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Directed by Dr. Yashomati M. Patel 49pp.

Estrogen receptor alpha positive (ER α +) breast cancer cells proliferate and survive by utilizing multiple pathways. Thus, combination chemotherapies targeting multiple pathways may be used to decrease ER α +

breast cancer cell density. Tamoxifen (Tam), an estrogen antagonist, has been used for over 30 years to inhibit ER α +

breast cancer proliferation. Naringenin (Nar), a flavonoid, has been shown to reduce the proliferation of ER α +

breast cancer cells by inhibiting extracellular signal-regulated kinases 1/2 (ERK 1/2) and AKT.

In our studies, we investigated the effects of a combination of Tam and Nar on MCF-7 ER α +

breast cancer cells. We showed that Tam and Nar significantly inhibited cellular proliferation and viability by flow cytometry analysis when compared to Tam alone. Proliferation was inhibited by the reduction of expression of AKT and ERK 1/2, as determined by immunoblot analysis.

We have investigated the combination of Nar and Tam on Tam resistant (Tam-R) cells. Our results demonstrate that Nar alone impaired cellular proliferation and viability in Tam-R cells. In the absence of lipophilic compounds, Nar eliminated the phosphorylation of ERK 1/2, as determined by immunoblot analysis.

Taken together, our results suggest that a combination treatment of Nar and Tam is more effective in inhibiting cellular proliferation and viability than Tam alone in ER+

breast cancer cells. This combination treatment has the potential to improve the efficacy of breast cancer chemotherapy regimens.

TARGETING MULTIPLE PROLIFERATION PATHWAYS AS A
NOVEL BREAST CANCER TREATMENT

by
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Approved by

Committee Chair

Dedicated to my fiancé, Frankie, and my mom, each whom inspire me to be a better person everyday.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Breast carcinoma is a devastating disease that is predicted to claim over 40,000 lives in the US in 2013 (1). Furthermore, over 240,000 new cases of breast cancer will be diagnosed in 2013 (1). Approximately two thirds of breast cancer is estrogen receptor positive (ER+) (2). There are two characterized estrogen receptors, ER α and ER β , yet ER α is the only receptor that promotes proliferation in ER+ breast cancer cells (3).

Estrogen receptor α domains

ER α is a nuclear receptor with six functional domains (A – F), four that regulate the ER α activity. On the N terminus of ER α , the A/B domain contains the ligand-independent transcriptional activation function 1 (AF1). Phosphorylation at the AF1 allows the ER α to be constitutively active, even in the absence of estrogen (4,5) (Figure 1).

On the other hand, the C terminus of ER α contains the E/F domains, which harbor the ligand-dependent transcriptional activation function (AF2). Estrogen stimulates transcriptional activity of the ER α by binding to the AF2 (4,5). Although these regions are activated by different stimuli, AF1 works with AF2 synergistically to promote ligand dependent transcription of estrogen responsive genes (6) (Figure 1).

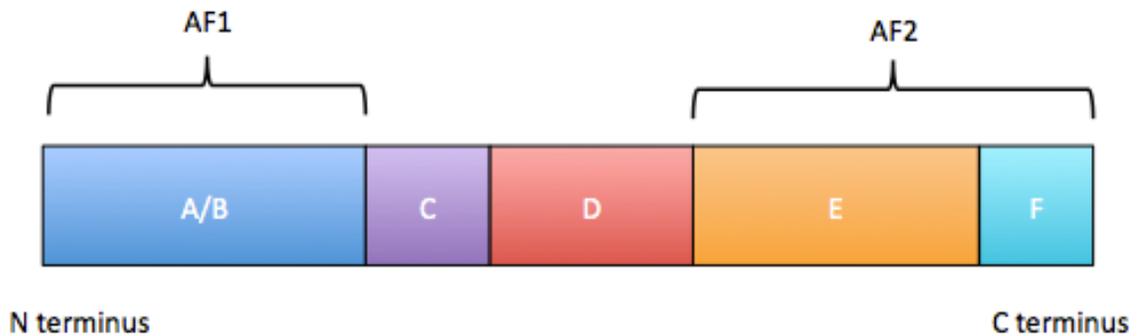


Figure 1. Schematic of ER α . ER α has 6 domains. A/B domain contains the AF1, C is the DNA binding domain, D is the hinge domain, E/F domains harbor the AF2.

There are various mechanisms that promote the survival and growth of ER α + breast cancer. Specifically, ER α + breast cancers can utilize the nongenomic and genomic pathways of the ER α , the phosphoinositide 3-kinase (PI3K), and the mitogen activated protein kinase (MAPK) pathways.

ER α + breast cancer proliferation and the estrogen/ER α complex

The primary pathway for ER α + breast cancer cellular proliferation begins with the formation of the estrogen/ER α complex. When estrogen enters its target cell, it binds to the AF2 domain of the ER α , and the estrogen/ER α complex can proceed down one of two pathways: the genomic or non-genomic pathway, both of which produce pro-proliferative effects.

The non-genomic pathway of the estrogen/ER α complex produces rapid responses after estrogen binds to the receptor. These rapid responses are mediated through ER α 's that are localized at the plasma membrane. Once estrogen binds to ER α ,

the complex disassociates from cell membrane and interacts with cytoplasmic kinase pathways (7,8).

Alternatively, the genomic pathway works over a period of hours. In the classical genomic model, the estrogen/ER α complex binds to the estrogen response element (ERE) on estrogen responsive genes. Coregulators are then recruited to the promoter region to alter the rate of transcription (9). Estrogen responsive genes include those involved in cell proliferation, cell cycle control, transcriptional regulation, and metabolic processes (10).

ER α + breast cancer proliferation and PI3K

In ER α + breast cancers, >30% of tumors develop a mutation in the PI3K pathway, resulting in uncontrolled proliferation and resistance to apoptosis (11-19). Activation of tyrosine kinase growth factor receptors (RTK) stimulates the PI3K pathway (20-25). The activated intracellular portion of the RTK serves as a “docking station” (26) for intracellular proteins such as PI3K. At the cell membrane, activated PI3K phosphorylates phosphatidylinositol-4,5-bisulfate (PIP₂), converting it to phosphatidylinositol-3,4,5-trisulfate (PIP₃) (27). Next, PIP₃ recruits AKT (28-31). Activation of AKT requires two phosphorylation events; first, PDK phosphorylates AKT at Thr³⁰⁸ resulting in partial activation. Next, proteins such as mTOR complex 2 (TORC2) (32) or integrin linked-kinase (ILK) (33) can phosphorylate AKT at Ser⁴⁷³ to achieve full activation. Once AKT is fully activated, it can activate its downstream substrates, which control cell cycle progression and transcription (34) (Figure 2).

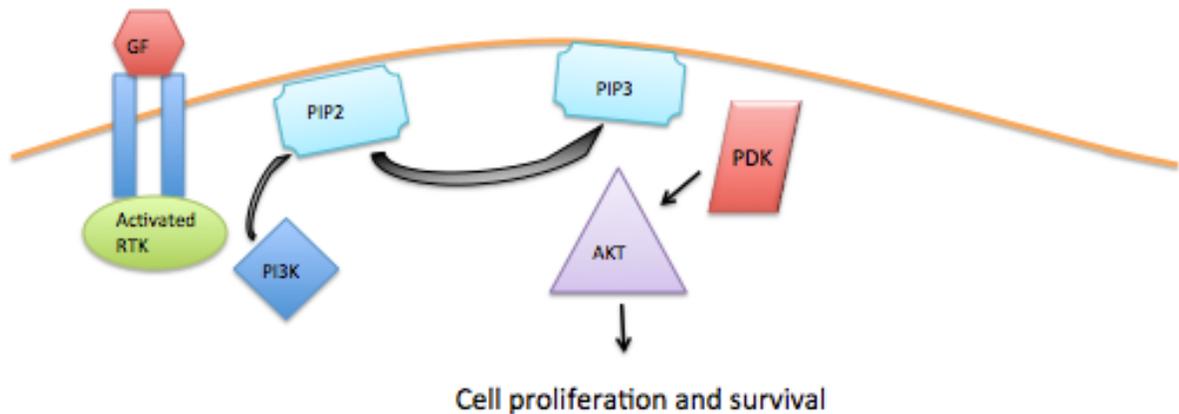


Figure 2. Overview of the PI3K pathway. Activated RTK recruits PI3K. Activated PI3K phosphorylates PIP2, resulting in an active PIP3, which recruits AKT. Phosphorylation events by various proteins, including PDK, activate AKT, resulting in cell proliferation and survival.

ERα+ breast cancer proliferation and MAPK

In breast cancer, the Mitogen Activated Protein Kinase (MAPK) cascade has been found to be constitutively active (35). Similar to the PI3K pathway, the MAPK pathways are involved in the regulation of survival and proliferation. The MAPK ERK 1/2 pathway, which is activated by growth factors such as estrogen (36), includes Raf, the MEK 1/2, and the ERK 1 and 2 (ERK 1/2). ERK 1/2 have numerous substrates, including RSK, which promote cellular proliferation and survival (37) (Figure 3).

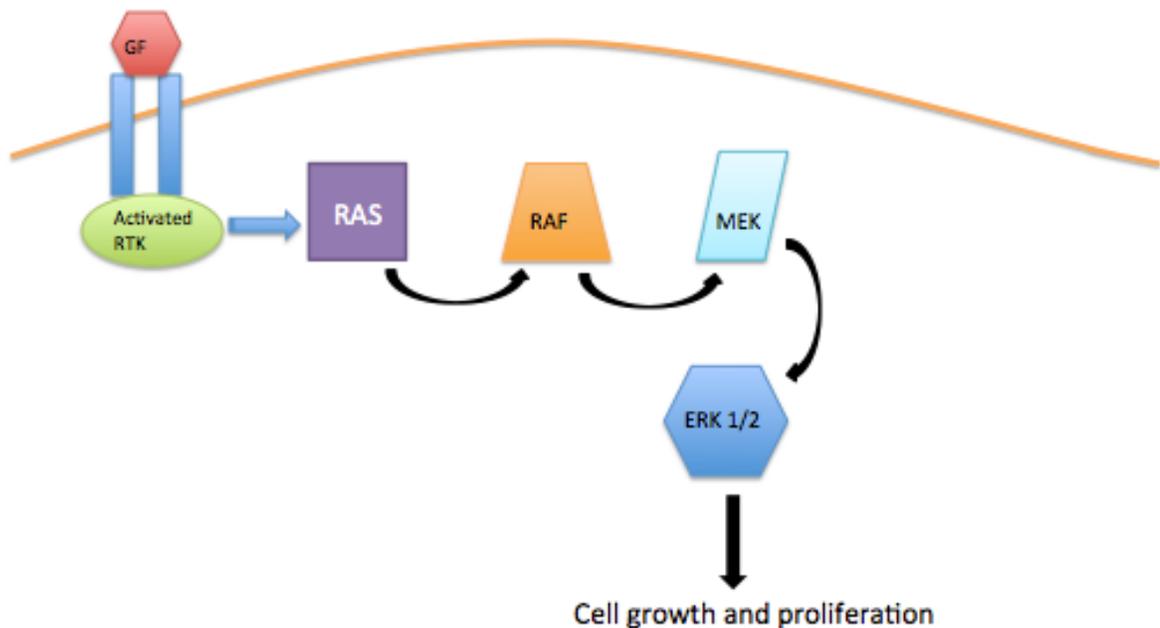


Figure 3. Overview of the MAPK ERK 1/2 pathway. An active RTK stimulates RAS, which activates Raf. Raf phosphorylates MEK 1/2, which phosphorylates ERK 1/2. ERK 1/2 can translocate to the nucleus to phosphorylate various transcription factors.

Cross talk between ER α and kinase pathways

ER α , PI3K, and MAPK pathways regulate one another. It is well documented that the kinase pathways influence the transcriptional activity of the ER α .

Phosphorylated ERK 1/2 can phosphorylate ER α on serine 118 (38), which leads to ligand-independent activation of ER α at the AF1 domain (39). Additionally, AKT phosphorylates ER α on serine 167, causing ligand-independent activation of ER α (40,41). ER α ligand-independent activation results in recruitment of co-activators to promote transcription of target genes (42) (Figure 4).

Furthermore, the action of the non-genomic ER α /estrogen complex activates numerous pathways, including the PI3K and MAPK pathways (43, 44) (Figure 4). This

bi-directional stimulation between ER α and kinase pathways renders a cancer that is difficult to treat.

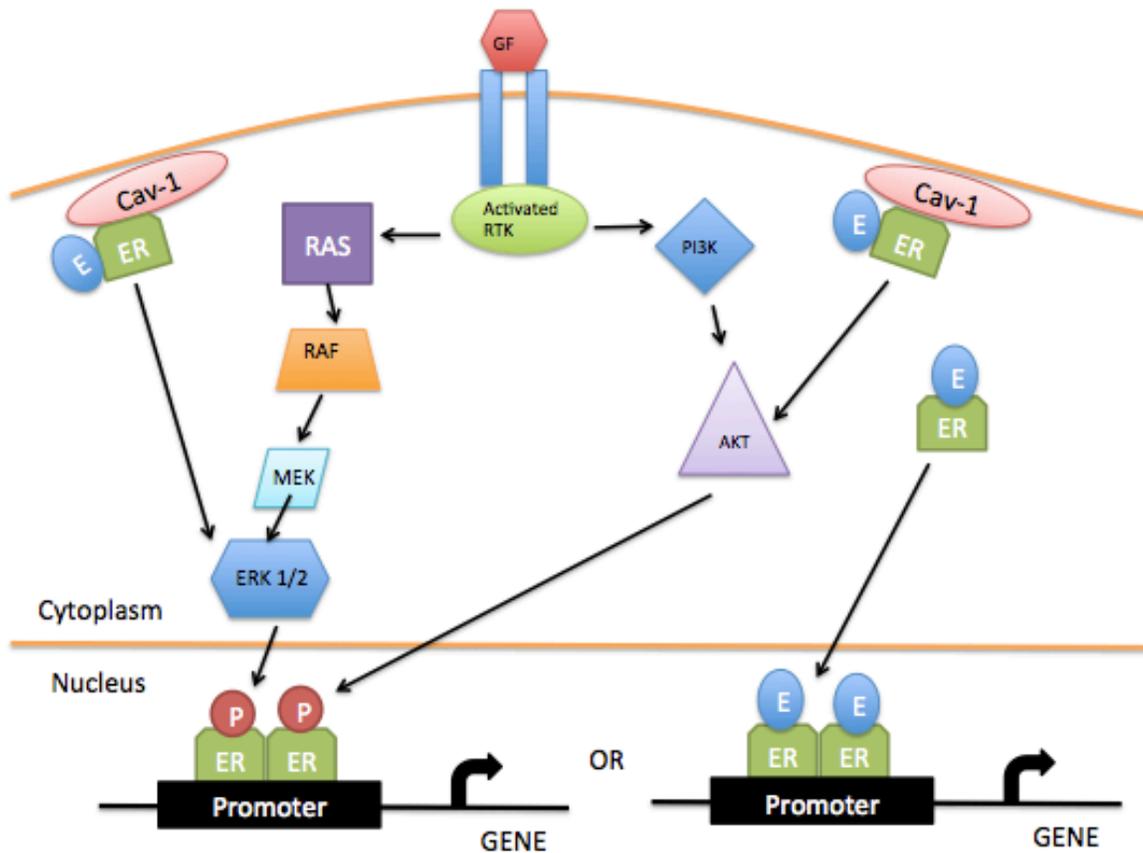


Figure 4. Crosstalk between kinase pathways and ER α . The cytosolic estrogen/ER α complex can activate ERK 1/2 and AKT, which further phosphorylate the ER α to promote ligand-independent transcription. P: phosphate group. E: estrogen.

ER α + breast cancer treatments: endocrine therapy

Endocrine therapy has resulted in reduced mortality and decreased recurrence of ER+ breast cancer (45). Because of high efficacy and tolerance, as well as more than 30 years of positive clinical results, tamoxifen (Tam) is the most prescribed endocrine therapy drug to treat ER+ breast cancer (46). Directly competing with estrogen, a

metabolite of Tam, 4-hydroxytamoxifen (4-OHT), binds to the ER α , and the resulting complex binds to the ERE, inhibiting expression of estrogen responsive genes, and ultimately causing cell cycle arrest (47-49).

Tam has been said to be “the most important drug to be developed in the history of breast cancer” (50). Yet, Tam is not without its limitations. Tam treatment does not result in cell death; rather it arrests the cells the G₁ phase of the cell cycle (51). In addition, thirty percent of ER+ breast cancer tumors do not respond to Tam, and those that do eventually develop resistance. Previous studies have found that over activation of the MAPK and PI3K pathways during Tam treatment may cause Tam resistance via ligand-independent activation of the ER α , decreasing the overall rate of ER+ breast cancer survival (52).

ER α + breast cancer treatment: flavonoids

Because of their ability to promote proliferation and activate the ER α , PI3K and MAPK pathways have become a therapeutic target for breast cancer (53,54). In our lab, we have identified naringenin (Nar), a grapefruit flavonoid, as an inhibitor of both the PI3K and MAPK pathways. Nar has been shown to inhibit the phosphorylation of both ERK 1/2 and AKT in MCF-7 ER α + breast cancer cells (55).

Additionally, studies have shown that Nar inhibits estrogen-dependent proliferation by competing with estrogen to bind to ER α (56). When bound to ER α , Nar decreases the rapid activation of ER α at the plasma membrane by quickly disassociating it from caveolin-1 at the cell membrane (Figure 4) (57). This interaction abolishes the

ability of ER α to activate the kinase pathways, and has been shown to promote apoptosis (58).

Apoptosis

Apoptosis is a pathway that is stimulated by various stimuli and is regulated by caspases (cysteine aspartyl-specific protease). Cytochrome c, a central player in apoptosis, resides in the inner membrane of the mitochondria. Pro-apoptotic signals affect the mitochondrial membrane, resulting in cytochrome c release into the cytosol, where cytochrome c associates with the Apaf-1 protein to create the apoptosome. The apoptosome then cleaves initiator caspases, such as caspase 9, resulting in activation of the caspase. From there, activated initiator caspases cleave to activate executioner caspases, such as caspases 3, 6, and 7. Active executioner caspases eventually cleave important cellular components such as ICAD (inhibitor of caspase-activated DNAase), which frees the DNase, resulting in DNA fragmentation (59).

Tam and Nar as combination therapy

Inhibitors of single kinases or pathways are rarely clinically successful (60), and estrogen-antagonists such as Tam often lead to resistance. Combination therapies have the potential to fully inhibit tumors by promoting apoptosis and overcome or avoid resistance (52). Currently, combination therapies using endocrine therapy and kinase inhibitors for breast cancer are being investigated (61) and may be the future of ER α + breast cancer treatments.

To inhibit ER α + breast cancer cell proliferation in cell culture, Nar in combination with Tam appears ideal. Tam inhibits ligand-dependent activation of the

ER α by acting as an estrogen antagonist. Nar inhibits ERK 1/2 and Akt phosphorylation, which prohibits ligand-independent activation the ER α . Together as a combination therapy, Tam and Nar (Tam+Nar) would target multiple pathways that promote ER+ breast cancer cell proliferation and survival.

Tam resistance and Nar

As mentioned earlier, endocrine therapy resistance often occurs in ER α + breast cancer cells. This has been speculated as the cause of the ERK1/2 pathway phosphorylating of the ER α (52). Studies have demonstrated that inhibition of the ERK 1/2 activity in Tam-R cells restored Tam sensitivity (60). Studies have also shown that the inhibition of PI3K has reversed hormone therapy resistance (62). Because Nar inhibits the activity of ERK 1/2 and AKT, Nar may restore Tam sensitivity to Tam-R cells.

In our lab, we have developed Tam-R MCF-7 ER α + breast cancer cells. These Tam-R cells were shown to have a constitutively active ERK 1/2 (unpublished data). By subjecting Tam-R cells to Nar treatment, ERK 1/2 phosphorylation may be inhibited and apoptosis could occur, demonstrating that Nar could be an effective treatment for Tam-R cells.

CHAPTER II

MATERIALS AND METHODS

Materials

The MCF-7 cell line was purchased from ATCC. Tissue culture materials were from Gibco (Grand Island, NY). Nar and 4-OHT were obtained from Sigma Aldrich (St. Louis, MO). Guava Via-Count Reagent was purchased from Millipore (Billerica, MA). Phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴), p44/42 MAP kinase, phospho-AKT (Ser⁴⁷³), and Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). ER α antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Actin antibody was obtained from Abcam (Cambridge, UK). Anti-mouse and anti-rabbit horseradish peroxidase conjugated secondary antibodies were purchased from Sigma Aldrich. AlexaFluor 488 conjugated Goat anti-Rabbit secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA). The enhanced chemiluminescence (ECL) detection kit was from ThermoScientific (Waltham, MA).

Cell culture

Various media formulations were used during these studies. MCF-7 and Tam-R cells were maintained in either Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 0.01 mg/mL bovine insulin, and 100 U/mL penicillin/streptomycin, or phenol red-free Dulbecco's Modified Eagle Medium

(PRF-DMEM) supplemented with 10% charcoal-stripped FBS. Tam-R media was supplemented with 100 nM Tam. MCF-7 and Tam-R cells were maintained at 37° C with 5% CO₂. Media was replaced every 2 days, and cells were passed once they attained 80% confluence.

Immunoblot analysis

Cell lysates were prepared as previously described (53). Protein concentrations of whole cell lysates were determined by Bradford Protein Concentration Assay (Bio-Rad, Hercules, California). Samples were heated at 95°C for five minutes, separated by 10% SDS-PAGE, and transferred overnight to Immobilon-P membranes (Millipore). Membranes were incubated with the antibody of interest and visualized by Bio-Rad ChemiDoc XRS system using an enhanced chemiluminescent (ECL) kit. The resulting bands were quantified using the densitometry feature in the Quantity One analysis software.

Via-Count viability assay

Treated cells were washed twice with PBS and subjected to trypsin. Cells were centrifuged at 5000 x g for 5 min, supernatants were discarded and cells resuspended in PBS. A 1:20 dilution of cells was prepared in ViaCount Reagent and incubated for 5 min in the dark. Viability was analyzed by Guava easy-Cyte™ Flow Cytometry (Millore) using the ViaCount software.

Immunofluorescence

Treated cells were fixed in 3.7% formaldehyde for 15 min, washed and then permeabilized in 0.25% Triton-X solution for 5 minutes. Cells were blocked for 15 minutes in a 5% goat serum, 1% BSA solution in PBS. Cells were treated with primary antibody of interest at a 1:100 dilution for an h at RT. Cells were washed 3 times in PBS and placed in secondary for an h in the dark. Cells were washed and treated with a 1:1000 DAPI solution for 5 min. Coverslips were mounted onto slides using Dako mounting solution.

Statistical analysis

Data are stated as means \pm SEM. The significance of comparing means was assessed by two-way analysis of Student's *t*-test (StatPlus, AnalystSoft).

CHAPTER III

RESULTS

Both Tam and Nar independently inhibit cell proliferation

Tam has been shown to inhibit MCF-7 cellular proliferation by inducing cell cycle arrest (47-49). Nar, a kinase inhibitor, has been shown to inhibit cellular proliferation in MCF-7 cells by inhibiting pro-proliferation pathways, PI3K and MAPK (55, 69). To determine the time course of inhibition, we treated MCF-7 cells with either 100 nM Tam or 250 μ M Nar for 2 – 5 days and assayed for cell density. Both Tam and Nar treatment reduced cell density on days 2, 4, and 5 of treatment when compared to control, with the greatest reduction on Day 4 (Figure 5A, B).

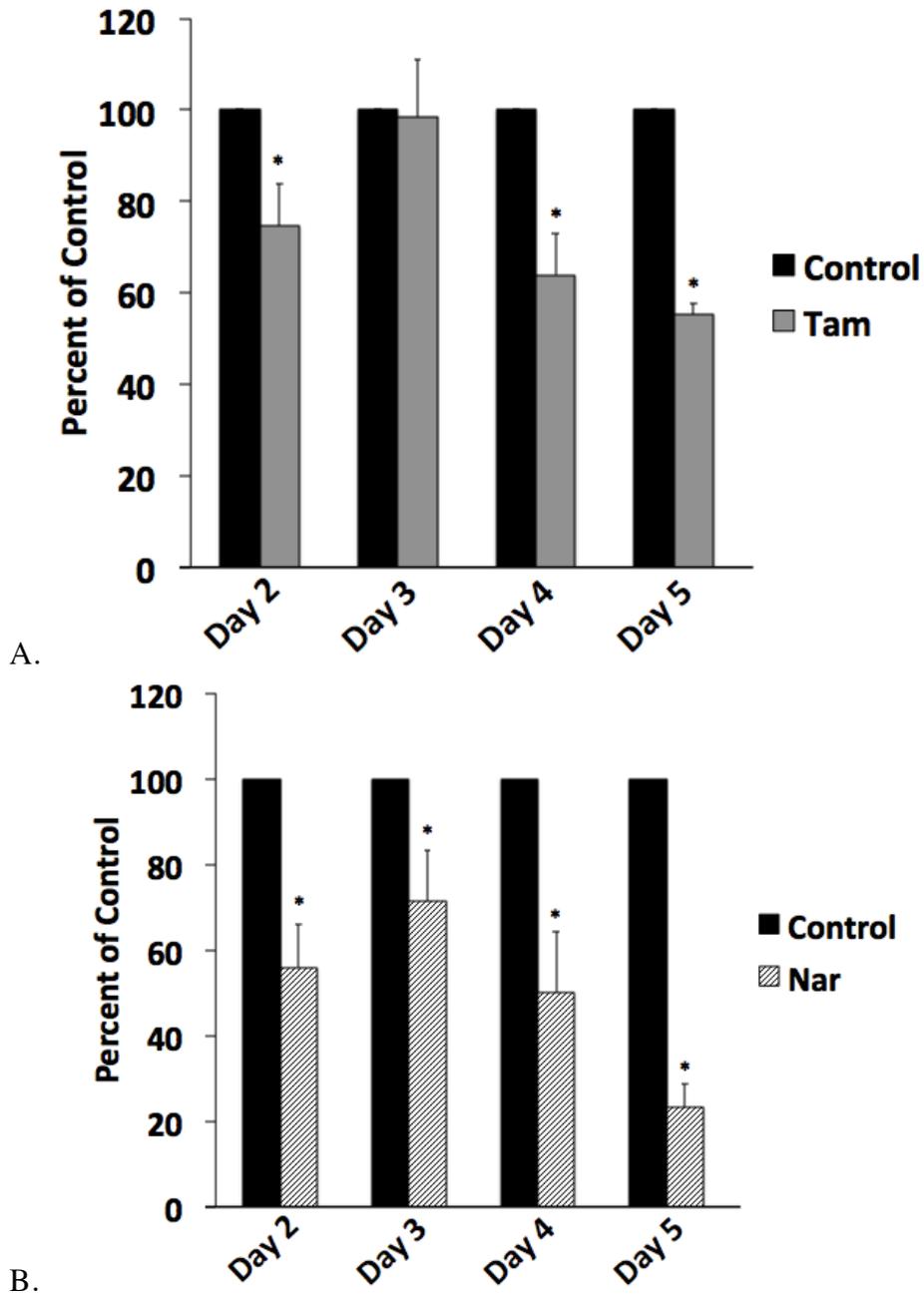


Figure 5. Tam and Nar alone inhibit cell density. MCF-7 cells were grown in full medium in the presence of 4-OHT (100 nM) (Panel A) or Nar (250 μ M) (Panel B). Cell density (cells/ml) was determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to vehicle control (*).

Tam+Nar impairs cell density greater than Tam alone

Combination treatments are often used to treat cancer. Particularly, a combination of kinase inhibitors and ER α inhibitors has been investigated in the treatment of ER α + breast cancer, in both experimental and clinical settings (61,62). Because Tam and Nar alone inhibit cell density, we wanted to determine the effects on cell density of a combination of Tam+Nar compared to the current clinical treatment: Tam alone. To date, there have been no studies on the use of Tam and Nar as a combination treatment.

To investigate whether Tam+Nar inhibits cell density greater than Tam alone, we treated MCF-7 cells with 100 nM Tam or a combination of 100 nM Tam and 250 μ M Nar for 2 - 5 days. When compared to Tam alone, Tam+Nar inhibited cell density on days 2 - 5 (Figure 6).

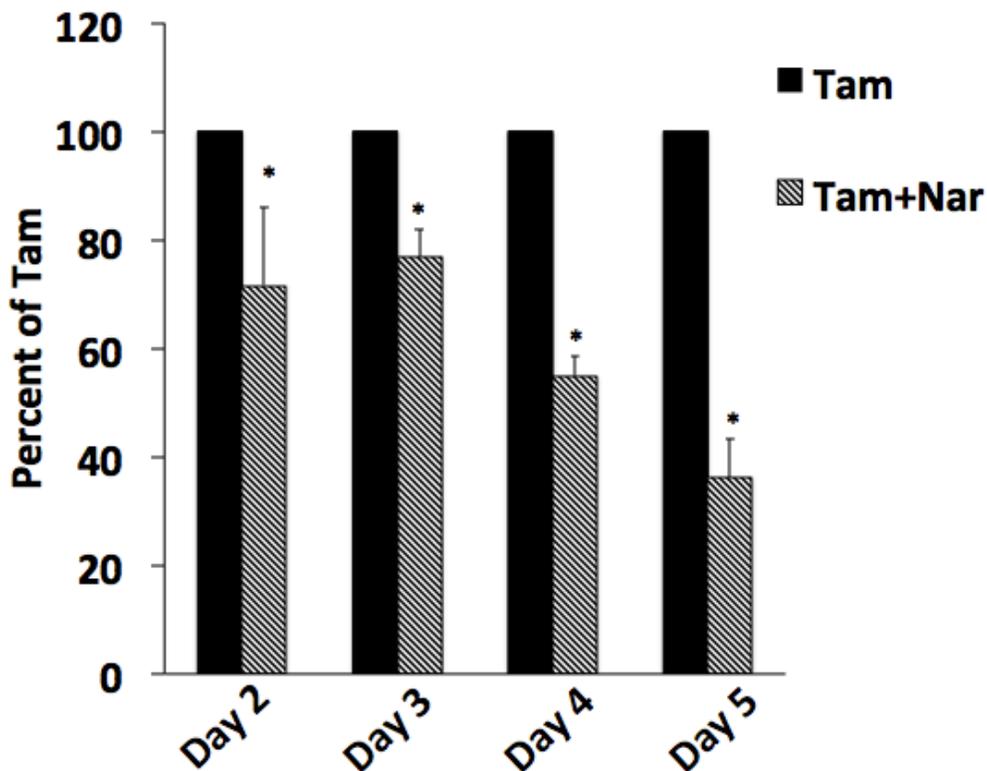


Figure 6. Tam+Nar inhibits proliferation greater than Tam alone. MCF-7 cells were grown in full medium in the presence of 4-OH-Tam (100 nM) or in 4-OH-Tam and Nar (250 μ M). Cell density (cells/ml) was determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to Tam (*).

Tam+Nar impairs cell viability greater than Tam alone

To investigate the effect of Tam+Nar on ER+ breast cancer cell viability, we treated MCF-7 cells with 100 nM Tam, 250 μ M Nar, or a combination of the two for 2 - 5 days. As expected, Tam treatment did not show a significant decrease in cell viability, which confirmed the cytostatic behavior of Tam. Nar treatment significantly reduced (40%) cell viability by day 5, confirming the cytotoxic behavior of Nar (Figure 7).

The combination treatment significantly reduced cell viability on days 4 and 5 when compared to Tam only. Furthermore, our results show that Tam+Nar treatment elicited a more cytotoxic effect than either treatment alone. Collectively our results indicate that Tam+Nar produced maximal effects on cell density and viability between days 4 and 5.

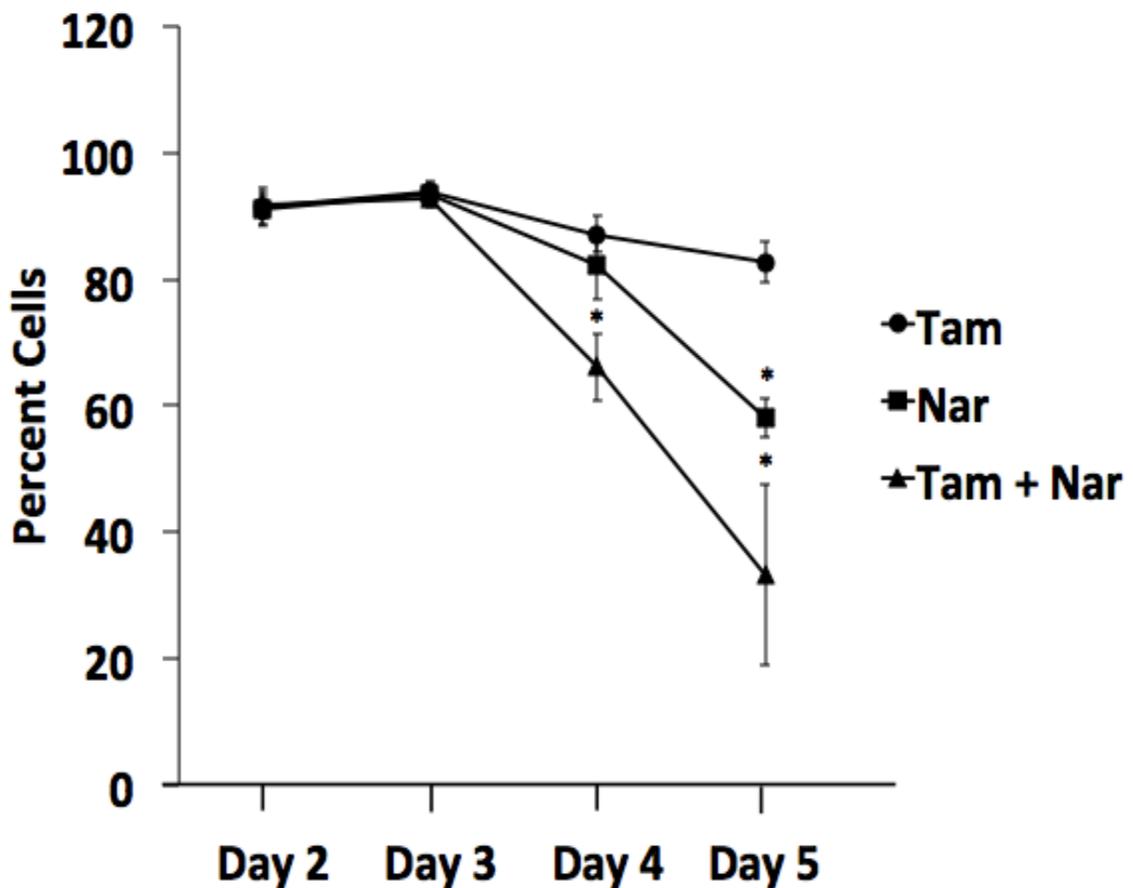


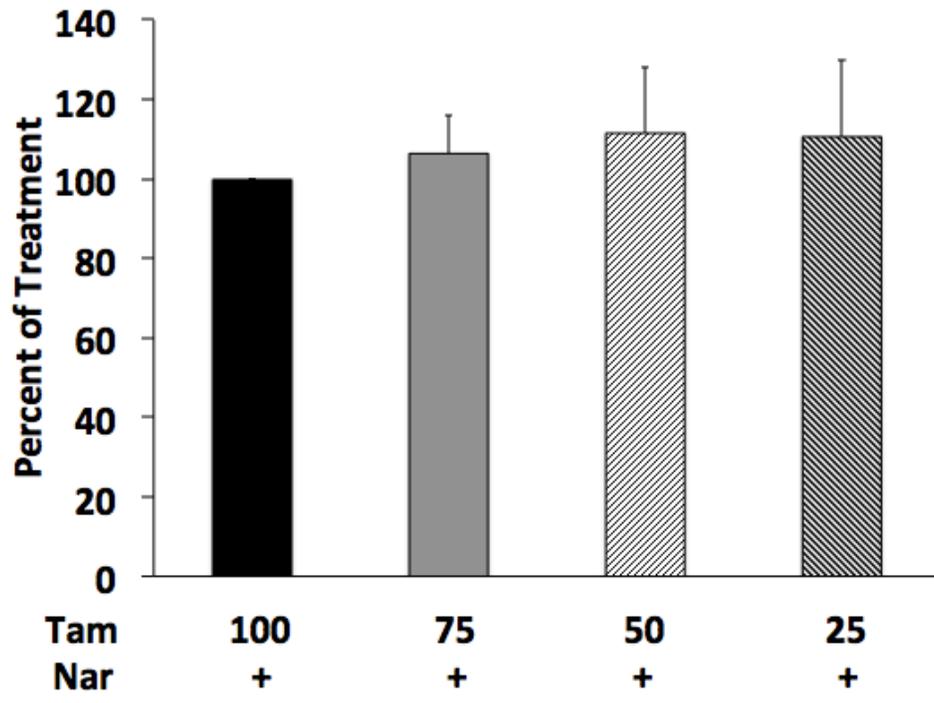
Figure 7. Tam+Nar elicits a cytotoxic effect. MCF-7 cells were grown in full medium the presence of 4-OHT (100 nM), Nar, (250 μ M) or a combination of the two. Cell viability was determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to Tam on individual days (*).

Determination of the optimal Tam+Nar concentration

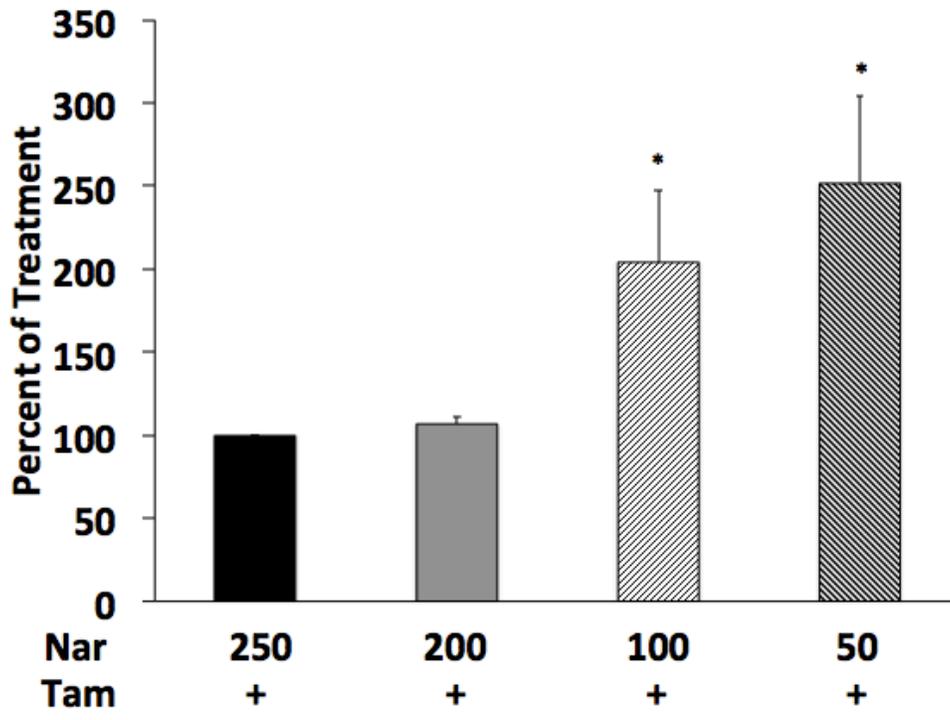
Next, we investigated whether a lower concentration of drugs would elicit similar effects as the reference (Ref) treatment (250 μ M Nar and 100 nM Tam on MCF-7 cells on day 4). To do this, we treated MCF-7 cells with 250 μ M Nar and decreasing concentrations of Tam for 4 days. Cell density did not change when Tam was reduced during the dual treatment (Figure 8A).

To determine the effects on cell density of a lower concentration of Nar during dual treatment, we treated MCF-7 cells with 100 nM Tam and decreasing concentrations of Nar for four days. A combination treatment of 200 μ M Nar and 100 nM Tam showed a minimal increase of 7% in cell density when compared to the Ref treatment (Figure 8B).

Next, we wanted to determine the lowest concentrations of both Nar and Tam. To do this, we treated cells for four days with varying concentrations of Nar and Tam. The optimal lowest concentration was 200 μ M Nar and 25 nM Tam. The other two treatments showed a significant increase in cell density (Figure 8C).



A.



B.

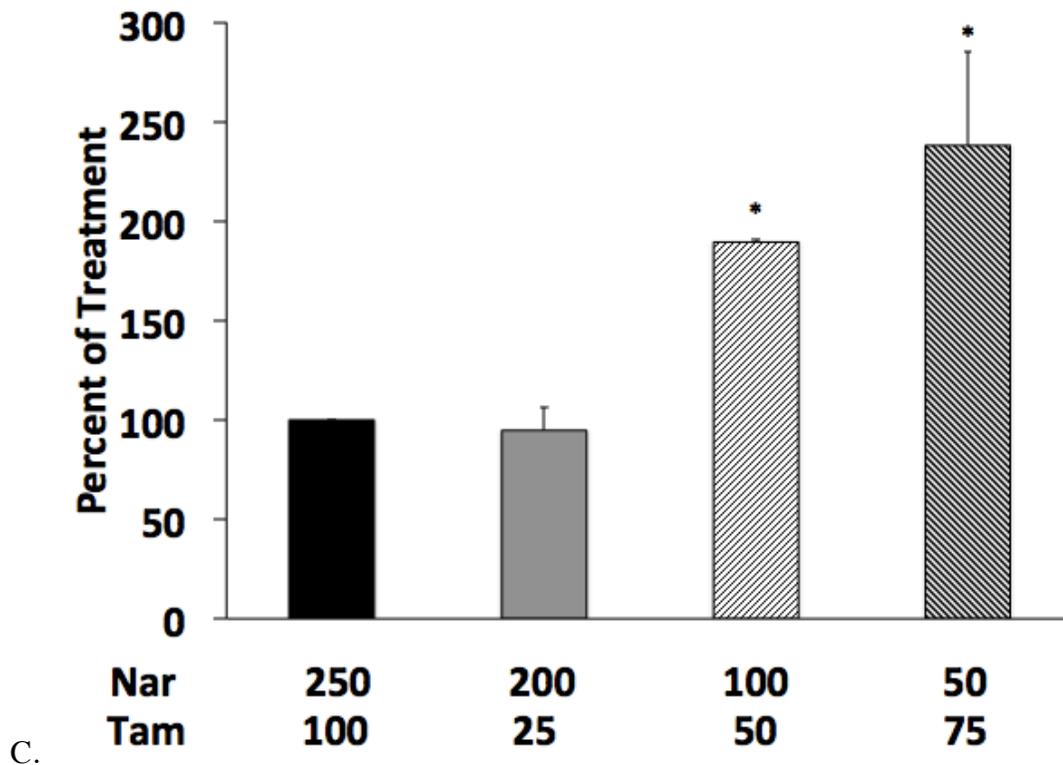


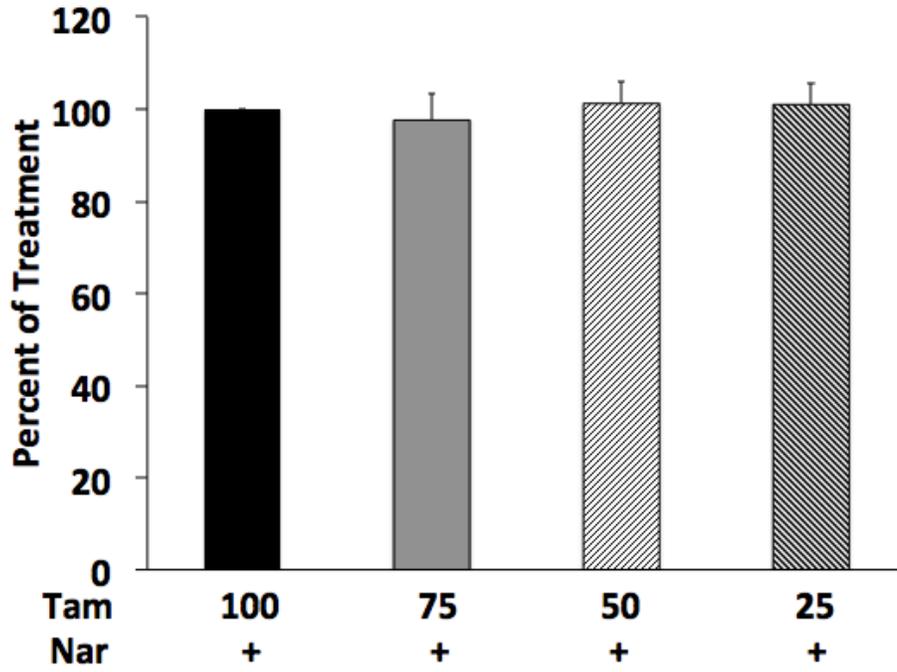
Figure 8. The determination of the optimal concentration of Tam+Nar on cell density. MCF-7 cells were grown in full medium the presence of varying concentrations of Nar and varying concentrations of 4-OHT for four days. Cell density (cells/ml) was determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to Ref (*).

We also investigated whether a lower concentration of drugs would elicit similar effects the Ref treatment on cell viability. To do this, we treated MCF-7 cells with 250 μ M Nar and decreasing concentrations of Tam for 4 days. Reduction of Tam did not affect cell viability (Figure 9A).

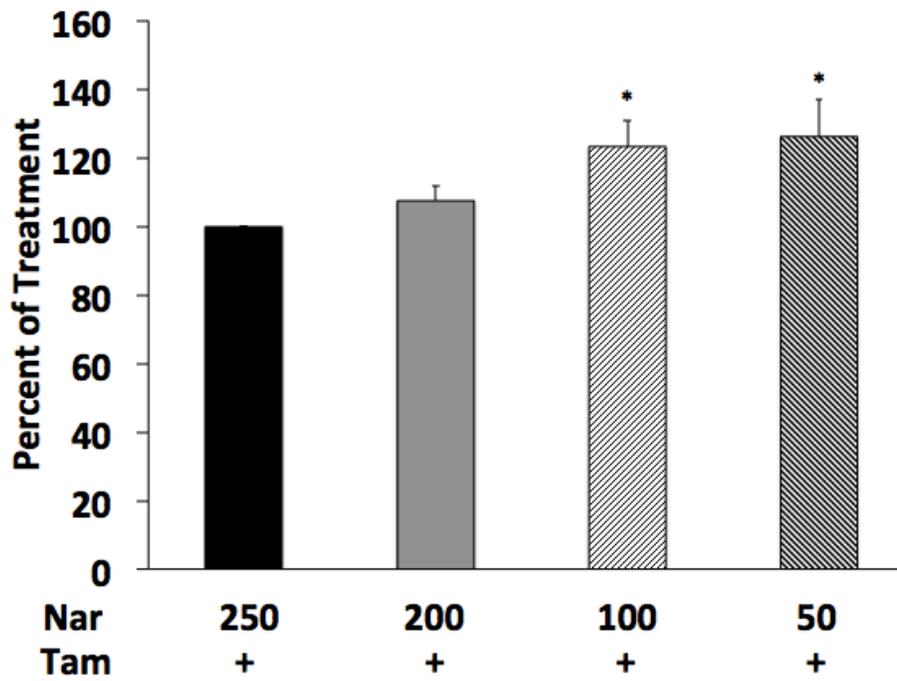
Next, we investigated the effects of varying concentrations of Nar on cell viability while in combination with Tam. When compared to the Ref treatment, a combination of 200 μ M Nar and 100 nM Tam showed no change in cell viability, while 100 μ M Nar in combination with 100 nM Tam showed a significant increase in cell viability (Figure 9B).

Lastly, we treated cells with varying concentrations of both agents for 4 days and assessed cell viability. There was a significant increase in cell viability in Nar concentrations lesser than 200 μM when compared to the Ref treatment (Figure 9C).

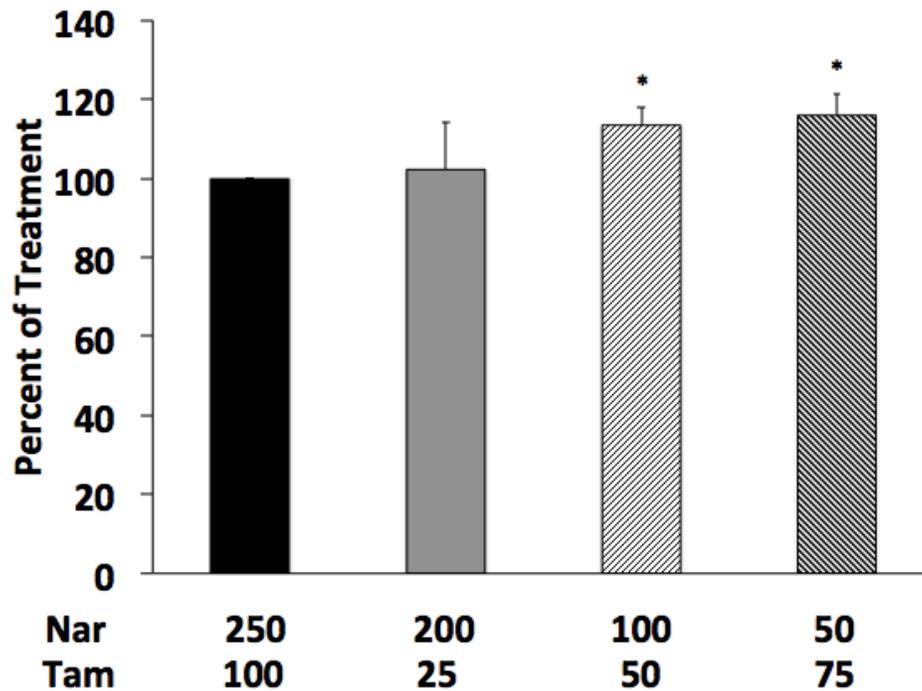
Our results suggest that Nar concentration is dose dependent on cell viability and density after 200 μM . Tam concentrations do not alter cell density or viability. Collectively, our data indicates that 25 nM Tam and 200 μM Nar is the optimal concentration. From here forward, we refer to 25 nM Tam and 200 μM Nar combination treatment as Tam+Nar.



A.



B.



C.

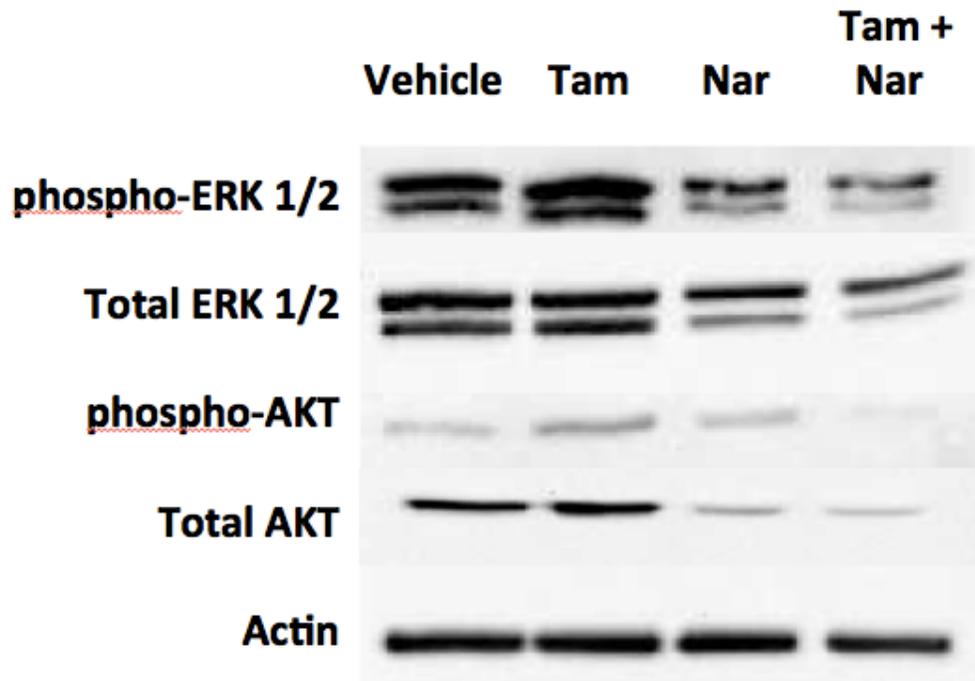
Figure 9. The determination of the optimal concentration of Tam+Nar on cell viability. MCF-7 cells were grown in full medium the presence of varying concentrations of Nar and varying concentrations of 4-OHT for four days. Cell viability was determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to Ref (*).

Tam+Nar reduces expression of ERK 1/2 and AKT

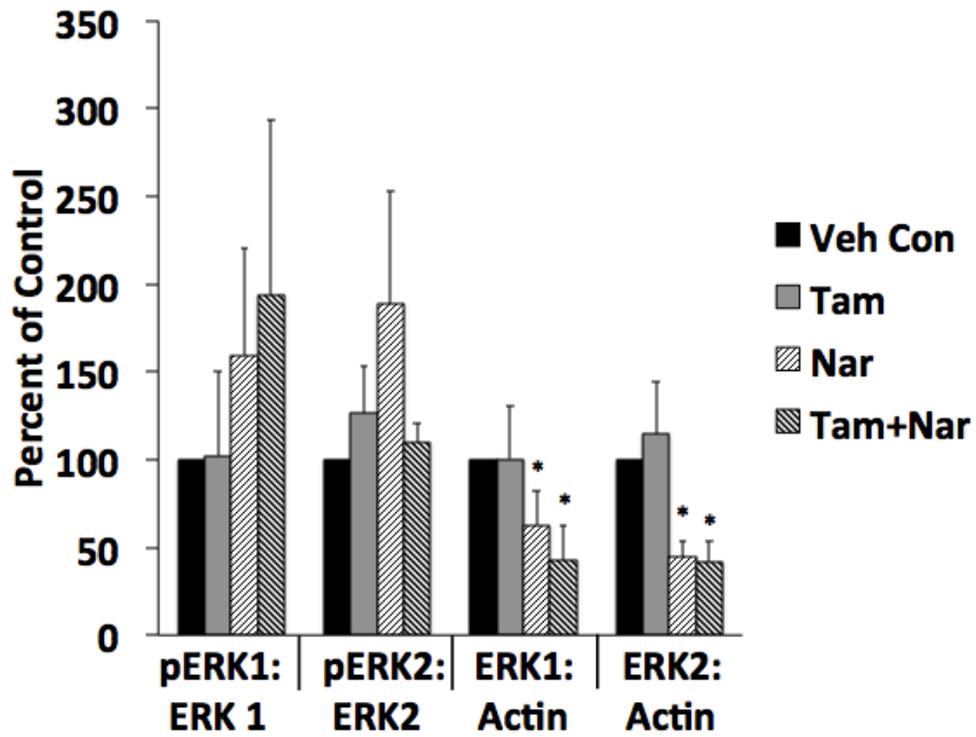
To determine the mechanism by which Nar elicits these effects on cell density and viability while in combination with Tam, we investigated the known targets of Nar. Nar has been shown to inhibit the phosphorylation of ERK 1/2 and AKT in MCF-7 cells in the absence of lipophilic compounds, such as estrogen (57, 70). In our studies, we wanted to simulate an environment similar to that present *in vivo*; so we performed our experiments in the presence of serum with lipophilic hormones. There are no studies

investigating the molecular mechanism of Nar in MCF-7 cells cultured in the presence of lipophilic hormones.

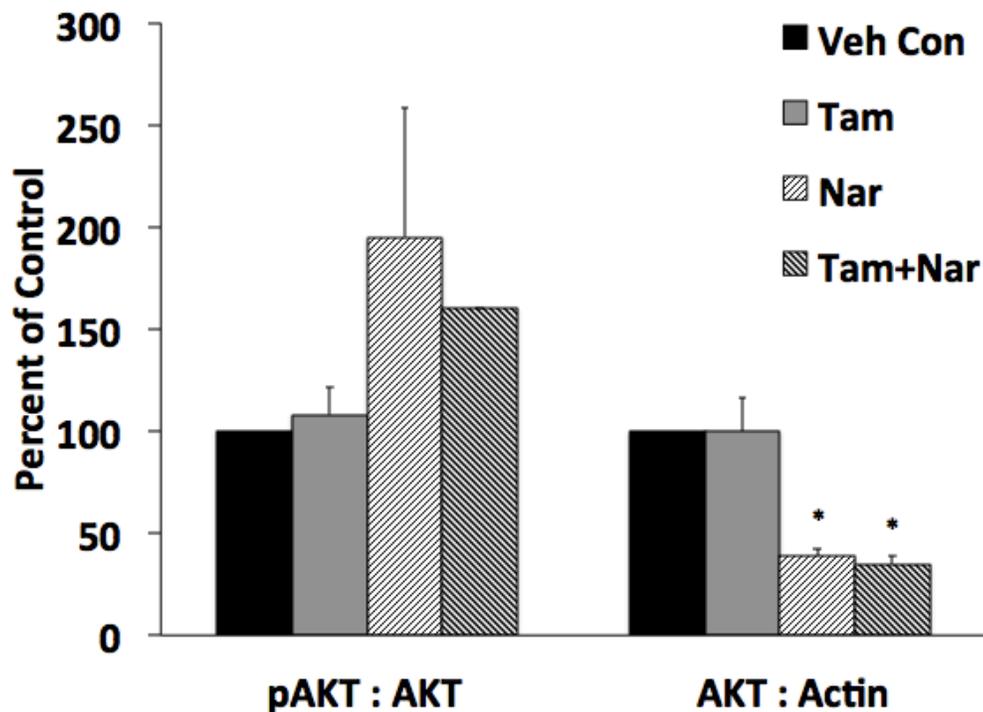
To investigate the effect Tam+Nar has on ERK 1/2 and AKT, we treated MCF-7 cells with Tam, Nar, and Tam+Nar for 4 days. We examined the phosphorylation and expression levels of ERK 1/2 and AKT. Tam treatment did not affect the expression of ERK 1/2 and AKT. On the other hand, Nar and Tam+Nar significantly reduced the expression of ERK 1/2 and AKT when compared to control (Figure 10A, B, C). None of the treatments significantly changed the phosphorylation of ERK 1/2 (Figure 10A, B) or the phosphorylation of AKT (Figure 10A, C) relative to total protein levels.



A.



B.



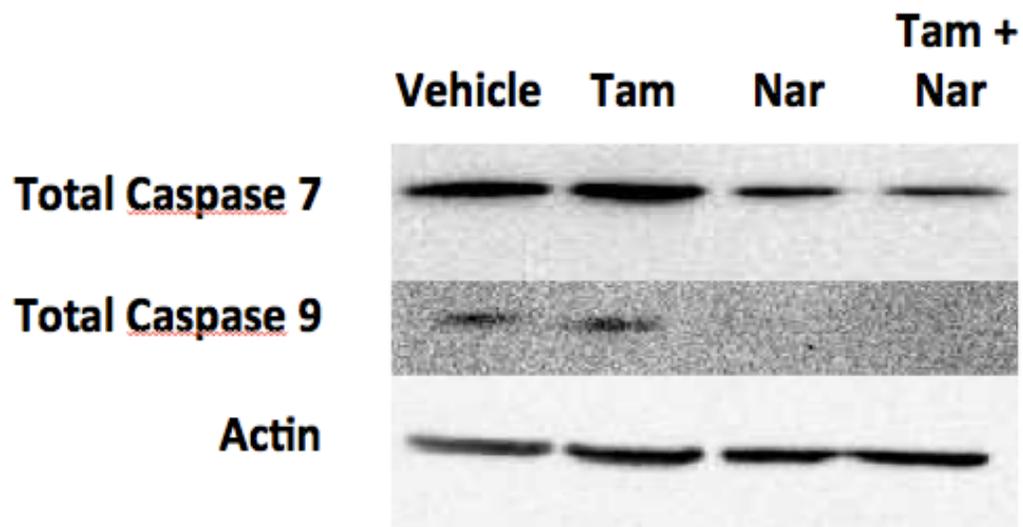
C.

Figure 10. Nar decreases the expression of ERK 1/2 and AKT. MCF-7 cells were grown in full medium in the presence of 4-OHT (25 nM), Nar (200 μ M), or a combination of the two for 4 days. Protein lysates were prepared. Lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK 1/2, ERK 1/2, phospho-AKT, AKT, and actin. Immunoblot results were quantified by densitometry (Panels B and C). ERK results are the means \pm SEM of four independent experiments. AKT results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to vehicle (*).

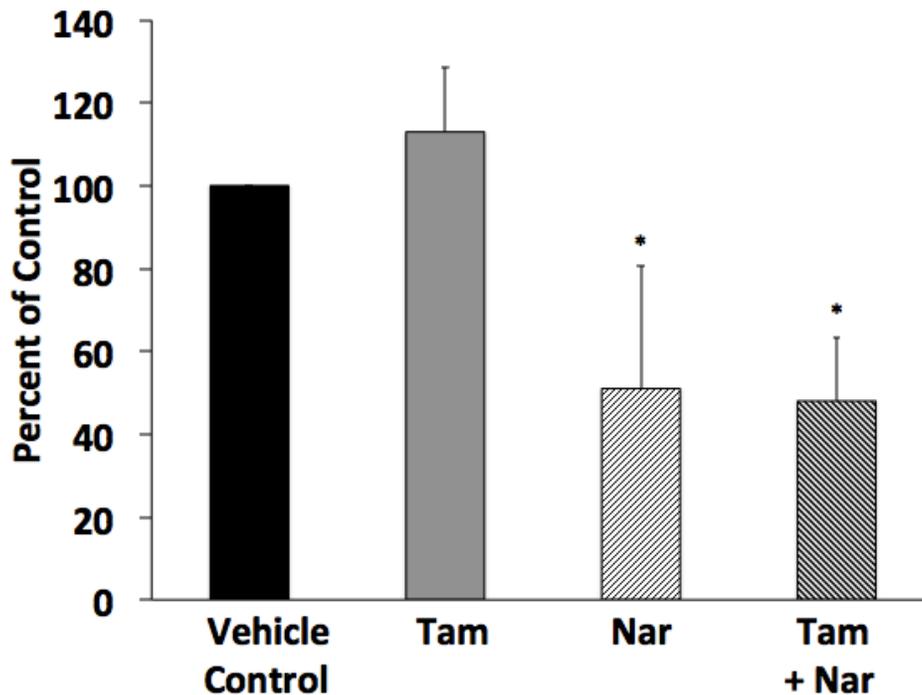
Tam+Nar promotes apoptosis via activation of caspases 7 and 9

Studies have shown that Nar induces apoptosis via caspase 3 activation in HeLa-cells transfected with ER α (63,64). Since MCF-7 cells do not express caspase 3 (65) but do express caspases 7 and 9, we wanted to investigate whether these caspases were involved in Nar induced apoptosis during Tam+Nar treatment (Figure 11A, B). Total caspase 9 was undetectable in Nar and Tam+Nar treated cells. Total caspase 7 was also

significantly reduced during Nar and Tam+Nar treatment. Furthermore, Tam alone has no effect on the expression of caspase 7 or 9. These results show that caspase 7 and 9 are cleaved and activated, and that apoptosis is occurring.



A.



B.

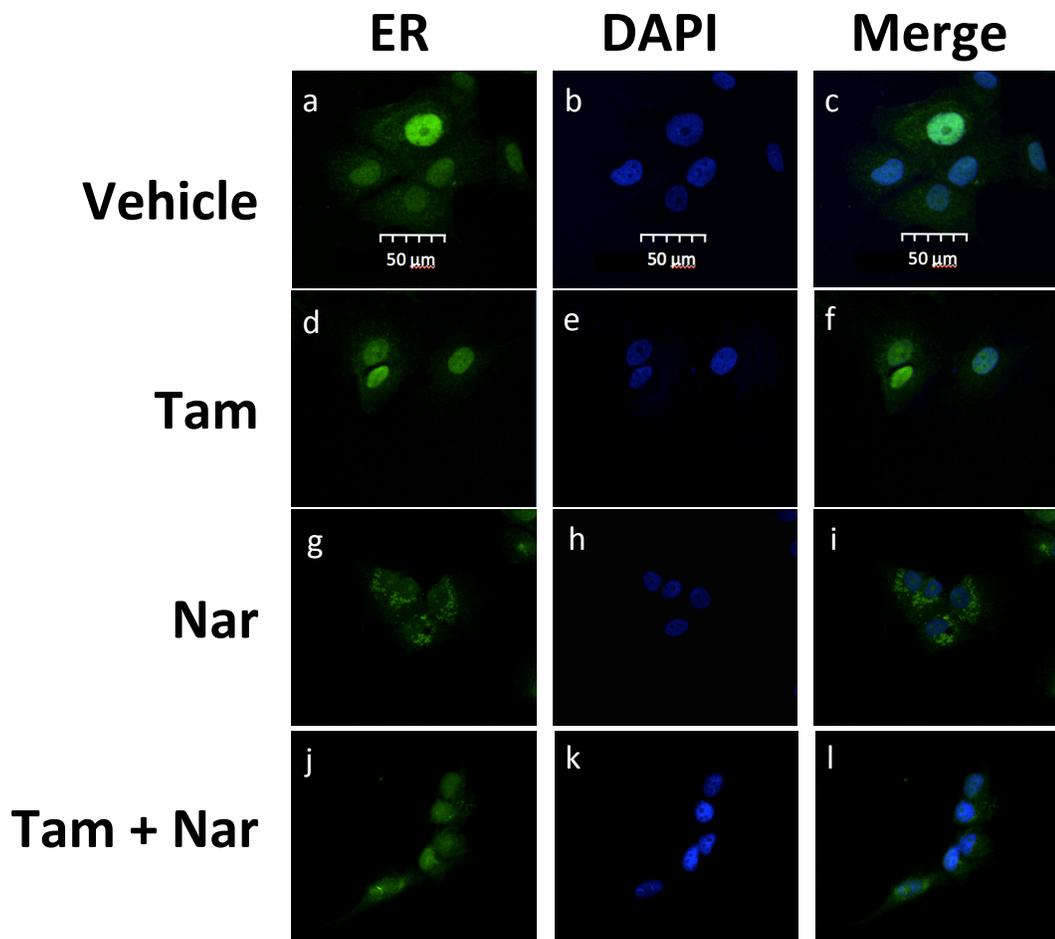
Figure 11. Nar promotes apoptosis via increased activation of caspases 7 and 9.

MCF-7 cells were grown in full medium in the presence of 4-OHT (25 nM), Nar (200 μ M), or a combination of the two for 4 days. Protein lysates were prepared. Lysates were subjected to SDS-PAGE and immunoblotted using antibodies against total caspase 7, total caspase 9, and actin (Panel A). Caspase 7 bands were quantified using densitometry (Panel B). Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to vehicle (*).

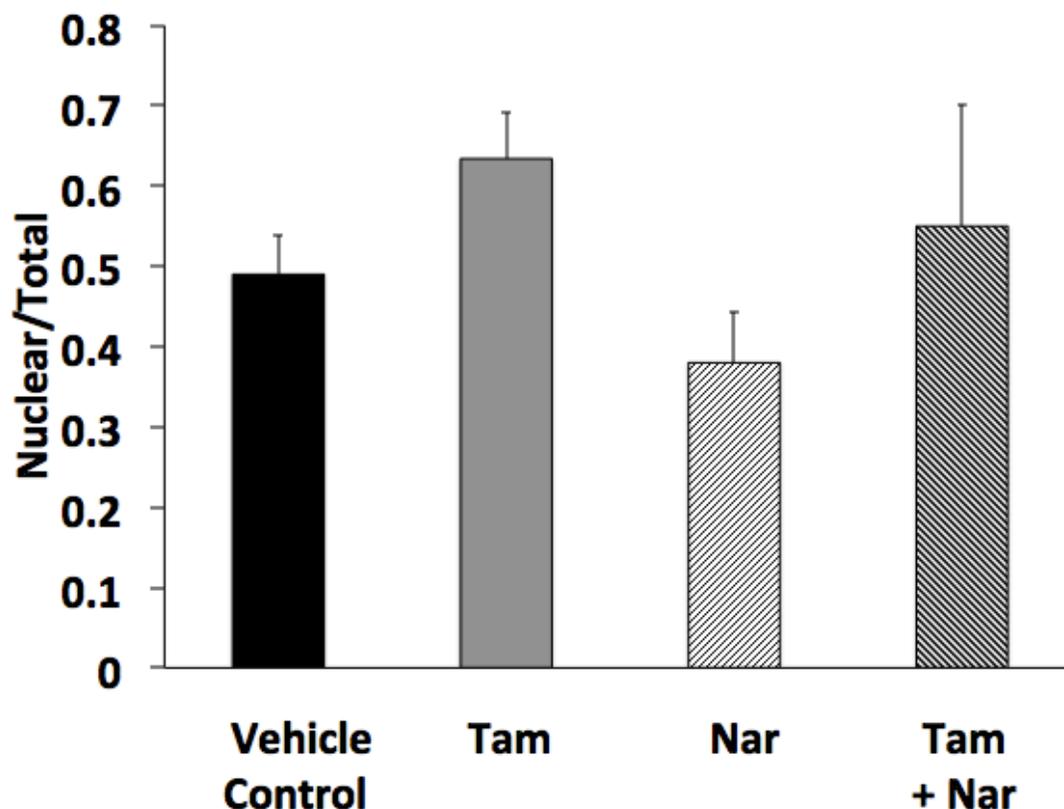
Tam+Nar influences estrogen receptor localization

Because both Tam and Nar are known to interact with the ER α (47-49, 56), we wanted to investigate the localization of ER α during the various treatments. Previous studies showed that Tam induces the translocation of the ER α from the cytosol into the nucleus (66). On the other hand, there have been no studies to determine the effect of Nar on ER α localization.

To investigate the localization of the ER α , we treated the cells with Tam, Nar, or Tam+Nar for 4 days and performed immunofluorescence (Figure 12A, B). In untreated cells, ER α was distributed throughout the cytosol and the nucleus. In Tam treated cells, ER α was predominately localized in the nucleus, while Nar treated cells showed an accumulation of ER α in the cytoplasm. Tam+Nar treated cells exhibited an even distribution of ER α in the nucleus and the cytoplasm, suggesting that both Nar and Tam independently influence the localization of ER α in the combination treatment.



A.



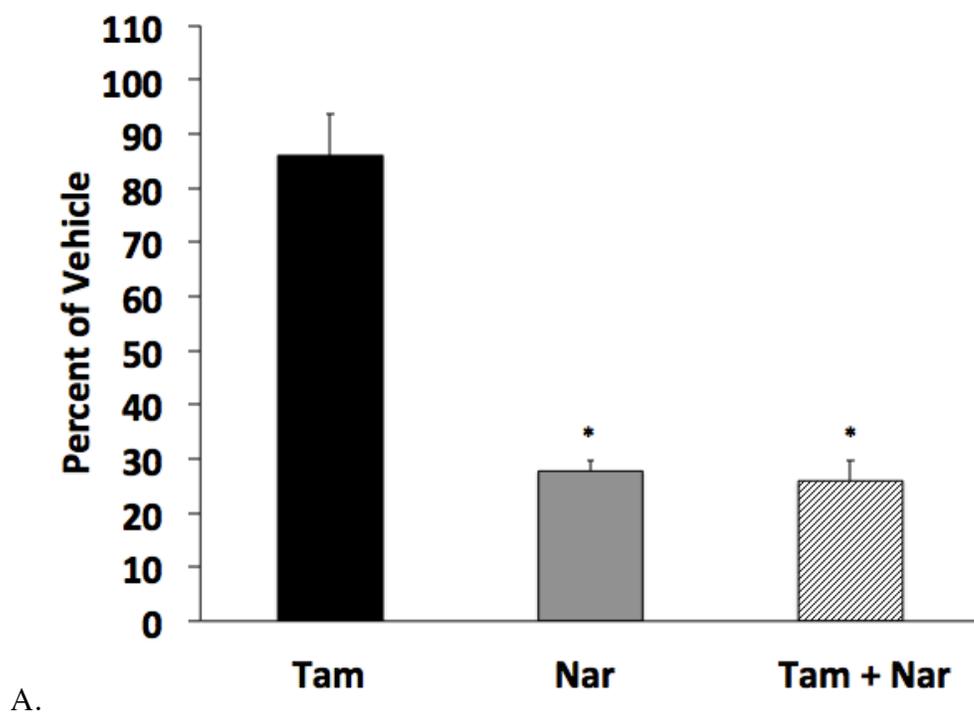
B.

Figure 12. Nar alone reduces ER α nuclear localization. MCF-7 cells were grown in full medium in the presence of 4-OHT (25 nM), Nar (200 μ M), or a combination of the two for 4 days on coverslips. At day 4, cells were fixed with formaldehyde and subjected to immunofluorescence using anti-ER α and DAPI. ER α localization was determined by confocal microscopy (Panel A). ER α nuclear localization was quantified using intensity parameters (Panel B). Results are the means \pm SEM of three independent experiments.

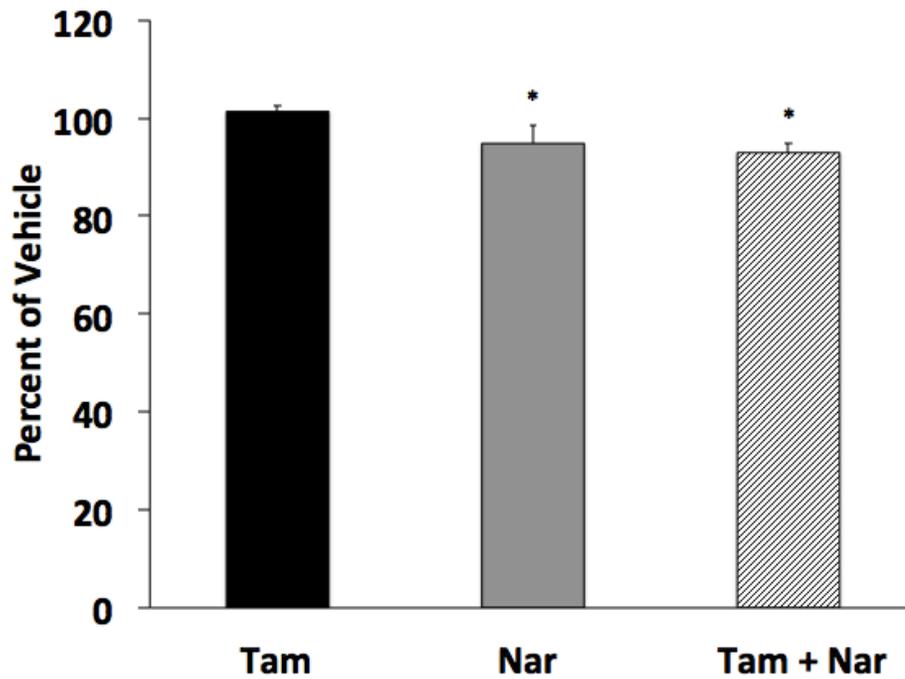
Nar inhibits cell proliferation and viability in Tam-R cells

Next, we investigated the effects of cell proliferation and viability on Tam-R cells. To determine the proliferation effects of Nar on Tam-R cells, we treated Tam-R cells with 25nM Tam, 200 μ M Nar, or Tam+Nar for four days. As expected, Tam treatment had little effect on Tam-R cell density, while Nar reduced cell density by 70%. Tam+Nar had the same effect as Nar treated cells (Figure 13A).

To investigate the viability effects of Nar on Tam-R cells, Tam-R cells were treated with 25nM Tam, 200 μ M Nar, or Tam+Nar for four days. Tam treatment had no effect on cell viability, while Nar treatment significantly decreased the viability of Tam-R cells. As expected, Tam+Nar cells exhibited the same viability as Nar treated cells (Figure 13B).



A.



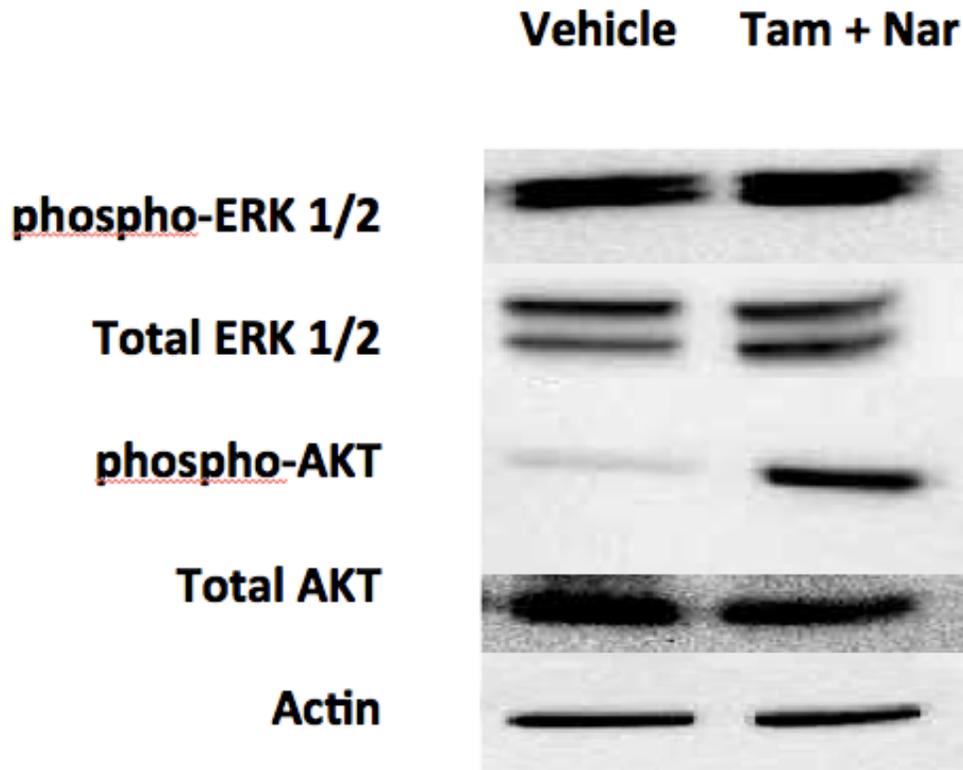
B.

Figure 13. Nar impairs cell density and cell viability. Tam-R cells were grown in full medium the presence of 4-OHT (25 nM), Nar (200 μ M), or Tam+Nar. Cell density at day 4 (Panel A) and cell viability (Panel B) were determined by flow cytometry. Results are the means \pm SEM of three independent experiments. Significance at $P < 0.05$ was compared to vehicle (*).

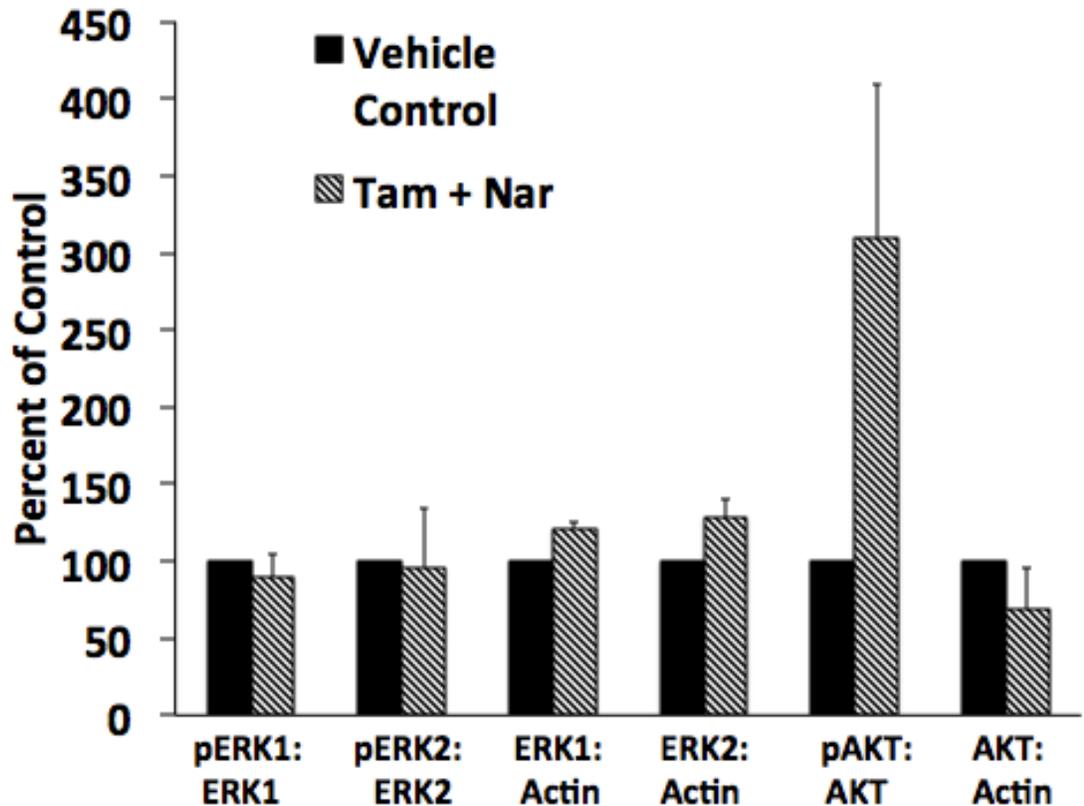
Tam+Nar increases the phosphorylation of AKT

Since Tam+Nar reduced total ERK 1/2 and AKT expression in Tam-sensitive cells, we wanted to determine whether the optimal concentration of Tam+Nar elicits the same effect on Tam-R cells. We treated Tam-R cells with Tam+Nar for 4 days, and cell lysates were assayed for phospho-ERK 1/2, ERK 1/2, phospho-AKT, AKT, and actin. Tam+Nar slightly increased the expression of ERK 1/2, while the levels of phospho-ERK 1/2 exhibited no significant change when compared to control levels. In contrast, the expression of AKT was reduced in treated cells when compared to control levels, and

AKT phosphorylation increased over 2 fold in Tam+Nar treated cells (Figure 14). All together, these results differ remarkably from Tam sensitive cells.



A.



B.

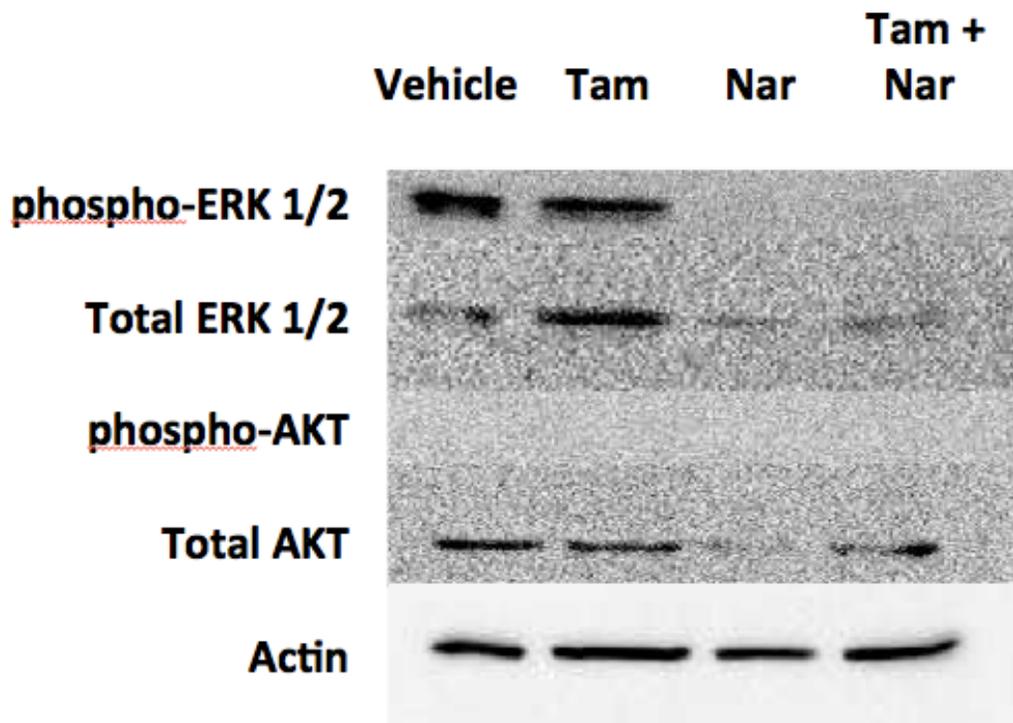
Figure 14. Tam+Nar increases the phosphorylation of AKT. Tam-R cells were grown in full medium in the presence of Tam+Nar for 4 days. Protein lysates were prepared. Lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK 1/2, ERK 1/2 phospho-AKT, AKT, and actin (Panel A). Immunoblot results were quantified by densitometry (Panel B). Results are the means \pm SEM of two independent experiments.

In the absence of lipophilic hormones, Nar eliminates the phosphorylation of ERK 1/2

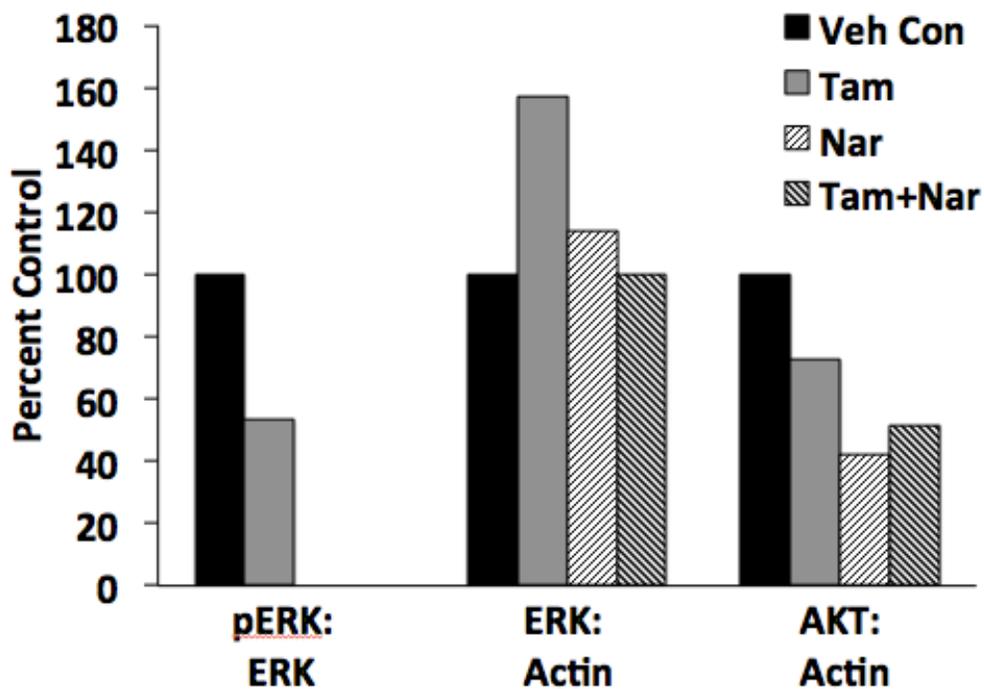
Our previous studies demonstrated that Nar inhibited the phosphorylation of ERK 1/2 in charcoal-stripped media (unpublished data). Charcoal-stripped media does not contain lipophilic compounds, such as as estrogen. We wanted to determine the effects of Tam, Nar, and Tam+Nar on phospho-ERK, total ERK, phospho-AKT, and AKT in the absence of estrogen. To do this, we maintained Tam-R cells in phenol red-free medium

supplemented with charcoal-stripped FBS and treated cells with Tam, Nar, and Tam+Nar for 4 days.

Tam treatment reduced the phosphorylation of ERK 1/2, yet increased the total expression of ERK 1/2. Nar, alone and in combination, resulted in undetectable levels of phospho-ERK 1/2. These results were accompanied with no change in ERK 1/2 expression. Surprisingly, without lipophilic compounds, Tam-R cells did not activate the AKT pathway, and the expression of AKT was significantly decreased when compared to control (Figure 15). These results differ drastically from Tam-R cells grown in the presence of lipophilic compounds.



A.



B.

Figure 15. Nar eliminates the phosphorylation of ERK 1/2. Tam-R cells were grown in charcoal stripped medium in the presence of 25 nM 4-OHT, 200 μ M Nar, or a combination of 25 nM 4-OHT and 200 μ M Nar. At day 4, cells were lysed. The protein lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK 1/2, ERK 1/2, phospho-AKT, AKT, and actin. Results were quantified using densitometry (Panel B).

CHAPTER IV

DISCUSSION

More than 65% of breast cancer diagnosed is ER+ (2), and Tam is the most prescribed anti-hormone therapy to treat this type of cancer (46). Tam functions to arrest cells in G1 of the cell cycle by competing with estrogen to bind to ER α (51). Once Tam is bound to ER α , the complex translocates to the nucleus, but the Tam/ ER α complex cannot properly bind to the ERE (47-49). Yet, ER+ breast cancer can overcome this block in cell cycle progression through the activation of the PI3K and MAPK pathways. Because Nar has been shown to inhibit the activity of these pathways (57) and inhibit ER α activity (55-58), a combination of Tam and Nar has the potential to be more effective than Tam alone.

Because Tam is well studied, effects of Tam on cell density and cell viability are widely documented. On the other hand, studies on Nar on these parameters are limited; studies have investigated the 24-hour effects of 1.0 μ M (63) and 100 μ M (55) Nar on MCF-7 cell proliferation. Yet the prolonged (2 – 5 days) effects of Nar on MCF-7 cell proliferation and viability had not been determined.

Although our studies indicate that Nar treatment alone has the ability to inhibit MCF-7 proliferation and reduce viability, single kinase inhibitors are rarely successful in a clinical setting (60). It has also been suggested that combination therapies of anti-

estrogen and kinase inhibitors are the future of ER+ breast cancer treatment (61). Thus, we wanted to investigate the effects of a combination of Nar and Tam.

Since Tam+Nar inhibits multiple pathways, we focused our studies on the effects of Nar on Tam sensitive MCF-7 cells. Overall, our studies indicate that Tam+Nar inhibited MCF-7 cell density and cell viability better than Tam alone, and the greatest effect occurred between days 4 and 5. To minimize undesired side effects, we investigated the optimal concentration of the Tam+Nar treatment on cell density and cell viability. The reduction of Tam concentration showed no effect on cellular proliferation and viability when compared to the Ref treatment. There was a dose-dependent effect as Nar concentration was reduced from 200 μM to 50 μM . Lastly, Nar concentrations were reduced in the presence of increasing Tam treatment. Again, a dose dependent effect was observed with Nar. Our studies show that an optimal treatment of 200 μM Nar and 25 nM Tam for four days of treatment inhibited cellular proliferation and reduced cellular viability in MCF-7 cells greater than Tam alone. Future studies could investigate lower concentrations of Tam on cellular proliferation and viability while combined with 200 μM Nar. Fewer undesired side effects could be a benefit of a lower concentration of Tam.

Next, we investigated the effects of Nar on Tam-R cells, because Nar targets the pathways that are upregulated in Tam-R cells that allow these cells to proliferation again. In Tam-R cells, Tam did not affect the density or viability, as expected. Nar and Tam+Nar significantly reduced cell density and viability. Studies have suggested that kinase inhibitors may reverse Tam-resistance (52). Because Nar and Tam+Nar elicit the

same effects on Tam-R on density and viability, our results suggest that Nar does not recover Tam sensitivity in Tam-R cells. Though the effects of Tam, Nar, and Tam+Nar on Tam-R viability (Figure 13B) do not appear as robust as the Tam-sensitive (Tam-S) experiment (Figure 7), the results do agree. In Tam-R cells, the combination treatment is essentially the same as the Nar treatment. Thus, in Tam-sensitive cells (Figure 7), we can only compare the Nar treatment on day 4 to the Tam-R experiment. Overall, Nar elicits the same density and viability effects on Tam-R cells as Tam sensitive cells.

Because the apoptotic mechanism of Nar has never been investigated in cells that do not express caspase 3, we investigated the apoptotic mechanism of Tam+Nar on MCF-7 cells after four days. Our results showed total reduction of full-length caspase 9, as well as a significant reduction in full-length caspase 7. Although we were unable to detect the cleaved forms of these caspases, other studies have shown that a reduction in full-length caspase indicates cleavage, and thus activation (67). To investigate the apoptotic mechanism of Nar treatment on Tam-R cells, future studies should analyze levels of caspase 7 and caspase 9 in these cells.

Because MCF-7 cells grow primarily through the activities of the ER α , we wanted to investigate the effect of Nar and Tam+Nar on the localization patterns of ER α . In untreated cells, ER α is distributed throughout the cytoplasm and nucleus. Nar sequestered ER α in the cytoplasm. Tam+Nar showed a redistribution of ER α in both the cytoplasm and the nucleus. This result suggests that Tam and Nar compete with one another to interact with ER α .

Previous studies in our lab demonstrated that Tam-R cells exhibited an even distribution of the ER α when treated with Tam, which is similar to localization pattern of ER α in untreated MCF-7 cells. Additionally, these previous studies showed that ER α is sequestered outside of the nucleus in Tam-R cells treated with Nar. These results are similar to the ER α localization in Nar treated Tam-S MCF-7 cells. Collectively, our results suggest that Nar treatment results in the accumulation the ER α in the cytoplasm. Next, we wanted to investigate the molecular mechanism of Tam+Nar by investigating the known targets of Nar, ERK 1/2 and AKT in Tam-S (MCF-7 cells) and Tam-R cells cultured in the presence or absence of lipophilic compounds. In Tam+Nar Tam-S cells, only total ERK 1/2 expression decreased. In Tam-R cells with lipophilic hormones, only the phosphorylation of AKT increased 300% of untreated. When cultured without lipophilic compounds, Tam-R cells did not express phosphorylated AKT, but these cells expressed high levels of phosphorylated ERK 1/2, which confirms the estrogen-independence in these cells. Tam+Nar treatment eliminated this phosphorylation (reviewed in Figure 22).

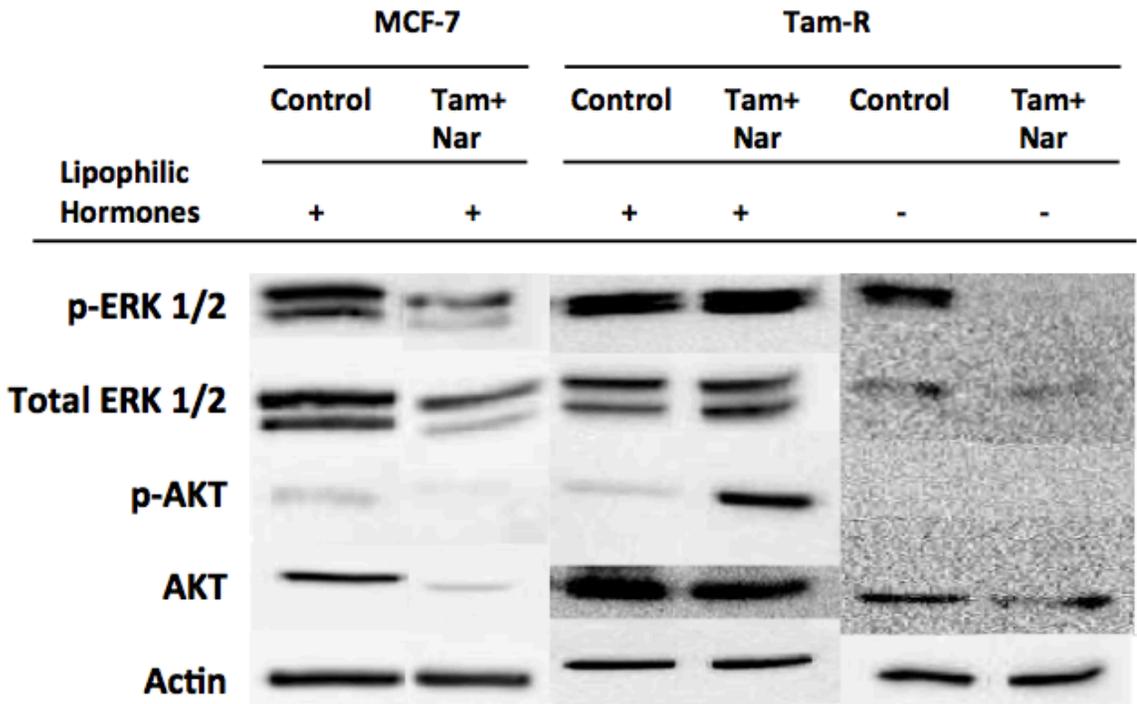


Figure 16. Overview of the known targets of Nar. MCF-7 and Tam-R cells were grown in full medium or in charcoal stripped medium with or without a combination of 25 nM 4-OHT and 200 μ M Nar. At day 4, cells were lysed. The protein lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK 1/2, ERK 1/2, phospho-AKT, AKT, and actin.

The mechanism for the inhibition of ERK 1/2 phosphorylation in Tam-R cells in the absence of estrogen is due to Nar treatment. Tam-R cells proliferate and survive independent of AKT and ER α /estrogen activity. Thus, a constitutively active ERK 1/2 pathway in Tam-R cells is a potential mechanism used by these cells for survival. A constitutively active ERK 1/2 can either directly affect transcription factors to induce proliferation, or ERK 1/2 can phosphorylate the ER α , activating ER α transcription independently of ligand binding. Because our confocal results suggest that Nar sequesters ER α in the cytoplasm, ER α ligand-independent transcription may not be

activated by ERK 1/2 during Nar treatment. Thus, without the influence of estrogen, Nar inhibits ERK 1/2 phosphorylation, directly affecting transcription factors that induce proliferation in Tam-R cells.

Furthermore, our results suggest that Tam+Nar impairs MCF-7 proliferation through the inhibition of the activity of ER α , either directly or indirectly. Because Nar sequesters ER α in the cytoplasm, the ER α transcriptional activity may be reduced, and when Tam is bound to the ER α , ER α cannot transcribe genes. This would explain the decrease in ERK and AKT proteins: both of these genes have transcription factor binding domains for ER α (68). Thus, if ER α transcriptional activity is decreased, the total protein of its targets would be decreased as well.

Previous studies suggest that Nar inhibits cellular proliferation by inhibiting the ER α /estrogen complex from activating the MAPK and PI3K pathways (64), yet when estrogen is present, our immunoblot results do not indicate this. On the other hand, Bulzome et. al showed that concentrations of Nar higher than 0.1 μ M inhibit cellular proliferation independent of this proposed mechanism (63). Thus, further studies need to be performed to understand the underlying mechanism of a Tam+Nar combination treatment.

While Nar inhibits the phosphorylation of AKT and ERK 1/2, it is uncertain if this is a direct effect or if Nar is targeting a kinase upstream in these pathways. It is also possible that neither is the real target eliciting these effects but just a byproduct. Future studies could investigate this by using combinations of ERK 1/2, MEK 1/2, AKT, and

PIK3 inhibitors. These studies should be performed in stripped media to eliminate the endogenous activation of these pathways.

Overall, our studies demonstrated that Tam+Nar is an effective inhibitor of ER+ breast cancer cell proliferation in MCF-7 Tam-S and Tam-R cells. Tam+Nar proved effective in reducing cell viability by inducing apoptosis. Although further research needs to be done to determine the exact mechanism of Tam+Nar in MCF-7 cells in the presence of estrogen, our studies suggest that ER α interactions may contribute to this mechanism.

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