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Naringenin Inhibits Proliferation and Survival of Tamoxifen-Resistant Breast Cancer Cells

Joseph Ramos, Talia Hatkevich, Lauren Eanes,
Idalys Santos-Sanchez and Yashomati M. Patel

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Abstract

The majority of breast cancers are estrogen receptor positive (ER+) and utilize estrogen to promote cell proliferation. Thus, the ER has been the target of many therapies. While this strategy has been successful, the long-term use of antiestrogen therapies, such as tamoxifen (Tam), frequently results in Tam resistance (Tam-R). Tam-R cells may proliferate due to the activation of the phosphatidylinositol-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways. Targeting these proliferation and survival pathways after the development of resistance is critical for the treatment of drug-resistant cancers. We have identified the flavanone Naringenin (Nar) as an inhibitor of both the PI3K and MAPK pathways. Here, we show that Nar impairs cell proliferation and induces apoptosis of Tam-R MCF-7 breast cancer cells. We also demonstrate that Nar treatment reduced the levels of both ERK and AKT in Tam-R cells. Furthermore, Nar treatment localized ER α to a perinuclear region in Tam-R cells. Nar may function by inhibiting both the PI3K and MAPK pathways as well as localizing ER α to the cytoplasm to impair cell proliferation of Tam-R MCF-7 cells. These studies provide insight into the molecular mechanisms involved in cell proliferation of Tam-R breast cancer cells.

Keywords: naringenin, tamoxifen-resistant, breast cancer, proliferation, MAPK

1. Introduction

The majority of breast cancers are estrogen receptor positive (ER+) and depend on estrogen for cell proliferation [1]. The majority of ER+ breast cancers respond to antiestrogen therapies such

as tamoxifen (Tam) [2]. Unfortunately, the long-term use of Tam frequently results in Tam resistance. Tam resistance is often accompanied by the activation of other proliferation promoting pathways such as growth factor receptor pathways and their downstream signaling molecules such as phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) [3]. Endocrine resistance and activation of growth promoting signaling molecules are indicative of a poor prognosis and increased mortality [4]. Thus, the identification of therapeutic compounds that regulate proliferation in Tam-resistant cancers could lead to more effective treatment options.

In order to impair proliferation in ER+ breast cancer cells, antiestrogen therapies such as Tam are utilized to target the ER [2]. Normally, estrogen binds the ER that results in dimerization, translocation into the nucleus, and regulation of gene transcription [5–8]. The estrogen-ER complex regulates numerous genes that affect cell proliferation and survival [5–8]. Tam acts as an agonist or antagonist to the ER depending on the cell type [9]. In breast tissue, Tam functions mainly as an antagonist to the ER. It does so by binding the ER and preventing it from transcribing estrogen-responsive genes [2, 9–11]. Inhibiting transcription of these genes impairs cell proliferation and survival. Previous studies have shown that overactivation of the MAPK and PI3K pathways during Tam treatment may be involved in Tam resistance via ligand-independent activation of the ER, decreasing the overall rate of ER+ breast cancer survival [6]. Both the MAPK and PI3K pathways regulate cellular growth and survival [12]. These pathways have also been shown to activate the ER via phosphorylation in a ligand-independent manner [13, 14]. Conversely, the ER can activate both the MAPK and PI3K pathways by a nongenomic mechanism [13, 14]. Taken together, these findings suggest that Tam resistance may be the result of complex interactions between the ER and components of kinase signaling pathways. Therefore, identification of compounds that inhibit the activity of the PI3K or MAPK pathways may restore growth arrest to Tam resistant cells. Chemical inhibitors of MEK and PI3K are currently being investigated as promising new strategy for breast cancer patients [15, 16].

Previous studies have identified the grapefruit flavanone, Naringenin (Nar) as an inhibitor of both the MAPK and PI3K pathways [15, 17–20]. Flavanones have low toxicity compared to other plant compounds and can function to impair cell proliferation, angiogenesis, and signaling cascades [21–26]. Previous studies have shown that Nar hinders cell proliferation and motility by interfering with the PI3K and MAPK pathways [26, 27]. Nar has also been shown to bind directly to the estrogen receptor and function as an ER antagonist [26, 27]. The ability of Nar to impair the MAPK and PI3K pathways as well as function as an antagonist to the ER suggests that Nar has the potential to growth arrest Tamoxifen-resistant cells (Tam-R). In this study, we show that Nar inhibits cell proliferation of Tam-R MCF-7 cells. Furthermore, we demonstrate that Nar impairs both the MAPK and PI3K pathways by reducing the levels of ERK and AKT. Nar treatment results in relocalization of ER α to a perinuclear location in Tam-R cells. Thus, Nar acts by impairing both the MAPK and PI3K pathways as well as functioning as an antagonist to the ER.

2. Materials and methods

2.1. Cell culture

MCF-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM)/10% fetal bovine serum (FBS), supplemented with insulin, or phenol red-free DMEM (PRF-DMEM) supplemented with 10% charcoal-stripped fetal bovine serum (CS-FBS). Cells were maintained at 37°C with 5% CO₂. Media was changed every 2 days and cells were passaged at 80% confluency.

2.2. Generation of Tam-R cells

Tam-R cells were generated by culturing MCF-7 cells in DMEM supplemented with 100 U/mL penicillin/streptomycin, 0.01 mg/mL bovine insulin, 10% FBS, and 10⁻⁶ M of 4-OH-tamoxifen for 10 months [28–30].

2.3. Naringenin treatment

Naringenin was purchased from Sigma Aldrich. Cells were treated with Naringenin (2,3-Dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 4',5,7-Trihydroxyflavanone) (Nar) or treated with a vehicle DMSO alone. Cells were treated with Nar (at the indicated concentration) a few hours after plating. Cells were treated for the indicated times and then assayed for a variety of parameters.

2.4. Cell density assays

Cells either treated with the vehicle DMSO alone or Nar (at the indicated concentrations and the indicated time points) were washed twice with 1×PBS, trypsinized and then centrifuged at 5000 × g for 5 min. Pelleted cells were resuspended in 1×PBS. Cells (1:20 dilution) were incubated in ViaCount Reagent for 5 min in the dark and analyzed by Guava easy-Cyte™ flow cytometry (Millipore) using the ViaCount software. The ViaCount Reagent determines cells density (a measure of all cells) as well as viable, apoptotic, and dead cells by using two dyes. The nuclear dye stains only nucleated cells and the viability dye stains only dying cells. Levels of the stains allows for accurate assessment of viable, apoptotic, and dead cells.

2.5. Immunoblot analysis

Cells either treated with the vehicle DMSO alone or Nar (250 μM) for 7 days were washed once with 1×PBS and lysed. Cell lysates were rocked for 20 min and then centrifuged for 20 min at 4°C. Proteins (30 μg) were subjected to 10% SDS-PAGE and Western blot analysis protein were immunostained with the indicated antibody and detected using ECL and a Bio-Rad ChemiDoc XRS system. Protein bands were analyzed using Quantity One software.

2.6. Immunofluorescence

Cells were cultured on sterilized glass coverslips for 7 days. Cells were either treated with the vehicle DMSO alone or Nar (250 μM) for 7 days. After treatment, cells were washed with 1×PBS,

fixed with 3.7% paraformaldehyde for 15 min, and then permeabilized in 0.25% Triton. Cells were incubated with an ER α antibody (1:100) for 1 h and then a secondary antibody for 45 min. Cells were incubated with DAPI (1:1000) for 5 min and then washed with 1 $\times$ PBS. Cells were visualized using an Olympus iX81 Motorized Inverted Confocal Microscope equipped with Fluoview FV500 software. To determine the effect of Nar on apoptosis, cells were stained with DAPI and cells containing condensed and fragmented nuclei (presented as punctate DAPI staining) were counted. Cells in 5–7 different fields/slides were counted and averaged. The experiment was performed three times.

2.7. Quantification of ER α

ER α levels were quantified by fluorescence intensity in both the cytoplasm and nucleus. The ratio of nuclear/cytoplasmic signal was measured for 5–7 fields under various conditions and averaged. The experiment was performed three times.

2.8. Nuclear and cytoplasmic fractionation

Cells either treated with the vehicle DMSO alone or Nar (250 μ M) for 7 days were washed with 1 $\times$ PBS and centrifuged at 8000 rpm for 2 min. The supernatant was removed and cells were resuspended in 1 mL of Hank's balanced salt solution. Cells were then centrifuged at 4000 rpm for 2 min. The supernatant was removed and the cells were resuspended in 100 μ L of CE buffer (10 mM Hepes pH7.6, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.7% NP-40). They were placed on ice for 5 min, and then centrifuged at 4000 rpm for 4 min. The supernatant was collected as the cytoplasmic extract. The remaining pellet was resuspended in 500 μ L of CE buffer without NP-40 and then centrifuged at 10,000 rpm for 4 min. The supernatant was removed and the remaining pellet was the nuclear extract.

2.9. Statistical analysis

Results are the means $\pm$ SEM of three independent experiments ($*p < 0.05$). The significance was assessed by two-way analysis of Student's *t*-test (StatPlus, AnalystSoft).

3. Results

3.1. Characterization of Tam-R MCF-7 cells

Previous studies have shown that growth factor pathways are upregulated in Tam-R cells [3]. Since Nar targets the MAPK and PI3K pathways, we wanted to determine the effect of Nar on Tam-R cells. In order to do this, we first had to establish a Tam-R cell line. Previous studies have shown that MCF-7 cells can become tamoxifen-resistant through prolonged exposure to 4-OH-tamoxifen [28–30]. We cultured MCF-7 cells in the presence of 4-OH-tamoxifen for 10 months as described in Section 2. After 10 months of 4-OH-tamoxifen treatment, cells were assayed for proliferation and compared to Tamoxifen-sensitive MCF-7 cells (Tam-S). Cells were grown in either full medium (10% FBS) or medium containing charcoal-stripped serum. Since

MCF-7 cell proliferation is primarily driven by estrogen, the untreated wild-type Tam-S cells had a 462% increase in cell density when grown in full medium and a low rate of proliferation in the charcoal-stripped serum compared to cells grown in full medium. The cell density of Tam-S cells cultured in charcoal-stripped serum only increased 37% over 7 days (**Figure 1A**). Furthermore, Tam-S cells treated with tamoxifen also had a low rate of proliferation. In contrast, cell density of Tam-R cells increased by 378% in full medium and 287% in the presence of charcoal-stripped medium in 7 days. Additionally, tamoxifen treatment had no effect on cell density. Thus, the level of cell proliferation observed in the presence of Tam indicated that the cells were Tam-resistant. In all treatments the vehicle control (EtOH) had no effect when compared to untreated cells.

Figure 1. Characterization of Tam-R MCF-7 cells. Tam-S (MCF-7 wild-type) and Tam-R cells were cultured in phenol red free media containing FBS (Full media) or charcoal-stripped FBS and either left untreated or treated with the vehicle (ethanol) or 4-OH-tamoxifen (100 nM) for 7 days. (A) Cell densities (cells/ml) were determined and compared to initial counts to calculate percent change. Results are the means $\pm$ SEM of three independent experiments. Differences between Full media and Charcoal-stripped media were tested for statistical significance ($*p < 0.05$). (B) Cell lysates were collected and proteins were subjected to SDS-PAGE. Proteins were immunoblotted using antibodies against p-ERK1/2, ERK1/2, p-AKT, AKT, and actin. Results are representative of five independent experiments. (C) Cells were fixed and stained for ER α and visualized using confocal microscopy. The results are representative of three independent experiments.

Previous studies have suggested that cell proliferation in Tam-R cells may be due to the activation of growth factor pathways [3]. In order to determine if the change in growth rate was associated with a change in the protein levels and/or phosphorylation of ERK1/2 and/or AKT, we assayed p-ERK1/2, ERK1/2, p-AKT, AKT, and actin. Tam-S MCF-7 cells express both ERK1/2 and AKT and low levels of both p-ERK1/2 and p-AKT were detected. Tam-R cells also express both ERK1/2 and AKT at similar levels when normalized to actin levels. In agreement with previous studies, we observed an increase in p-ERK1/2 in the Tam-R cells when compared to Tam-S (**Figure 1B**) [31]. Surprisingly, Tam resistance did not stimulate the phosphorylation of AKT when normalized to actin levels in our cells (**Figure 1B**).

Another observed difference present in Tam-R cells is the redistribution of ER α to the cytoplasm upon tamoxifen resistance [31]. We wanted to determine whether our Tam-R cells exhibited any alteration in ER α localization when compared with Tam-S cells. To investigate the localization pattern of ER α , both Tam-S and Tam-R MCF-7 cells were assayed for ER α localization by confocal microscopy (**Figure 1C**). In Tam-S cells, ER α was localized primarily to the nucleus (72 ± 4 of total ER α) with lower levels present in the cytoplasm (28 ± 7 of total ER α). In contrast, Tam-R cells exhibited increased levels of ER α in the cytoplasm (47 ± 6 of total ER α) compared to Tam-S cells. This increased level of ER α was evenly distributed throughout the cytoplasm. ER α was still present in the nucleus of Tam-R cells although at lower levels (53 ± 7 of total ER α) than that observed in Tam-S cells.

3.2. Nar impairs cell density of Tam-R MCF-7 cells

Next we wanted to determine if Nar could inhibit cell proliferation in Tam-R MCF-7 cells. Previous studies suggested that Tam-R cells utilize PI3K and/or MAPK pathways for cell proliferation. Since Nar inhibits both these pathways, it should result in impaired growth of Tam-R cells. We first wanted to determine the time- and concentration-dependent effects of naringenin on Tam-R cells. As shown in **Figure 2A**, Nar treatment decreased cell density within 2 days when compared to untreated cells and cell density further declined at 4 and 7 days. There was a significant difference in cell density at day 4 and 7, so we conducted all of our studies on day 7. We then wanted to determine the effect of Nar concentration on cell density (**Figure 2B**) and viability (**Figure 2C**) of Tam-R cells. While Nar treatment (at all concentrations) decreased both the cell density and viability of Tam-R cells in 7 days only a Nar concentration of 250 μ M had a significant effect on both cell density and cell viability when compared to untreated Tam-R cells. In our studies, we determined that Nar inhibited cell proliferation of both Tam-S and Tam-R cells with an IC₅₀ value of 237 μ M. While previous studies have shown that lower concentrations of Nar impaired the proliferation and viability of MCF-7 cells, our studies here demonstrate that Tam-R MCF-7 cells require higher concentrations of Nar to impair proliferation and viability [15, 17]. Higher concentration of Nar in cell culture as well as in animal studies have been employed in other studies and may reflect the specific sensitivities of the targets of Nar to elicit specific physiological effects [32–35].

Figure 2. Nar inhibits cell proliferation in a concentration- and time-dependent manner. Tam-R cells were cultured in phenol red free media containing charcoal-stripped FBS with 4-OH-tamoxifen (100 nM). (A) Cell densities (cells/ml) were determined in the presence or absence of Nar (250 μM) for the indicated time points. (B) Cell density (cells/ml) and (C) cell viability were determined at various Nar concentrations and compared to initial counts to calculate percent change. Results are the means ± SEM of three independent experiments. Differences between untreated and Nar treated were tested for statistical significance (* $p < 0.05$).

3.3. Nar induces apoptosis in Tam-R cells.

To determine whether the potential effect of Nar on cell proliferation were similar in Tam-S and Tam-R cells, both cell types were grown in media containing Tam in the presence or absence of Nar [34–37]. As shown previously, Tam impaired the proliferation of Tam-S cells. Nar treatment of Tam-S cells not only further impaired cell proliferation it also decreased viability (**Figure 3A**). As expected the Tam-R cells exhibited increased proliferation in the presence of Tam when compared to Tam-S cells (**Figure 3A**). However, the increase in proliferation was completely reversed by the addition of Nar. Nar impaired viability of Tam-R cells to a similar extent as that seen in Tam-S MCF-7 cells (**Figure 3A**). Next, we assayed for apoptotic and dead cells upon Nar treatment of Tam-R cells (**Figure 3B and C**). There was an increase in both apoptotic and dead cells in Nar treated cells over 7 days when compared to untreated cells. In complementary studies, we assayed for condensed and fragmented nuclei by DAPI

staining (**Figure 3D** and **E**). Nar treatment of Tam-R cells resulted in a 16% increase in nuclear apoptosis when compared to untreated cells.

Figure 3. Nar is cytotoxic to Tam-R cells. (A) Tam-S and Tam-R cells were cultured in phenol red free media plus charcoal-stripped FBS (PRF-DMEM + CS-FBS) containing 4-OH-tamoxifen (100 nM) in the presence or absence of Nar (250 μ M). After 7 days, cells were collected, cell densities quantified, and growth rate calculated. Results are the means $\pm$ SEM of five independent experiments. Tam-R cells were cultured in the presence or absence of Nar for the indicated time points and assayed for (B) apoptotic and (C) dead cells. Percent apoptosis and percent dead cells were determined by flow cytometry. Results are the means $\pm$ SEM of three independent experiments. Differences between untreated and Nar treated at the indicated time points were tested for statistical significance (* $p < 0.05$). (D) Tam-R cells were cultured in the presence or absence of Nar for 7 days and then stained with DAPI and visualized by confocal microscopy. Condensed and fragmented nuclei are indicated by arrows. (E) Quantification staining is expressed as % apoptotic cells in Nar treated cells compared to untreated cells. Results are the means $\pm$ SEM of three independent experiments. * $p < 0.05$.

3.4. Nar decreases the levels of ERK and AKT protein in Tam-R cells

Previous studies have shown that short-term exposure to Nar reduces both AKT and ERK1/2 phosphorylation in MCF-7 cells. Our recent studies demonstrated that long-term (days) exposure to Nar decreased the protein levels of ERK1/2 and AKT in Tam-S MCF-7 cells [20]. We wanted to determine whether Nar had similar effects on ERK1/2, and AKT in Tam-R MCF-7 cells. To determine if Nar altered the levels and/or the phosphorylation of ERK1/2 and AKT,

we incubated Tam-R cells with Tam alone, Nar alone, or a combination of Nar and Tam. While Tam-R MCF-7 cells expressed both AKT and ERK1/2, as shown previously, the addition of Nar in the presence or absence of Tam in Tam-R cells resulted in significantly lower levels (30–40%) of both ERK1/2 and AKT (**Figure 4A and B**). Next, we examined the effect of Nar on the phosphorylation status of ERK1/2 and AKT in Tam-R cells. Our findings show that Tam-R cells have increased levels of p-ERK1/2 but unchanged levels of p-AKT when compared to Tam-S cells as seen in **Figure 1B**. As shown in **Figure 4A**, Nar alone and in combination with Tam resulted in undetectable levels of p-ERK1/2 in Tam-R cells. This may be due in part to the reduced levels of total ERK1/2. Phosphorylated AKT was undetectable in all samples.

Figure 4. Nar impairs the expression of ERK1/2 and AKT. Tam-R MCF-7 cells were grown in phenol red free media plus charcoal-stripped FBS (PRF-DMEM + CS-FBS) in the presence of 4-OH-tamoxifen (100 nM), Nar (250 μM), or a combination of the two. (A) Following 7 days of treatment, cells lysates were collected. Proteins were subjected to SDS-PAGE and immunoblotted using antibodies against p-ERK1/2, ERK1/2, p-AKT, AKT, and actin. (B) Protein levels were quantified using densitometry. Results are the means ± SEM of three independent experiments. Differences between Tam-treated and Nar or Nar-Tam treated were tested for statistical significance (* $p < 0.05$).

3.5. Nar alters ERα localization in Tam-R MCF-7 cells

Since ER localization changes upon tamoxifen resistance and Nar is known to bind ERα, we wanted to determine whether Nar had an effect on ERα localization in Tam-R cells [31]. To investigate the localization pattern of ERα, Tam-R MCF-7 cells were cultured in the presence or absence of Nar and ERα localization was determined by confocal microscopy. Cells were also stained with DAPI. In untreated Tam-R cells, ERα was uniformly distributed in the cytoplasm (**Figure 5A and B**). ERα was also present although at lower levels in the nucleus when compared to levels present in the cytoplasm. Surprisingly, Nar treatment resulted in a redistribution of ERα to a perinuclear localization in Tam-R MCF-7 cells. Significantly, lower levels of ERα were present in the nucleus (19%) as well as throughout the cytoplasm in Nar treated cells when compared to untreated cells. In complimentary studies, we fractionated Tam-R cells incubated in the presence or absence of Nar into cytosolic and nuclear fractions and assayed for ERα localization (**Figure 5C**). In untreated Tam-R cells there was a relatively even distribution of ERα and in contrast, Nar treatment reduced the levels of ERα in the

nucleus. Since our fractionation studies do not distinguish region of the cytoplasm, the total cytoplasmic ER α levels include the perinuclear ER α levels and thus higher total cytoplasmic ER α levels.

Figure 5. Effect of Nar on ER α localization in Tam-R cells. Tam-R MCF-7 cells were grown in phenol red free media plus charcoal-stripped FBS containing 4-OH-tamoxifen (100 nM) in the presence or absence of Nar (250 μ M) for 7 days. (A) Cells were fixed, stained for ER α and DAPI and visualized using confocal microscopy. The results are representative of three independent experiments. (B) Quantification of ER α nuclear localization. Results are the means $\pm$ SEM of three independent experiments. * p < 0.05. (C) Cells were fractionated into nuclear and cytosolic fractions and assayed for ER α by Western blot analysis. ER α levels were quantified and expressed as % of total ER α . Results are the means $\pm$ SEM of three independent experiments. * p < 0.05.

4. Discussion

Since ER+ breast cancers utilize estrogen to promote proliferation, pharmaceutical treatments have targeted the ER. One of the most widely used and successful breast cancer treatments is the antiestrogen, Tam. The optimal Tam treatment duration needed to decrease recurrence and improve survival is 5 years. Unfortunately, prolonged Tam treatment leads to Tam resistance. Resistance may in part be due to the activation of other proliferation promoting pathways.

Tam-R cells activate signal kinase pathways to promote cellular proliferation. Currently, the use of Tam in conjunction with multiple kinase inhibitors is being investigated for the treatment of breast cancers [38]. Since Nar also has been shown to have antiproliferative effects, we investigated the ability of Nar to impair cell proliferation of Tam-R breast cancer cells. Our findings suggest that Nar targets both ERK1/2 and ER α to impair cell proliferation of Tam-R MCF-7 cells.

While initially Tam binds to the ER and acts in an antagonist to prevent the ER from interacting with coactivators on the promoters of estrogen responsive genes that regulate cell proliferation and survival, eventually with prolonged treatment cells become Tam resistant [10, 11]. Previous studies have implicated the overactivation of the MAPK and PI3K pathways as contributors of acquired Tam resistance [4]. The ER is able to activate both the MAPK and PI3K pathways [3, 13, 31, 39–43]. In turn, the MAPK and PI3K pathways activate the ER in a ligand-independent manner [3, 13, 31, 39–43]. In order to determine the effects of Nar, we first generated a Tam-R MCF-7 cell line by culturing MCF-7 cells in the presence of 4-OH-tamoxifen for 10 months [28–30]. We monitored the cells for changes in growth rate, ERK1/2 and AKT and ER α localization. Following 10 months of treatment with Tam, the proliferation rate of the treated cells began to increase. These cells were classified as Tam-R. Since the cells were cultured in charcoal-stripped serum, the Tam-R cells appear to be mediating their proliferation through pathway(s) other than the estrogen requiring pathway. Previous studies have shown the activation of both the MAPK and PI3K pathways in Tam-R cells [44–47]. Since Nar impairs both MAPK and PI3K pathways, we wanted to determine whether Nar could reduce Tam-R cell proliferation. Nar treatment caused a complete reversal of proliferation in our Tam-R cell line. Not only did Nar abolish cell proliferation, but it also resulted in a lower cell density than was initially plated. We further show that Nar decreased viability and increased levels of apoptotic and dead cells. In complementary studies, we show that Nar treatment resulted in fragmented and condensed nuclei suggesting apoptotic cell death. Previous studies have documented the ability of Nar to fragment and condense nuclei [32]. These results demonstrate that Nar induces cell death in Tam-R cells.

Since a possible mechanism promoting cell proliferation and survival in the Tam-R cells is the MAPK and PI3K pathways, we investigate the effect of Nar treatment on ERK1/2 and AKT. Previous studies have shown that both the MAPK and PI3K pathways can facilitate proliferation in MCF-7 cells following estrogen deprivation [3, 48]. Furthermore, PI3K and MAPK pathways are upregulated in Tam-R cells [3]. While previous studies have shown that Nar treatment reduced the phosphorylation of both AKT and ERK1/2, our studies show that Nar significantly reduced the levels of both AKT and ERK1/2 in Tam-R cells [15, 17]. Our studies examined the effects of Nar over longer time periods and thus examined the longer term effects of Nar. We have similar effects of Nar on ERK1/2 and AKT levels in MCF-7 cells [20]. Reduced levels of ERK1/2 and AKT activation have been shown to contribute to impaired proliferation and survival of cells. These findings suggest that inhibition of the MAPK and PI3K pathways by Nar may contribute to the impairment of cell proliferation and survival in Tam-R cells.

In Tam-R cells ER α is relocalized from the nucleus to the cytoplasm [31]. This relocalization of ER α may allow for its interaction with kinase signaling pathways such as the PI3K and

MAPK pathways [31]. Both the MAPK and PI3K pathways in these cells may be activated by ER α and in turn ERK1/2 and AKT may activate ER α in the cytosol. This may support the idea that the ER α is more active in the cytosol in the Tam-R cells exhibiting nongenomic effects by interacting with the kinase signaling pathways. In addition, p-ERK1/2 has been shown to activate ER α by direct phosphorylation allowing ER α to resume transcription of estrogen-responsive genes [13, 39]. In this way, ER α would be active in both the cytoplasm and the nucleus. These data suggest that Tam-R cells increase cell proliferation not only through effects on estrogen-responsive genes, but also through activation of the MAPK and/or the PI3K pathways. While the Tam-R cells exhibited an even distribution of ER α throughout the cytoplasm, the addition of Nar localized ER α to a perinuclear region of the cell with significantly lower levels in the nucleus. One interpretation of the mechanism of Nar action is that the Tam-ER α complex that may have been activating components of the MAPK and PI3K signaling pathways in the cytosol was now ineffective because Nar treatment results in reduced levels of ERK1/2 and AKT. Conversely, reduced levels of ERK1/2 and AKT decrease the levels of phosphorylated ER α and thus decrease the transcriptional activity of ER α . Nar may also function by competing with Tam for the ER and unlike the Tam-ER α complex which can translocate into the nucleus the Nar-ER α complex may be unable to enter the nucleus as seen in the perinuclear localization of ER in Nar treated Tam-R cells.

5. Conclusion

In summary, our studies demonstrate that Nar inhibits cellular proliferation and induces apoptosis in Tam-R MCF-7 cells. We show that Nar treatment reduced the levels of ERK1/2 and AKT and resulted in a perinuclear localization pattern of ER α in Tam-R cells. Since Nar can reduce the protein levels of ERK1/2 and AKT as well as reduce the levels of ER α in the nucleus in Tam-R cells, this may explain the reduced cell proliferation/survival. These studies also suggest that Nar may be a potential candidate therapy for Tam-R ER+ breast cancers.

Author details

Joseph Ramos, Talia Hatkevich, Lauren Eanes, Idalys Santos-Sanchez and Yashomati M. Patel*

*Address all correspondence to: ympatel@uncg.edu

Department of Biology, University of North Carolina at Greensboro, Greensboro, North Carolina, USA

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