

RESEARCH ARTICLE

Effects of Rapamycin on Cell Apoptosis in MCF-7 Human Breast Cancer Cells

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Abstract

Background: Rapamycin is an effective anti-angiogenic drug. However, the mode of its action remains unclear. Therefore, in this study, we aimed to elucidate the antitumor mechanism of rapamycin, hypothetically via apoptotic promotion, using MCF-7 breast cancer cells. **Materials and Methods:** MCF-7 cells were plated at a density of 1×10^5 cells/well in 6-well plates. After 24h, cells were treated with a series of concentrations of rapamycin while only adding DMEM medium with PEG for the control regiment and grown at 37°C, 5% CO₂ and 95% air for 72h. Trypan blue was used to determine the cell viability and proliferation. Untreated and rapamycin-treated MCF-7 cells were also examined for morphological changes with an inverted-phase contrast microscope. Alteration in cell morphology was ascertained, along with a stage in the cell cycle and proliferation. In addition, cytotoxicity testing was performed using normal mouse breast mammary pads. **Results:** Our results clearly showed that rapamycin exhibited inhibitory activity on MCF-7 cell lines. The IC₅₀ value of rapamycin on the MCF-7 cells was determined as 0.4µg/ml (p<0.05). Direct observation by inverted microscopy demonstrated that the MCF-7 cells treated with rapamycin showed characteristic features of apoptosis including cell shrinkage, vascularization and autophagy. Cells underwent early apoptosis up to 24% after 72h. Analysis of the cell cycle showed an increase in the G0/G1 phase cell population and a corresponding decrease in the S and G2M phase populations, from 81.5% to 91.3% and 17.3% to 7.9%, respectively. **Conclusions:** This study demonstrated that rapamycin may potentially act as an anti-cancer agent via the inhibition of growth with some morphological changes of the MCF-7 cancer cells, arrest cell cycle progression at G0/G1 phase and induction of apoptosis in late stage of apoptosis. Further studies are needed to further characterize the mode of action of rapamycin as an anti-cancer agent.

Keywords: Rapamycin - MCF-7 breast cancer cells - apoptosis

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Introduction

Breast cancer is the commonest type of cancer that causes high morbidity and mortality in US women (Centers for Disease Control and Prevention, 2010). In 2000, 1,050,346 cases of breast cancer were reported with 372,969 deaths occurred worldwide. In Malaysia, a report from the College of Radiology, Academy of Medicine of Malaysia identified breast cancer as the most common cancer as well as the most common female cancer in all races ages of 20 years from 2003 until 2005. A report that was published by the National Cancer Registry (NCR) in 2003-2005 mentioned that the total 67,792 new cases were diagnosed among 29,596 males (43.7%) and 38,196 females (56.3%). The most frequent cancer during this period was breast cancer (18%) followed by large bowel cancer (11.9%) and lung cancer (7.4%).

Normal breast development is governed by the

equilibrium between cell proliferation and apoptosis, and there is a strong evidence that tumour growth is not just a consequence of uncontrolled proliferation but also of reduced apoptosis. The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumour in response to chemotherapy and radiotherapy. Apoptosis or programmed cell death (PCD) is an active, energy-dependent process of cell death, which occurs during development, in response to certain physiologic stimuli, and secondary to cell injury and stress (Gewies, 2003; Elmore, 2007; Giansanti et al., 2011). The purpose of this type of cell death is for regulating the removal of damaged and unwanted cells in discrete tissues, a critical function in embryonic development and normal tissue homeostasis. It differs from necrotic cell death because the damaged cells are eliminated by PCD in a fashion, without eliciting intense inflammatory response (Ashkenazi and Salvesen, 2014).

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Rapamycin is a drugs that use as alternative mechanisms to inhibit the breast cancer growth (Yu et al., 2001; Noh et al., 2004; Idris et al., 2014). Rapamycin, a macrolide fungicide, was first isolated from *Streptomyces hygroscopicus* in the early 1970s and was originally known to have potent immunosuppressant properties (Noh et al., 2004). As a result, lots of interest was placed upon rapamycin as a potential antitumor drug which eventually led to further endeavours for therapeutic development (Law, 2005). Rapamycin was originally used as an antifungal agent and a suppressor of the immune system, rapamycin has recently been studied as an anticancer agent. In breast cancer, rapamycin offers significant promise as many of the pathways dysregulated in this disease are mTOR related (Hidalgo and Rowinsky, 2000). The most pathways studied is the P13K/AKT/mTOR pathway (Khemapech et al., 2012), which can be activated by different membrane tyrosine kinase receptors, including the epidermal growth factor (EGFR) family of growth receptors, insulin-like growth receptors (IGF-R) and ER α (Gibbons et al., 2009).

The MCF-7 cell line is an ideal model to study the pathway of malignant progression (Aranovich et al., 2012). Prior to the MCF-7 discovery, there was no mammary cell line that could live longer than a few months. MCF-7 has diverse appearances of differentiated mammary epithelium such as the capability of forming domes and the capacity to process estradiol via cytoplasmic estrogen receptors (Bateman et al., 2010; Yaacob et al., 2013). TNF alpha inhibits the growth of MCF-7 breast cancer cell line (Wang et al., 2012).

Rapamycin is an effective anti-angiogenic drug (Guba et al., 2002). However, its mechanistic properties still remain unclear. Therefore, we aim to elucidate the antitumor mechanism of rapamycin, hypothetically via apoptotic promotion, using MCF-7 breast cancer cell lines.

Materials and Methods

Cytotoxicity test

Approximately 1×10^5 normal breast cells were seeded in each well of 6-well plates. After an overnight incubation, the rapamycin at 50% inhibitory concentration (IC_{50}) was added and cells incubated for 72h. Untreated and treated cells were examined for morphological changes by inverted phase contrast microscope. Number of viable cells were counted under an inverted microscope using hemocytometer by Trypan Blue Exclusion Assay (TBEA) (Lai et al., 2011). The viability of the untreated and treated cells was compared and the cytotoxicity of the compound on the cells was determined.

Cell lines and Culture

The MCF-7 or Human Breast Adenocarcinoma cancer cell line was used throughout this study. This cell line was obtained from the American Type Culture Collection (ATCC) (Product # HTB-22). Stock culture of MCF-7 cells was grown in T25-cm² tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) as a differentiation media, supplemented with 0.5% (v/v) fetal bovine serum, and 1% (v/v) penicillin and streptomycin.

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% CO₂. The cells were passed twice a week.

IC₅₀ determination

MCF-7 cells were plated at a density of 1×10^5 cells/well in 6-well plates. After 24h, cells were treated with a series of concentrations of Rapamycin with DMEM medium was only added to the untreated division. These cells were subsequently grown at 37°C, 5% CO₂ and 95% air for 72h. To determine cell viability, the trypan blue exclusion assay was used using 20 μ l of cell suspension in DMEM medium which were mixed with 20 μ l of trypan blue. The numbers of stained (dead cells) and unstained (live cells) cells were subsequently counted using a hemocytometer.

Cell proliferation assay

Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. After 1 day, rapamycin at IC_{50} concentration was added into the wells. Untreated were exposed to culture medium without rapamycin. Then, the cells were further incubated for 8 days. Cell proliferation was evaluated using a trypan blue exclusion method. Cells were counted every day until the eighth day to identify the growth pattern of the treated and untreated cells. Results were expressed as a percentage of the control.

Cell Morphological analyses

Cells were plated in 6-well plates at the density of 1×10^5 cells/well and grown for 24h. Rapamycin at IC_{50} concentration was again added and the cells were then grown at 37°C in a humidified 5% CO₂ for 72h. Untreated MCF-7 cells were examined for morphological changes with a phase contrast microscope later on.

Flow cytometric analysis

Exponentially growing cells were treated with the rapamycin at the inhibitory concentration (IC_{50}) for 72h. Specimens were collected and prepared in triplicate by FITC Annexin V Apoptosis Detection Kit I for apoptosis analysis and by Cycle TEST PLUS DNA Reagent Kit for cell cycle analysis and analyzed by flow cytometer (BD FACSCanto II) immediately.

Statistical analysis

Data values were expressed as means \pm SD. Differences were compared by one-way analysis of variance (ANOVA) followed by Bonferroni correction. P values were considered to be statistically significant when $p < 0.05$.

Results

Effect of rapamycin on cell viability

The effects of various concentrations of rapamycin on the growth of MCF-7 cells were examined by TBEA and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer. Rapamycin concentrations at the dose of 0.1, 0.2, 0.3,

0.4, 0.5, 0.6 and 0.7 $\mu\text{g/ml}$ (Figure 1). The IC_{50} value of rapamycin in the MCF-7 cells was determined as 0.4 $\mu\text{g/ml}$ and significantly different ($p < 0.05$). Data are reported as means \pm SD of representative experiments.

Cell proliferation assay

The treatment with the rapamycin at inhibitory concentration (IC_{50}) were significantly suppressed the viability of the cell concentration at the time-dependent manner until eight days. The percentage different of treated and untreated cells was almost 70% (Figure 2).

Cell morphological alterations

Direct observations by inverted microscopy demonstrated that the MCF-7 cells treated with rapamycin showed some morphological changes compared with the untreated cells. Rapamycin-treated MCF-7 cells exhibit characteristic features of apoptosis, including cell shrinkage, vascularization and autophagy features-related to apoptosis. After treated with 0.4 $\mu\text{g/ml}$ after 24h, some cells were floating and become rounder and smaller and refraction also decreased (Figure 3C). Some cell debris was observed in the medium (Figure 3C). The cells left on the wall became rounder and smaller (Figure D). In the untreated MCF-7 most cells were adhesive and grown well (Figure 3A). The MCF-7 cells left underwent significant changes in morphology; the original shapes was gone, cells became rounder and larger and the cytoplasm became rougher (Figure 3D).

Apoptosis and cell cycle analysis

The flow cytometric analysis was used to characterize the activities of rapamycin apoptosis inducing in MCF-7 cells as shown in Figure 4. The rapamycin was known as an apoptosis inducer in MCF-7 cells, as shown in Figure 4 by an increasing of apoptotic activity almost 24% in the

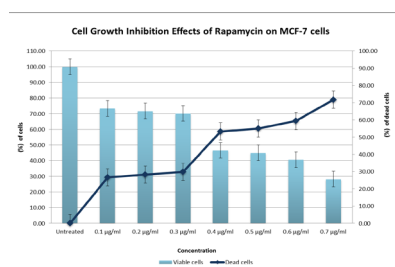


Figure 1. Effect of Rapamycin on the Growth of MCF-7 Cells. Cell growth inhibition effects in the MCF-7 cells treated with Rapamycin. Cells were treated with different concentrations of rapamycin for 72hrs. The percentage cell death was calculated from the untreated (data are reported as means \pm SD)

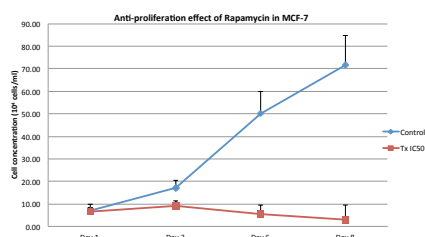


Figure 2. Anti-proliferative Effect of the Rapamycin (Data are Reported as means \pm SD)

late apoptosis stage in cells treated with the rapamycin. The patterns of the cell cycle were also analyzed by the flow cytometry. As shown in figure 5 there was an increase in the G0G1 phase cell population and a corresponding decrease in the S and G2M phase populations. After 72h of treatment, the percentage of cells in the G0G1 phase increased from 81.5% to 91.3% c and a corresponding decrease in the S and G2M phase populations from 17.3% to 7.9% compared to untreated cells. Rapamycin extract at concentration 0.4 $\mu\text{g/ml}$ induced accumulation and delayed MCF-7 cells transition from the G0G1 phase of the cell cycle.

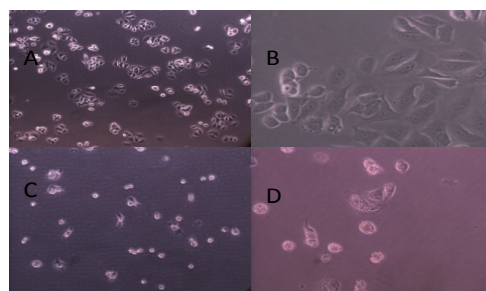


Figure 3. Morphology of MCF-7 Cells Visualized under Phase-contrast Microscope. **A:** Untreated MCF-7 cell (10x), **B:** Untreated MCF-7 cell (40x), **C:** Rapamycin treated MCF-7 cell (10x) and **D:** Rapamycin treated MCF-7 cell (20x). The morphology of untreated cell is flat polygonal with cell-cell contacts (B) meanwhile rapamycin treated cell has lose its original shape and shrink (C,D)

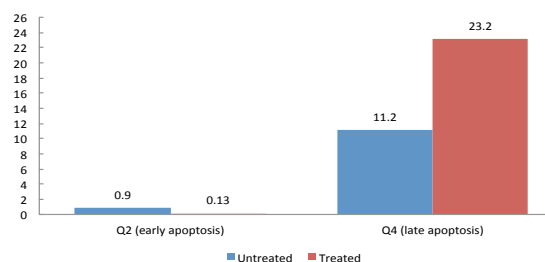


Figure 4. Cells Undergo Apoptosis after Cell Cycle Arrest at the Cell Cycle Phase Treatment with Rapamycin at 0.4 $\mu\text{g/ml}$ Extract Resulted in an Increase of the Cells in the Early Apoptosis up to 23.2% after 72h

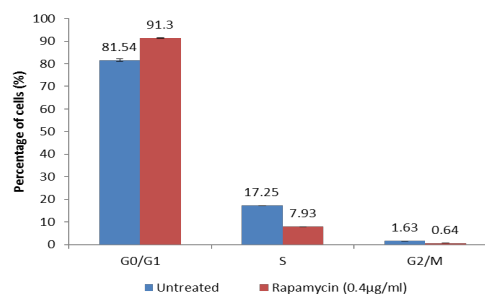


Figure 5. Treatment Resulted in an Increase in the G0G1 Phase Cell Population and a Corresponding Decrease in the S and G2M Phase Populations. After 72h of treatment, the percentage of cells in the G0G1 phase increased from 81.5% to 91.3% compared to control. Rapamycin extract at concentration 0.4 $\mu\text{g/ml}$ induced accumulation and delayed MCF-7 cells transition from G0G1 phase of the cell cycle

Cytotoxicity test

The cytotoxic activity of the rapamycin was determined on normal breast mouse mammary pad cells. A viability of 97.5% of cells treated with the compound was perceived, we found that results show not significantly different from the viability of untreated cells. Absence of effect of the compound on the cell morphology and cell number clearly showed that the compound is non-cytotoxic to the normal cells at an inhibitory concentration (IC_{50}).

Discussion

Rapamycin is currently in clinical trials as an anticancer agent that has the potential to potently inhibit tumor cell proliferation by using the mTOR pathway (Noh et al., 2004; Rizzieri et al., 2008). Although rapamycin show activity in clinical trials, however, only a few of the treated patients respond. Therefore, in this study, rapamycin was used to investigate the apoptosis activity in MCF-7 breast cancer cell line. Previous reports suggested that rapamycin could induce the differentiation of human myeloid leukaemia cells (Sykes et al., 2011) (Hashemolhosseini et al., 1998), lung cancer (Boffa et al., 2004) and breast cancer (Noh et al., 2004). Rapamycin has now been considered as an effective immunosuppressant (Law, 2005). Moreover, as in post-transplant immunosuppression protocols, rapamycin is presently used as one of the constituents in a multidrug treatment to prevent graft rejection (Rizzieri et al., 2008). Law (2005) proposed that rapamycin has a potent anti-tumor action against certain tumor cell types. Current studies have shown that rapamycin demonstrated very effective anti-tumor effects in a large variety of cancers such as lung cancer (Boffa et al., 2004), gastrointestinal cancer (Wiedmann and Caca, 2005), renal cell carcinoma (Rathmell, Wright and Rini, 2005) and others. Several studies have shown that rapamycin can induce apoptotic cell death of some tumor cells, partly by blocking insulin-like growth factor-I-mediated cell growth (Mahalati and Kahan, 2001).

The antiproliferative activity of rapamycin was first determined by the value of IC_{50} that was derived from various concentrations of rapamycin tested in MCF-7 cells. Here, we showed that rapamycin decreased the percentage of viable MCF-7 cells and that the effect is dose-dependent. The concept of IC_{50} is fundamental to drug toxicology which is defined as the concentration of an inhibitor where the response (or binding) are reduced by half. From this study, we have shown in Figure 1 that 50 percent of MCF-7 cell dies when rapamycin concentration was at $0.4\mu\text{g/ml}$, signifying rapamycin's strong antiproliferative activity. Hence, the IC_{50} of rapamycin was established to be at $0.4\mu\text{g/ml}$.

MCF-7 has been reported the first hormone-responsive breast cancer cell line (Simstein et al., 2003) and respond to estrogens and anti-estrogens, differential expression of estrogens receptor (ER), ER mRNA, and progesterone receptor and differences in tumorigenicity and proliferation rate. Clinical studies have shown that therapeutic agents preventing the synthesis and activity of estrogens are essential in the breast cancer treatment. The addition of

estradiol which is one of the fractions of estrogen to the medium of MCF-7 cells induces a proliferative response (Hamelers et al., 2003). The characteristics of MCF-7 cells like the estradiol-dependence of growth and low metastatic potential has led to the statement that they represent an early epithelial adenocarcinoma of breast (Osborne et al., 1987).

Apoptosis is a progressive programmed cell death that takes place in multicellular organisms. Biochemical measures direct to attribute cell morphological changes and death (Zhang et al., 2005). These changes consist of blebbing, cellular vacuolization, defeat of cell membrane irregularity and attachment, cellular shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Simstein et al., 2003). Therefore, induction of vacuolization and cellular shrinkage of the treated cells as observed in our study are significant features of apoptotic cell death. Direct observations by inverted microscopy demonstrated that the MCF-7 cells treated with rapamycin showed morphological changes of cells undergoing apoptosis when compared to the untreated cells.

Rapamycin prevented cell cycle progression of MCF-7 cells from G1 to S phase and decreased their proliferation. Our observation extends prior observations that cell cycle arrest by rapamycin occurs at the G1 checkpoint (Hashemolhosseini et al., 1998). Our IC_{50} for rapamycin was $0.4\mu\text{g/ml}$, whilst Mahalati et al, 2001 reported that the therapeutic range of rapamycin concentration is between 5 and $15\mu\text{g/L}$ for transplant recipients (Mahalati and Kahan, 2001). Thus we can hypothesize that rapamycin exhibited anti-proliferative potential at a much lower concentration than the concentration required to prevent transplant rejection. Besides that, other studies have also shown that the major mechanism by which rapamycin suppressed tumor cell growth was by inhibiting of cell cycle progression in the G1 phase (Hashemolhosseini et al., 1998; Kawamata et al., 1998).

Rapamycin has been shown to induce apoptotic cell death of some tumor cells, in part by blocking insulin-like growth factor-I-mediated cell growth (Thimmaiah et al., 2010). Our assessment, by using of Annexin V binding to externalized phosphatidylserine as a marker of apoptotic cells, has successfully identified apoptosis as a mechanism for the rapamycin-induced reduction of tumor progression in MCF-7 cell line. Our observation found that MCF-7 cells undergo apoptosis after cell cycle arrest at the cell cycle phase treatment with rapamycin at $0.4\mu\text{g/ml}$ extract resulted in an increase of the cells in the late apoptosis up to 23.2% after 72h. Rapamycin also found to induce apoptosis in certain tumor types, they reported that rapamycin induced apoptosis in p53-deficient rhabdomyosarcoma cell as a consequence of continued cell cycle progression during mTOR inhibition (Hosoi et al., 1999; Huang et al., 2001). Furthermore, the reports that rapamycin enhances the cytotoxicity of chemotherapeutic agents support the hypothesis that rapamycin induces or enhances apoptosis in certain tumor types or conditions (Georger et al., 2001; Grünwald et al., 2002).

As a conclusion, this study demonstrated that rapamycin may potentially act as an anti-cancer agent

via the inhibition of growth with some morphological changes of the MCF-7 cancer cells, arrest cell cycle progression at G0/G1 phase and induction of apoptosis in late stage of apoptosis. Further studies are needed to further characterize the mode of action of rapamycin as an anti-cancer agent.

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