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Memantine inhibits cell proliferation and activates LKB1-AMPK pathway in breast carcinoma

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Abstract

Drug repositioning that is a screening of presently approved drugs for already unknown indications is therapeutically necessary and influential for drug discovery. In this study, it was aimed to research whether memantine as a repositioned drug can activate the LKB1-AMPK pathway in breast carcinoma cells by triggering tumor suppressor genes *LKB1, AMPK*, its downstream targets 40S ribosomal S6 kinases (*S6K1* and *S6K2*), and eukaryotic initiation factor 4E-binding protein 4E-BP1. It was also evaluated its apoptotic effect by detecting the gene expressions of *Caspase 7* and *NOXA*. Thus, MCF-7 cells were treated with 250 µM memantine for 48 h, and its cytotoxic effect was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. *AMPKα1, AMPKα2, S6K1, S6K2, 4E-BP1, Caspase 7* and *NOXA* gene expression levels were measured by quantitative real-time polymerase chain reaction. The results clearly revealed that memantine inhibited MCF-7 cell proliferation and activated the LKB1-AMPK pathway by reducing *S6K1, S6K2,* and *4EBP1* gene expressions. Memantine also augmented the gene expressions of *Caspase 7* and *NOXA*. The findings reveal a molecular mechanism for the first time that may contribute to the anti-cancer effect of memantine to prevent or treat breast cancer. But further research should be performed to better understand its anti-cancer action.

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1. Introduction

Drug development is a time consuming, costly, and complicated process with an approximate success ratio of just 2%. To decrease drug development failure ratio, cost and time, drug repositioning purposes to discover novel evidence for present drugs [1]. Memantine, a repositioned drug, is the US Food and Drug Administration (FDA)-approved drug that blocks N-methyl-D-aspartate (NMDA) type glutamate receptors in neurons and clinically efficient on Alzheimer's dementia and Parkinson's disease [2,3]. It has also possessed specific pharmacological characteristics such as plasma half-life, rapid and easy absorption, quickly passing a blood-brain barrier, well tolerability, moderate affinity, and strongly voltage-dependency [4,5]. Recent researches about drug repurposing applications have revealed that memantine could be a promising drug to treat neonatal sepsis, bacterial meningitis, and Chagas disease caused by *Trypanosoma cruzi* [6,7]. It has been also reported that memantine stimulates apoptosis, autophagy, and cell death caused by chemotherapeutic cytarabine and demonstrates anti-proliferative effects on different cancer cell types [3, 8-11].

Functional NMDARs are significant agents to sustain cell growth and viability and expressed on the surface of various cancer cell lines like breast carcinoma. The use of memantine as an NMDA receptor antagonist can be considered as a therapeutic strategy to improve new and efficient treatment for breast cancer [8,12]. Breast cancer is commonly diagnosed with malignancy and brings about cancer-related mortality among women worldwide. Its treatment options are multidisciplinary including hormonal therapy, radiotherapy, surgical resection, chemotherapy, which could be challenging or even toxic for patients to exterminate breast cancer [13]. Molecular target treatment has recently been an appealing therapy strategy for breast cancer. In this treatment option, particular molecular pathways that vary in breast carcinoma can be used by molecularly targeted therapy agents and necessary for tumor progress and survival. Purposing of molecular pathways of adenosine monophosphate-activated protein kinase (AMPK) might be crucial to treat effectively breast cancer [14].

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AMPK is a key sensor of intracellular energy status, cell survival, and apoptosis. Its function is partially mediated tumor suppressor proteins which are related to AMPK signaling pathways like Liver kinase B1 (LKB1). LKB1 serves as a main upstream kinase by directly activating AMPK and AMPK-related kinases, which are contained in numerous events like cell polarity, energy metabolism, proliferation, and apoptosis. AMPK is also the best-described substrate of LKB1 and many functions of AMPK clarify the works of LKB1 in tumor suppression. Additionally, the LKB1-AMPK molecular pathway modifies metabolic pathways among anabolism and catabolism in reply to diverse energy insults and regulates cell growth and autophagy to sustain energy and nutrient homeostasis in cells [15,16].

The downstream pathways of LKB1-AMPK can be activated by pharmacological agents and these agents can directly bind to trigger the LKB1-AMPK activity, and thus obstruct the progression of tumor growth in preclinical and early clinical models [17]. In this study for the first time, it was aimed to investigate whether NMDA receptor antagonist memantine can activate the LKB1-AMPK pathway by triggering tumor suppressor genes *LKB1, AMPK* cata-lytic subunits, (*AMPKα1* and *AMPKα2*), and its downstream targets 40S ribosomal S6 kinases (*RPS6KB1* and *RPS6KB2*) and eukaryotic initiation factor 4E-binding protein (*4EBP1*). Additionally, it was evaluated the apoptotic activity of memantine by detecting the gene expression levels of *Caspase 7* and *NOXA* in breast adenocarcinoma cells.

2. Materials and Methods

2.1. Cell culture and chemicals

MCF-7 (ATCC® HTB-22™) cells were flourished in RPMI medium added with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA) and cultured in 75-cm^2 Tissue Culture flasks in an incubator in 5% CO_2 at 37°C. Memantine was supplied from Sigma-Aldrich, St Louis, MO, USA, and dissolved in sterile, non-pyrogenic distilled water.

2.2. Cell cytotoxicity assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with some modifications was used to evaluate the cytotoxic effect of memantine [18,19]. $4x10^3$ cells/well were grown into 96 well plates and produced for overnight. When attached after 24 h, MCF-7 cells were treated with memantine for 24 and 48 h. Then, MTT solution (5 mg/ml in PBS) was added to each well, and the plate was incubated for 4 h at 37°C. DMSO was put into each well in order to solubilize the formazan crystals and the plate was kept at 37°C for 30 min. Absorbance rate was quantified via the SpectraMax M3 (Molecular Devices, USA) microplate reader at 570 nm. Each treatment was performed at least five times, and IC50 values were computed using GraphPad Prism 8 software package.

2.3. Detection of gene expressions via quantitative real-time PCR (qRT-PCR)

2.3.1.Primer design

The specific oligonucleotide primers were designed according to DNA sequences of *RPS6KB1 (S6KB1), RPS6KB2 (S6KB2), 4E-BP1, AMPKα1, AMPKα2, LKB1, Caspase 7, NOXA* and *GAPDH* genes deposited in NCBI reference sequence database (Table 1). NCBI and BLAST (Basic Local Alignment Search Tool) algorithms were used to confirm the designed primers.

Table 1. Designed forward and reverse primers for quantitative real-time PCR

Gene Name	Forward Primer (5'-3' sequence)	Reverse Primer (5'-3' sequence)
S6KB1	AGAAGATGCAGGCTCTGA	TTACCAAGTACCCGAAGTA
S6KB2	GAGCCTGGGAGCCCTGATGTA	GAAGCCCTCTTTGATGCTGTCC
4E-BP1	TAGCCCTACCAGCGATGAGCCT	GTATCAACAGAGGCACAAGGAGGTAT
AMPKα1	CAGGGACTGCTACTCCACAGAGA	CCTTGAGCCTCAGCATCTGAA
AMPKα2	CAACTGCAGAGAGCCATTCACTT	GGTGAAACTGAAGACAATGTGCTT
LKB1	GAGCTGATGTCGGTGGGTAT	GCCCTGGATTTGGTGCTC
Caspase 7	GGACCGAGTGCCCACTTATC	TCGCTTTGTCGAAGTTCTTGTT
NOXA	AAGAACGCTCAACCGAGCC	CTGCCGGAAGTTCAGTTTGTC
GAPDH	CTGACTTCAACAGCGACACC	TAGCCAAATTCGTTGTCATACC

2.3.2. RNA extraction

To evaluate the expression of selected genes, total RNA was isolated by using Trizol reagent (Invitrogen, Thermo Fischer Scientific, USA) method [20]. Then, the extracted RNA was treated using RNase-free DNase I and stored at −80 °C. RNA concentrations were evaluated using a Nanodrop 2000 spectrophotometer (Thermo Fisher scientific).

2.3.3. cDNA synthesis and qRT-PCR

2000 ng total RNA was reverse transcribed by Superscript III cDNA Synthesis Kit (Invitrogen, Thermo Fischer Scientific, USA). DNA concentrations were detected using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Forward and reverse designed primer sequences were used as shown in Table 1. 10–20 cycles of specific target amplification was performed by SYBR Green qPCR master mix with the following thermocycler conditions: 95°C for 5 min, then 50 cycles of 95°C for 15 sec, 60°C for 30 sec (amplification), followed by 72 °C for 10 min. Reactions were prepared in 10 µl total volume, including 5 µl SYBR Green qPCR Master Mix, 0.5 µl of each forward and reverse primers, 2.5 µl nuclease-free water and 2 µl of template cDNA. RT-qPCR analysis was carried out in Roche LightCycler qPCR. GAPDH was utilized as an internal control when calculating Cq value. ΔΔCq method was performed to measure the expression levels of genes [21].

2.3.4. Statistical analysis

Experimental data were presented as mean ± standard deviation (SD) for at least four independent experiments. The data were assayed via one-way ANOVA followed by Tukey post hoc test using SPSS 20.0 software (Chicago, USA). The level of significance (p-value) was expressed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results and Discussion

3.1. Memantine displayed an antiproliferative activity in MCF-7 cells

To detect cytotoxic effects of memantine on MCF-7 cells, the cells were treated with 100-500 µM range of memantine for 24 and 48 h and evaluated the percentage of viable cells. Memantine significantly ($p<0.01$ and $p<0.001$) decreased MCF-7 cell proliferation in a dose-dependent manner for 24 and 48 h (Figure 1.). IC50 values of memantine were calculated as 300 µM and 250 µM for 24 and 48h, respectively. Cell viability at 100-500 µM dose range of memantine varied from 90.50% to 6.80% for 24 and 48h. As a result, memantine showed an antiproliferative activity in MCF-7 cells. For gene expression analysis, further 48h was preferred as a time-point because the mediators that induce cell death, tumor suppression or growth may take some more time to act in cells. Antiproliferative effect of memantine on metastatic breast cancer cells was also reported in the previous studies [8,9]. North et. al (2010) found that by use of Alamar Blue assay, memantine inhibited SKBR3 and MCF-7 breast cancer cell proliferation with IC50 at 48 h of approximately 250 µM and 200 µM, respectively [8]. Although different assays were used for the detection of MCF cell viability, IC50 concentrations of memantine were found similar in each experiment for MCF-7 cells. Additionally, Seifabadi et. al (2017) reported that the IC50 value of memantine was exhibited at >100 µM concentration and memantine (100 µM) combined with paclitaxel (100 nM), anti-cancer chemotherapy drug, displayed significantly synergistic antiproliferative effect on MCF-7 cells [9]. It was also clarified that memantine in combination with cytarabine that is a chemotherapy agent enhanced cell death of acute lymphoid and myeloid leukemia cell lines and demonstrated antiproliferative effect on T-98 G human glioma and LNCaP prostate cancer cell lines [3,10,11]. Albayrak et. al. (2018) also proved the antiproliferative effect of memantin by discussing with the effect of metformin, an another repositioning drug, and found that memantin hindered LNCaP prostate cancer cell growth rate at almost 10 times lower dose than metformin [11]. As a result, the previous *in vitro* studies about anticancer effects of memantine on breast cancer cells were found to be consistent with the results of this study, which shows a significant inhibition against breast cancer cell progress.

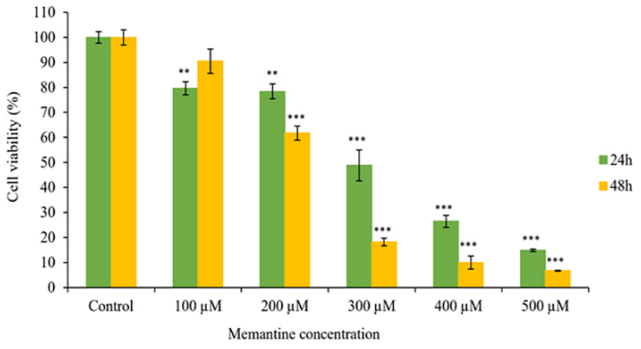

Figure 1. The viability of MCF-7 cells treated with different concentrations of memantine for 24-48 h. Cell viability was detected using MTT assay. The IC50 value was calculated as 250 µM for 48 h. The data are displayed as the percentage mean of cell viability ±SD. **$p<0.01$ and ***$p<0.001$ demonstrate significant values according to control.

3.2. Memantine stimulated apoptotic cell death by enhancing the expression of Caspase 7 and NOXA genes

Apoptosis is a programmed cell death, which has been considered to be serious for cancer prevention. So any drug or compound that can stimulate apoptosis might be beneficial for cancer therapy and apoptosis initiation is presumably the most effective defense toward cancer development [22]. Caspases are a family of proteins that are central effectors of apoptosis and their activation is also a characteristic of apoptosis [23]. Caspase-7 is the major effector caspase in MCF-7 cells, which lack caspase-3. The cells contain a premature stop codon mutation in the *Caspase 3* gene and *Caspase 7* demonstrates the same *in vitro* substrate specificity as *Caspase 3* [24]. Another apoptotic cell death indicator protein in tumor cells is NOXA. It is a pro-apoptotic member of the Bcl-2 family, which intercede the triggering of apoptosis by activation of the intrinsic and mitochondria apoptosis signaling pathways. Cell death stimulation via cytotoxic drugs is essentially regulated by the intrinsic apoptosis signaling cascade. NOXA plays a significant role in apoptosis stimulation by cytotoxic drugs [25].

In the present study, *Caspase 7* and *NOXA* gene expressions of MCF-7 cells treated with memantine for 48 h were evaluated. In Figure 2. it was demonstrated that the treatment of the cells with 250 µM memantine for 48h significantly increased gene expression levels of *Caspase 7* and *NOXA* (2.185±0.111, $p<0.001$ and 1.460± 0.036, $p<0.01$ as fold changes, respectively) versus the control without memantine. In a previous study, it was found that memantine also triggered apoptotic cell death on prostate cancer cells by enhancing pro-apoptotic Caspase 3, Caspase 9, Bax protein levels, and reducing anti-apoptotic Bcl-2, survivin protein expression levels. It was also revealed that memantine displayed antineoplastic activity through inducing of Bax-dependent pathway in apoptosis [11]. Morever, Lowinus et. al (2019) reported that memantine increased the chemotherapy drug cytarabine-induced cell death in several subtypes of acute leukemia cells by stimulating Caspase 9 and Caspase 3, thus enhancing intrinsic apoptosis [3]. It was also reported that memantine-derived drugs exhibited antitumor effects in human U87MG glioblastoma cell line by triggering apoptosis [26]. These previous reports were in agreement with the results of this study.

The stimulation of *Caspase 7* and *NOXA* gene expressions by memantine was revealed for the first time in this study. These findings suggest that memantine may cause apoptotic cell death by enhancing the gene expressions of *Caspase 7* and *NOXA* in MCF-7 cells. In this study, however, it could not be demonstrated the apoptotic mechanisms including mitochondrial intrinsic and extrinsic pathway and also the role of pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Therefore, further studies are required to clarify the action of these apoptotic mechanisms and the role of apoptotic proteins.

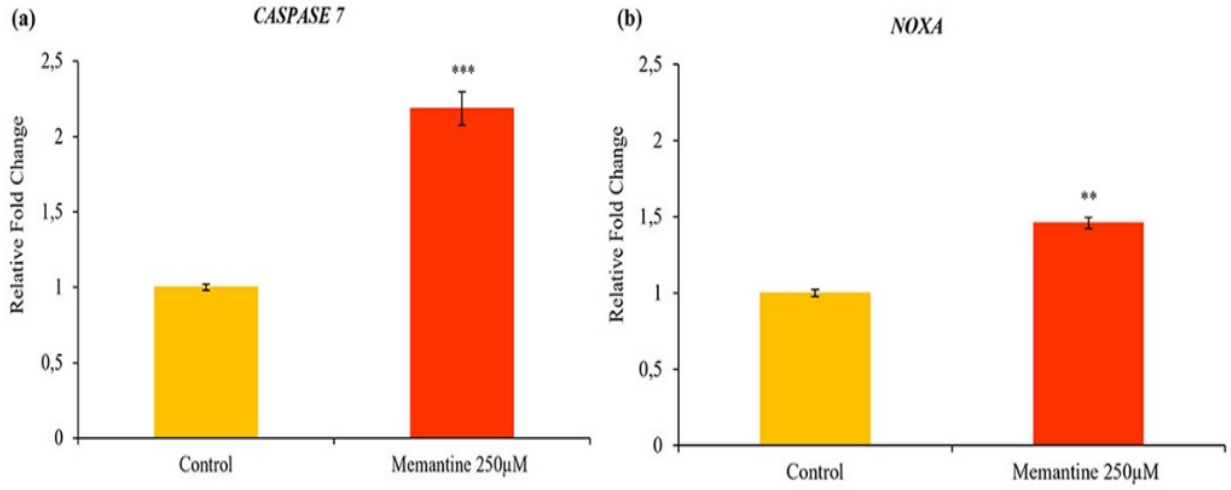

Figure 2. Gene expression levels of Caspase 7 and NOXA. The relative fold changes of (a) Caspase 7 and (b) NOXA genes (2.185±0.111 and 1.460± 0.036 as fold changes, respectively) quantified by RT-PCR. The data are shown as the relative expression level compared to GAPDH and indicated as mean ± SD. **$p<0.01$ and ***$p<0.001$ demonstrate significant values according to control.

3.3. Memantine activated the LKB1-AMPK pathway through reducing the gene expression levels of *RPS6KB1, RPS6KB2*, and *4EBP1*

Liver kinase B1 (*LKB1*) is a tumor suppressor gene that includes the control mechanisms of tumorigenesis and metabolism [13]. When *LKB1* is involved in an interaction with AMP-activated protein kinase (*AMPK*), it cancels signal transduction via the mammalian objective of rapamycin (*mTOR*) to block cell progress, thus acting as a tumor suppressor. Moreover, attenuated *LKB1* expression was related to enhanced metastatic and invasive potential. Therefore, *LKB1*-associated pathways could ensure potential targets to reduce the invasive and metastatic features of breast cancer and the drugs activating these pathways might be beneficial as cancer therapeutics [27,28]. To define the molecular signal transduction, which is liable for the anticancer action of memantine, gene expressions of *LKB1, AMPK* catalytic subunits (α1 and α2), and LKB1-AMPK pathway-related downstream genes, 40S ribosomal S6 kinases (*RPS6KB1* and *RPS6KB2*) and eukaryotic initiation factor 4E-binding protein (*4EBP1*) were investigated in this study. MCF-7 cells were treated with 250 µM memantine for 48 h and gene expression levels were shown in Figure 3. The results showed that memantine significantly ($p<0.001$) enhanced *LKB1, AMPKα1*, and *AMPKα2* gene expression levels of 2.609±0.000, 2.842±0.017, 4.252±0.012 as fold changes, respectively. However, memantine caused dramatically ($p<0.01$ and $p<0.001$) decreases of *RPS6KB1, RPS6KB2*, and *4E-BP1* gene expression levels in the fold change values of 0.617±0.000, 0.016±0.000 and 0.241±0.000, respectively (Figure 3).

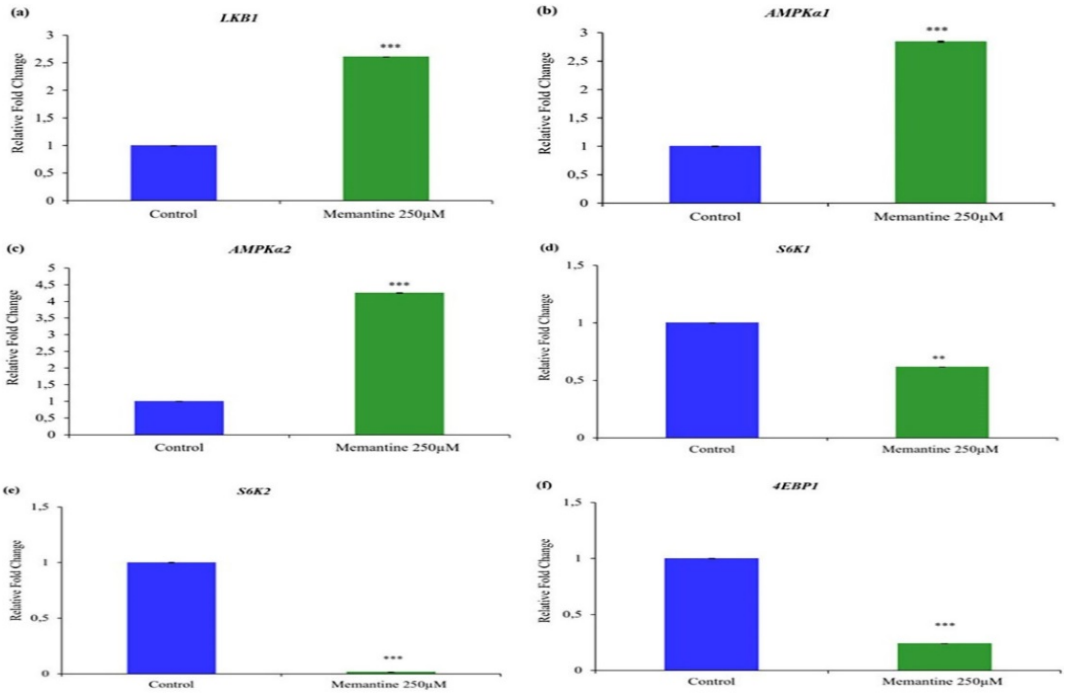

Figure 3. Effect of memantine (IC50: 250 µM, for 48 h) on gene expression levels of (a) *LKB1*, (b) *AMPKα1*, (c) *AMPKα2*, (d) *S6K1*, (e) *S6K2* and (f) *4E-BP1* evaluated by RT-PCR. The data are shown as the relative expression level compared to GAPDH and indicated as mean ± SD. **P<0.01 and ***P<0.001 demonstrate significant values according to control.

In a previous study, it was shown that decreased LKB1 protein expression in the samples obtained from local breast carcinoma patients was associated with higher histological stage, larger tumor gravity, the existence of lymph node metastasis and shorter survival [29]. Morever, Baek et. al (2020) have reported that *AMPK* activation extinguish the progress of several cancers by the arrangement of cell cycle process, autophagy, apoptosis, the prevention of protein and fatty acid synthesis. They have also signified that *AMPK* activity in patient tumors is related to aggressive clinical phenotype or poor prognosis [30]. AMPK is formed as a heterotrimer consisted of α1, α2, β1, β2, γ1, and γ2 subunits. *AMPKα1* is extensively expressed in cells; however, *AMPKα2* is only expressed in skeletal muscle, hepatocytes, and cardiac muscle cells. Yi et. al (2020) have recently demonstrated that transcriptional inhibition of *AMPKα1* expression leads to a

breakdown of cell–cell adhesion and enables cancer metastasis. Additionally, they have found that *AMPKα1* mRNA levels are dramatically decreased in breast cancer subtypes and its decreased expression is associated with breast cancer metastasis and poor clinical results [31]. It was also reported that *AMPKα2* is greatly suppressed in MCF-7 cells and breast cancer tissues [32]. These results in common impress on the importance of decreased *AMPK* activity promoting human carcinogenesis and, the function of *AMPKα2* relating to its control of normal mammary epithelial cell growth and its decreased expression in breast cancer. In contrast to these results, memantine caused an increase of *LKB1, AMPKα1,* and *AMPKα2* mRNA expression levels in MCF-7 cells and exhibited anti-cancer action. Therefore, the findings of this study suggest that memantine activates *LKB1* and *AMPK* to hinder the malign characteristics of MCF-7 cells. To evaluate the LKB1- AMPK pathway activation by memantine, the expression of its downstream proteins are also evaluated in this study.

mTOR (rapamycin), also recognized as the mammalian aim of rapamycin, is a significant target for breast cancer treatment because of acting a crucial role in tumorigenesis. mTORC1, one of the distinct complex named as raptor, mediates its performance by its downstream targets ribosomal S6 kinases (S6K1 and S6K2) and 4E-BP1. *S6K1* and *S6K2* are coded by ribosomal S6 kinase *RPS6KB1* on chromosome 17 and *RPS6KB2* on chromosome 11, respectively [33]. Perez-Tenorio et. al. (2011) reported that *S6K1 (RPS6KB1)* and *S6K2 (RPS6KB2)* genes were overexpressed in breast cancer tissues and the overexpression of these genes possess prognostic and treatment predictive importance in breast cancer [34]. Karlsson et. al. (2011) also demonstrated that *S6K2* was often co-expressed with *4E-BP1*, a candidate oncogene in breast cancer, refering synergy among the *mTOR* targets *S6K2* and *4EBP1* in breast cancer growth and development [35]. Moreover, overexpression of *4E-BP1* is considered to be almost universal in breast cancer progression since it is a part of a genomic area that, when overexpressed, presents a poor prognosis for patients [36,37].

The results of this study suggest that memantine arrests MCF-7 cell proliferation by activating LKB1-AMPK pathway and blocks the overexpression of its downstream genes *4E-BP1, S6K1* and *S6K2*, thus suppressesing the breast cancer progression (Figure 4). In the present study, the findings in MCF-7 cells treated with memantine were in contrast to these results as there was a decrease in *4E-BP1, S6K1* and *S6K2* expressions. The significant ($p<0.001$) decreases of *S6K2* and *4E-BP1* expressions together in the cells treated with memantine might also display the existence of *mTORC1* in the LKB1-AMPK pathway. But it could not detect *mTOR* expression in this study. Therefore, further investigations should be performed to reveal whether the activation of this pathway by memantine is *mTOR* dependent or not. Moreover, the function of memantine in association with the LKB1–AMPK pathway in tumor suppression should be more investigated.

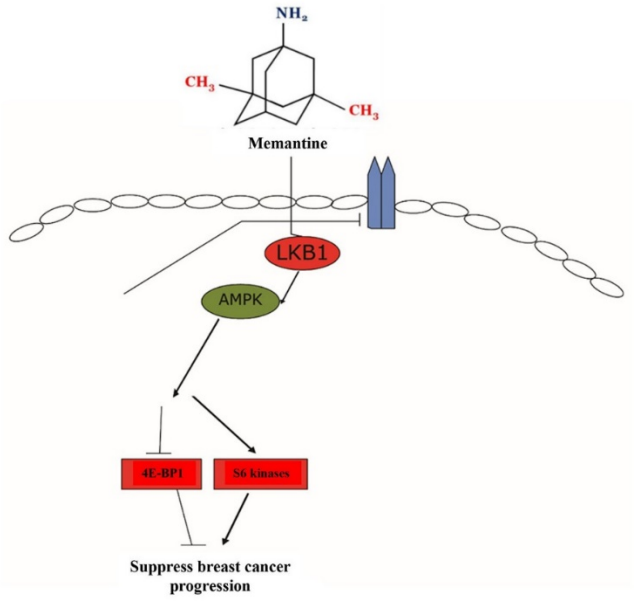

Figure 4. Activation of the LKB1-AMPK signaling pathway by memantine in MCF-7 cells

4. Conclusions

In this study for the first time, it was highlighted that memantine is capable of activating the LKB1-AMPK pathway and reduces gene expression levels of *S6K1, S6K2*, and *4EBP1*. The data obtained from this study reveal a molecular mechanism that may contribute to the anti-cancer action of memantine to prevent or treat breast cancer. Enhanced *Caspase 7* and *NOXA* expressions may point out that memantine could cause apoptotic cell death. But the cell death mechanism of memantine on MCF-7 cells is required to be clarified. Further research should be carried out to exhibit whether memantine causes the reduction of cell-cell adhesion, the inhibition of cancer metastasis, and the activation of LKB1-AMPK pathway-dependent mTOR or not. The mechanisms linked to this pathway should be revealed in detail to better understand the anti-cancer action of memantine.

Conflicts of interest

The author state that did not have conflict of interests

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