

The cytotoxic effect of memantine and its effect on cytoskeletal proteins expression in metastatic breast cancer cell line

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ABSTRACT

Objective(s): Breast cancer is an important leading cause of death from cancer. Stathmin and tau proteins are regulators of cell motility, and their overexpression is associated with the progression and bad prognosis of breast cancer. Memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is the potential inhibitor of tau protein in neurons. This study determines the effect of memantine on breast cancer cell migration and proliferation, tau and stathmin gene expression in cancer cells and its synergistic effect with paclitaxel.

Materials and Methods: The cell proliferation was evaluated by MTT assay and for this purpose, MCF-7 breast cancer cells were treated with various concentration of memantine (2, 20 and 100 µg/ml). Tau and stathmin mRNA expression was evaluated through quantitative real time RT-PCR method. The migration of cancer cells treated with memantine for 24 hr was compared to non-treated cells using an *in vitro* transmembrane migration assay.

Results: Incubation of breast cancer cells with memantine resulted in a dose dependent reduction in cell survival ($P=0.0001$). Paclitaxel (100 nM) showed synergistic effect with memantine ($P=0.0001$). Memantine significantly decreased tau and stathmin mRNA expression (by RT-PCR), so that 100 µmol/l of memantine decreased tau and stathmin expression by 46% ($P=0.0341$) and 33% ($P=0.043$), respectively. Migration of cells was also decreased by memantine ($P=0.0001$).

Conclusion: The presented data shows that memantine reduced mRNA levels of tau and stathmin proteins and also reduced cellular migration.

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Introduction

Breast cancer is known as the most common cancer and the second leading cause of death from cancer in women around the world (1). According to the latest data from the National Cancer Institute, 61% of breast cancers are diagnosed before metastasis, 31% of them after spreading to the lymph nodes nearby or outside the chest, and nearly 6% of them are diagnosed after distant metastasis (2). Existence of cancer stem cells has stated new theories about metastasis of cancer. High mobility in these cells leads to metastasis (3). Cytoskeletal changes in cancer stem cells are the main key to metastasis. Microtubule proteins play an important role in cell movement and may also compete with taxanes (4). Microtubules are components of the cytoskeleton, responsible for various actions such as intracellular transport, cell signaling, preserving the cell shape and mitosis (5). Therefore, the drugs with anti-microtubule activity prevent the proliferation of cancer cells (6). Microtubules are polymers made of

heterodimers of alpha and beta tubulin (5). Microtubule-associated proteins (MAP) are connected to microtubule organizing center (MTOC). Some of them will stabilize the microtubule structure (MAP2, MAP4, TUA, STOP, Mip-90 and stathmin), and others are responsible to set up the interior space of microtubules (MAP1, MAP2 and TUA) (7).

Stathmin, also known as oncoprotein 18, is also one of the regulators of mitotic spindle and microtubule cytoskeleton during cell cycle (8, 9). The expression and phosphorylation of stathmin is dependent upon a variety of signals altering both proliferation and differentiation of eukaryotic cells (10). Stathmin phosphorylation is significantly increased as the cell progresses from the S phase to the G2-M phases of cell cycle (11). Stathmin is highly expressed in breast cancer as well as other carcinomas, and its overexpression leads to the progression and bad prognosis of the disease (12).

Tau protein is the product of alternate processing of a single gene considered as microtubule-associated tau

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protein (MAPT) (13). Tau protein is expressed in normal epithelial and breast cancer cells. Expression of tau protein may be important for optimizing chemotherapy (14). Negative expression of tau protein can be an independent predictor for chemotherapy containing taxanes (15). MAPT attaches to both inner and outer surfaces of microtubules and results in the assembly of tubulin and stabilization of microtubule (4). Taxanes and tau protein have the same joints and as a result tau protein competes with these drugs. Taxane binds to microtubules and prevents their moving and spindle formation resulting in the cell cycle halt at the G2 / M phases (16).

The activity of tau phosphorylation is regulated by serine-threonine kinase (17). Over-phosphorylation of this protein occurs mainly in axons and probably leads to degeneration of neurofibrillary, cell dysfunction and death. They have remarkable performance in the pathogenesis of Alzheimer's disease (18).

Memantine, a drug used to treat the symptoms of Alzheimer's disease, is in the category of N-methyl-D-aspartate (NMDA) receptor antagonist drugs (19). Memantine is also an inhibitor of internal ribosome entry site (20). Memantine can inhibit the level of amyloid precursor protein (APP) and the expression of tau protein by cap-independent translational initiation mechanism in Alzheimer's disease (21). This study determines the effect of memantine on breast cancer cell motility, tau and stathmin gene expression in cancer cells and its synergistic effect with paclitaxel.

Materials and Methods

Cell culture

MCF-7 breast cancer cell line, purchased from the national Cell Bank of Iran (NCBI), were cultured in DMEM (supplemented with 4 mM L-glutamine, 4.5 g/l glucose, 10% FBS, 100 µg/ml streptomycin, and 100 µg/ml penicillin) under 37 °C humidified air containing 5% CO₂ until the third passage before performing the experiments. All of the cell culture materials were from Gibco, Pittsburgh, USA.

Cytotoxicity assay

In vitro cytotoxicity was evaluated through plating out breast cancer cells (1×10^4 cells/well in 96 well plates) in 100 µl of medium per well, and allowed to attach. Memantine at the doses of 2, 20 and 100 (µmol/l) were added to the cells and incubated for 48 hr. Percentage of viable cells in each well was determined by the MTT assay and compared with untreated cells. The experiments were carried out in triplicates and mean percentage of the viable cells is reported. To investigate the synergistic effect of memantine and paclitaxel on MCF-7 cell line, 50 and 100 nM concentrations of paclitaxel were added to memantine and then the MTT assay was performed.

Plates were read using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek, Winooski, USA) at 540 nm with a reference wavelength of 630 nm. The cell viability was determined by the following formula:

$$\% \text{Cell viability} = \frac{\text{Mean absorbance in test wells} \times 100}{\text{Mean absorbance in control wells}}$$

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from breast cancer cells, treated for 24 hr using RNeasy Mini plus Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. The RNA quality was verified by spectrophotometer and gel electrophoresis. cDNA was synthesized by using RevertAid™ Reverse Transcriptase (Fermentas, Vilnius, Lithuania) with oligo-dT primers (22). Quantitative real time RT-PCR was performed by using specific primers for tau and stathmin mRNAs as an internal control with the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) and the amplification was run on the Rotor-gene 6000 (Qiagen, Hilden, Germany). The PCR cycling conditions for the genes consisted of an initial denaturation at 95 °C for 10 min, followed by 45 amplification cycles including denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec and an extension at 72 °C for 30 sec. The identity of PCR products was verified by a 1.5% agarose gel, stained with ethidiumbromide, followed by visualization under the ultraviolet light.

Transwell migration assays:

Cell migration was determined as described previously (23) using Transwell Boyden chambers from Corning (New York, NY).

Results

Cell growth inhibition

As shown in Figure 1, memantine resulted in cell viability reduction ($P=0.0001$). Moreover, paclitaxel 100 nM showed synergistic effect with memantine ($P=0.0001$) (Figure 2).

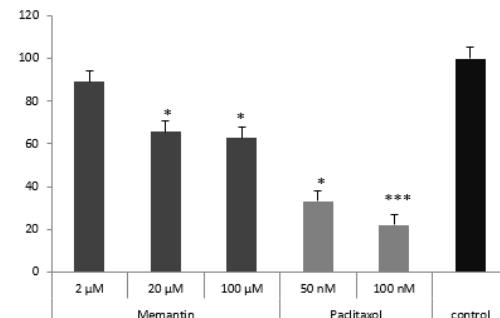


Figure 1. Effect of memantine on cell viability. MCF-7 breast cancer cells were treated with memantine and the viability of cells was determined by the MTT assay. The data are expressed as the percentage of cell viability±SEM. * $P<0.05$ ** $P<0.01$ and *** $P<0.001$ demonstrate significant values versus control

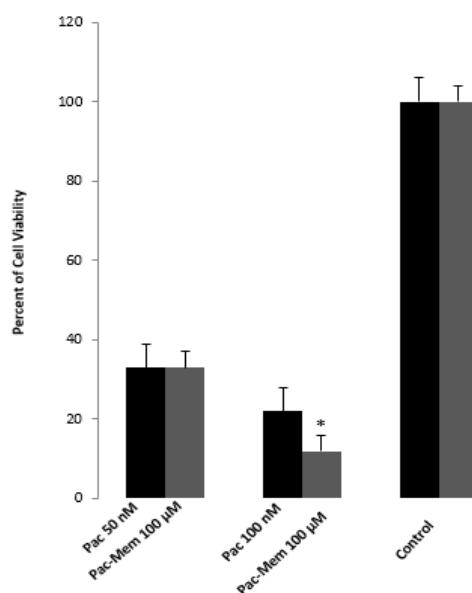


Figure 2. Cytotoxic effect of combination of memantine and paclitaxel on breast cancer cell line. Cells were incubated with memantine and paclitaxel. The cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The combination of paclitaxel (PAC) and memantine improves efficacy in breast cancer cells (MCF-7). * $P<0.05$ demonstrates significant values versus control

Memantine inhibits expression of tau and stathmin mRNA in breast cancer cells

After optimizing the RT-PCR, expression of tau and stathmin genes was determined using quantitative RT-PCR in the treated and control breast cancer cells.

Memantine at the concentrations of 2, 20 and 100 $\mu\text{mol/L}$, resulted in 8 ($P=0.471$), 14 ($P=0.184$) and 46% ($P=0.0341$) decrease in tau expression, respectively (Figure 3). Memantine also resulted in 6 ($P=0.627$), 9 ($P=0.346$) and 33% ($P=0.043$) decrease in stathmin expression in the mentioned doses, respectively (Figure 4).

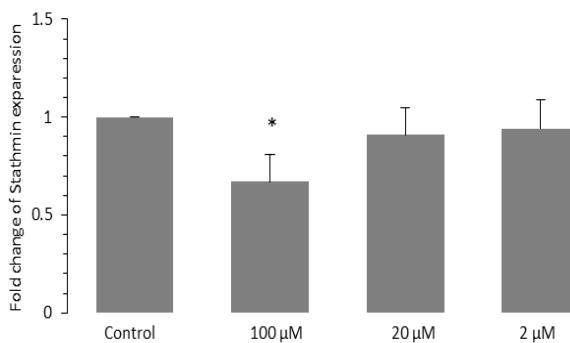


Figure 4. Fold change of stathmin expression in MCF-7 cell expression after 24 hr incubation of cells with memantine. Quantitative reverse transcription polymerase chain reaction analysis demonstrated that memantine decreased stathmin expression significantly. $P<0.05$ demonstrates significant values versus control

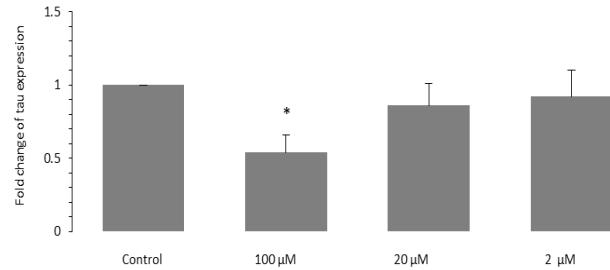


Figure 3. Fold change of tau expression in MCF-7 cell expression after 24 hr incubation of cells with memantine. Quantitative reverse transcription polymerase chain reaction analysis showed that memantine decreased tau expression significantly. $P<0.05$ demonstrates significant values versus control

Memantine represses breast cancer cell migration

Transwell migration assays showed that memantine at the concentrations of 2, 20 and 100 $\mu\text{mol/l}$, decreased the number of migrating cells by 10%, 50%, and 78%, respectively ($P=0.0001$) (Figure 5).

Discussion

Here we showed that memantine (100 $\mu\text{mol/L}$) decreased stathmin and tau gene expressions in MCF-7 cells. Incubation of breast cancer cells with memantine resulted in a dose dependent reduction in cell survival. Moreover, coadministration of paclitaxel and memantine showed more antiproliferative effect than paclitaxel alone. The migration of breast cancer cells was also decreased by memantine *in vitro*.

Previous studies have shown that tau is a predictive factor for tumor metastasis (24), which allows the dissemination of the malignancy (25). Tau protein localizes to microtentacles (McTNs) and it is important for the promotion of their extension in detached breast cancer cells (25). McTNs are microtubule-based membrane protrusions in circulating tumor cells, which can increase metastasis by enhancing their reattachment potential (26). Tau-induced McTNs promote reattachment of cells and lead to retention of circulating cancer cells in lung capillaries (25). Analysis of patient tumors demonstrated that tau expression is increased by 52% in cancer cells (25). Increase of tau protein is also associated with poor prognosis of patients (24, 27).

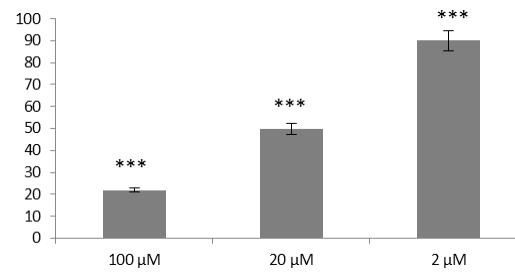


Figure 5. Boyden chamber data showing the number of cells present on the lower compartment of a transwell membrane after 24 hr incubation with memantine. MCF-7 cells displayed the greatest number of cells present. Median value ($n=3$) is shown. *** $P<0.001$ demonstrates significant values versus control (100%)

Although targeting the actin cytoskeleton for the reduction of tumor invasion and motility seems to be effective, it is demonstrated that tumors with high tau expression significantly produce McTNs. In a similar way, tubulin stabilizers including paclitaxel are not advised in patients with tau overexpression due to McTN formation. In fact, in clinical studies it has been demonstrated that high tau expression leads to resistance to chemotherapy with paclitaxel and may increase the risk of recurrence (27).

In this study for the first time, we highlighted that memantine improves the effects of paclitaxel. The combination of paclitaxel and memantine might also prevent toxicities associated with full therapeutic dose of paclitaxel.

Stathmin is overexpressed in several recurrent and metastatic cancer types. Overexpression of stathmin is also shown to be associated with poor prognosis (28).

Stathmin induces cell motility in the extracellular matrix *in vitro* and metastasis of sarcoma cells *in vivo* (29, 30). Furthermore, adenovirus-mediated gene transfer of anti-stathmin ribozyme has led to the inhibition of proliferation and clonogenicity associated with G2/M arrest, increase of apoptosis in both ER-positive and ER-negative breast cancer cells and also inhibition of mammary tumor growth in nude mice (31). Stathmin is negatively regulated by tumor suppressor protein p53, and its transcription is repressed through function of p53 and derepressed by mutation of p53. Silencing of stathmin has shown to induce tumor suppression functions including cell-cycle arrest and apoptosis in breast cancer cells harboring p53 mutations that are invasive and resistant to treatment (32).

These studies showed the importance of stathmin and tau proteins in metastasis and also as a target for novel investigations into breast cancer. Moreover, low tumor expression of stathmin resulted in high response to neoadjuvant chemotherapy regimens containing docetaxel and better prognosis of breast cancer, indicating the beneficiary effect of stathmin expression decrease in response to chemotherapy (33).

Taxanes are mitotic inhibitors stabilizing microtubules that organize mitotic spindle. High stathmin expression in breast cancer leads to taxane resistance. Therefore, stathmin expression strongly influences activities of taxanes in breast cancer (34). Coadministration of agents that commonly involve in microtubules have more profound inhibitory effect than those involving different pathways (31). In other words, the synergistic effect of paclitaxel and memantine could be relevant to their similar mechanism of action on microtubules.

Conclusion

We showed that memantine reduced mRNA levels of tau and stathmin and also estrogen positive

breast cancer cell line migration *in vitro*. However, it needs much more preclinical and clinical evidence to use memantine in clinical studies in the future.

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