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The role of calcium and nitrite in triple negative breast cancer

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Abstract: Background: In 2017, approximately 252,000 of American women were diagnosed with breast cancer of which ~25% of these cases were classified as estrogen receptor-negative (ER-). A substantial subset of these breast tumors do not express estrogen or progesterone receptors nor do they overexpress HER2 and have been therefore designated as triple negative breast cancers (TNBC). TNBC has been shown to be more aggressive and have poor prognosis. Androgen receptor (AR) is expressed in ~70% of ER- breast cancer. Even though, the ER role in breast cancer is well known and recognized, little is known about the clinical importance of androgens and AR in breast carcinogenesis. Ligand bound AR translocates to the nucleus, and then a cascade of events occurs culminating with transcription of target genes.

Aim: Hypothesizing that activation of androgen receptor (AR) by calcium or nitrite results in increased proliferation in AR+ TNBC, hence creating an opportunity to be able to attack triple negative breast cancer cells via the androgen receptor and thereby be able to shrink the cancerous cells.

Methods: Estrogen receptor negative, androgen receptor positive MDA-MB 453 breast cancer cells were treated with synthetic androgen (R1881); non-steroidal anti-androgens casodex, flutamide, or hydroxyl flutamide; calcium channel blockers mibefradil (T type) and methoxyverapamil (L type); or nitric oxide synthase inhibitor nitroarginine (LNNA) for 96h.

Results: Cells treated with calcium showed a higher proliferation rate in comparison to control cells. In contrast cells treated with methoxyverapamil or mibefradil at higher concentrations, showed decreased cell growth.

Conclusion: Data suggest that the overexpression of calcium channels and nitric oxide synthase, one or both of them, is a major contributor in activating androgen receptor leading to cell growth and survival of androgen receptor-positive breast cancer, and hence androgen activation in triple negative breast cancer. The data also suggest that FDA approved calcium channel blockers and NOS inhibitors maybe useful for the treatment of AR+ TNBC.

Keywords: breast cancer, estrogen receptor-negative, calcium, nitrite.

Introduction:

Breast cancer is the most commonly diagnosed cancer among American women, besides skin cancer (1). In 2017, the number of new cases of breast cancer in women in United States was 252,710 of which breast cancer caused death in 40,610 cases (2). Numbers between the United States and Saudi Arabia are not varying, but the difference is apparent in the age of patient and the stage of disease when it was first discovered, in Saudi Arabia the total number of new cases of cancer is 2741 including about 19.9% of breast cancer in women that is ranked first figures (3). Estrogen receptor (ER) is a member of the steroid hormone receptor family. As estrogen receptor (ER) has an important role in regulating growth

and differentiation of normal mammary gland, it also has as an important role in the progression of most breast cancers. The direct transcriptional regulation of target genes helps ER to mediate its downstream effect. Targeting estrogen receptor has been a powerful cornerstone in the treatment of ER positive breast cancer (4). Estrogen receptor negative (ER-) breast cancers are a group of tumors with poor prognosis and less cancer prevention and treatment strategies compared to ER positive tumors (5).

Tumors that do not express ER, progesterone receptors (PR) and HER2 represent approximately ~25%-30% of all breast cancer and are classified as triple negative breast cancers (TNBC). Patients with TNBC have not benefited from the endocrine directed therapy; unfortunately, there is no optimal standard treatment for TNBC. Nevertheless, there are several pathways currently being studied regarding ER- breast cancer one of which is the role of androgen receptor in proliferation of ER-negative breast cancer (4). Although estrogen is considered to be a female-specific sex hormone and androgen a male one, both exist and play important developmental roles in both sexes. Even though estrogen is widely recognized for its part in breast cancer, little is known regarding a potential role for androgen receptor in this disease (6).

Recent studies have shown that the androgen receptor (AR) is expressed in approximately 70% of all breast cancers regardless of the tumor's ER status (7). Furthermore, 90% of ER+ and 50% of ER- tumors express AR (7). MDA-MB 543 cell line proliferation induced by AR suggesting that AR is positively associated with certain tumor characteristics (7). Androgen receptor is a member of the steroid hormone receptor family and like other steroid hormone receptors, it consists of a single polypeptide that has different domains, mediating its functions: (i) an androgen –independent activation function 1 domain (AF1), (ii) DNA binding domain that interacts with androgen response elements, (iii) a ligand-binding domain (LBD) that androgens and anti-androgens bind to, (iv) a hinge region connects the DNA-binding domain with the ligand –binding domain. When the AR is not bound to a ligand, it interacts with heat shock proteins (HSP), but once it is bound to a ligand, AR disassociates from chaperone proteins and translocates to the nucleus, then a cascade of molecular events occurs that lead to the target gene activation (4).

Figure1: Structural domain of the AR: extreme amino terminal domain is called the A/B domain contains (AF1) domain, followed by the conserved DNA binding domain (DBD), and linked by a hinge region with the ligand binding domain (LBD) (12).

The interaction of AR with co-regulators and the phosphorylation of AR and AR regulators modulate the androgen-induced transcriptional activation of AR in response to growth factors, the endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (11). Androgens have been shown to be involved in breast cancer and the recent data suggested that

AR has a prognostic role in triple negative breast cancer, and that the pathogenesis of breast cancer is affected critically by the androgen-signaling pathway (4).

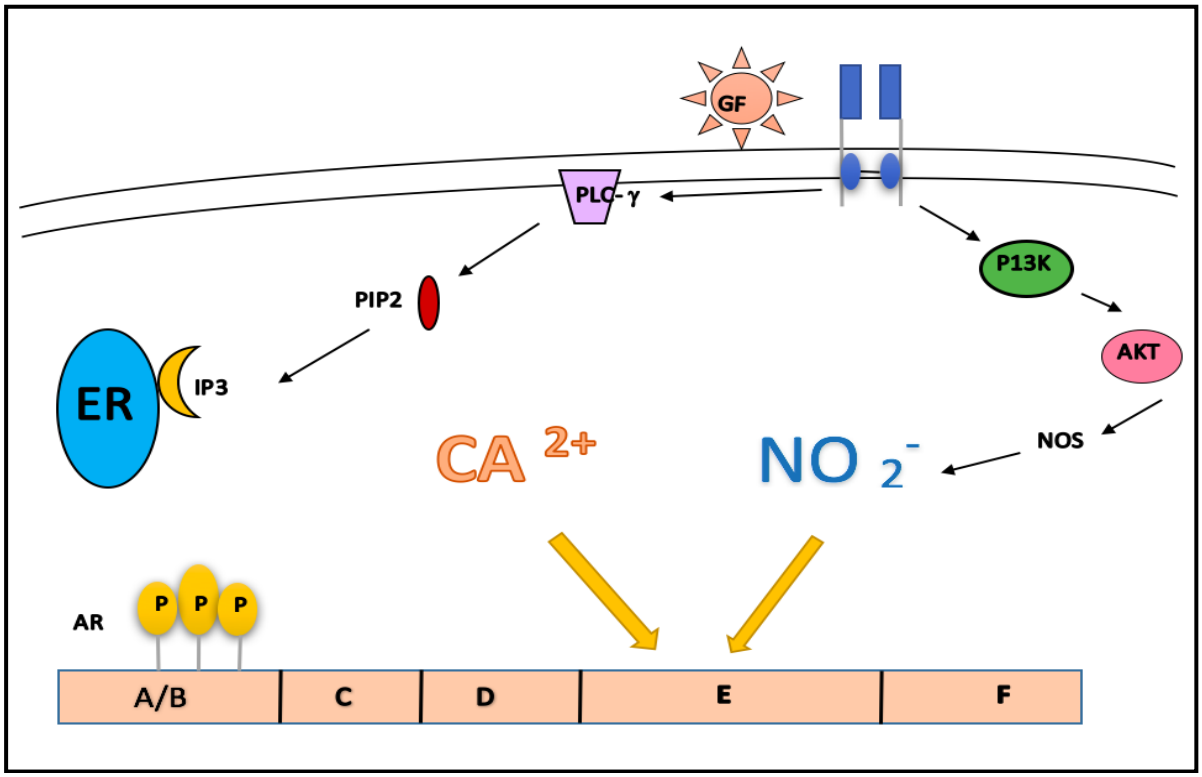

Figure 2: Proposed pathways of AR activation. Increased intracellular calcium via the PLC-γ pathway leads to the activation of AR. Activation of NOS via AKT produces nitrite, which activates AR.

The rise of intracellular and extracellular free Ca^{2+} have been shown to have a major role in cellular interactions and processes. Calcium signaling is a character of many diseases one of which is cancer. Calcium signaling has an important role in cancer processes such as migration and proliferation (8). The consequences of alteration of calcium signaling may be massive in cancer cells and it could contribute to tumor progression, although the alteration in calcium transport may not be a requirement for the cancer initiation. Cancerous cells use the same calcium channels, exchangers and pumps as the healthy cells. (8)

Previous data from this laboratory has shown that in the absence of estradiol, metalloestrogens, such as calcium and nitrite, are capable of mimicking the role of estrogen, activating ER. Calcium (Ca^{2+}) acts a mediator between the PLC-γ pathway and ligand binding domain (LBD) of ERα by forming a high affinity complex with the LBD of the receptor (9). The enzyme nitric oxide synthase (NOS) has a major role in transforming the L- arginine to nitric oxide. Nitrite has been shown to increase cell proliferation in the breast cancer cell line MCF-7 suggesting a role for nitrite in activating estrogen receptor leading to proliferation and growth of breast cancer cells (10). Preliminary data from my study in the AR+ MDA-MB 543 cells have shown increased growth in cells that were treated with calcium, and their proliferation rate

was higher compared to control cells. In contrast, cells treated with methoxyverapamil or mibefradil at higher concentrations, showed decreased cell growth.

Hypothesis:

hypothesized that activation of androgen receptor by calcium (via PLC- γ Pathway) or nitrite (via the AKT pathway) results in increased proliferation in AR+ triple negative breast cancer cells, hence creating an opportunity to be able to attack triple negative breast cancer cells via the androgen receptor and thereby be able to shrink the cancerous cells

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Material and Methods:

Study design

Estrogen receptor-negative, androgen receptor-positive (ER-/AR+) MDA-MB-453 cells were obtained from the Tissue Culture Shared Resources, Georgetown. Cells were maintained in phenol red containing IMEM media supplemented with 5% Fetal Bovine Serum (FBS) at 37° C with 5% CO_2. Cells stimulated with hormone were allowed to attach overnight in phenol red containing IMEM media supplemented with 5% FBS. Media was then changed to phenol red-free IMEM media supplemented with 5% charcoal- stripped calf serum (CCS) 48h prior to treatment. Cells were treated with synthetic androgen R1181 (1 nM), non-steroidal anti-androgens casodex (1 μM), flutamide (10 μM), hydroxyl flutamide (OH-Flut; 10 μM), L type calcium channel blocker methoxyverapamil (MV; 50-75 μM), T type calcium channel blocker mibefradil (MF; 5-7.5 μM), calcium (Ca^{2+}; 1-3 mM), cadmium (Cd; 1 μM), nitrite

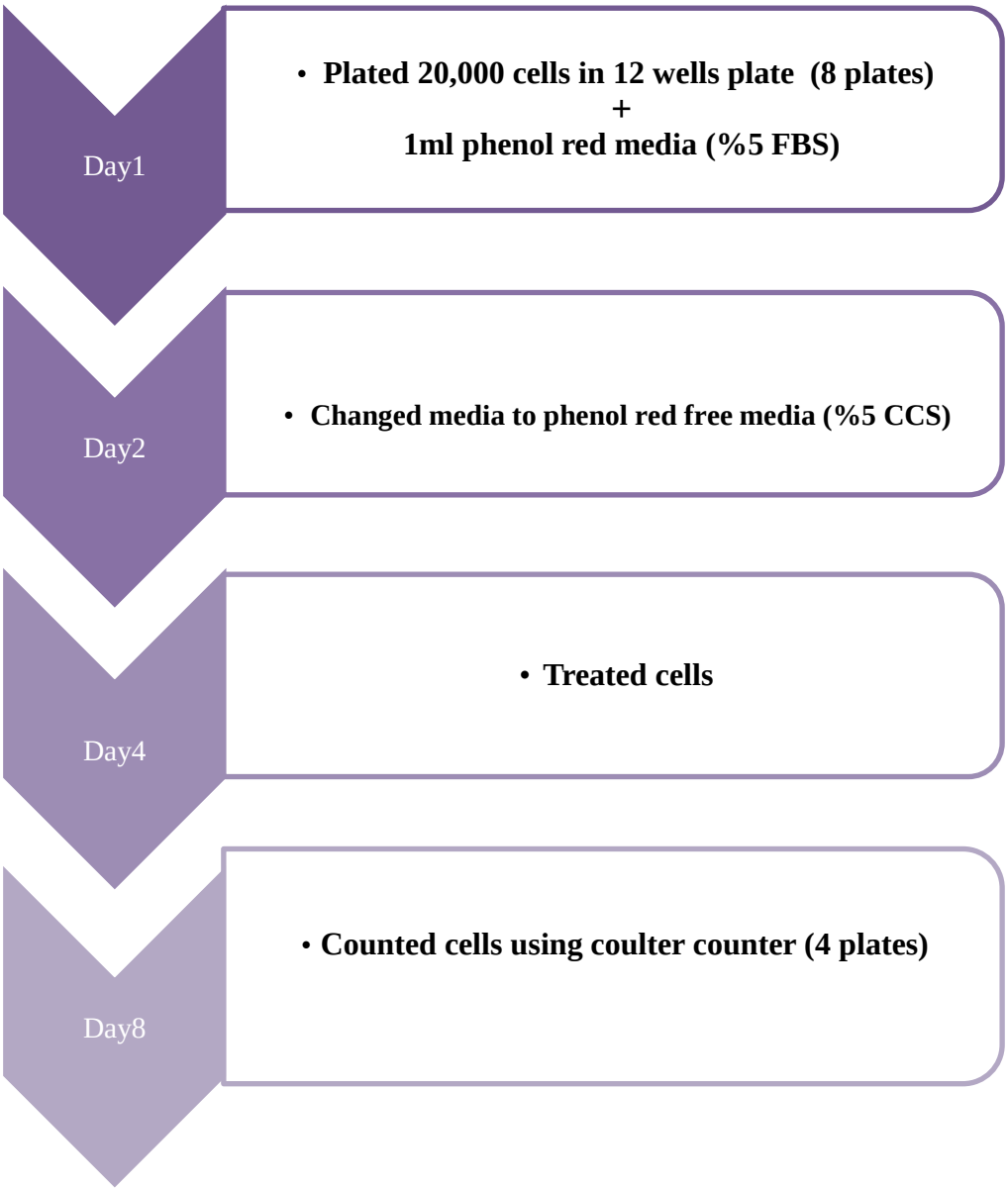
(NO_2^-;1 μM), or nitric oxide synthase inhibitor nitroarginine (L-NNA; 10-50 μM).

Setting and sampling

In order to evaluate the ability of calcium and nitrite to activate AR in ER-/AR+ cells, cells were stimulated for 96 h followed by cell growth assessment. Cells were cultured overnight in phenol red containing IMEM media supplemented with 5% fetal bovine serum at a density of 2×10^4 cells per well in 12-wells plates at 37° C with humidified 5% CO_2. Cells were treated with R1881, flutamide, hydroxyl flutamide, casodex, Calcium (Ca^{2+}), Cadmium (Cd), Nitrite (NO_2^-), MF, MV, L-NNA for 96 h. Cells were then rinsed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin for 5 minutes. Cells were then collected and counted using a coulter counter.

Data collection:

Growth Experiment:



Troubleshooting:

Contamination with some of the flasks containing MDA-MB 453 cell line while incubating them, it was thought that the contamination was from the 5% IMEM (FBS) media used. In order to test that a 10cm flask was obtained and was filled with 5 ml of the media. After a few days the media reminded clear, suggesting that the cells were not contaminated. The problem was solved by asking for more cells from my co-mentor and maintaining extra back up cells after splitting them in addition more sterilizing procedures were employed for the pipette used in the experiments. Cell death was observed while conducting 4 days'

growth assay, this problem could be due to the overgrowth of the cells or the starting number of cells were too high per well.

Results:

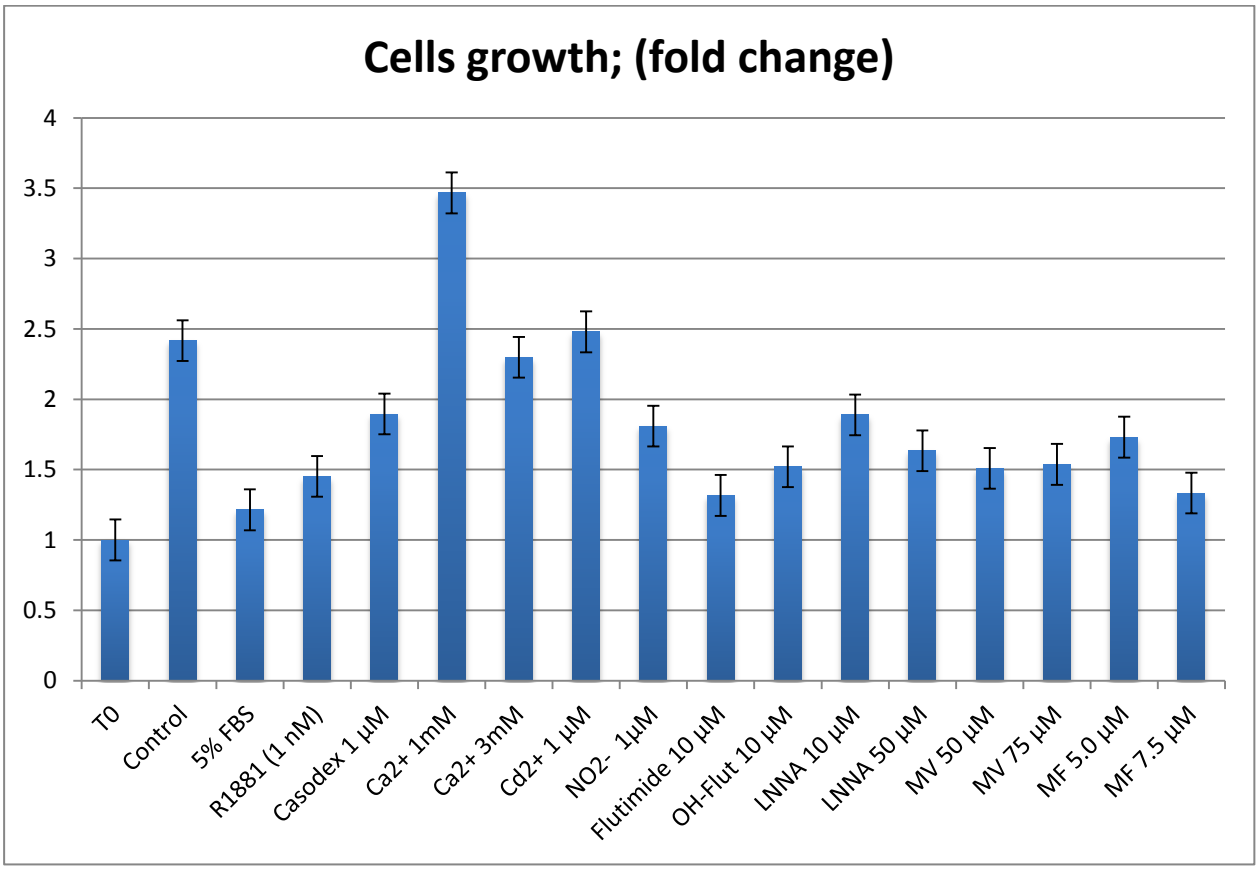


Figure 1: Effect of treatments on MDA-MB 453 cell line treated for 96-h.

MDA-MB453 cells were treated for 96 hours R1181 (1 nM), casodex (1 µM), calcium (Ca^{2+}; 1-3 mM), cadmium (Cd; 1 µM), nitrite (NO_2^-;1 µM), flutamide (10 µM), hydroxyl flutamide (OH-Flut; 10 µM), nitric oxide synthase inhibitor nitroarginine (L-NNA; 10-50 µM), L type calcium channel blocker methoxyverapamil (MV; 50-75 µM), T type calcium channel blocker mibefradil (MF; 5-7.5 µM). Error bars represent standard errors.

In the first experiment (figure1) synthetic androgen at 1 nM unexpectedly inhibited the cell growth by about 1-fold change compared to the control. Casodex also inhibited the growth to about 0.5-fold change less than the control. In contrast, calcium at 1mM gave the highest proliferation rate compared to the other treatments while it is a 1-fold change higher than the control. The other concentration of calcium at 3mM has no obvious effect on the cell growth and the same result was observed for the cadmium. The anti-androgens, flutamide and hydroxyl flutamide, lowered the growth by ~1-fold change. The NOS inhibitors decreased the cell growth by ~1-fold change compared to the control. The calcium channel blockers methoxyverapamil (L type) & mibefradil (T type) were also tested. In methoxyverapamil, cells growth declined almost equally at the different doses of 50 and 75µM. Another inhibitor was mibefradil,

the lower dosage at 5 µM inhibited the growth by 0.75-fold change, but the higher concentration at 7.5 µM had the most notable inhibition rate besides the flutamide.

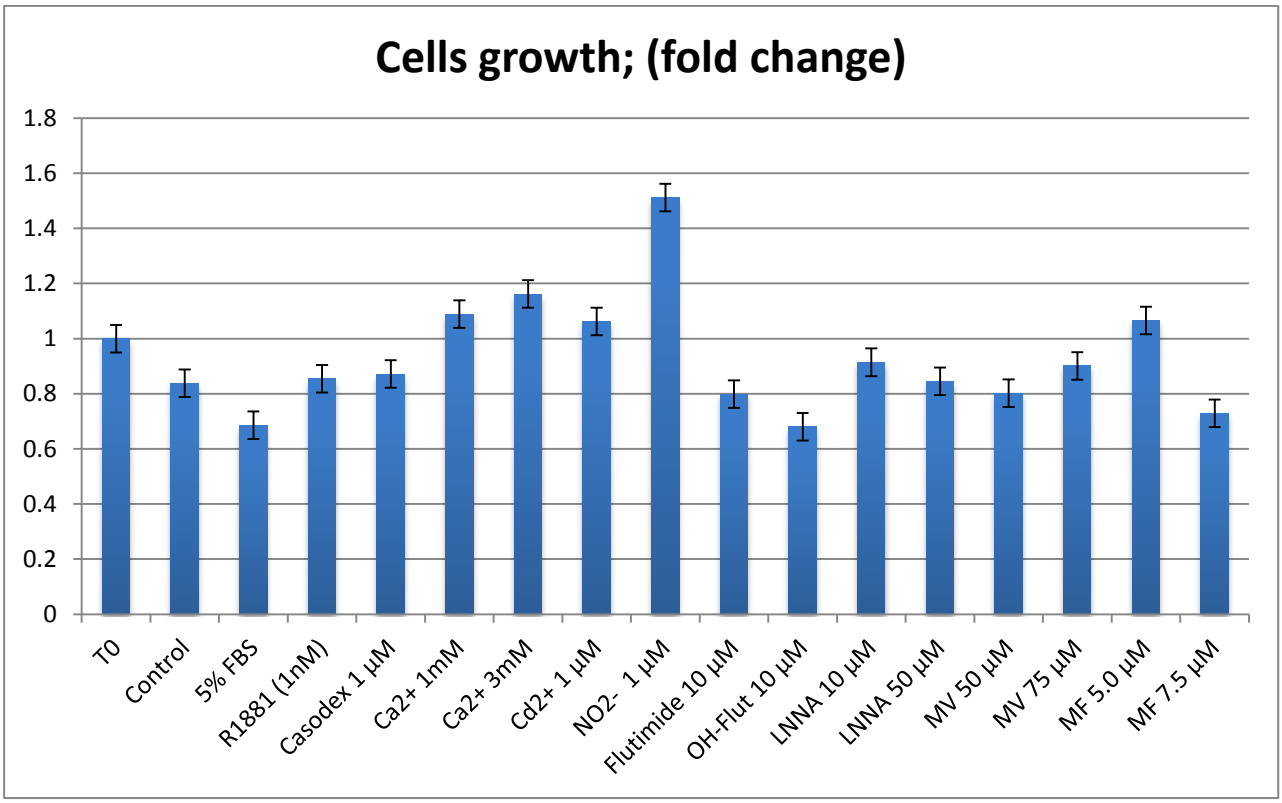


Figure 2: Effect of treatments on MDA-MB 453 cell line treated for 96-h.

MDA-MB453 cells were treated for 96 hours with R1181 (1 nM), casodex (1 µM), calcium (Ca^{2+}; 1-3 mM), Cadmium (Cd; 1 µM), nitrite (No_2^-;1 µM), flutamide (10 µM), hydroxyl flutamide (OH-Flut; 10 µM), nitric oxide synthase inhibitor nitroarginine (L-NNA; 10-50 µM), L type calcium channel blocker methoxyverapamil (MV; 50-75 µM), T type calcium channel blocker mibefradil (MF; 5-7.5 µM). Error bars represent standard errors.

As seen in the second experiment (figure2), the growth of the androgen was slightly over the growth rate of the control, casodex as well had the same effect. Calcium at two different concentrations (1-3 mM) and cadmium at 1 µM caused cells to grow well over the control cells. The nitrite treatment at 1 µM caused the higher cell proliferation by 0.7-fold change compared to the other treatments. Flutamide had no effect on the cells, while hydroxyl flutamide lowered cell growth. LNNA with two concentrations 10 uM and 50 uM showed no change in the growth. Calcium channel blocker mibefradil was used with two different concentrations 5 uM and 7.5 uM, there was no change in growth above the control for 5 uM concentration while the higher concentration 7.5 uM inhibited the growth. Another calcium channel blocker methoxyverapamil was used at two different concentrations 50 uM and 75 uM, and there was no growth above or below the control growth.

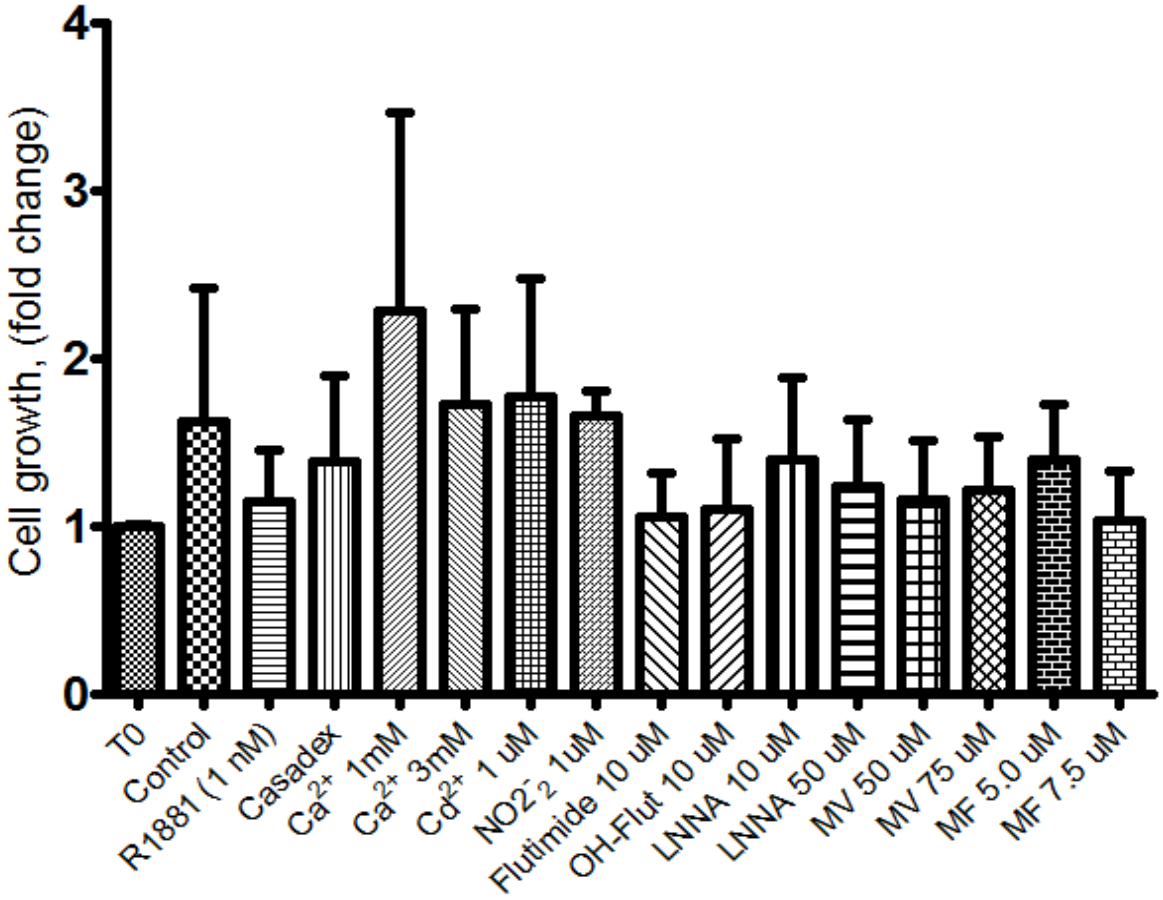


Figure 3: Effect of treatments on MDA-MB 453 cell line for 96-h.

MDA-MB453 cells were treated for 96 hours with R1181 (1 nM), casodex (1 μM), calcium (Ca^{2+}; 1-3 mM), cadmium (Cd; 1 μM), nitrite (NO_2^-;1 μM), flutamide (10 μM), hydroxyl flutamide (OH-Flut; 10 μM), nitric oxide synthase inhibitor nitroarginine (L-NNA; 10-50 μM), L type calcium channel blocker methoxyverapamil (MV; 50-75 μM), T type calcium channel blocker mibefradil (MF; 5-7.5 μM). Error bars represent standard errors of the mean.

Effects of androgen, calcium, nitrite, calcium channels blockers, and NOS inhibitors on the growth of MDA-MB-453 cells.

The data were combined and plotted in figure 3. When triple negative breast cancer cell line (MDA-MB 453) was treated with R1881 (synthetic androgen 1 nM) there was slight decrease in cells growth. 1 μM of casodex as well reduced the growth rate, but the fold change difference was less than R1881 when compared to the control. Calcium at a 1mM dose increased the fold change of cell growth by approximately 1-fold change, and it is the highest response to a treatment compared to the control. The higher dosage of calcium, which was 3mM, did not have the same impact on the cells and their growth remained close to the control growth rate. Cadmium and nitrite treatments, each at 1 μM had no

considerable effect on cell growth. 10 µM of flutamide and 10 µM of hydroxyl flutamide inhibited the cell growth by approximately half fold change. LNNA at 10 µM, slightly lowered cells growth rate, but with the higher concentration of LNNA at 50 µM, cell growth was inhibited further. Following treatment with methoxyverapamil an L type calcium channel blocker, cell growth declined almost equally at the different doses of 50 and 75µM. Another inhibitor was mibefradil a T type calcium channel blocker, the lower dosage at 5 µM inhibited the growth slightly, but the higher concentration at 7.5 µM had the most notable inhibition rate besides the flutamide.

Discussion:

Recent studies showed evidence that androgen is capable of driving the proliferation in cells that are negative for estrogen, progesterone and HER2 receptors, also called triple negative breast cancer (TNBC). In our laboratory we hypothesized that either calcium or nitrite has the ability to activate androgen receptor in the AR+/ER- breast cancer cells preliminary data from this laboratory suggest a role for calcium and nitrite in increasing proliferation in AR+ breast cancer cells.

The preliminary results further suggest that activation of AR+ was may be through two pathways, one of them through PLC- γ pathway by increasing the intracellular and extracellular calcium through the calcium channels, and the other by increasing nitrite concentration in the cells via PI3K then AKT.

Androgen agonist and antagonist

The AR positive breast cancer MDA-MB 453 has been shown to response to androgens. However, the conducted growth assays suggest that androgen receptor is active. In the MDA-MB 453, treatment with the synthetic androgen, R1881, showed a slight inhibition in the growth compared to the control. This difference in the results might be due to the use of synthetic androgen or to the low concentration of the androgen used, considering higher dosage of R1881 is an option in upcoming experiments. Unexpectedly, the antiandrogens, casodex flutamide and hydroxyl flutamide, had an obvious inhibition of the cells suggesting that the AR was activated and was driving the growth of MDA-MB 453.

Calcium and nitrite

The different concentration of calcium had different impact on the cells; the 1mM concentration increased the growth significantly, among the other treatments it has the most notable increase in the growth rate of the cells, while the other concentration at 3 mM did not affect the cell growth as much, suggesting that at lower concentrations calcium enters the cells and induces proliferate. Nitrite and cadmium unexpectedly did not have much of effect on the cells, and that may be due to multiple reasons such as low uptake of cadmium by the cells or metabolism of nitrite to nitrate.

Nitric oxide synthase (NOS) inhibitors

When the cells were treated with L-NNA at concentrations of 10 and 50 uM for 4 days growth, LNNA lowered the growth rate of the cells compared to the control. In addition, the higher concentration inhibited the growth more suggesting that the NOS pathway was inhibited and that the 50 uM was sufficient to block growth. These results indicate a possible nitrite activation that lead to the activation of AR, and hence cell proliferation and survival.

Calcium channel blockers

Considering that MDA-MB 453 cell line have L type calcium channels, the L type calcium channel inhibitor methoxyverapamil at concentrations of (50 uM, 75 uM) inhibited the growth equally suggesting that an increase in intercellular calcium leads to cells proliferation by activating the AR. On the other hand, the T type calcium channel inhibitor mibefradil decreased the cells growth at 5 uM, but the higher concentration at 7.5 uM decreased furthermore, suggesting that the higher dosage of T type calcium channel blocker has a greater effect on blocking the calcium channels.

Conclusion:

Data suggest that the overexpression of calcium channels and nitric oxide synthase, one or both of them, is a major contributor in activating androgen receptor leading to cell growth and survival of androgen receptor-positive breast cancer, and hence androgen activation in triple negative breast cancer. The data also suggest that FDA approved calcium channel blockers and NOS inhibitors maybe useful for the treatment of AR+ TNBC.

Recommendations:

- Further investigate the role of androgen receptor activation in triple negative breast cancer.
- Investigate the possible mechanisms by which AR is activated.
- Obtain more data regarding calcium and nitrite and their effect in AR pathways.

Abbreviations list:

ER-	estrogen receptor-negative
TNBC	triple negative breast cancers
AR	Androgen receptor
MDA-MB 453	Breast cancer cells
R1881	synthetic androgen
LNNA	nitric oxide synthase inhibitor nitroarginine
NOS	nitric oxide synthase
ER	estrogen receptor

PR	progesterone receptors
AF1	function 1 domain
LBD	ligand-binding domain
HSP	heat shock proteins
DBD	conserved DNA binding domain
FBS	Fetal Bovine Serum
CCS	charcoal- stripped calf serum
PBS	phosphate-buffered saline

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الملخص: يهدف هذا البحث الي التعرف على دور الكالسيوم والنتريت في سرطان الثدي السلبي الثلاثي، في هذا النوع من السرطان، لا يوجد مستقبلات على سطح الخلايا السرطانية لهرمون الاستروجين وهرمون البروجيسترون، كما انه لا توجد بها مستقبلات من نوع HER2، بينما تتواجد مستقبلات الاندروجين في ٧٠٪ تقريبا من خلايا سرطان الثدي التي لا تحوي مستقبلات لهرمون الاستروجين. وقد تم الافتراض انه بتفعيل مستقبلات الاندروجين عن طريق الكالسيوم او النتريت ينتج عنه زيادة في انتشار مستقبلات الاندروجين في سرطان الثدي السلبي الثلاثي. بناء عليه تمت معامله الخلايا السرطانية من نوع MDA-MB 453 والتي لا تحتوي على مستقبلات هرمون الاستروجين، وتحتوي على مستقبلات لهرمون الاندروجين، باستخدام الاندروجين الاصطناعي (R1881)، مضاد للأندروجين الغيرستيرويديه Casodex، الفلوتاميد، أو الهيدروكسيل فلوتاميد، حاصرات قنوات الكالسيوم mibeferadil (نوع T)، Methoxyverapamil (نوع L)، او النيتروجين المثبط للإنزيم المصنع لأوكسيد النيتريك (LNNA) لمده ٩٦ ساعه، وقد أظهرت الخلايا المعالجة بالكالسيوم معدل انتشار اعلى بالمقارنة مع خلايا التجربة الضابطة (control). في المقابل الخلايا المعالجة مع Methoxyverapamilو Mibeferadilفي تركيزات اعلى، أظهرت انخفاض ملحوظ في نمو الخلايا. وتشير البيانات إلى أن الإفراط في التعبير عن قنوات الكالسيوم والانزيم المصنع لأوكسيد النيتريك، واحد أو كلاهما، يساهم بشكل رئيسي في تفعيل مستقبلات الاندروجين مما يؤدي إلى نمو الخلايا وبقاء سرطان الثدي ذو مستقبلات الاندروجين فاعلا، وبالتالي تنشيط الاندروجين في سرطان الثدي الثلاثي السلبي. بالتالي استخدام حاصرات قنوات الكالسيوم ومثبطات الانزيم المصنع لأوكسيد النيتريك قد تكون مفيدة لعلاج سرطان الثدي السلبي الثلاثي المحتوي على مستقبلات اندروجين.

الكلمات المفتاحية: سرطان الثدي، مستقبلات هرمون الاستروجين السلبية، الكالسيوم، النتريت.