Assessment of Rapamycin effect on apoptosis in MCF-7 breast cancer cell line

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Abstract:

Background: Rapamycin was the first inhibitor of mTOR (mechanistic previously named mammalian target of rapamycin, also known as FRAP or RAFT 1) to be discovered and studied as a therapy for a wide variety of diseases. Since the phosphoinositide 3 kinase (PI3K)/Akt (protein kinase B) /mTOR (PAM) pathway is frequently activated/dysregulated in breast cancer, rapamycin was explored as an effective target therapy in breast cancer, however the mode of its action remains unclear, therefore our study aimed at understanding the antitumor effect of rapamycin, using MCF-7 breast cancer cells.

Methods: Trypan blue dye exclusion method and MTT assay were performed to determine the IC50 value of rapamycin. Trypan blue dye exclusion method was also used to determine cell growth in untreated versus rapamycin treated MCF-7 cells, these cells were examined for morphological changes using inverted phase microscope.

Results: Rapamycin showed inhibitory activity on MCF-7 cell line via cell growth inhibition as determined by the IC50 value. The IC50 value was determined as 75µg/ml measured by trypan blue dye exclusion method and confirmed by MTT assay. Treatment of MCF-7 cells with rapamycim at IC50 resulted in induction of apoptosis up to 41% as determined by both Annexin-V/Propidium iodide dual staining assay and flowcytometry. Direct examination by inverted phase microscope showed that MCF-7 cells treated with rapamycin at IC50 for 72 hrs. resulted in characteristic morphological changes in the form of cell shrinkage and loss of cell contacts, also the cells became rounded and some of them were floating in the medium.

Conclusions: This study demonstrated that rapamycin acts as an anticancer drug via inhibition of cell growth in a dose dependent manner as well as induction of apoptosis .However further studies are needed to characterize the mode of action of rapamycin as an anticancer agent.

Keywords: Rapamycin, MCF-7 breast cancer cells, mTOR.

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Background:

Breast cancer is the most commonly occurring cancer in women [1]. Although mortality trends are declining in high income countries, it is still rising in low- and middle-income countries [2]. About one million new cases will be discovered annually and 400.000 deaths worldwide [3].

International Agency for Research on Cancer, considering geographic variability across 20 world regions reported that the incidence of female breast

cancer is 11.6% [4]. Breast cancer is the most prevalent cancer among women in Egypt [5]. National Cancer Registry Program (NCRP) in Egypt reported that breast and liver are the commonest sites of cancer in females (32% and 13.5%, respectively) [6].

Breast cancer development depends on the disequilibrium between cell proliferation and apoptosis, and there is a strong evidence that tumor growth depends on reduced apoptosis. The equilibrium between proliferation and apoptosis is critical for determining the growth or regression of the tumor in response to chemotherapy and radiotherapy [7] .Apoptosis is an active process that aims at removal of damaged cells due to cell injury or other stimuli [8]. Rapamycin is a drug that uses as alternative mechanisms to inhibit the breast cancer growth [9].

Protein kinase is considered one of the most abundant gene families in human, as it controls cell signaling and function through protein phosphorylation. There are 634 protein kinases encoded in human genome and 85% of kinase dysregulation is linked with the incidence of many human disorders including breast cancer, among these kinases is mTOR [10].

mTOR, a conserved serine/threonine protein kinase which function as central regulator of cell growth and proliferation, by integrating multiple signals from growth factors and nutrient signals [11].

PIK3CA gene mutations are found in approximately 30-40% of estrogen receptor (ER) positive breast cancer resulting in activation of mTOR pathway [12]. mTOR hyperactivity is often associated with resistance to endocrine therapy, human epidermal growth factor receptor 2 (HER-2) directed therapy and cytotoxic therapy in breast cancer [13].

Rapamycin, is a macrocyclic lactone, isolated from the bacterium Streptomyces hygroscopicus that initially had strong anti-fungal effect [14]. Then it was found to have strong immunosuppressive effects through blocking T-cell activation, in 1999 it was approved for use as an immunosuppressant drug in USA [15]. It is used in procedures such as kidney transplantation, to prevent acute rejection, reduce the risk of infections and to lower the incidence of post-surgery cancer [16].

Rapamycin has an inhibitory effect on mTOR, It acts via inhibiting cellular proliferation and growth, so it was explored as an anti-cancer agent [17]. In breast cancer, rapamycin offers a significant promising anticancer activities against dysregulated signaling pathways which are mTOR related [18].

MCF-7 is one of the most commonly used breast cancer cell lines which proved to be a suitable model cell line for breast cancer investigations worldwide, including anticancer drug experiments [19]. It retained several characteristics of differentiated mammary epithelium such as the capability of forming domes and the ability to process estradiol via cytoplasmic estrogen receptors [20, 21].Tumor necrosis factor (TNF) alpha inhibits the growth of MCF-7 breast cancer cell line [22].

Since the effect of rapamycin as a monotherapy is still modest with variable degrees of response to treatment in cancer patients [13], more studies are needed to detect mechanism of action of rapamycin. In this study, we investigated the antitumor effect of rapamycin using MCF-7 cell line through detection of its effect on cell viability and apoptosis.

Materials and Methods:

Cell lines and cell culture

Human breast adenocarcinoma MCF-cell line was obtained from the American Type Culture Collection and cells were cultured using RPMI -1640 (1x) by the Holding Company for Biological Products and Vaccines, Egypt (VACSERA).

Reagents

Rapamycin was purchased from Sigma- Aldrich (CAS no. 53123-88-9), RPMI -1640 (1x), fetal bovine serum (FBS), antibiotic- antimycotic (Anti-Anti 100×100), 0.4% trypan blue dye, PBS (phosphate – buffered saline, PH 7.6, wo Ca, Mg) DMSO and0.25%(w/v) Trypsin 0.53 mM EDTA with phenol red were purchased from Gibco (Invitrogen by Life Technologies Ltd., Paisley, UK), MTT reagent kit was purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany, Cat No. A9201), Annexin v/ propidium iodide staining kit was also obtained from Sigma- Aldrich (Merck KGaA, Darmstadt, Germany, Cat No. A9201).

Cell culture protocol

Human breast adenocarcinoma MCF-7 cell lines were cultured in RPMI (1x) 1640 medium supplemented with L-glutamine (containing 10 % heat inactivated FBS and 1% penicillin/streptomycin) in 25 cm^2 tissue culture flasks. The culture medium was changed daily and cell morphology was observed daily by inverted phase microscopy.

Upon receipt, the culture medium was removed to a centrifuge tube, the cell monolayer was rinsed briefly with 2-3 ml of PBS. After that, 2-3 ml of 0.25% Trypsin-EDTA was added to the flask, further incubated for 3 minutes at 37°C 5% CO_2 and observed for cell dispersal by an inverted phase microscopy.

As the cell layer is detached, 6-8 ml of complete growth media was added to the flask and the cells were aspirated by gentle pipetting up and down, transferred to 15 ml conical tube and centrifuged at 1500 rpm for 5 min. supernatant was removed and the cell pellet was resuspended in fresh complete growth medium and appropriate aliquots of cell suspension was added to new culture vessels.

The cells were passaged upon reaching a confluency of > 80% and were split at different ratios based on the experiments being performed. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Cells were passed twice a week.

All the procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA).

IC50 determination (cell viability assay)

Trypan blue dye exclusion method

Trypan blue staining solution is a vital stain that stain dead tissues or cells blue. Since cells are very selective, in a viable cell, the trypan blue will not pass through the membrane; however, it traverses the membrane in a dead cell.

MCF -7 cells were plated at a density of 1×105 cells/well in 2ml culture medium in 6-well tissue culture plate. After 24 hrs. Incubation, triplicate wells were treated with different concentrations of rapamycin (25, 50, 75,100,200 and 500 µg/ml) while only adding media and DMSO (Serva Electrophoresis GmbH) (conc. less than 0.1%) to the control group. After 72 hrs. old media was removed, cells washed with PBS, rinsed with 0.25% Trypsin 0.53mM EDTA for 2-3 minutes then neutralized with fresh culture medium and collected in 15 ml conical tubes then, cells were centrifuged at 1500 rpm for 5 min, and cell pellet was resuspended in fresh media.

To determine cell viability, 20µl of cell suspension in RPMI medium were mixed with 20µl of 0.4% trypan blue dye (Cat. no.15250061, Thermo Fisher scientific, New York). The numbers of stained (dead cells) and unstained (live cells) cells were subsequently counted using a haemocytometer and an Olympus Inverted Trinocular Microscope (model: CKX-41) (10× objective), 4 large corner squares were counted and the number of viable cells /ml were calculated according to the following formula:

Conc. of Viable cells/ml = average no of cells per square × dilution factor×104

MTT assay protocol

Cell viability was also confirmed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan.

Cells were seeded at a density of 1x103 cells/well in a volume of 100ul fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 hrs. Then, Media was aspirated, fresh medium (without serum) was added, and triplicate wells were incubated either alone (negative control) or with different concentrations of Rapamycin to give a final concentration of (500-200-100-75-50-25-12.5-6.25-3.125-0.78 and 1.56 µg/ml). After 72 hrs. of incubation, medium was aspirated, 40ul MTT salt (2.5µg/ml) were added to each well and incubated for further four hours To stop the reaction and dissolving the formed crystals, 200µL of 10% Sodium dodecyl sulphate (SDS) in deionized water was added, followed by gentle mixing in a gyratory shaker and incubated overnight .

After that, the absorbance was measured using a micro plate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at wavelength 595nm while background absorbance was measured at wavelength 620nm. The experiment was repeated 3 times, and the mean OD value was calculated. The percentage of change in viability was calculated according to the formula: (Reading of extract / Reading of negative control) -1) x 100.

with fresh complete growth media only added to cells. Then the cells were further incubated for 6 days at 37°C 5% CO_2. Cell proliferation was evaluated by trypan blue dye

Morphological analysis

Cells were plated in 6-well plates at the density of 1×105 and incubated overnight. Rapamycin was added at IC50 which is 75 µg/ml followed by 72 hrs. Incubation. Treated and untreated MCF-7 cells were examined for morphological changes with a phase contrast microscope (OLYMPUS, CK X 41).

Apoptotic analysis using Flowcytometry

Annexin –V/Propidium iodide dual staining assay followed by flowcytometry were used for quantitative assessment of apoptosis. It is based on the use of annexin- V conjugated with fluorescein isothiocyanate (FITC) to label phosphatidyl serine sites on the membrane surface of apoptotic cells, it also includes propidium iodide to label the cellular DNA in necrotic cells where the cell membrane has been totally compromised.

MCF-7 cells were plated at a density of 1×105 cells/well in 6-well plates and incubated overnight. Then cells in triplicates were treated with rapamycin at IC50 value of 75µg/ml then again incubated for 72 hrs. After treatment, the cells were rinsed, trypsinized and washed with medium RPMI, collected in falcons in which respective supernatant was collected. The cell pellet were washed twice in PBS. Then, 100µl of cell suspension was transferred to a FACS tube and then mixed with 5µl annexin V-FITC (mg/ml) and 5µl propodium iodide (2.5 mg/ml). Tubes were gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400µl) was added to the FACS tube, and the tubes were run through a FACS machine within 1h.

The cells were analyzed for the expression of annexin-V and PI to identify apoptosis. Cells were grouped into four different populations: viable cells that were unlabeled, early apoptotic cells bound to annexin-V FITC only, late apoptotic cells bound to both annexin-V FITC and PI and necrotic cells bound to PI only. Analysis was done using the FACS Calibur system (BD, San Jose, CA). Data were analyzed with Cell Quest software (BD, San Jose, CA). Each experiment was performed in triplicate.

Statistical analysis

All microbiological tests were run in triplicate. Data was analyzed using the IBM SPSS, Statistics for windows, version 22. Differences between two groups were compared by student t test and between more than two groups by one-way analysis of variance (ANOVA). A statistical value < 0.05 was considered significant.

Results:

Effect of rapamycin on cell viability

IC50 determination by trypan blue dye exclusion method

The effect of different concentrations of rapamycin (25, 50, 75, 100,200 and 500 µg/ml) on MCF-7 cells were assessed by trypan blue dye exclusion method, the number of viable (unstained) cells and dead (stained) cells were counted by a haemocytometer using an inverted phase microscope. The IC50 (lethal concentration of the drug which causes death of 50% of cells in 72 hrs.) value of rapamycin in the MCF-7 cells was 75µg/ ml and these results are statistically significant ($p<0.05$). Data are reported as mean ± SD of representative experiments (figure 1).

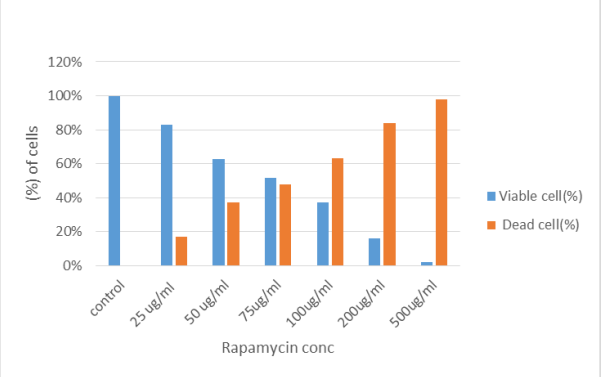

Figure (1): Inhibitory effect of different concentrations of Rapamycin on MCF-7 breast cancer cell line. Cell growth inhibition of MCF-7 cells treated with different concentrations of rapamycin (25, 50, 75, 100,200 and 500µg/ml) for 72 hrs. The IC50 value of rapamycin was determined as 75µg/ ml by trypan blue dye exclusion method. The results are statistically significant ($p<0.05$). The percentage cell death was calculated from the untreated.data are reported as means ± SD.

IC50 determination by MTT assay

The cytotoxic effect of rapamycin on MCF-7 cells was also evaluated by MTT assay. Triplicate wells were treated with rapamycin at different concentrations (500-200-100-75-50-25-12.5-6.25-3.125-0.78 and 1.56 µg/ml). Rapamycin significantly inhibited the proliferation of MCF-7 cells after 72 hrs. Higher concentrations of rapamycin induced greater inhibitory effects (figure 2).

The cytotoxic and antiproliferative effect of rapamycin on MCF-7 cells was concentration dependent and was highly significant (P value < 0.001). Data are reported as means ± SD (table 1).

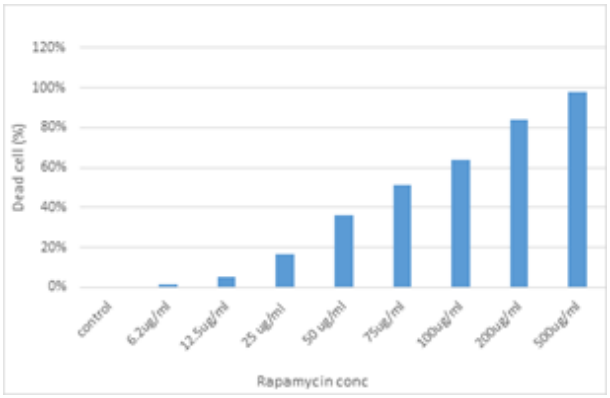

Figure (2): The cytotoxic effect of different concentrations of rapamycin on MCF-7 cells. Triplicate wells were treated with different concentrations of rapamycin for 72 hrs. Higher concentrations of rapamycin induced greater inhibitory effects. The cytotoxic and antiproliferative effect of rapamycin on MCF-7 cells was concentration dependent and was highly significant (P value < 0.001).Data are reported as means ± SD.

Table 1: the cytotoxic effect of different concentrations of rapamycin in MCF-7 cells

Rapamycin concentration	Inhibitory ratio %
6.2 µg/ml	1.8 ± 0.2
12.5 µg/ml	5.1 ± 0.36
25 µg/ml	16.6 ± 0.6
50 µg/ml	36± 1.0
75 µg/ml	51 ± 1
100 µg/ml	63.6 ± 1.5
200 µg/ml	84 ± 0.01
500 µg/ml	98 ± 0.01

**P value < 0.001*

Cell morphological analysis

Treatment of MCF-7cells with rapamycin at IC50 75µg/ml for 72 hrs. resulted in significant morphological changes compared with untreated cells as observed by inverted phase microscopy, cells treated with 75µg/ml of rapamycin showed features of apoptosis in the form of cell shrinkage and vascularization, also some cells became rounded and were floating in the media with some cell debris was observed in the culture media (figure 4B). While the untreated cells were flat polygonal with cell contacts, they were adherent and growing well (figure 4A).

Apoptotic analysis by flow cytometry

Annexin V/PI staining and flowcytometry were applied to determine whether the inhibitory effect of rapamycin on MCF-7 cell growth is due to induction of apoptosis or not. Cells were plated at a density of 1×105 cells/well in six –well tissue culture plates and treated

with rapamycin at IC50 which is 75µg/ml for 72 hrs. While only adding DMSO and culture media to control cells. Cells were harvested and stained with annexin V-FITC and PI followed by flowcytometry analysis.

Our study revealed that treatment of MCF-7 cells with rapamycin at IC50 for 72hrs resulted in an enhanced apoptosis and had significant effect ($p< 0.05$) compared to untreated cells. This experiment was performed in triplicate and the results were expressed as the mean percentage of apoptotic cells ± SEM. The analysis of annexin V/PI double stained cells was performed after gating on the homogenous cell population, the percentage of early and late apoptotic cell population was significantly increased from11.5±0.64 to 41.30±1.01.

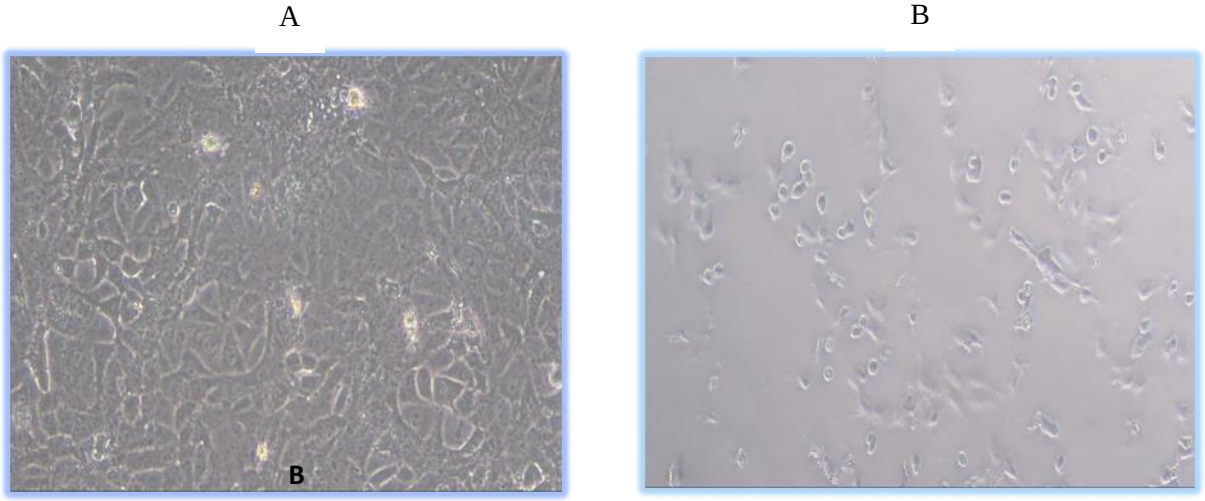

Figure (3): Morphology of MCF-7 cells visualized under inverted phase microscope
A) Untreated MCF-7 cells (40x objective), morphology of MCF-7 cells is flat polygonal with cell contacts. **B)** MCF-7 cells treated with rapamycin at IC_{50} for 72hrs. (40x objective), cells undergo shrinkage and become rounded.

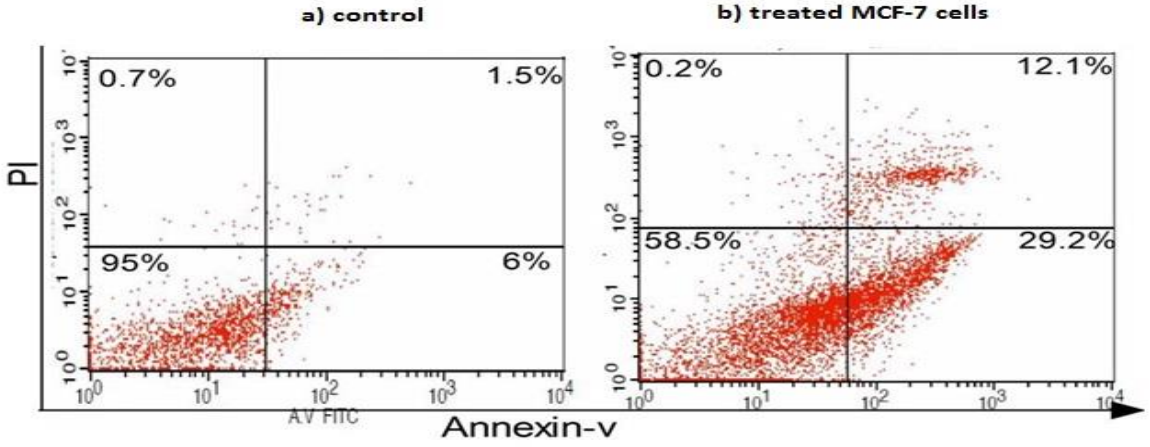

Figure (4): Annexin-V/PI dual staining assay of control **(a)** and rapamycin treated MCF-7cells **(b)** at IC50 which is 75µg/ml for 72 hrs. The y-axis represents the PI labelled cells while the x-axis represent annexin-V/FITC labelled cells. The left lower portion of the flouro-cytogram represents viable cells, the right lower portion represents cells in early apoptosis while the right upper portion represents cells in late apoptosis. The percentage of the apoptotic cell population (early and late apoptotic cells) was significantly increased from11.5±0.64 to 41.30±1.01 (the experiment was done in triplicate, data are reported as mean ± SD, $p < 0.05$).

Discussion:

Breast cancer is one of the most significant health problems worldwide and remains the most frequent cancer in women and the second most frequent cause of cancer deaths [4]

mTOR pathway is the key kinase that controls cell growth and proliferation in response to environmental conditions. mTOR activity is frequently de-regulated in a number of human cancers including breast, prostate, lung, liver, and renal carcinomas. Up regulation of mTOR signaling can lead to tumor growth and progression through diverse mechanisms including the promotion of growth factor receptor signaling, angiogenesis, glycolytic metabolism, lipid metabolism, cancer cell migration and suppression of autophagy [23].

Several cancers overexpress or have mutated forms of mTOR or some of the targets of mTOR kinase signaling. Thus, mTOR signaling has been recognized as a promising target for anticancer treatment [24].

Also, hyper activation of AKT (upstream effector of mTOR) and subsequent activation of mTOR may lead to resistance to endocrine therapy in breast cancer patients. Clinically, AKT activation is associated with worse prognosis. It was shown that there was an inverse correlation between activation of AKT and partial response rates [25].

Rapamycin because of its inhibitory effect on mTOR pathway, and on cellular growth, was explored as an anti-cancer agent. It was shown to inhibit cellular proliferation and/or be effective in several types of cancer including pancreatic and breast [26].

Si, Chu [27] demonstrated that Rapamycin significantly inhibited the proliferation of human VM endothelial cells at 48 and 72 hrs. Higher concentrations of rapamycin induced greater inhibitory effects.

Wang, Su [28] also reported that the proliferation of human retinoblastoma Y79 cells was inhibited by rapamycin in a dose-dependent manner after treatment of cells with rapamycin with different concentrations for 48 hrs.

Many rapalogues including rapamycin have now made their way into clinical use or are being investigated for therapeutic use in breast cancer patients. It has shown little efficacy in the treatment of breast cancer when used as a combination therapy. Phase II trial data in HER-2 positive patients suggested that adding rapamycin may benefit trastuzumab treatment [29], also the combination of resveratrol with rapamycin may stop Akt feedback activation in breast cancer cells [30].

Therefore, in this study Rapamycin was used to test its antitumor properties in MCF-7 breast cancer cells and to find if there is a correlation between growth inhibitory effect of rapamycin in MCF-7 cell line and mTOR gene deletion.

The antiproliferative activity was first detected by determination of the value IC50 of rapamycin which is critical for drug toxicology. Here, we showed that rapamycin decreased the percentage of viable MCF-7 cells with increasing drug concentrations (dose dependent inhibition). The IC50 value of rapamycin was determined as 75µg/ml by trypan blue dye exclusion method and confirmed by MTT assay.

This result agrees with Din T et al. who also reported that that rapamycin decreased the percentage of viable MCF-7 cells after treatment with various concentrations for 72 hrs. and that the effect was also dose-dependent [7].

Citi, Del Re [31] Citi et al. also reported that Everolimus, which is the hydroxyethyl derivative of rapamycin, also suppressed the proliferation of MCF-7 cell line in a concentration dependent manner after 120 hrs. of treatment.

Some studies also demonstrated the effect of combination therapies on inhibition of MCF-7 cell growth. Liu, Zhang [32] reported that treatment with everolimus or letrozole resulted in growth inhibition of stem cells in a dose dependent manner compared with single-agent therapy, the combination of everolimus with letrozole was more effective in the inhibition of cell growth ($p < 0.001$) and tumorigenicity ($p < 0.01$).

Significant apoptotic morphological changes were observed in our study in the form of blebbing, cellular shrinkage and loss of cell contacts in MCF-7 cells treated with rapamycin at IC 50 for 72 hrs. Compared to untreated cells when observed by inverted phase microscopy, a finding which could be explained by the findings of Thimmaiah, Easton [33] who reported that rapamycin can induce apoptotic cell death in part by blocking insulin like growth factor- I-mediated cell growth.

Our study found that treatment of MCF-7 cells with rapamycin at IC50 resulted in induction of apoptosis which was supported by flowcytometry where both early and late apoptotic cells found to be increased after 72 hrs. in comparison to untreated cells.

Our result is consistent with Tengku Din, Seeni [7] who have reported that rapamycin is an inducer of apoptosis and treatment of MCF-7 cells with rapamycin at IC50 for 72h resulted in an increased rate of late apoptosis and cell cycle arrest at the G1 checkpoint.

Also, these results were in agreement with Noh, Mondesire [14] who have stated that decreased cell growth induced by rapamycin could be attributable to decreased cell cycle progression or increased apoptosis. They examined cell line after 4 days of treatment with rapamycin and found that the percentage of cells in the G1 phase was increased in the rapamycin-sensitive cell lines including MCF-7 cell line.

These results also were in concordance with Kasukabe, Okabe-Kado [34] who reported that the sensitivity of rapamycin to MCF-7 cells was markedly affected by cotylenin A. This treatment induced growth arrest of the cells at the G1 phase, rather than apoptosis.

In addition, Geoerger, Hero [35] and Grunwald, DeGraffenried [36] reported that rapamycin enhances the cytotoxicity of chemotherapeutic agents support the hypothesis that rapamycin induces or enhances apoptosis in certain tumor types or conditions.

Rapamycin also found to induce apoptosis in other tumor types, Si, Chu [27] reported that rapamycin enhanced apoptosis conditions in human VM

endothelial cells, exposed to rapamycin at different concentrations. In comparison with the blank group, apoptosis of human VM endothelial cells was significantly elevated when treated with rapamycin at 1, 10, 100 and 1,000 ng/ml.

Wang, Su [28] also reported that Rapamycin induced G1 cell cycle arrest in human retinoblastoma Y79 cells.

Conclusion:

In conclusion, our study demonstrated that rapamycin has antitumor properties and can potentially act as an anticancer agent via the inhibition of growth with some morphological changes of the MCF-7 cancer cells, and induction of apoptosis both in early and late stages .further studies are needed to characterize the mode of action of rapamycin.

List of Abbreviations

AKT: akt murine thymoma viral oncogene/protein kinase B
ER: Estrogen receptor
FRAP: FKBP-12- rapamycin-associated protein
HER2: Human epidermal growth factor receptor 2
IC50: Half-maximal inhibitory concentration
PAM: Phosphoinositide 3 kinase (PI3K)/Akt /mammalian target of rapamycin
PI3K: Phosphoinositide 3 kinase
PI3K: Phosphoinositide 3 kinase
mTOR: Mammalian/mechanistic target of rapamycin
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NCRP: National Cancer Registry Program
PIK3CA: phosphatidylinositol 4, 5 –biphosphate 3 - kinase catalytic subunit alpha
PTEN: phosphatase and tensin homologue deleted on chromosome ten
TNF: Tumor necrosis factor

Availability of data and materials

The data used in the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors report no conflicts of interest associated with this work

Authors' Contributions:

WT: performed study design, interpreted and analyzed the data and was the major contributor in writing the manuscript.
AS: performed study design, analyzed, interpreted the data and was the major contributor in statistical analysis of data.
NH: was the major contributor in analysis of results of flow cytometry.
EM: was the major contributor in analysis of results of FISH technique, draft manuscript preparation.
NAH: helped conduct of the study.
SGM: helped conduct of the study.
All authors read and approved the final manuscript.

Consent for publication

The consent to publish had been taken from each participant in this work.

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Competing interests

The authors declare that they have no competing interests

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