

## EXPERIMENTAL STUDY

# Alzheimer's drug Memantine inhibits metastasis and p-Erk protein expression on 4T1 breast cancer cells

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**ABSTRACT**

**OBJECTIVES:** Drug repurposing studies enable shorter routes to the clinic by skipping the steps like *in vitro* *in vivo* screening, chemical optimization and toxicological studies. In our study, we investigated the potent anti-cancer effect of Alzheimer's drug Memantine on 4T1 breast cancer cells.

**METHODS:** Memantine's effect on proliferation of 4T1 cells was evaluated by using the MTT assay. Memantine inhibited 4T1 cell proliferation in a concentration-dependent manner at 24 and 48 hours. We investigated the drug's effect on the protein expressions of Bax, Bcl-2, Casp-3, Casp-9, E-Cad, Vimentin, B-Cat, GSK3B, p-ERK, ERK, p-GS, GS that are involved in apoptosis, metastasis and cell survival.

**RESULTS:** Memantine altered the Bcl-2, Bax, Casp3, Casp-9 apoptotic protein expression levels. We found that memantine inhibited p-Erk expression and that result suggested a plausible mechanism of action for memantine's antineoplastic effect. Memantine also inhibited wound closure at 24 h, significantly ( $p = 0.0055$ ).

**CONCLUSIONS:** Memantine inhibited 4T1 breast cancer cell proliferation at significantly lower doses than mostly studied re-purposed drug Metformin. Therefore, we believe that memantine might hold a great promise as a new repositioned drug in cancer treatment and it is our further interest to investigate its effects *in vivo* (Fig. 3, Ref. 22). Text in PDF [www.elis.sk](http://www.elis.sk)

**KEY WORDS:** Memantine, anti-cancer, drug re-purposing.

**Introduction**

Breast cancer is the most common cancer among women. Beyond that, high number of new cases are estimated in the upcoming years (Siegel et al, 2015). Breast cancer is a heterogenous disease that exhibits different histological sub-types. The most challenging sub-type in treatment is triple-negative breast cancer (TNBC), where tumours lack three commonly found receptors (Collignan et al, 2016). One of the hallmarks of cancer cell is to reprogram the energetic needs by an increased uptake and the utilization of glucose. This is a well-known phenomenon as the Warburg effect (Libertia et al, 2016). Metabolic differences between normal and cancer cells need to be well understood, so that these differences might contribute to the development of better treatment options.

Drug re-positioning is the process of finding new indications for the already approved drugs (Wurth et al, 2016). Repurposing studies, which enable shorter paths to the clinic, are becoming more popular especially in the oncology field. The driving force for the development of cancer therapeutics from non-cancer drugs stems from the fact that different diseases share common mo-

lecular pathways, targets and ontologies in key cellular processes (Gupta et al, 2013).

Glucose has been in the centre of attention in the study of cancer metabolism. There are several ongoing clinical trials for using metformin as glucose metabolism interfering agent in the treatment of different types of cancers (Chae et al, 2016). Although, Metformin has taken the lead as a potential repositioned drug in cancer treatment, there have been compelling ideas and confusions about metformin's clinically achievable concentration and efficacy (Kourelis et al, 2012). Cancer metabolism research recently widened interest beyond the glucose and now, glutamine metabolism in cancer cells is under investigation (Altman et al, 2016). Glutamine is the most abundant amino acid that is found in blood and it has a critical role in the cell growth and proliferation related biological activities such as: energy production, macromolecular synthesis, mTOR activation and ROS homeostasis (Chen et al, 2015). Alzheimer's drug memantine is used for suppressing the excitotoxic effect of glutamate in brain as N-methyl-D-aspartate receptor blocker. Memantine is also known to increase complex I and decrease complex IV mitochondrial activity (Mitrakas et al, 2014). Glutamine serves as a nitrogen source required for the biosynthesis of purine and pyrimidine nucleotides that is especially hypercritical for rapid cell divisions as in cancer cells (Pavlova et al, 2016). Therefore, the use of memantine as an excess glutamate blocker in cancer treatment might hold benefit in preventing cancer cells from using this source.

There is a recent experimental evidence that memantine has anti-neoplastic activity over different cancer cells. In addition to

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that, memantine has been found to exert the same activity at nearly 10 times lower dose than metformin *in vitro* (Albayrak et al, 2017, Seifabadi et al, 2017, Yoon et al, 2017). Therefore, we aimed to investigate the memantine's potential anti-cancer effect on 4T1 metastatic breast cancer cells. We evaluated this drug's effect on cell proliferation, wound healing and protein expression levels of Bax, Bcl-2, Casp-3, Casp-9, E-Cad, Vimentin, B-Cat, GSK3B, p-erk, Erk, p-GS, GS and Gapdh that play important roles in several cellular processes.

## Materials and methods

### Cell culture and chemicals

4T1 breast cancer cell line was kindly provided by Prof Dr Gunes Esendagli. 4T1 cells were grown in RPMI medium supplemented with 10 % Fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA). Cells were grown in an incubator in 5 % CO<sub>2</sub> at 37 °C. Memantine was supplied from Sigma-Aldrich, St Louis, MO, USA. Cells were treated with memantine for 6, 24 and 48 hours. Memantine was dissolved in sterile, non-pyrogenic distilled water.

### Cell cytotoxicity assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxic effect of memantine. Cells were seeded into the 96 well plate and cultured overnight. 4T1 cells were treated with memantine for 24 and 48 hours. MTT solution (5mg/ml in PBS) was added to each well at the end of the treatment and the plate was incubated for 3 h at 37 °C. DMSO was added to each well in order to solubilize the formazan crystals, followed by incubation at 37 °C for 30 min. The absorbance ratio was measured using the SpectraMax M3 (Molecular Devices, USA) microplate reader at 570 nm.

### Western blotting

After 6 h, 24 h and 48 h treatment of 4T1 cells with memantine, cells were washed with PBS and scraped into the RIPA lysis buffer containing 1mM PMSF followed by sonication for 15 seconds. Samples were centrifuged for 15 minutes at 14000 rpm at 4 °C and the supernatant was collected. Proteins were quantified by using the 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) and then loaded into the 10 % Tris-glycin gels. The gels were transferred to the PVDF membrane (Merck Millipore, Darmstadt, Germany) at 250 mAmp for 90 minutes. Membranes were blocked with 5 % non-fat milk powder in TBS-T for 1 hour at room temperature and incubated overnight at 4 °C with the primary antibodies for Bax, Bcl-2, Casp-3, Casp-9, E-Cad, Vimentin, B-Cat, GSK3B, p-ERK, ERK, p-GS, GS and Gapdh at 1:1000 dilution (Thermo Pierce, Rockford, IL, USA). Blots were washed with TBS-T subsequently. Protein bands were detected by using the secondary antibody (Thermo Pierce, Rockford, IL, USA) and the blots were visualized by BioVision ECL Western Blotting Substrate Kit (Bio-vision, California, USA). Chemiluminescent signals of immunob-

lots were detected by using the Gel Logic 2200 Pro (Carestream Health, Rochester, NY, USA).

### Wound healing

4T1 cells were seeded into a 6 well plate at  $5 \times 10^5$  density per well for an overnight incubation. Then the cells were scratched by using a pipette tip (P200). The wells were washed with PBS to remove the excess cell debris and then the plate was incubated in complete media containing 125 and 250 µM concentrations of memantine. The wounded area was photographed by using Olympus IX71 microscopy (USA) at 0 and 24 hours. The migration area was calculated by using the Image J software (NIH, Bethesda, MD, USA).

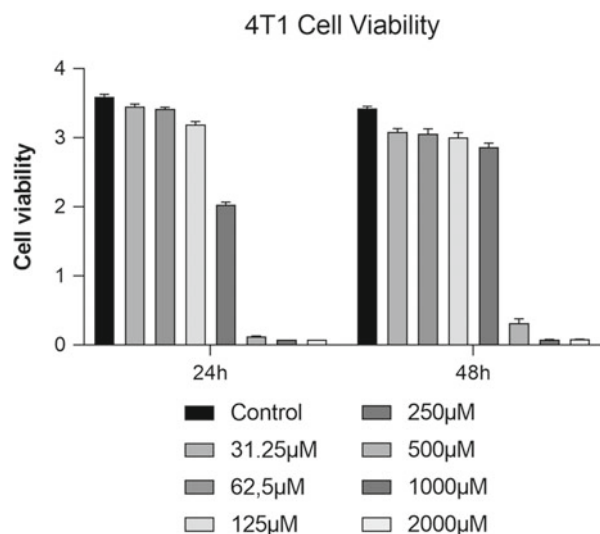
### Statistical analysis

Experiments were done in triplicates. Statistical analysis was performed by using GraphPad Prism software. Differences between the control and treatment groups were analysed by using the t-test. The results were expressed as the mean ± standard error of the mean.

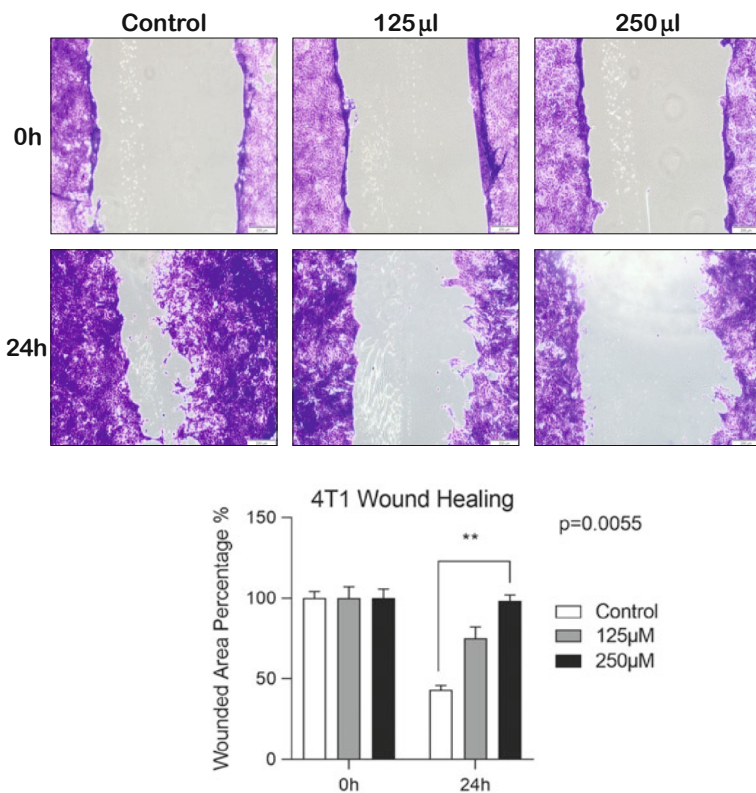
## Results

### Effect of Memantine on cellular toxicity of 4T1 breast cancer cells

In order to determine the cytotoxic effect of Memantine, 4T1 cells were treated with varying concentrations (31,25–2000 µM) for 24 and 48 hours. As shown in Figure 1, Memantine inhibited 4T1 cell proliferation in a concentration-dependent manner at both 24 and 48 hours. All tested concentrations of Memantine significantly decreased cell viability at both treatment times in 4T1 cells (Fig. 2). A significant decrease in cell viability was observed in 250 µM concentration of Memantine at 48 h.



**Fig. 1. Memantine's effect on 4T1 cell proliferation at 24 and 48 hours with different memantine concentrations (31,25–2000 µM). All concentrations of memantine affected cell viability statistically significantly ( $p < 0.05$ ) both at 24 and 48 hours.**



**Fig. 2.** The effect of Memantine on wound healing at 24 hours (\* $p < 0.05$ ), when compared to 0 h and 24 h, paired sample t test was used ( $n = 6$ ), when compared to different time courses.

#### Memantine inhibits wound closure at 24 hours

The wound healing assay is an *in vitro* technique for probing collective cell migration in two dimensions (Jonkman et al, 2014). The effect of Memantine over metastasis was assessed by using the wound healing assay. 125 and 250  $\mu\text{M}$  concentration of Memantine was used in order to visualize its effect on cellular migration of 4T1 cells. Both 125  $\mu\text{M}$  and 250  $\mu\text{M}$  concentrations of Memantine were found to have negative effect over wound closure in a dose-dependent manner as statistically significant.

#### The effects of Memantine on apoptosis and metastasis related protein expression levels

Apoptosis is highly desired for cancer cells in cancer treatment. We observed that memantine increased apoptotic protein Bax expression levels. Total Caspase-3 protein expression level was decreased at 48 hours, whereas Caspase-9 protein expression level increased at 48 hours. In order to assess the correlation between the wound healing and protein expression levels, we investigated the epithelial-mesenchymal transition related protein expressions like E-Cadherin and Vimentin. Consistently with the wound healing data, we found that memantine increased E-Cadherin protein expression level. We evaluated memantine's effect on B-Cat and GSK3 and we observed that memantine treatment increased B-catenin expression level at 48h (Fig. 3).

#### Memantine inhibits p-ERK protein expression level

Extracellular-signal-regulated-kinase (ERK) signalling pathway is involved in several fundamental cellular processes like cell proliferation, differentiation and survival (Munshi and Ramesh, 2013). We investigated the effect of memantine on cell survival pathway related p-ERK and total ERK protein expressions. We have found that memantine inhibits p-ERK protein expression levels at 48 h. Therefore, our results indicated that memantine inhibited ERK phosphorylation. Glycogen synthase is an important enzyme in glycogen synthesis. We also observed that memantine increased the glycogen synthase protein expression levels (Fig. 3).

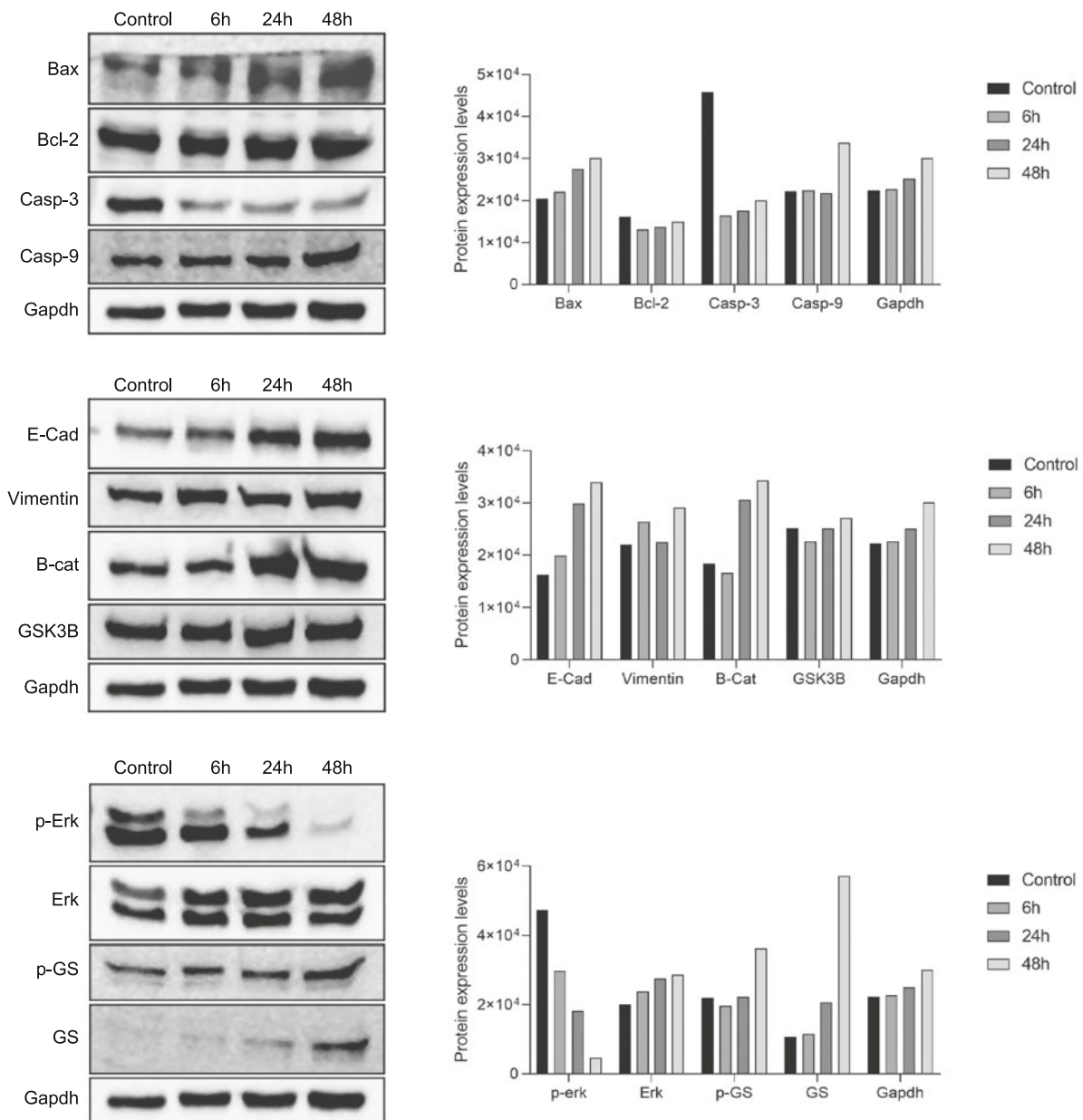
#### Discussion

Repurposing non-cancer drugs as potential treatment options for cancer patients is highly favourable due to the increasing unmet medical needs (Verbaander et al, 2017). There is an increasing number of researches that investigates the mechanism of action for tumour cell metabolism targeting agents like Metformin. Metformin exerts its anticancer effect through the regulation of glucose metabolism (Salani et al, 2014).

Epidemiological and experimental studies underline the association between metformin usage and reduced cancer incidence and mortality rates (Heckman-Stoddard, 2017). However, Metformin usage in cancer treatment remains controversial due to the inability of having clinically achievable concentrations. This study offers a potential re-purposed use of Alzheimer's drug memantine as having 10 times lower IC50 level. Ongoing early phase clinical trial indicated that repurposed agents like metformin and memantine could be used safely as an adjuvant therapy in combination with temozolamide in GBM treatment (Maraka et al, 2018). However, it could also be used in other type of cancers, in this study we investigated memantine's potential effect over 4T1 breast cancer cells. We identified that Alzheimer's drug Memantine inhibits cellular cytotoxicity of 4T1 breast cancer cell line at concentrations as low as 250  $\mu\text{M}$  at 24 h and 48 h. Memantine significantly inhibited the viability of the cells. At 48 h treatment of cells with memantine, it was even harder to harvest cells for further investigation as that concentration was too toxic for the cells.

Epithelial-mesenchymal transition plays a critical role in cancer metastasis. Therefore, the inhibition of metastasis remains critical during tumorigenesis. We found that the treatment of 4T1 cells with different concentrations of drug (125 and 250  $\mu\text{M}$ ) inhibited wound healing significantly even after 24 hours. This data suggests that memantine might be used in the inhibition of metastasis.

Memantine also increased the apoptotic proteins like Bax and Caspase 9 at protein expression levels. We found that meman-



**Fig. 3.** Western blotting for memantine’s effect on 4T1 metastatic breast cancer cell line on 6 h, 24 h and 48 h. for Bax, Bcl-2, Casp-3, Casp-9, E-Cad, Vimentin, B-Cat, GSK3B, p-erk, Erk, p-GS, GS and Gapdh protein expression levels. Gapdh was used as a loading control.

tine triggered Bax-dependent pathway of apoptosis in 4T1 breast cancer cells. ERK molecular signalling pathway is important in cellular survival of the cancer cells and interrogating with this pathway remains crucial in cancer treatment. Memantine inhibited p-ERK protein expression in a time dependent manner. Our results highlight a potential anti-neoplastic mechanism of action for this drug. However, these findings need further investigation *in vivo*. This study is limited as investigating the effects *in vitro* only. It is our further interest to investigate memantine’s anticancer effect *in vivo*.

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