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Rapamycin sensitizes Akt inhibition in malignant human breast epithelial cells

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Abstract

Akt and mTOR are therapeutic targets for the treatment of cancer. The effects of inhibiting mTOR, with rapamycin, and Akt, with A-443654, concurrently, on cell morphology, cell proliferation, the cell cycle, and apoptosis were examined using the benign MCF10A and malignant MCF10CA1a human breast epithelial cells. Rapamycin and A-443654 in combination produced the greatest morphological changes and inhibited cell proliferation by G2/M arrest. Rapamycin and A-443654 in combination induced apoptosis at earlier times and at lower A-443654 concentrations in MCF10CA1a tumor cells than in the benign MCF10A cells. Rapamycin and A-443654 increased p53 and p15^{INK4B} protein levels, decreased anti-apoptotic Bcl-2 levels, and increased Bad levels in the MCF10CA1a tumor cells by ~ 5-fold. These results suggest that the combined inhibition of Akt and mTOR may have beneficial therapeutic and safety margin effects.

Keywords

combined mTOR and Akt inhibition; rapamycin; MCF10CA1a cells; combined mTOR and Akt inhibition effects on MCF10A and MCF10CA1a cell morphology; cell cycle and apoptosis; combined effects of mTOR and Akt inhibition on cell function

1. Introduction

Breast cancer is the second leading cause of cancer-related deaths among women in the United States. The estimated rate of developing breast cancer in American women is about one in seven [1]. Most patients die from breast cancer metastasis rather than the primary tumor. The goal of therapy is to delay the progression of disease and to prolong survival [2]. Because the therapeutic options are becoming increasingly complex, in order to achieve high efficacy with minimal toxicity, the use of adjuvant therapy regimens are developing rapidly [3].

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Conflicts of Interest

None of the authors have any conflict of interests with the institution, financially or with other relationships that may inappropriately influence the author's work.

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The MCF10A human breast epithelial cell lineage includes the non-transformed benign 10A cell line and the 10CA1a tumor cell line [4]. The 10CA1a cell line is poorly-differentiated and invasive [5;6;7]. In this study, the benign 10A and the 10CA1a tumor cells were used to examine the effects of inhibiting Akt and mTOR, two kinases that play a critical role in regulating cell growth, cell proliferation, the cell cycle, and apoptosis.

During breast cancer development, overactivation of the PI3K/Akt/mTOR survival pathway is frequently observed. Akt is a serine/threonine kinase, which is activated by upstream PI3K, and regulates the expression of genes involved in cell function, including apoptosis. Progressive elevation of phosphorylated Akt levels has been detected in the 10A, 10AT and 10CA1a cells [8] and frequently occurs in breast cancer [9]. One of the major downstream targets of Akt is mTOR (mammalian target of rapamycin), which is a serine/threonine kinase that regulates translation through its downstream targets, the ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Thus, mTOR regulates cell growth, cell proliferation and cell cycle progression [8;9]. The mTOR inhibitor rapamycin was initially developed as an immunosuppressant. However, rapamycin and its analogues have been identified as promising anti-tumor drugs [10;11;12]. Rapamycin directly binds to FK506 binding protein 12 (FKBP12), and this complex then binds to mTOR, inhibiting the mTOR-raptor interaction [13]. By inhibiting mTOR, rapamycin decreases protein synthesis and inhibits cell growth, proliferation and survival [14]. Currently, rapamycin and its derivatives, such as CCI-779 [15], RAD-001 [16] and AP-23573, are being tested in phase I or II clinical trials [8]. Thus, inhibition of overactivated Akt and mTOR simultaneously by specific inhibitors constitutes a potential approach for treatment of breast cancer.

A-443654, a novel Akt inhibitor, is a derivative of indazole-pyridine compounds which bind to the ATP binding site of Akt. The binding of A-443654 to Akt is potent, ATP competitive and reversible [17]. A-443654 is a pan Akt inhibitor with equal potency against Akt1, Akt2 and Akt3 isoforms, but it is highly selective for Akt versus other kinases [17]. A-443654 greatly slows the growth of tumors and induces apoptosis in various cell lines and *in vivo* tumor models [17]. Thus, A-443654 inhibition of Akt may be useful in breast cancer therapy. The therapeutic window of A-443654 is narrow, however, with an effective dose only 2-fold lower than the maximal tolerated dose. In addition, cessation of A-443654 results in rapid re-growth of the tumors examined in several tumor models [17], suggesting that A-443654 is cytostatic.

It has been reported that rapamycin is able to induce Akt-Ser 473 phosphorylation in a subset of cancer cell lines through inactivation of S6K1, and phosphorylation takes place in a delayed manner [18]. The mechanism of Akt phosphorylation induced by A-443654 is completely different from the mechanism of rapamycin-mediated Akt phosphorylation through mTORC2. Akt phosphorylation induced by A-443654 is extremely rapid and robust, which is independent of mTORC1 inhibition, requires PI3K activity and does not require S6K1 inhibition [18]. Since Akt and mTOR are both downstream targets of PI3K, and rapamycin is much less toxic than A-443654, the combination of these agents may lower the therapeutic dose of A-443654 required for efficacy and consequently decrease its toxicity within its effective dose range. Our hypothesis is that the combination of rapamycin and A-443654 can improve A-443654 efficacy in breast tumor cells and simultaneously decrease its toxicity, such that lesser effects are observed in benign cells, while maintaining or increasing efficacy in tumor cells. In support of this hypothesis, studies examining the combined effects of rapamycin and A-443654 on tumors in a MiaPaCa-2 pancreatic cancer xenograft explant scid mouse model, revealed that efficacy was better for the combination of rapamycin and A-443654 than either agent alone [17].

The effects of rapamycin in combination with A-443654 on cell morphology, cell proliferation, cell cycle progression and apoptosis, using the benign MCF10A and malignant 10CA1a cells have been examined. The results of this study reveal that rapamycin increases the efficacy of A-443654 in causing morphologic changes, cell cycle arrest and apoptosis, and that the malignant 10CA1a cells are more sensitive to the combination of rapamycin and A-443654 than the benign 10A cells, suggesting this combination may have beneficial therapeutic effects with less toxicity.

2. Materials and methods

2.1. Cell culture

The MCF10A and MCF10CA1a cell lines were obtained from Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI). MCF10A cells, the progenitor line of the MCF10A cell series, are spontaneously immortalized breast epithelial cells obtained from a woman with fibrocystic breast disease [19]. The 10A cells were transfected with a mutated T₂₄ Ha-Ras gene to generate the 10AT cells [20]. The 10CA1a cell line was generated from a xenograft developing from a MCF10AT lesion and selected for sequential passage by trocar isolation and passaged through two additional generations before cells were established in culture. The 10CA1a cells are malignant and invasive, whereas the 10A cells are benign and used as controls.

The MCF10A series of cells were cultured in Dulbecco's Modified Eagle Medium/F-12 medium (DMEM/F-12, Invitrogen, Carlsbad, CA) supplemented with 10 µg/mL of human insulin (Invitrogen), 20 ng/mL of epidermal growth factor (Invitrogen), 0.5 µg/mL of hydrocortisone (Sigma, St. Louis, MO), 5% horse serum (Invitrogen), 100 U/mL of penicillin (Invitrogen), and 100 µg/mL of streptomycin (Invitrogen). Cells were maintained in a humidified environment of 5% CO₂/95% air at 37°C as described previously [21]. The cell lines were passaged and cultured in 60 mm tissue culture dishes and were 80% confluent at time of harvest.

2.2. Cell morphology assay

Following treatment, the cells were visualized and photographed with an Olympus PD70 imaging system (Olympus America Inc.). Phase contrast images were taken at 200x and 200×1.6 magnification.

2.3. MTT assay

MCF10A cells (2.5×10^4) and 10CA1a cells (1.0×10^5) were seeded in 60mm dishes and were cultured with rapamycin (CALBIOCHEM, San Diego, CA) for 24, 48 and 72 h. Cells treated with DMSO (Sigma, St. Louis, MO) were used as control. The relative percentage of metabolically active cells was determined on the basis of mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazine, monitored at A550 nm. The percentage of metabolically active cells was compared with the percentage of control cells treated with DMSO.

2.4. Cell proliferation assay

MCF10A cells (2.5×10^4) and 10CA1a cells (1.0×10^5) were seeded in 60mm dishes and were cultured with DMSO or rapamycin for 48 h. A-443654 (obtained from Dr. Giranda, Abbott Laboratories, Abbott Park, IL) then was added to cells at different concentrations for various time periods. Controls consisted of the respective cells treated with DMSO. Viable cell numbers were determined using the Trypan blue dye exclusion assay.

2.5. Cell cycle analysis by flow cytometry

MCF10A cells (1.0×10^5) and 10CA1a cells (1.5×10^5) were plated in 60mm dishes and were treated with DMSO or 100 nM rapamycin for 48 h. Subsequently, 1, 2 or 5 μ M A-443654 was added for 4, 8 or 12 h. Cells were harvested by trypsin/0.1% EDTA. Cells were centrifuged for 5 min at 500 g at 4°C, washed and fixed with 75% ethanol overnight, and resuspended in 1 ml of propidium iodide staining solution for 1 h at room temperature. Cell cycle distribution was determined by analyzing 10,000 cells using a FACScalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). *2.6 PARP cleavage:*

MCF10A cells (1.0×10^5) and 10CA1a cells (1.5×10^5) were plated in 60mm dishes and treated with DMSO or 100 nM rapamycin for 48 hrs. Subsequently, 1, 2 or 5 μ M A-443654 was added for 4, 8 or 12 h and cell lysates isolated. Protein lysates (20 μ g) were separated by SDS-PAGE using a 7.5% gel and transferred to nitrocellulose membrane as described below. Blots were probed with anti-poly adenosine diphosphate-ribose polymerase (PARP) antibody (Cell Signaling Technology, Beverly, MA) overnight and protein bands were detected with a horseradish peroxidase-conjugated secondary antibody. When PARP is cleaved, an 85kd band is generated during apoptosis. Cells treated with mouse anti-Fas monoclonal antibody (Fas mAb clone CH11; Upstate Biotechnology, Lake Placid, NY), which causes apoptosis, were used as positive controls.

2.6. Immunoblot analysis

Immunoblot analysis of whole-cell lysates was used to examine p53, cyclin D1, cyclin E, p15^{INK4B}, p21^{Cip/WAF1} and P27^{kip1}, Bcl-2, Bcl-X_L, Bad and Bax protein levels (antibodies from Cell Signaling Technology, Beverly, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Beverly, MA) was used as an internal standard for normalization of protein loading in the immunoblot analysis. Protein lysates (20 μ g) were electrophoresed on 10% or 15% SDS-PAGE gels, and were transferred to nitrocellulose membrane (Pall Corporation, Ann Arbor, MI). After blocking in 5% milk in TBST (20 mM Tris-HCl and 500 mM NaCl, pH 7.5; 0.1% Tween 20), the membranes were incubated with the appropriate diluted primary antibodies in 5% bovine serum albumin (BSA) in TBST at 4°C overnight with gentle shaking, followed by incubation with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. The protein bands were detected by chemiluminescence (Amersham Life Science Inc., Piscataway, NJ) on Kodak X-OMAT film (Sigma). Exposed films were scanned and the band density was quantified using KODAK 1D Image Analysis (Eastman Kodak Company, Rochester, NY) equipped with analysis software program. The levels of proteins were normalized to GAPDH.

2.7. Statistical analysis

Statistically significant differences ($p < 0.05$) between groups were determined by analysis of variance, followed by a Tukey-Kramer multiple comparison analysis using PRISM software (version 3.0).

3. Results

3.1. Effects of rapamycin and A-443654 alone or in combination on cell morphology

The effect of rapamycin or A-443654 alone, and in combination, on the morphology of the benign 10A cells and 10CA1a tumor cells was examined. Rapamycin did not induce any notable morphological changes in either the 10 or 10CA1a cell line up to 48 h. Consequently, all studies were performed with rapamycin present for 48h. The Akt inhibitor A-443654 alone, however, was able to produce morphological changes in both 10A and 10CA1a cells rapidly, which largely consisted of cell retraction and blebbing, as previously

reported [22]. A-443654-induced morphological changes occurred very rapidly (within 2 to 4 h) in both 10A and 10CA1a cells (data not shown), with 10CA1a cells more sensitive to A-443654 than the 10A cells. A-443654 alone at 2 μ M caused the 10CA1a cells, but not the 10A cells, to detach from the plate after 8 h, whereas 1 μ M of A-443654 caused 10CA1a cells to detach from the plate after 12 h (Fig. 1A). In contrast, 10A cells only detached from the plate at 5 μ M of A-443654 after 12 h, and even then the number of detached cells was much less than that of the 10CA1a cells (Fig. 1A).

The combination of rapamycin with A-443654 sensitized cells to undergo morphological changes more rapidly and at lower concentrations of A-443654. With rapamycin pretreatment of cells (100 nM for 48 h), A-443654, at 1 μ M, caused a small percentage of 10CA1a cells to detach from the plate at 8 h, and substantial numbers of 10CA1a cells to detach from the plate at 12 h (Fig. 1B). Virtually all 10CA1a cells were floating in the media with rapamycin and 5 μ M A-443654 at 12 h (Fig. 1B). The 10A cells that were pretreated with rapamycin and then treated with A-443654 were induced to detach from the plate at 5 μ M of A-443654 at 8 h, or were induced to detach from the plates at 2 μ M A-443654 at 12 h (Fig. 1B). These data show that 10CA1a tumor cells are more sensitive to the effects of inhibiting Akt with A-443654 than the benign 10A cells, and that rapamycin sensitizes both the 10A and 10CA1a cells to undergo morphologic changes at lower A-443654 concentrations and an increased response in A-443654-induced cell death (Fig. 1A and B).

3.2. Effects of A-443654 or rapamycin alone and in combination on cell proliferation

To examine the effects of rapamycin and A-443654 alone and in combination, on cell proliferation, 10A and 10CA1a cells were treated with rapamycin for 48 h, in the presence or absence of A-443654 at 1 or 5 μ M, and cell proliferation monitored over the period 4 to 24 h. Rapamycin alone inhibited 10A cell proliferation by ~30% at 24 h, and by >50% at 48h (Fig. 2A). Rapamycin inhibition of 10A cell proliferation was more efficacious than that of the 10CA1a cells (Fig. 2A). The Akt inhibitor A-443654 inhibited 10A and 10CA1a cell proliferation in a concentration-dependent manner (Fig. 2B). The 10CA1a tumor cells, however, were more sensitive to the growth inhibitory effects of A-443654 than the 10A cells. A-443654 greatly decreased 10CA1a cell proliferation after 8 h, but did not significantly decrease 10A cell proliferation, as compared to the respective DMSO controls (Fig. 2B). The combination of rapamycin and A-443654, however, markedly inhibited both 10A and 10CA1a cell proliferation relative to cells treated with A-443654 alone. In addition, 10CA1a cell proliferation was inhibited to a greater extent by the combination treatment as compared to the benign 10A cells. The number of existing viable 10CA1a cells following the treatment of cells with rapamycin and A-443654, at either 1 or 5 μ M, was substantially decreased at 8h and virtually no viable cells existed at 24 h (Fig. 2B).

3.3. Rapamycin and A-443654 in combination alter the cell cycle

Examination of the effects of rapamycin and A-443654 on the cell cycle was performed with a view of identifying the mechanism(s) underlying the inhibitory effects of the combination of rapamycin and A-443654 on cell proliferation. Because no notable morphologic changes, but anti-proliferative effects, were observed with rapamycin at 48 h, the treatment of cells with rapamycin was performed for 48h prior to addition of A-443654. The 10A and 10CA1a cells were treated with 1, 2 or 5 μ M A-443654 for 4, 8 or 12 h, in the presence or absence of rapamycin, and the effects on the cell cycle was analyzed by flow cytometry. Rapamycin alone at 48h slightly increased S phase in the 10A cells and marginally decreased S phase in the 10CA1a cells (Fig. 3A and B). A-443654 induced G2/M arrest in a dose- and time-dependent manner in both the 10A and CA1a cells, although the concentration response and percentage of cells in G2/M arrest in the 10CA1a tumors cells was greater than the concentration response and percentage of cells in G2/M arrest in the benign 10A cells. For

example, after 8 h, the percentage of 10A cells in G2/M phase in the DMSO-treated controls and the cells treated with A-443654 at 1, 2 or 5 μ M were ~14.4, 19.3, 21.7 and 24.5% respectively, whereas the percentage of 10CA1a tumor cells in G2/M in the DMSO control and those treated with A-443654 at 1, 2 or 5 μ M were ~21.3, 36.5, 40.1 and 41.9% respectively. A-443654 also showed a time-dependent increase in G2/M arrest in both the 10A and CA1a cells (Fig. 3A and B). For example, the percentage of 10A cells in G2/M in the DMSO control and in those treated with 1 μ M A-443654 for 4, 8 and 12h were ~14.4, 18.8, 19.3 and 30.1%, respectively, whereas the percentage of 10CA1a cells in G2/M in the DMSO control and those treated with 1 μ M for 4, 8 and 12 h were ~21.3, 26.3, 36.5 and 39.1%, respectively (Fig. 3B). These data suggest that 10CA1a cells are more sensitive to A-443654-induced G2/M arrest than the 10A cells (Fig. 3B). The combination of rapamycin with A-443654 did not further increase the G2/M phase (Fig. 3A and B). However, the percentage of cells in G2/M was substantially decreased by the combination of rapamycin and A-443654 in a concentration- and time-dependent manner, with more cells undergoing cell death (sub-G1 peak) with the combined rapamycin and A-443654 treatment, relative to A-443654 alone (Fig. 3A and B).

A-443654 alone induced the appearance of the sub-G1 peak. In the 10A cells, the sub-G1 peak appeared after 8 h treatment at 5 μ M A-443654 (~0.7%), whereas the sub-G1 peak appearing after 8 h of 1 μ M A-443654 treatment in the 10CA1a cells was ~0.18%, followed by ~2.5% at 2 μ M A-443654 and ~5.0% at 5 μ M A-443654 (Fig. 3C). After 12 h A-443654 treatment, the percentage of 10A cells in sub-G1 was ~2.2, 4.7 and 4.7% at 1, 2 and 5 μ M A-443654, respectively, whereas the sub-G1 percentage in 10CA1a cells was ~6.4, 18.5 and 18.8% at 1, 2 and 5 μ M A-443654, respectively (Fig. 3C). The combination of rapamycin and A-443654 increased the sub-G1 peak in 10A cells to ~3.1% at 8 h and 5 μ M A-443654, an ~4.4- fold increase, relative to cells treated with A-443654 alone (~0.7%). In the 10CA1a cells, rapamycin and A-443654 in combination increased the sub-G1 peak to 1.21% at 8 h of 1 μ M A-443654, an ~6.7- fold increase relative to cells treated with A-443654 alone (~0.18%) (Fig. 3C). These data show that both A-443654 and the combination of rapamycin and A-443654 increased the percentage of cells in sub-G1 in a concentration- and time-dependent manner, with the sub-G1 percentage of 10CA1a cells being ~ 2.9- to 5-fold greater than that of the 10A cells (Fig. 3C).

3.4. Rapamycin sensitizes A-443654-mediated apoptosis

PARP cleavage was used to examine whether the cell death observed in cell morphology studies, and that associated with the sub-G1 peak observed in flow cytometry, resulted from apoptosis. Rapamycin alone did not significantly increase PARP cleavage in either the 10A or 10CA1a cells. In contrast, A-443654 at 1 μ M produced some evidence of apoptosis, as shown by the presence of the cleaved 85kd PARP band, although very light and not clearly visible in the figure, in the 10CA1a cells (Fig. 4). However, A-443654 at 2 or 5 μ M caused PARP cleavage, as clearly demonstrated by the presence of the cleaved 85 kd band (Fig. 4). The intensity of the cleaved PARP band was increased in the presence of rapamycin and A-443654 in both the 10A and 10CA1a cells, as compared to the A-443654-treated 10A and 10CA1a cells, respectively (Fig. 4). Further, the magnitude of the cleaved PARP band density was considerably greater for the 10CA1a cells (Fig. 4). These results show that the 10CA1a tumor cells are more susceptible to apoptosis in the presence of rapamycin and A-443654 than the benign 10A cells.

3.5. Effects of rapamycin and A-443654 alone or in combination on the expression of apoptosis-related proteins

The effects of the rapamycin and A-443654 combination on the levels of key proteins that control the progression of apoptosis was examined, since apoptotic cell death was shown by

PARP cleavage in response to A-443654 alone, and in combination with rapamycin. Rapamycin alone failed to cause apoptosis (Fig. 3C) in either the 10A or 10CA1a cells. No significant change in Bcl-2 was observed in the 10A cells with A-443654 at either 8 or 12h (Fig. 5A). In contrast, A-443654 at 2 and 5 μ M decreased Bcl-2 levels by 30 to 40% in the 10CA1a cells at 8h, with only a marginal 10 to 20% decrease noted for 12h, relative to the respective DMSO control (Fig. 5A). The combination of rapamycin with 2 or 5 μ M A-443654, however, markedly decreased Bcl-2 protein levels by ~40 to 50% in the 10A cells and by ~70% in the 10CA1a cells, respectively, compared to the respective DMSO controls (Fig. 5A).

Bad, a pro-apoptotic protein was not significantly elevated in the 10A cells by A-443654 or by the rapamycin and A-443654 combination (Fig. 5B). In the 10CA1a cells, A-443654 alone significantly increased Bad levels by ~3.4- to 4.2-fold at 8h and by ~4.5- to 5.5-fold at 12 h post-treatment, respectively, relative to the respective DMSO control (Fig. 5B). The combination of rapamycin and A-443654, however, induced Bad levels in the 10CA1a cells by ~4.3- to 5.1-fold at 8h and by an ~5.2- to 5.7-fold at 12 h post-treatment, respectively, relative to the respective DMSO control (Fig. 5B). No significant effect on Bcl-X_L or Bax protein levels was observed for cells treated with A-443654 or the combination of rapamycin and A-443654 (data not shown). These data suggest that apoptosis produced by the combination of rapamycin and A-443654 in the 10A cells likely occurs through decreased Bcl-2 expression. In contrast, apoptosis produced by the combination of rapamycin and A-443654 in 10CA1a cells may be mediated through a decrease in Bcl-2 and an increase in Bad.

3.6. Effects of rapamycin and A-443654 alone or in combination on the expression of cell cycle-related proteins

To examine whether rapamycin and A-443654-induced alterations in the cell cycle were associated with altered expression of cell cycle progression-regulating proteins, cells were treated with 1, 2 or 5 μ M A-443654 for 8 and 12 h in the presence or absence of rapamycin, and protein levels of p53, cyclin D1, cyclin E, p15^{INK4B}, p21^{Cip/WAF1} and p27^{kip1} were determined. Cyclin E, p21^{Cip/WAF1} and p27^{kip1} protein levels were not significantly altered by rapamycin and A-443654 treatment under these conditions (data not shown).

p53 plays a key role in mediating anti-tumor effects by inducing cell cycle arrest and/or apoptosis. Loss of p53 accelerates tumor progression in transgenic mice [23]. Protein levels of p53, however, were increased ~1.5- to 1.6- fold by A-443654 alone in the 10A cells at 8 and 12 h post-treatment, as compared to the respective DMSO control (Fig. 6A). The combination of rapamycin and A-443654 did not further increase p53 expression levels in the 10A cells (Fig. 6A). The 10CA1a cells, however, showed a greater response to A-443654, which increased p53 levels by ~1.9- to 2.4-fold, relative to the DMSO control cells. Further, rapamycin and A-443654 in combination, increased p53 levels at either 8 or 12 h in the 10CA1a cells by an additional ~1.3- to 1.5-fold over that monitored for the cells treated with A-443654 alone at the respective time points (Fig. 6A).

Cyclin D1 is one of the major proteins that regulate cell cycle progression from G1 to S phase. In these experiments, treatment of 10A and 10CA1a cells with rapamycin for 48h increased cyclin D1 levels by ~2.6- and 3.3-fold, respectively, relative to the corresponding DMSO controls (Fig 6B). A-443654 alone elevated cyclin D1 comparably by ~1.5- to 2.0-fold in the 10A cells at either 8 or 12 h, and by ~1.8- to 2.4-fold in the 10CA1a cells at 8 and 12h, respectively, compared to the corresponding DMSO controls (Fig. 6B). Surprisingly, A-443654 at 1 μ M exerted a maximal effect on cyclin D1 expression in the 10A cells, with higher concentrations decreasing cyclin D1 levels (e.g. an ~2.0-fold increase at 1 μ M) decreasing to a 1.5-fold increase at 5 μ M at 8 h) (Fig. 6B). However, this inverse

concentration-response was not observed in the 10A cells at 12 h or in the 10CA1a tumor cells at either 8 or 12 h. The combination of rapamycin and A-443654 elevated cyclin D1 levels by ~2.7- to 2.9-fold in the 10A cells at either 8 or 12 h (Fig. 6B). The combination of rapamycin and 1 μ M A-443654 however, increased cyclin D1 in 10CA1a cells by an ~5.1-fold at 8 and 12 h relative to the corresponding DMSO controls (Fig. 6B). In the combined rapamycin and A-443654-treated 10A cells, cyclin D1 levels remained elevated at 1, 2 and 5 μ M A-443654 concentrations (Fig. 6B). However, in the combined rapamycin and A-443654 treated 10CA1a cells, the elevation of cyclin D1 levels progressively diminished with increasing A-443654 concentration, with cyclin D1 levels decreasing by ~30 and 70% at 2 μ M at 8 and 12 h, respectively, and by ~50 and ~75% at 5 μ M A-443654, at 8 and 12 h respectively, relative to the combined rapamycin and 1 μ M A-443654-treated cells (Fig. 6B).

p15^{INK4B} protein levels were not increased by either rapamycin or A-443654 alone, but were significantly elevated by the combination of rapamycin and A-443654 at 12 h in both the 10A and 10CA1a cells. Rapamycin and A-443654 in combination at all concentrations increased p15^{INK4B} levels by ~1.7- and 2.0-fold in the 10A and 10CA1a cells, respectively, at 12h relative to the corresponding DMSO controls (Fig. 6C).

4. Discussion

Breast cancer patients mainly succumb to the metastatic spread of cancer rather than to the primary localized tumor. Unlike the primary tumor which can be removed by surgery and treated with radiation therapy [24], metastatic carcinoma is generally more difficult to cure, and can only be controlled and palliated by systemic treatment, including endocrine therapy, chemotherapy and targeted treatment [2]. However, endocrine therapy is effective only on estrogen-receptor positive tumors. The development of taxane-based regimens has led to the increased use of combined chemotherapy and targeted treatment, notably trastuzumab, which targets ErbB2 (HER2) [3]. Combination chemotherapy and targeted treatment has been demonstrated to increase the efficacy in prolonging survival in breast cancer patients. Despite the advantages of combination therapy, the emergence of resistance to the chemotherapeutic agents and the apparent toxicity of trastuzumab remain as primary obstacles for successful treatment [25]. Nevertheless, targeted chemotherapy and combination regimens are emerging as a new direction for breast cancer treatment.

Over activation of PI3K/Akt pathway, and most notably, hyperphosphorylation of Akt, has been reported with increasing proliferative and invasive potential associated with progression of the disease in breast tumor tissue [26]. Constitutively activated or overexpressed Akt is oncogenic, and hence, inhibition of Akt constitutes an attractive target for therapy. Unlike the commercially available Akt inhibitors, which function through other mechanisms rather than competing with ATP-binding site, A-443654, a novel and potent pan Akt inhibitor, competitively and reversibly binds to the ATP site of Akt with a high degree of specificity [17].

In this study we have examined the effects of the combination of the TORC1 inhibitor rapamycin with the Akt inhibitor A-443654 on the benign 10A and 10CA1a tumor human breast epithelial cells. Pretreatment of these cells with rapamycin resulted in altered morphology, and increased apoptosis in the 10CA1a tumor cells relative to the benign 10A cells at earlier time points and lower concentrations of the Akt inhibitor. Cell proliferation was also decreased and cell cycle arrest occurred at G2/M, with the more pronounced effects of the combination treatment on the 10CA1a tumor cells. Examination of PARP cleavage as evidence of apoptosis was accomplished and the levels of proteins involved in apoptosis were examined. The apoptotic effects on the 10CA1a cells associated with the combination

treatment reflect, in part, decreased Bcl-2 and increased Bad protein levels. Protein levels of the major regulators of the cell cycle were also examined and differences in the levels of key regulators (e.g. p53, cyclin D1) noted between the two cell lines in response to the combination of rapamycin and A-443654.

These results show that A-443654 produces morphologic effects fairly rapidly, such that within 2 h of treatment cells exhibit altered morphology, and by 8 h the 10CA1a tumor cells (~2.5%) start to undergo apoptosis. Increased duration of treatment (i.e. 12 h) of A-443654 (5 μ M) increases efficacy reflected by a larger percentage of apoptotic 10CA1a cells (18.8%). However, this concentration also exhibits toxicity to the benign 10A cells (~4.7% apoptotic cells). These data suggest that A-443654 may have a very narrow therapeutic window. In order to examine whether inhibition of mTOR (TORC1) by treatment with rapamycin would decrease toxicity to the 10A cells, while sensitizing 10CA1a tumor cells to A-443654, a series of studies was performed using rapamycin and A-443654 in combination. These data suggest that inhibition of mTOR with rapamycin increases the efficacy of A-443654 at lower concentrations, and potentially alters the therapeutic safety margin. Clearly, more in depth studies are required to establish this phenomenon but such an effect might prove advantageous in the treatment of tumors with hyperphosphorylated Akt and responsive to inhibition of Akt. Rapamycin appears to be well tolerated. A recent report provided evidence that feeding rapamycin to mice at any age prolongs survival [27]. Subsequently, it was reported that the ribosomal protein S6 kinase, a downstream target of mTOR, also regulates mammalian life span [28]. Thus, treatment with rapamycin, or a related derivative, followed by treatment with an inhibitor of Akt, such as A-443654, may provide the combination therapy required to increase the efficacy, safety margin, and duration of Akt inhibition required to achieve productive results. Studies examining the combined use of rapamycin and A-443654 in treating tumors from a MiaPaCa-2 pancreatic acid mouse explant model showed that the combination of drugs exhibited greater efficacy, as compared to either agent alone [17]. The combination of A-443654 and rapamycin also effectively inhibited hypoxia-driven increases in VEGF production, in cell lines derived from pediatric solid tumors [29]. Thus, the targeted inhibition of two major components in a signaling pathway appears to produce greater efficacy than inhibition of the individual signaling components alone.

Rapamycin and its analogues are in clinical trials for the treatment of cancer. Since mTOR is downstream of PI3K and Akt, possible side effects from inhibiting the broader upstream effectors could be avoided through targeted inhibition of Akt and mTOR [30,31]. We previously reported that rapamycin alone inhibited cell proliferation [30]. The effects of rapamycin in combination with other agents, such as cotylenin A [10], radiation [14], paclitaxel [32] and TGF- β [33] on potentiating cytotoxicity have been examined. Since rapamycin is well tolerated by patients, it can be used for a longer period of time and may prevent tumor relapse. It has been reported that mTOR forms two structurally and functionally distinct complexes, mTORC1 and mTORC2. While mTORC1 is formed by mTOR, raptor and mLST8 and is rapamycin sensitive, mTORC2 is rapamycin insensitive and contains mTOR, rictor and mLST8, mSIN1 and PRR5 [34]. The elevation of Akt serine 473 phosphorylation following inhibition of mTORC1 by rapamycin has been reported to be dependent on rictor and PI 3K [35]. However, evidence has been presented that mTORC2 may be sensitive to prolonged treatment with rapamycin in selected tumor cell lines as well [36]. The distinct roles of these two complexes in A-443654 induced Akt phosphorylation [18] provide a future direction for treating rapamycin insensitive tumors potentially by simultaneously inhibiting rictor or associated proteins.

By virtue of the heterogeneity associated with different types of cancers, chemotherapy is becoming more complex and personalized. For this purpose, the molecular alterations

including signaling targets and aberrance should be monitored for each patient. Inhibition of PI3K/Akt pathway exemplifies one targeted pathway for development of therapeutic agents, and one of critical importance, especially in cases where there is hyperactivation of this pathway or individual kinase components. In recent years, the analysis of gene and protein expression levels by genomic and proteomic techniques, respectively, have led to the discovery of more patient-specific targets. Thus, a multiple-target strategy directed towards sequential components of critical signaling pathway(s) is likely required for personalized treatment, and seems to be well-tolerated with fewer side effects [37].

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Fig. 1. Effects of rapamycin and A-443654 alone and in combination on 10A and 10CA1a cell morphology

Fig. 2A

Fig. 2B

Fig. 2.

Fig. 2A. Effects of rapamycin alone on 10A and 10CA1a cell viability

Fig. 2B. Effects of rapamycin and A-443654 combination on 10A and 10CA1a cell proliferation

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3.
Fig. 3A. FACSscan Analysis of rapamycin and A-443654 effects on DNA content in 10A and 10CA1a cells
Fig. 3B
Fig. 3C. Apoptosis induced by rapamycin and A443654 in 10A and 10CA1a cells

Fig. 4. Rapamycin and A443654 effects on apoptosis as assessed by PARP cleavage

Fig. 5A

Fig. 5B

Fig. 5.
Fig. 5A. Effects of rapamycin and A443654 in combination on Bcl-2 protein levels in 10A and 10CA1a cells
Fig. 5B. Effects of rapamycin and A443654 in combination on Bad protein levels in the 10A and 10CA1a cells

Fig. 6A

Fig. 6B

Fig. 6.
Fig. 6A. Effects of rapamycin and A-443654 in combination on p53 protein levels in the 10A and 10CA1a cells
Fig. 6B. Effects of rapamycin and A443654 in combination on cyclin D1 protein levels in the 10A and 10CA1a cells
Fig. 6C. Effects of rapamycin and A443654 in combination on p15^{INK4B} protein levels in the 10A and 10CA1a cells