetes treatment (18). Metformin treatment is found to be associated with a lower risk of several cancers, however, its effects on GBM has not been well characterized (4,24). Metformin treatment decreases Temozolomide resistance in GBM cells (26). Metformin, however, has safety concerns in clinical setting as most of the preclinical works used supra-physiological doses of metformin (25). In this study we aimed to overcome this challenge by investigating the effect of metformin by combining it with dichloroacetate (DCA) that targets cancer cell metabolism via pyruvate dehydrogenase kinase inhibition (11). Targeting cancer cell metabolism might have implications for the treatment of aggressive GBM. Furthermore, we aimed to investigate the apoptotic protein expression profiles to better understand GBM at a molecular level. We also aimed to interfere cancer cell metabolism using metformin, DCA and memantine in T98G and U87-MG GBM cell lines.

■ MATERIAL and METHODS

Cell Culture and Chemicals

T98G and U87-MG human glioblastoma cell lines were provided from ATCC. T98G and U87-MG cells were grown in DMEM/F12 medium supplemented with 10% Fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA). Cells were grown in a 5% CO₂ incubator at 37°C. Metformin, dichloroacetate and memantine were provided from Sigma-Aldrich, St Louis, MO, USA. Cells were treated with these agents for 48h. to determine IC50 levels. All agents were dissolved in sterile distilled water.

Cell Cytotoxicity Assay

3X10³ T98G and U87-MG cells were seeded into a 96-well plate and cultured overnight. Different concentrations of metformin, DCA and memantine were used in MTT assay. MTT solution (5 mg/ml in PBS) was added and cells were further incubated for 4 hours at 37°C. DMSO was added to solubilize crystals. Absorbance ratio was measured using SpectraMax M3 (Molecular Devices, USA)

GBM Patient Samples and Protein Isolation From Tumor Tissues

A total of 17 GBM tumor tissue samples were collected from Gazi University, Department of Neurosurgery, over the period April 2015–April 2016 with the approval of the Gazi University Clinical Research Ethics Committee. Tumor samples were fresh-frozen at -80°C. Two of the tumor tissues were excluded from the study as their pathology reports were found as GBM (-). 0.1 gram of tumor tissue sample was homogenized in RIPA lysis buffer. The lysate was centrifuged for 15 minutes at 14,000 rpm and supernatant was collected to perform BCA protein quantification assay. Samples were stored at -80 °C for further processing.

Western Blotting

T98G and U87-MG cells were washed with PBS and scraped into RIPA lysis buffer containing 1mM PMSF followed by sonication. Samples were centrifuged for 15 minutes at 13500 rpm at 4°C and supernatant was collected. Proteins were quantified by using BCA Assay Kit (Thermo Pierce, Rockford, IL, USA). Protein lysates (20 µg) were heated for 5 minutes at 95°C in LDS non-reducing sample buffer (Pierce, Rockford, IL, USA) then loaded to 10% Tris-glycine gels, transferred to PVDF membrane (Pierce, Rockford, IL, USA). Membranes were blocked for 1 hour at room temperature and incubated overnight at 4°C with the primary antibodies for Casp3, Bcl-2, Bad, Bax, GSK-3B, C-Myc and β-actin (Thermo Pierce, Rockford, IL, USA). Blots were visualized by Luminata Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany). Chemiluminescent signals of immunoblots were documented using Gel Logic 2200 Pro (Carestream Health; Rochester, NY, USA).

Statistical Analysis

Experiments were performed in triplicates. Metformin, dichloroacetate and memantine IC50 levels for T98G and U87-MG cell lines were calculated by using the Graph-Pad

Prism7. The results were expressed as the mean ± standard deviation. p<0.05 was considered as statistically significant.

■ RESULTS

Effects of Metformin, DCA, Metformin+DCA and Memantine on Cytotoxicity of T98G and U87-MG GBM Cells

Metformin decreased the cellular viability of T98G and U87-MG human GBM cancer cells in a dose-dependent manner as shown in Figure 1A, treatment with 45mM and 25mM metformin reduced cell viability for 48 hours (p<0.01). We investigated the effect of DCA on T98G and U87-MG cells for 48 hours, 10-60 mM concentration range of DCA was tested over cells. DCA reduced cell viability at 45mM concentration as shown in Figure 1B (p<0.01). Different concentrations of metformin+DCA were also tested over GBM cells in order to decrease the IC50 doses. Metformin+DCA combination decreased cell viability to lower doses, IC50 dose for combination treatment was found as 20mM DCA and 20mM metformin for T98G and U87MG cells for 48 hours as shown in Figure 1C (p<0.01). In addition, memantine affected cellular toxicity in a dose-dependent manner as shown in Figure 1D. 0.125–5 mM memantine concentration was tested over T98G and U87MG cells for 48 hours, and, IC50 value was determined as 0.5 mM for T98G and for U87MG cells (p<0.01).

Effects of Metformin, DCA, Metformin+DCA and Memantine on Casp3, Bcl2, Bax, c-Myc, GSK3B Protein Expression Levels

T98G cells were treated with 45mM metformin, 45mM DCA, 20mM metformin in combination with 20mM of DCA and 0.5mM memantine for 48 hours. U87-MG cells were treated with 25mM metformin, 45mM DCA, 20mM of metformin in combination with 20mM of DCA and 0.5mM memantine for 48 hours. The expressions of apoptosis and cell-cycle progression related proteins i.e. Casp3, Bcl2, Bax, c-Myc and GSK3B, were evaluated by western blot. Metformin and metformin-DCA combination decreased Casp-3 protein expression in T98G cells (Figure 2), whereas Casp-3 protein expression was increased in all treatments (metformin, DCA, metformin+DCA, memantine) in U87-MG cells (Figure 3). Bcl-2 protein expression was found to increase with DCA, metformin+DCA, and memantine treatment; whereas Bax protein expression was decreased in all treatments for T98G cells. c-Myc protein expression was decreased by the aforementioned treatments (metformin, DCA, metformin+DCA, memantine) in T98G cells. Memantine treatment inhibited GSK3B protein expression in T98G cells (Figure 2).

Differential Protein Expression Profiles of GBM Positive Tumor Tissue

GBM positive tumor tissue samples were characterized in terms of apoptosis as this might be an indicator of the disease progression. Fifteen GBM+ tumor tissue samples were investigated for the Casp-3, Bcl-2, Bad, Bax protein expression profiles. Investigated apoptotic proteins were found to be differentially expressed at each patient (Figure 4).

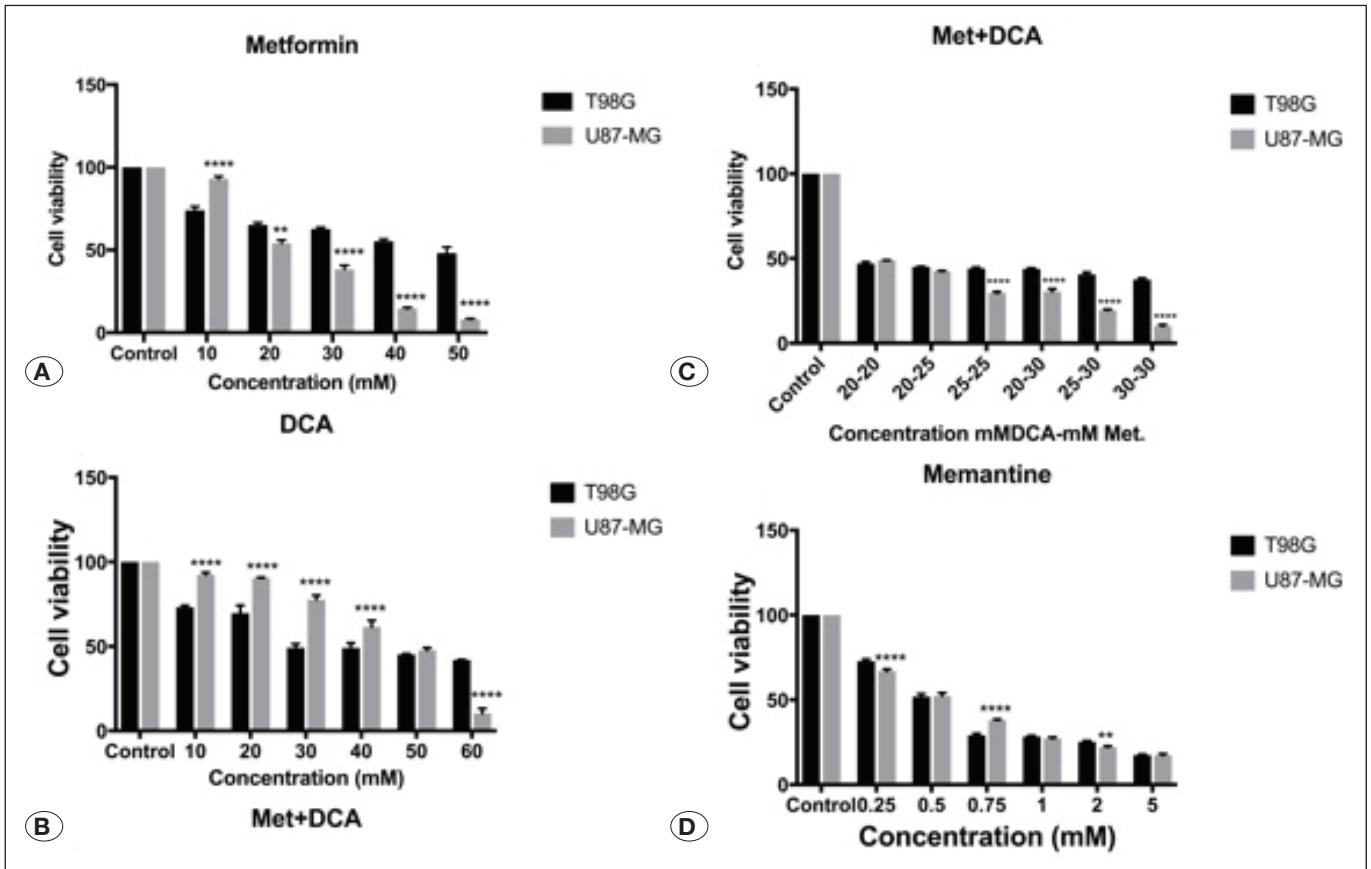


Figure 1: **A)** Metformin was tested over the range of 10-50 mM on T98G and U87-MG cells for 48 hours (h). IC50 value was determined as 45 mM for T98G cells and 25 mM for U87-MG cells ($p < 0.01$). **B)** Dichloroacetate (DCA) was tested over the range of 10-60 mM on T98G and U87-MG cells for 48 h. IC50 value was determined as 45 mM for T98G and U87-MG cells ($p < 0.01$). **C)** Metformin and DCA combination were tested over different ranges on T98G and U87-MG cells for 48 h. IC50 value was determined as 20mM DCA and 20mM Metformin for T98G and U87-MG cells ($p < 0.01$). **D)** Memantine was tested over the range of 0.125–5 mM T98G and U87-MG cells for 48 h. IC50 value was determined as 0.5 mM for T98G and U87-MG cells ($p < 0.01$).

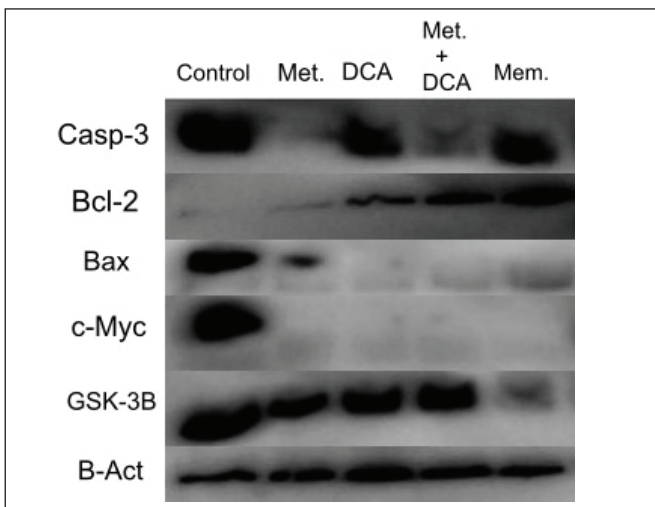


Figure 2: Effect of 48h treatment of T98G cell line with Metformin (45mM), DCA (45mM), Metformin+DCA combination (20mM DCA+20mM metformin) and Memantine (0.5 mM) on the expression levels of: Casp3, Bcl-2, Bax, c-Myc and GSK-3B. B-Act protein expression was used as loading control.

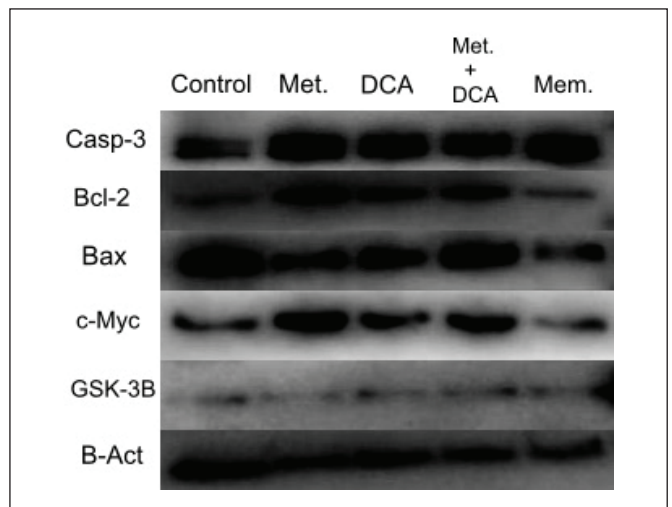


Figure 3: Effect of 48 hours treatment of U87-MG cell line with Metformin (25mM), DCA (45mM), Metformin+DCA combination (20mM DCA+20mM metformin) and Memantine (0.5 mM) on the expression levels of: Casp3, Bcl-2, Bax, c-Myc and GSK-3B. B-Act protein expression was used as loading control.

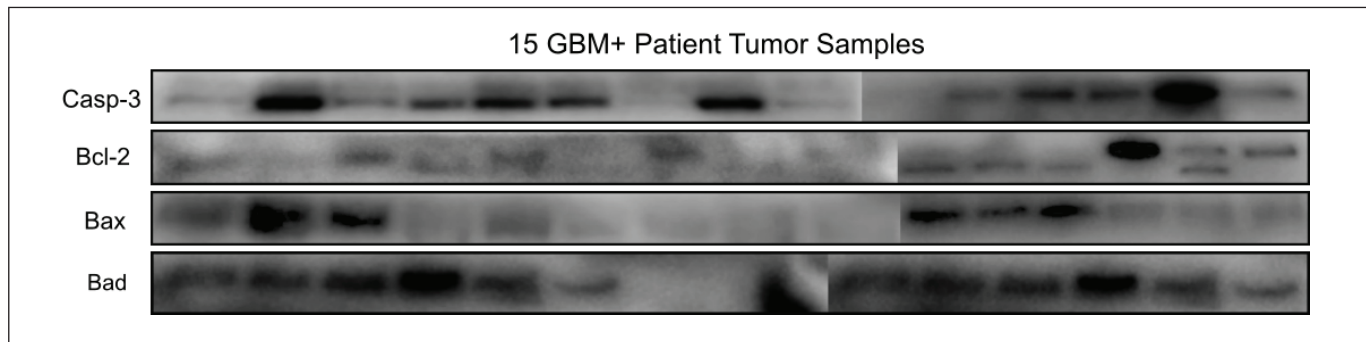


Figure 4: Differential protein expression profiles of 15 GBM+ tumor tissue samples for apoptosis genes Casp-3, Bcl-2, Bad, Bax.

DISCUSSION

Anti-angiogenic drug bevacizumab approval in GBM showed improvement in the overall survival rate among patients; however, this finding remains controversial (6). There is a need for alternative treatment regimens for such an aggressive tumor GBM in this personalized medicine era.

Glucose and glutamine addiction of cancer cells is known as the hallmark of cancers (16). Aberrant glucose metabolism is defined as the drivers of GBM aggression and chemoresistance. Targeting tumor metabolism in cancer treatment has now widened interest beyond glucose metabolism to other molecules such as glutamine. It is, therefore, crucial to gain more insights into glutamine metabolism in cancer cells (2). It is thus the aim of the current study to target glucose and glutamine metabolism using metformin, DCA and memantine.

Diabetes drug metformin's mechanism of action over cancer cells is known as its selectivity in targeting cancer stem cells. This mechanism might be dependent on glutamine metabolism (8). DCA is a pyruvate dehydrogenase kinase inhibitor that reverses the Warburg effect (20). Sensitizing tumor cells to glycolysis inhibition might be achieved by dual targeting via metformin and DCA. In addition, Alzheimer's drug memantine has a much lower IC50 doses when compared to that of metformin. The relatively lower IC50 dose for memantine (0.5mM) might thus overcome the supra-physiological dose related concerns of metformin in clinical setting. Newly synthesized memantine derivatives were also found to have anti-tumor activities in U87-MG GBM cell line (3). Targeting NMDA receptor associated signalling pathways using memantine might enhance the treatment of glioma and GBM (1,14). Enlightening memantine's anti-cancer mechanism over GBM cells, therefore, holds promise as a new repurposed drug.

Differential protein expression profiles of 15 GBM+ tumor tissues for apoptotic genes Casp-3, Bcl-2, Bad, Bax also confirms the heterogenic nature of the disease. Positive expression of Bax was associated significantly with the better survival rate in a large series of tumors (19). Bax protein expression profile, therefore, might be prognostic in GBM treatment response. Caspase-3 activation is found to be a predictor of progression-free survival in GBM patients (13).

Molecular stratification of patients is indispensable to target patient groups that are most likely to get benefit from the selected treatment protocol.

In a recent study that transition into Phase II trial, researchers investigated the maximum tolerated doses of memantine, mefloquine, and metformin in combination with Temozolomide. Maraka et al. concluded that those drugs could be combined safely with Temozolomide in newly diagnosed GBM (10). Some side effects, however, were reported on the combined therapy regimens and the approach was scrutinized, raising the confusions on whether memantine or mefloquine caused the observed side effects (15). It remains crucial to understand the drug-to-drug and/or pharmacokinetic interactions when using drug combinations.

CONCLUSION

Our in vitro findings provide proof of concept that Alzheimer's drug memantine might be used in GBM treatment at lower doses compared to metformin. Brain blood barrier penetrating properties of memantine is also promising in GBM treatment. It is of further interest that memantine could provide an alternative neoadjuvant therapy especially for elderly cancer patients. This claim requires further investigation using randomized controlled trials.

ACKNOWLEDGEMENTS

This study has been supported by a scientific grant from the Scientific and Technological Research Council of Turkey, TUBITAK (Project No: SBAG- 214S578).

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