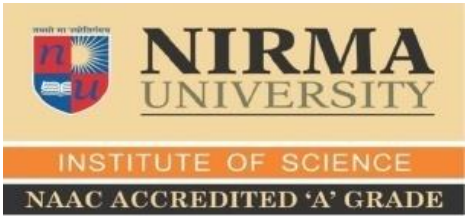
SMADs as a driver of TGF-β mediated signaling during antiestrogen treatment in Triple Negative Breast Cancer

A dissertation thesis submitted to Nirma University in partial fulfillment for the degree of

Master of Science
in
Microbiology / Biochemistry



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AHMEDABAD

MAY-2017

Acknowledgement

We thank our dear Almighty for enabling us with immense strength and courage during our dissertation project. We are extremely grateful to our parents for their constant support and care.

We are highly indebted and thoroughly grateful to our guide **Dr. Heena V. Dave** for her constant guidance which enabled us to carry out our work with sincerity and dedication. Her motivation, expertise and zeal helped us to develop scientific approach in our work. We sincerely thank her for all the generous advices that helped us to troubleshoot our problems. Truly, her depth of knowledge has enabled us to be not only good researchers but also good human beings in life.

We are grateful to our director **Prof. Sarat Dalai** and all the faculty members **Dr. Shalini Rajkumar, Dr. Sonal Bakshi, Dr. Rajeev Tyagi, Dr. Pranati Sar, Dr.Vijay Kothari, Dr. Sriram Seshadri, Dr. Amee Nair and Dr. Nasreen Munshi** for their valuable support in our project work.

We would like extend our sincere gratitude to NCCS, Pune for providing us cell lines. We also thank Dr. Umesh Yadav (Central University of Gujarat, Gandhinagar) for gifting us cell line and growth media.

We also thank all Ph.D. students especially Mr. Hardik Patel, Mr. Manoj Patidar, Mr. Naveen Yadav, Ms. Vishaka Bhurani for their constant guidance and support in our work.

We extend our heartfelt thanks to our lab assistants Mr. Sachin Prajapati and Ms. Sweta Patel for allowing us to use lab equipments, chemicals and necessary lab materials for our work.

We thank the library staff for helping us to widen our knowledge by letting us use library facilities. We also thank Mr. Hasit Trivedi from student section for all the help.

We sincerely thank all our dissertation mates- Ankit Naik, Aishwarya Joshi, Bhakti Tilva, Pankti Dave, Falaknaajjahan Babi and Arpit Soni for all the support throughout or project work.

Lastly, we extend our sincere gratitude and love to everyone whom we may have missed unknowingly, but has helped us with their valuable support.

Thank you.

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Contents

Abbreviations

BSA: Bovine Serum Albumin

CD4+: Cluster of Differentiation 4+

CD8+: Cluster of Differentiation 8+

$CuSO_4$: Copper Sulphate

DCIS: Ductal Carcinoma In Situ

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme Linked Immunosorbent Assay

EMT: Epithelial-Mesenchymal Transition

ER: Estrogen Receptor

FBS: Fetal Bovine Serum

FCS: Fetal Calf Serum

HER-2: Human Epidermal Growth Factor Receptor-2

IBC: Inflammatory Breast Cancer

IDC: Invasive Ductal Carcinoma

ILC: Invasive Lobular Carcinoma

MAPK: Mitogen-Activated Protein Kinase

MTT: 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide

Na_2CO_3: Sodium Carbonate

NaCl: Sodium Chloride

PBS: Phosphate Buffer Saline

PI3K: Phosphatidylinositol-3-Kinase

PR: Progesterone Receptor

SERM: Selective Estrogen Receptor Modulator

SMAD-3: SMA (*sma* gene for small body size) + MAD (mothers against decapentaplegic)-3

SMAD-4: SMA (*sma* gene for small body size) + MAD (mothers against decapentaplegic)-4

TAM: Tamoxifen

4-OH-TAM: 4-Hydroxy Tamoxifen

TGF-β1: Transforming Growth Factor-β1

TGF-β2: Transforming Growth Factor-β2

TNBC: Triple Negative Breast Cancer

VEGF: Vascular Endothelial Growth Factor

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Abstract

Triple Negative Breast Cancer (TNBC) is a complex heterogeneous disease characterized by the absence of Estrogen Receptor (ER^-), Progesterone Receptor (PR^-), and HER-2 receptor ($HER\text{-}2^-$). It represents approximately 15-20% of all breast cancer cases and believed to have aggressive phenotype and poor prognosis. Currently no adjuvant intervention is procurable for treating TNBC patients. Due to heterogeneity of this cancer subtype, there has been a major limitation to targeted therapy. In TNBC, because of absence of hormone receptors, antiestrogens which are meant to mimic the action of hormone thereafter cannot be used as target for treatment purpose. Therefore, the need for molecular prognostic markers is emerging. There are two areas yet to be explored: Lack of adjuvant treatment for triple negative breast tumors and non-responsiveness of antiestrogen.

Many tumor markers such as growth factors, signaling proteins, transcription factors are reported to be under clinical trials so as to find their utility for therapeutic purpose. TGF-β is one of those potential biomarkers which play a crucial role during breast tumorigenesis. The ability of TGF-β as an immunosuppressant has been reported to be a possible index to breast tumour progression. However, it has been reported that on activating alternate pathway may increase the effectiveness of antiestrogen in TNBC cells. This might further help to establish a crosstalk between Estrogen Receptor (ER) and TGF-β signaling through SMADs. Wherein in SMAD-dependent TGF-β signaling, SMAD-3 gets phosphorylated and binds with co-SMAD (SMAD-4) and forms a SMAD complex, which thereby enters the nucleus and initiates transcriptional activity.

The prime focus of our study was to find out the possibilities of the usefulness of antiestrogens as potential drug targets using Tamoxifen and its active metabolite 4-OH-Tamoxifen for TNBC cell lines (**MDA-MB-468 and MDA-MB-231**). Tamoxifen is primarily a selective modulator of the ER pathway. SMADs are primary intracellular signaling molecules that mediate the TGF-β signaling pathway.

In the current study, we have evaluated the phenotypic expression of TGF-β mediated signaling mediators during the antiestrogen treatment in TNBC cells (**MDA-MB-468 and MDA-MB-231 cell lines**).

Introduction

1.1. Cancer:

Cancer is a disease that is caused by an uncontrolled division of abnormal cells in the body. It is also termed as “Neoplasm”- new growth of tissue in the body. Cancer develops progressively, with tumors demonstrating different gradations of abnormality. Cancer is a genetic disease that may cause any change or mutation in the DNA wherein a single mutation is not sufficient to induce cancer.

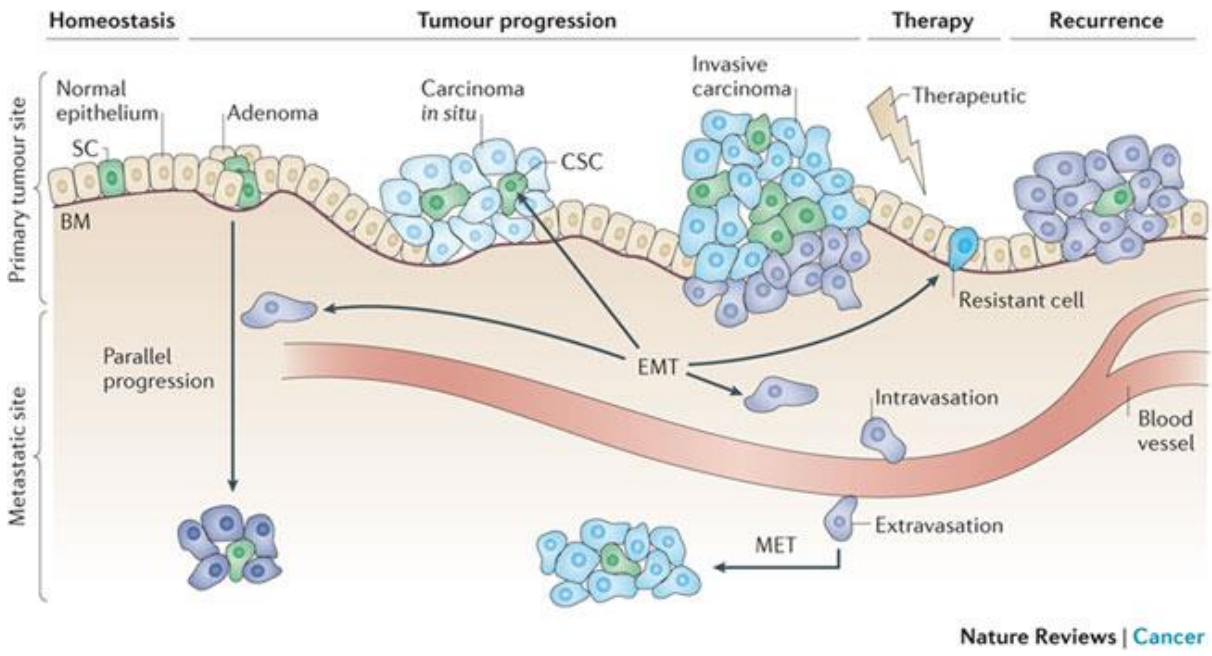

Figure 1: Stages of Cancer development

(Nature Reviews Cancer - February 2013)

Cancer tumors are classified as benign and malignant tumors:

- **Benign Tumors:** The tumor is said to be benign when it is localized and does not metastasize. These tumors are usually encapsulated and thereby not invade the surrounding tissues.
- **Malignant Tumors:** The tumor is said to be malignant when it invades the adjacent normal tissue. Malignant tumors tend to metastasize through blood vessels or lymphatic system to other parts of the body.

- **Staging of Cancer tumors:**

  Following the American Joint Committee on Cancer (AJCC) guideline, staging of cancerous tumors done till date is as following:

Table 1: Staging of Cancer tumors

Tumours	**T0/Tis**	**T1**	**T2**	**T3**	**T4**
Tumour Size	**T0**: No primary tumour. **Tis**: Tumour only in breast ducts or lobules.	0-2 cm	2-5 cm	>5 cm	Tumor of any size with extension to chest wall/skin or ulceration ****inflammatory breast cancer** is staged as T4.
Nodes	**N0**	**N1**	**N1mi**	**N2**	**N3**
	No lymph node metastases.	Cancer cells present in 1-3 axillary lymph nodes.	Lymph node tumor > 2 mm.	Cancer cells present in 4-9 axillary lymph nodes.	Cancer cells in infra or supraclavicular lymph nodes, or in >10 axillary lymph nodes.
Metastasis	**M0**	**M1**			
	No evidence of cancer metastasis.	Cancer found in other areas of body.			

(K Al Muhrib, SN Al-Raheem et al., IJC 2010)

1.2. Breast Cancer:

I. Incidence of Breast Cancer:

- Breast Cancer is one of the commonest cancer types that is prevalent in women. It is the second leading cause of death in women. In U.S. about 1 in 8 women have the chance of developing invasive breast cancer during her lifetime. In India, breast cancer cases have been exponentially increasing and have become one of leading cause of death in women after cervical cancer. (Bharath Rangarajan, Tanuja Sheth et al, 2016). In 2017, about 180 new cases of invasive breast cancer have been reported till date.

- Breast cancer develops when the cells of the breast tissue starts growing invariably and undergo uncontrolled cell proliferation.
- Various factors that contribute to the progression of Breast Cancer are as follows:

(i)**Endogenous Factors**: Genetic predisposition family history, breast density, endogenous hormone level, post-menopausal hormones, obesity

(ii)**Exogenous Factors:** Environmental factors, occupational exposures, radiation, smoking

❖ **Types of Breast Cancer** : Based on location and physiological structure, different types of breast cancer can be classified as follows:

(a) Ductal Carcinoma in situ (DCIS)

(b) Invasive Ductal Carcinoma

(c) Invasive Lobular Carcinoma

(d) Inflammatory Breast Carcinoma

Most breast cancers are *carcinomas* - cancer that begins at the epithelial lining of the organ. Breast cancer can also be *adenocarcinoma*, wherein the cancer has glandular origin. Other type of breast cancer that occurs is *sarcomas*- cancer that initiates in the cells of muscle, fat or even connective tissue.

- Ductal Carcinoma in situ (DCIS): This is an early form of breast cancer which can be pre-invasive or non-invasive cancer. Ductal carcinoma in situ can also be referred to as intraductal carcinoma. In DCIS, the cells that line the duct of the breast tend to have a change in their morphology.
- Invasive/Infiltrating ductal carcinoma: In invasive ductal carcinoma, the cancer begins in the milk duct of the breast initially, and then breaking the wall of the ducts grows and metastasizes to the nearby fatty tissues.
- Invasive/Infiltrating lobular carcinoma: Invasive Lobular carcinoma is the second common type of breast cancer, after invasive ductal carcinoma. It usually begins from the milk-producing lobules and then metastasizes to other sides.
- Inflammatory Breast carcinoma: Inflammatory breast carcinoma (IBC) is the rare type of breast cancer, where the breast appears to be swollen or inflamed.

Generally, most of the inflammatory breast carcinomas are invasive ductal carcinoma. This cancer type tends to progress rapidly and can be diagnosed only at stage III or stage IV.

❖ **Characteristics of Triple Negative Breast Cancer** :

- Lack of ER/ PR/ HER-2 receptors
- Represents 15-20% of all breast cancer cases
- Heterogeneous and aggressive phenotype resulting into high histological grade and poor prognosis.
- Higher expression levels of epidermal growth factor receptors (EGFRs) and basal cytokeratins (CK5/6 and CK14)

❖ **Molecular Classification of Breast Cancer:**

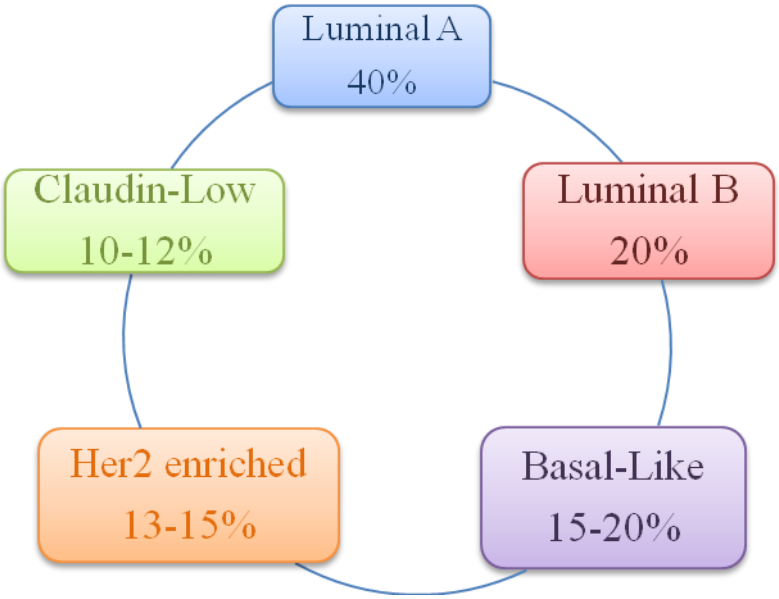

Figure 2: Molecular classification of Breast Cancer

Breast cancer has been distinctly classified based on global gene expression pattern into different four different classes: Luminal, Basal-like, HER-2 enriched, Claudin-low and normal breast like. (Yersal O et al. 2014). Luminal class have been further sub classified into two classes: Luminal A and Luminal B. (Yersal O et al. 2014).

❖ Luminal A: This is the most common breast cancer sub-type and accounts for approximately 50-60% of all breast cancer cases. These tumors are

characterized by low histological grade and good prognosis rate. Luminal A tumors are known to have higher levels of ER and low levels of HER-2.

- Luminal B: This sub-type accounts for 15-20% of all breast cancer cases characterized by higher histological grade with poor prognosis. The tumors have high proliferation rate with lower ER, HER-2 expression levels.
- Basal-like: It represents for 15-20% of all breast cancer. Basal-like cancers are associated with higher histological grade, aggressive phenotype and poor prognosis. Basal-like tumors express high levels of myoepithelial markers and do not express ER, PR and HER-2. Hence, Basal-like tumors are referred to as triple negative breast tumors.
- Claudin-Low: It accounts for 10-20% of all breast cancer cases. These tumors have low levels of cell-cell junctions: claudin3/claudin 4. Claudin-low tumors are characterized by higher histological grade and poor prognosis.

- **Diagnosis and Treatment of Breast Cancer** :

Based on clinical examination, various different diagnostic techniques are available that is confirmed along with molecular imaging and pathological assessment.

Treatment available for treating breast cancer patients all over the world is majorly classified into two categories: (a) Local Treatment (b) Systemic treatment.

- **Surgery**: This is the most common type of treatment available for all breast cancer patients. This localized treatment is further segmented to (i) Lumpectomy or partial mastectomy: In this type of surgery only some part of the cancerous cells from the breast is removed. (ii)Mastectomy- This is the widely used treatment option wherein entire breast affected with cancer is removed including the nearby tissues.
- **Radiation**: Radiation therapy is subjected to breast cancer patients where high-energy rays are is given to cancer patients in order to destroy the cancerous cells.
- **Chemotherapy**: With help of drugs or certain chemical agents, cancerous cells are killed. These drugs are either injected directly into the blood or intravenously given. **Adjuvant** chemotherapy is given to patients after the surgery so as to decrease the rate of recurrence of breast cancer (E. Senkus et

al 2013**)**. **Neoadjuvant** therapy is subjected to patients prior to any surgery so as to reduce the size of tumor or to stop the cancerous cells from growing.

- **Targeted Therapy (Hormonal therapy):** Cancers that are hormone sensitive or hormone dependent need hormones to grow or develop. Hormonal therapy can stop or slow down the growth of breast cancer either by stopping hormones being produced or preventing hormones from making cancer cells grow and divide.

- Antiestrogen drugs are widely used for the treatment of breast cancer such as:

- **Selective Estrogen Receptor Modulators (SERMs):** They block the binding site for estrogen and can slow down the growth of estrogen stimulated cancer. E.g. Tamoxifen and 4-Hydroxy-Tamoxifen.

- The antiestrogen tamoxifen is the most commonly used endocrine treatment for estrogen receptor positive breast cancer patients. However, response is variable and 33% of patients do not benefit from tamoxifen treatment. Differences in formation of biologically active tamoxifen metabolites could represent one important reason for variable treatment responses. Tamoxifen itself has only minor antiproliferative activity. 4-Hydroxytamoxifen is regarded as the biologically most important metabolite with strong antiproliferative activity (Cornelius Knabbe et al., 2008).

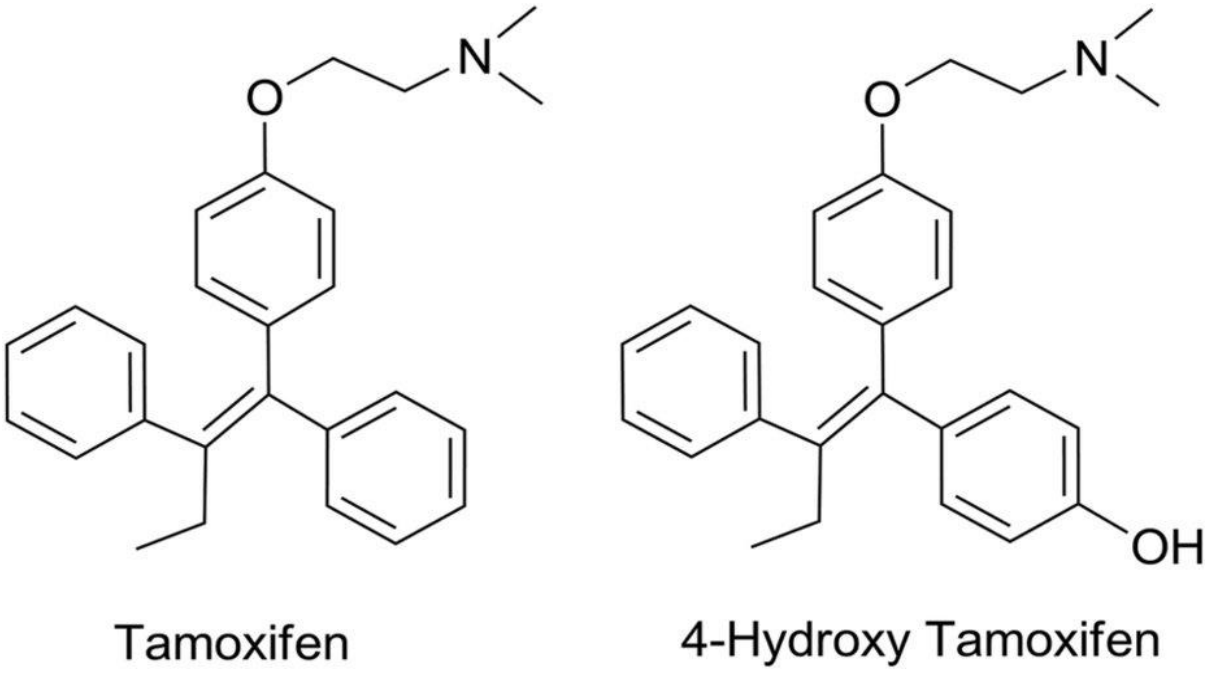

Figure 3: Structures of TAM and 4-OH-TAM

- **Angiogenesis inhibitors:** They prevent angiogenesis and reduce the rate of tumor development. E.g. Bevacizumab is a monoclonal antibody that specifically recognizes and binds to VEGF which plays critical role in angionesis.
- **Immunostimulants:** They enhance the normal immune response.
    - **Growth Factor Receptor Inhibitors:** Antibodies that bind to HER-2 receptor on tumor cells, preventing the binding of growth factor. E.g. Herceptin.

1.3. Current Management in Triple Negative Breast cancer:

Triple negative breast cancer is a heterogeneous breast cancer with higher histological grade, aggressive phenotype and poor prognosis. Absence of molecular prognostic markers has made therapeutic treatment for triple negative breast cancer a major limitation. Due to lack of targeted therapy, Chemotherapy is the only conventional treatment available for triple negative breast cancer patients. The need for molecular prognostic markers in treating triple negative breast cancer is therefore an emerging need.

- **TNBC associated with Epithelial-Mesenchymal Transition (EMT):**

The metastatic nature of TNBC tumors has been associated with epithelial-mesenchymal transition (EMT). In cancer, EMT occurs when the cancerous cells loose cell-cell adhesion, invade and metastasize to nearby tissues and convert to mesenchymal cells. Unlike the non-TNBC cells, TNBC tumors have higher invasing capacity and thus undergo transition from epithelial to mesenchymal cells. Triple Negative breast cancer tumors have been reported to have elevated levels of mesenchymal marker expression due to their invasing and highly metastatic nature. (Sangmin Kim and Jeongmin Lee, 2014)

Elevated levels of transforming growth factors (TGF-β) have been associated with the increasing capacity of cancer cells to invade and metastasize throughout the body. (Sangmin Kim and Jeongmin Lee, 2014)

❖ **Triple Negative Breast Cancer Cell line:**

Table 2: Molecular Characteristics of TNBC cell lines

Cell line	Molecular characteristics
MDA-MB-231	Basal mesenchymal cell line, Stellate-class, low expression of claudin-low
BT-20	Amplification of EGFR gene
MDA-MB-468	Basal-like epithelial cell line, Grape-like cluster morphology, high expression of EGFR,
MDA-MB-435	Basal cell line, express epithelial specific proteins

(Kathryn J. Chavez1, Sireesha V. Garimella et al 2010)

1.4. Transforming Growth Factor-β

Transforming Growth Factor-β (TGF-β) is a pleiotropic cytokine regulating a variety of cellular processes such as cell growth, differentiation, apoptosis, migration, cell adhesion, and immune response (Xiaohua Yan et al., 2009). TGF-β and the large family of close to 40 related growth factors are known to be critically involved in embryonic development, tissue homeostasis in the adult, and most disease states. There are three isoforms of TGF-β: TGF-β1, 2 and 3 found in mammalian species. The chromosomal localization of each isoform is distinct. Many of the proteins of the TGF-β family are involved in regulation of developmental processes and some such as the BMPs and activins, have been implicated in disease pathogenesis. TGF-β has prominent roles in wound healing, carcinogenesis (Haddow, 1972), fibrosis (Roberts et al., 1986) and in a host of other diseases.

1.5. TGF-β Signaling Pathway

The TGF-β homodimer transduces its signal upon activation by bringing together two types serine/threonine kinase receptors- two type I receptors and two type II receptors. TβRI (ALK5) and TβRII are specific for TGF-β. Upon TGF-β binding, ALK5 is phosphorylated and activated by the constitutively active TβRII at the GS region, which is important for the activation of its kinase domain as well as the recruitment of R-SMADs. The phosphorylation

of R-SMADs is an essential step for signal transduction. Of these R-SMADs, SMAD-2 and 3 are engaged in signal transduction of TGF-β, activin and Nodal group cytokines. Once activated, R-SMADs form complexes with a common SMAD (Co-SMAD, SMAD-4) and enter the nucleus and regulate transcription of target genes along with different cofactors (Shi Y and Massague J., 2003). Besides the canonical SMAD-mediated signaling pathway, TGF-β can also activate mitogen-activated protein kinase (MAPK), PI3K, protein phosphatase 2A, Rho family proteins as well as the epithelial polarity protein Par6 independent of SMAD signaling (Derynck and Zhang, 2003).

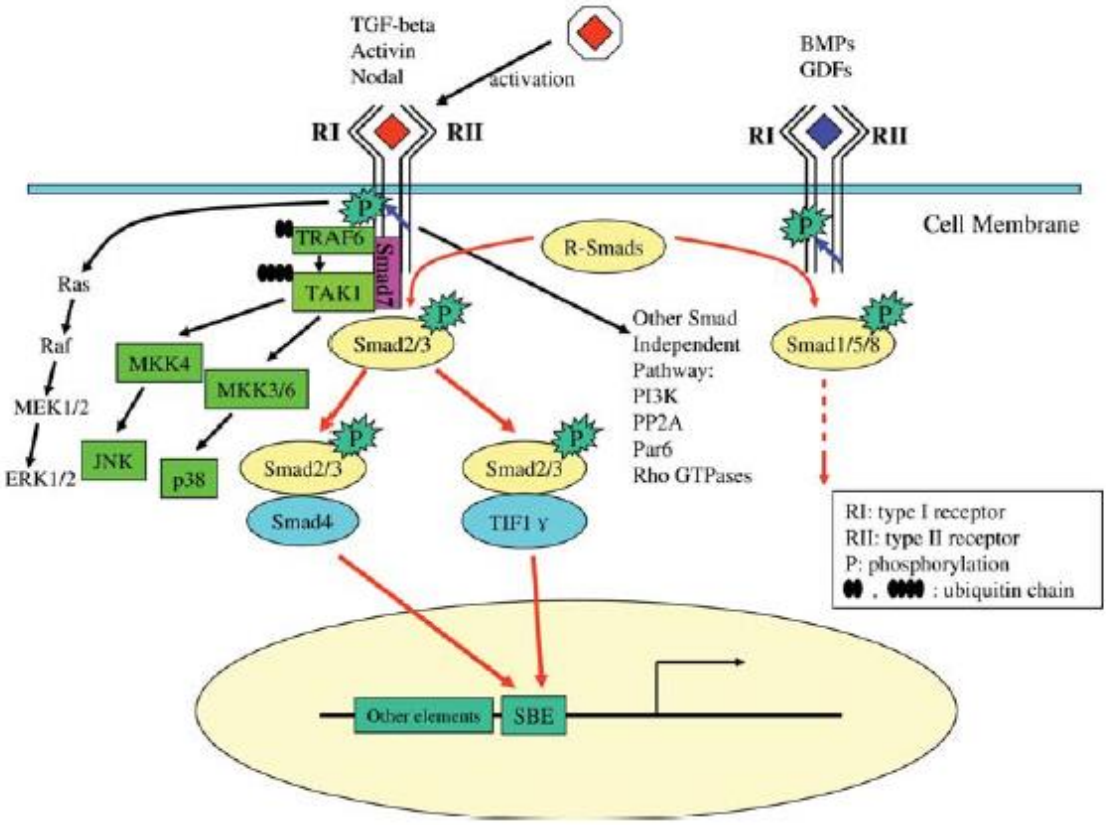

Figure 4: The TGF-β Signaling Pathway

1.6. Dual Role of TGF-β in Breast Carcinogenesis

TGF-β has dual role as tumour suppressor and tumour promoter in breast carcinogenesis.

a) TGF-β as tumour suppressor

Mechanistically, TGF-β has several operating arms to achieve its tumour-suppressive effect which include regulation of cell proliferation, apoptosis and indirectly through the tumour stroma. TGF-β controls cellular proliferation of most epithelial and hematopoietic cells by inhibiting cell cycle progression through G1 arrest (Massague and Gomis, 2006). TGF-β inhibits transcription factors involved in cell proliferation and differentiation (Warner BJ et al., 1990).

b) TGF-β as tumour promoter

TGF-β switches from tumour suppressor during the early stages of tumour development to tumour promoter at later stages of cancer by Epithelial-to-Mesenchymal Transition (EMT), tumour invasion, metastatic dissemination and evasion of the immune system. TGF-β induces EMT by SMAD-dependent transcriptional events and SMAD-independent Ras signaling in later stages of tumorigenesis (Kalluri R, Weinberg RA, 2009). In addition, TGF-β also supports tumour progression by evading the immune system. TGF-β can inhibit CD4+ and CD8+ T cells as well as Natural Killer cells by inhibiting the function of antigen presenting cells (Arteaga et al., 1993).

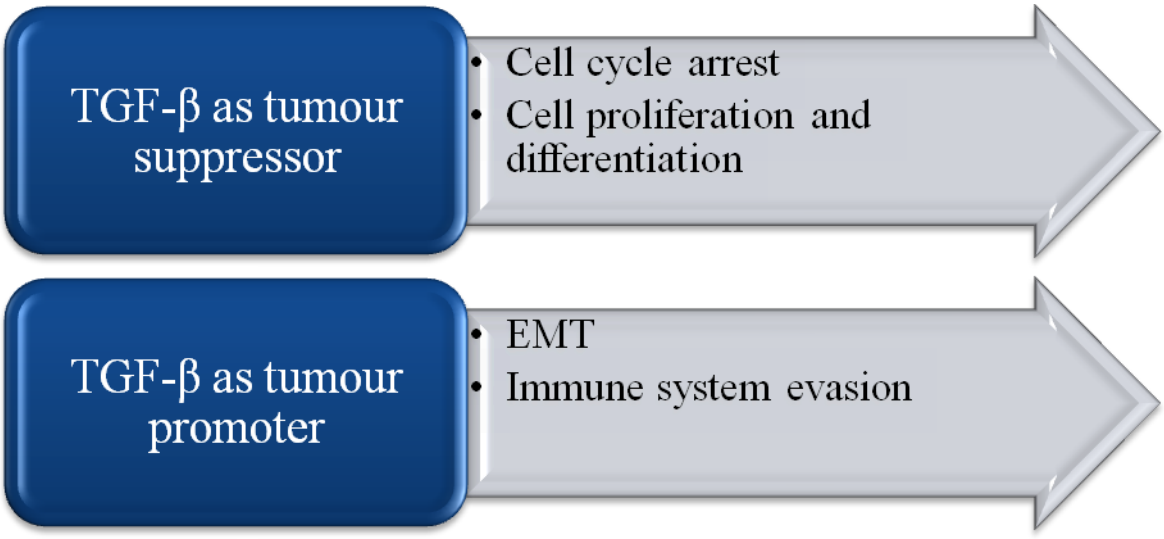

Figure 5: Dual Role of TGF-β

1.7. Crosstalk between Estrogen Receptor Pathway and TGF-β Pathway

Transforming Growth Factor-β and Estrogen Receptor-β signaling pathways are major regulators during mammary gland development and its function. They both play critical role in breast tumorigenesis. These signaling pathways have opposing roles: The TGF-β pathway induces apoptosis whereas ER-β signaling pathway initiates cell proliferation and differentiation. These regulatory pathways intersect and ER-β blocks TGF-β pathway by multiple means including direct interactions of its signaling components, SMADs. Estrogen receptor activation has been reported to inhibit transcriptional activity of TGF-β reporter assays by up to 60%. It inhibits TGF-β induced cell migration. Antiestrogens may induce immunosuppression in the tumor microenvironment in a manner that depends on TGF-β signaling (Joffroy et al., 2010). Estrogen inhibits TGF-β signaling by multiple mechanisms. SnoN is the negative regulator of TGF-β signaling and is widely expressed in adult and embryonic cells. The level of SnoN is directly linked to its ability to repress TGF-β signaling.

It is reported that estrogen receptor activation inhibits transcriptional activity of TGF-β and TGF-β induced cell migration through SMAD-2/3 degradation. ER-β may inhibit TGF-β signaling by enhancing SMAD-2/3 degradation by Estrogen dependent manner (Ito et al., 2010). This interaction of ER with regulatory SMADs is independent of DNA binding domain and transcriptional activity. Thus, ER may inhibit TGF-β pathway through non-genomic action.

❖ Aim:

To determine the change in SMADs (TGF-β mediated signaling) during antiestrogen treatment in Triple Negative Breast Cancer

❖ Objectives:

2.1. To evaluate the cytotoxic effects of TAM and 4-OH-TAM in TNBC cell lines (MDA-MB-468 and MDA-MB-231).

2.2. To determine phenotypic expression of TGF-β1, TGF-β2, SMAD-3 and SMAD-4 during antiestrogen treatment in time dependent manner.

Materials and Methods

3.1. Cell Cultures:

Triple negative (ER^-, PR^-, $HER\text{-}2^-$) breast cancer cell lines MDA-MB-231 and MDA-MB-468 (obtained from NCCS, Pune, India) were grown at 37 °C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Catalog#AL111) containing 4.5 g glucose per litre supplemented with 10% Fetal Bovine Serum (Catalog#RM112), 2 mM L-Glutamine (Catalog#TCL012), 0.1 mM MEM non-essential amino acids (Catalog#ACL006) and 1% antibiotic solution (Penicillin and Streptomycin, HIMEDIA Catalog#A007). Cultures were regularly examined using inverted microscope.

(MDA-MB-231) **(MDA-MB-468)**

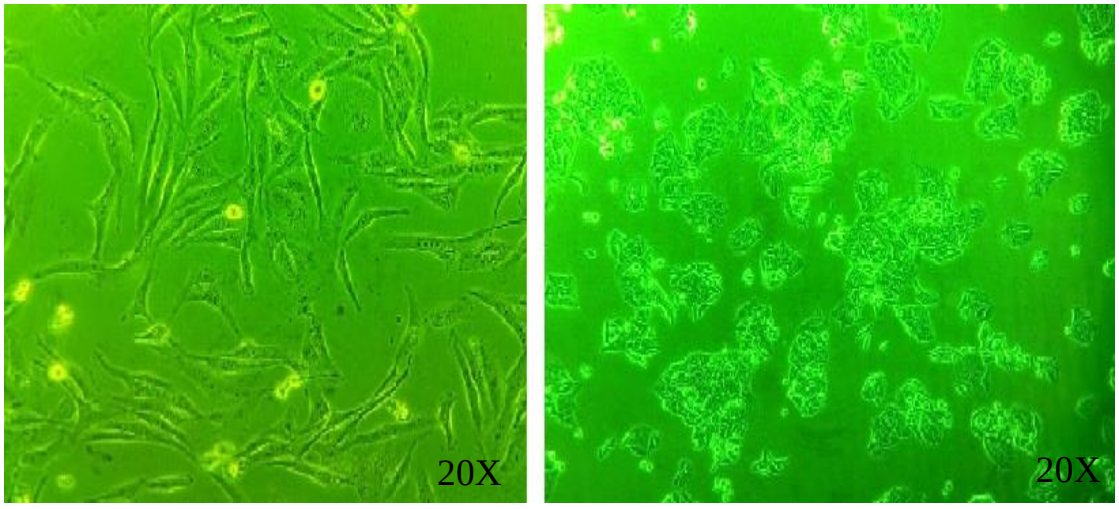

Figure 6: Morphology of MDA-MB-231 and MDA-MB-468 cell lines

	MDA-MB-231	**MDA-MB-468**
Molecular Classification	Claudin-low	Basal-like
Population Doubling Time	38 h	22 h
Morphology	Stellate-class	Grape-like clusters
Other Characteristics	Low expression of claudin, Ki67	High expression of EGFR

3.2. Growth Curve Analysis:

Growth curve experiment was established for 5 days to determine the population doubling time of the cells. On day-0, total 5 T25 flasks were seeded with seeding density of 10^4 cells per flask in complete growth medium. Each day one T25 flask was trypsinized and cell count was done manually using Haemocytometer. The graph was plotted using Graph Pad Prism 6 to determine population doubling time.

3.3. Proliferation-cytotoxicity (MTT) Assay:

Materials

Flat bottom 96-well tissue culture plate

Tamoxifen (Catalog#5648, Sigma)

4-OH-Tamoxifen (Catalog#H6278, Sigma)

MTT solution (Catalog#RM 1131 1G, HIMEDIA)

DMSO solvent

Method

- DAY 1: Culture flask having 90-95% confluency of cells was trypsinized and cell count was done. From cell suspension 96-well plate was seeded with 10^4 cells in each well and plate was incubated at 37°C in CO_2 incubator for 24 h. No cells were added in blank well.
- DAY 2: Five different concentrations (10^{-6} M to 10^{-10} M) of TAM and 4-OH-TAM were prepared in complete growth medium and added to its respected wells in triplicates (No drug was added in Blank and Control wells).
- DAY 3: MTT solution (1 mg/ml) was prepared in sterile PBS and filtered by 0.2 µm syringe filter. 50 µL of MTT solution was added in each well including blank and control wells. Plate was incubated at 37°C in CO_2 incubator for 3 h. After incubation, media was removed carefully and 150 µL of DMSO solvent was added in each well. Plate was covered with aluminium foil and incubated for 20 min at room temperature. Absorbance was taken at 570 nm using ELISA reader. The graph of absorbance

versus concentration of drug was plotted. The effective cytotoxic doses of TAM and 4-OH-TAM, and cell survival rate were determined using Graph Pad Prism 6.

- Cell Survival Rate and Cytotoxicity were calculated using following formulas:

$$\text{Cell Survival Rate} = \frac{(A_{\text{sample}} - A_{\text{Blank}})}{(A_{\text{control}} - A_{\text{Blank}})} \times 100$$

$$\text{Cytotoxicity (\%)} = \frac{(A_{\text{control}} - A_{\text{Blank}}) - (A_{\text{sample}} - A_{\text{Blank}})}{(A_{\text{control}} - A_{\text{Blank}})} \times 100$$

3.4. Growth Inhibition of MDA-MB-231 during Antiestrogen drug treatment:

Experimental set up was designed to determine the cytotoxic effect of effective doses of TAM and 4-OH-TAM on triple negative breast cancer cells.

- Short term drug exposure assay: To determine single dose effect of antiestrogen drugs on MDA-MB-231 cell line.

3.4.1. Short term drug exposure assay:

T25 culture flasks were seeded with cell density of 10^4 cells per flask in complete growth medium and incubated at 37°C in CO_2 incubator for 24 h. After incubation, antiestrogen drugs (TAM 10^{-6} M and 4-OH-TAM 10^{-7} M) were added to respective flask and incubated for 5 days at 37°C in CO_2 incubator. Cells were trypsinized on day-6 and cell count was done. The results were plotted as Mean ± SEM in graph.

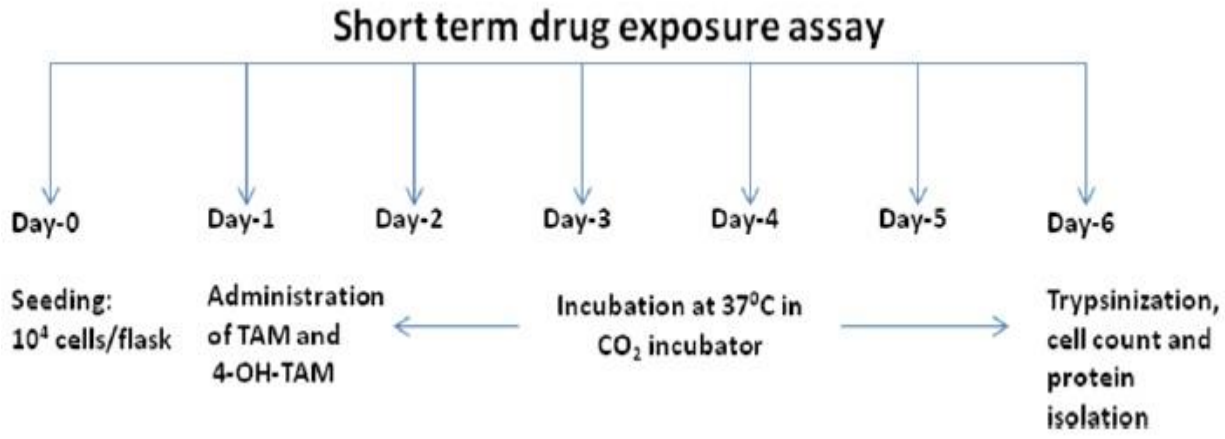

Figure 7: Schematic presentation of Short Term drug exposure assay

3.5. Enzyme Linked Immunosorbent Assay (ELISA) to determine phenotypic expression of growth factors and signaling mediators:

3.5.1. Extraction of Cytoplasmic and Nuclear proteins

Materials

Cytoplasmic lysis buffer

Nuclear lysis buffer

Method

Culture flask was trypsinized and the cell suspension was centrifuged at 1000 rpm for 7 min. Cell pellet was dissolved in 1 ml ice cold cytoplasmic lysis buffer and kept on ice for 1 h (vortexed 2-3 times in between). Cell lysate was centrifuged at 12,000 rpm at 4°C for 15 min. Supernatant obtained was used for cytoplasmic protein estimation. After removal of the supernatant, cell pellet was resuspended in 62 µl of nuclear lysis buffer and incubated on ice for 30 min. Cell lysate was centrifuged at 17,000 rpm at 4°C for 10 min. Supernatant obtained was used for nuclear protein estimation.

3.5.2. Estimation of total protein by Folin-Lowry method

Materials

Cytoplasmic protein samples

Bovine Serum Albumin (BSA)

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 1% Na-K Tartrate in Distilled Water

Reagent C: 0.5% $CuSO_4$

Reagent 1: 48 ml of Reagent A + 1 ml of Reagent B + 1 ml of Reagent C

Reagent 2: 1:1 Folin-Phenol [2N]: Distilled Water

Method

Working solutions of BSA (0.2 to 1.0 mg/ml), blank and cytoplasmic protein samples were taken in test tubes. 4.5 ml of Reagent 1 was added in each tube and incubated for 10 min. After incubation, 0.5 ml of Reagent 2 was added and incubated for 30 min in dark. Absorbance was measured at 570 nm and graph was plotted for standard concentrations of BSA. The protein concentrations of unknown samples were determined as mg/ml using the following formula:

$$\text{Protein concentration (mg/ml)} = \frac{\text{O.D of sample} \times \text{Standard Concentration}}{\text{O.D. of standard}}$$

3.5.3. Estimation of phenotypic expression of TGF-β1 by Sandwich ELISA

Reagents

- Primary (Coating) Antibody: Anti-TGF-β1,2,3 Mouse Monoclonal Antibody (Catalog#MAB1835, R&D Systems)

- Secondary (Detection) Antibody: Anti h-TGF-β1 (Catalog#AF-101NA, R&D Systems)
- Labelling Antibody: Alkaline Phosphatase labelled Goat anti-chicken IgG (Catalog#151-24-06, KPL)
- Peptide: TGF-β1 (Catalog#100B, R&D Systems)

Method

- ❖ DAY 1: Flat bottom ELISA plate was coated with primary antibody (100 µl/well). Plate was covered and incubated overnight at 4°C.
- ❖ DAY 2: Primary antibody was removed by inverting the plate. Blocking buffer (250 µl/well) was added and incubated at room temperature for 2 h. Plate was washed 3 times with washing buffer (250 µl/well). 100 µl of blank, standards and samples were added to respective wells. Plate was covered and incubated overnight at 4°C.
- ❖ DAY 3: Plate was decanted and washed thrice with wash buffer (250 µl/well). 100 µl of detection antibody (diluted in Binding Buffer II) was added to each well with final concentration of 5 µg/ml. Plate was incubated on shaker for 1 h at room temperature. After incubation, plate was washed 3 times with wash buffer. Alkaline phosphatase labelled Goat anti-chicken IgG antibody (diluted in Binding buffer I) was added to each well (100 µl/well) with final concentration of 500 ng/ml and plate was incubated for 1 h at room temperature. Plate was washed thrice with washing buffer. p-NPP substrate was added to each well (100 µl/well). Plate was incubated for 1 h at room temperature. Absorbance was measured at 450 nm in ELISA reader (Readings were taken after 30 and 60 min of incubation).

3.5.4. Estimation of phenotypic expression of TGF-β2 by Sandwich ELISA

Reagents

- Primary (Coating) Antibody: Anti-TGF-β2 polyclonal Goat IgG Antibody (Catalog#AB-112-NA, R&D Systems)
- Secondary (Detection) Antibody: Anti h-TGF-β2 polyclonal Rabbit IgG (Catalog#AB-12-NA, R&D Systems)

- Labelling Antibody: HRP conjugated Goat anti-rabbit IgG (Catalog#A16096, Invitrogen)
- Peptide: p-TGF-β porcine platelet derived (Catalog#102-B2, R&D Systems)

Method

- ❖ DAY 1: Flat bottom ELISA plate was coated with primary antibody with final concentration of 10 µg/ml diluted in 1X PBS. Plate was covered and incubated overnight at 4°C.
- ❖ DAY 2: Plate contents were removed by inverting the plate. Blocking buffer (250 µl/well) was added and incubated at room temperature for 2 h. Plate was washed 3 times with washing buffer (250 µl/well). 100 µl of blank, standards and samples were added to respective wells. Plate was covered and incubated overnight at 4°C.
- ❖ DAY 3: Plate was decanted and washed three times with washing buffer. 100 µl of detection antibody (diluted in Binding Buffer II) was added to each well (final concentration of 5 µg/ml). Plate was incubated on shaker for 1 h at room temperature. After incubation, plate was washed 3 times with washing buffer. HRP labelled Goat anti-rabbit IgG antibody (1:1000 diluted) was added to each well (100 µl/well). Antibody was diluted in 2% BSA in 1X PBS. Plate was incubated for 1 h at room temperature. Plate was washed 2 times with washing buffer and once with 1X PBS (200 µl/well). TMB-H_2O_2 substrate was added to each well (100 µl/well) and absorbance was measured at 650 nm after 10 min of incubation. 100 µl of stop solution (0.16 M H_2SO_4 in D/W) was added and absorbance was measured at 450 nm.

3.5.5. Estimation of phenotypic expression of SMAD-3 by Direct ELISA

Reagents

- Primary Antibody: SMAD-2/3 (FL-425) rabbit polyclonal IgG Antibody (Catalog#SC-8332, SCBT)
- Labelling Antibody: HRP conjugated Goat anti-rabbit IgG (Catalog#A16096, Invitrogen)
- Peptide: SMAD3 peptide (Catalog#204884, Abcam)

Method

- ❖ DAY 1: Flat bottom ELISA plate was coated with blank, SMAD-3 standard peptide (prepared in Binding buffer I) and protein samples (diluted in 1X PBS). Plate was covered and incubated overnight at 4°C.
- ❖ DAY 2: After incubation plate contents were removed by inverting the plate. Blocking buffer (250 µl/well) was added and the plate was incubated at room temperature for 2 h. The plate was washed thrice with washing buffer. Primary antibody (100 µl/well) was added and plate was incubated overnight at 4°C.
- ❖ DAY 3: Plate was decanted by inverting the plate and washed 3 times with washing buffer. HRP labelled Goat anti-rabbit IgG antibody (1:1000 dilution) was added to each well and incubated at room temperature for 1 h. Plate was washed twice with washing buffer and once with 1X PBS. TMB-H_2O_2 substrate was added to each well (100 µl/well) and absorbance was measured at 650 nm after 10 min of incubation. 100 µl of stop solution (0.16 M H_2SO_4 in D/W) was added and absorbance was measured at 450 nm.

3.5.6. Estimation of relative expression of SMAD-4 by Direct ELISA

Reagents

- Primary Antibody: SMAD-4 (B-8) mouse monoclonal IgG_1 Antibody (Catalog#SC-7966, SCBT)
- Labelling Antibody: HRP-labelled Goat anti-mouse IgG_1 (Catalog#A10551, Thermofisher Scientific)

Method

- ❖ DAY 1: Flat bottom ELISA plate was coated with blank and protein samples (diluted in 1X PBS). Plate was covered and incubated overnight at 4°C.
- ❖ DAY 2: After incubation plate contents were removed by inverting the plate. Blocking buffer (250 µl/well) was added and the plate was incubated at room temperature for 2 h. The plate was washed thrice with washing buffer. Primary antibody (100 µl/well) was added and plate was incubated overnight at 4°C.

- DAY 3: Plate was decanted by inverting the plate and washed 3 times with washing buffer. HRP labelled Goat anti-mouse IgG_1 antibody (1:1000 dilution) was added to each well and incubated at room temperature for 1 h. Plate was washed twice with washing buffer and once with 1X PBS. TMB-H_2O_2 substrate was added to each well (100 µl/well) and absorbance was measured at 650 nm after 10 min of incubation. 100 µl of stop solution (0.16 M H_2SO_4 in D/W) was added and absorbance was measured at 450 nm.

3.6. Statistical Analysis

Statistical analysis was done using Graph Pad Prism 6. P values <0.05 were considered as significant (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Student's t-test was performed to determine significance of the data obtained using MS Office Excel.

Results and Discussion

In the present study, we have determined the effects of antiestrogen on Triple Negative (ER^- /PR^-/$HER\text{-}2^-$) Breast Cancer cell lines MDA-MB-468 and MDA-MB-231.The dose response and the effect of antiestrogen drugs (TAM and 4-OH-TAM) were determined by MTT assay. The phenotypic levels of growth factors (TGF-β1 and TGF-β2) and signaling mediator (SMAD-3 and SMAD-4) were determined by ELISA to evaluate the effect of antiestrogens on triple negative cells as well as to determine the crosstalk between ER and TGF-β signaling pathways.

4.1. Establishment and maintenance of human breast cancer cell lines MDA-MB-468 and MDA-MB-231

MDA-MB-468 and MDA-MB-231 cell lines were selected for the current study since the main objective of the study was to evaluate the antiestrogen treatment in TNBC and to identify the crosstalk between ER and TGF-β pathways. Both the cell lines are adherent mammalian epithelial breast cancer cell lines. All the primary steps of cell line maintenance including reviving, passaging, freezing, cell counting and preservation were performed according to ATCC guideline (ATCC® HTB-132™ and ATCC® HTB-26™).

4.1.1. Cell Characteristics of MDA-MB-468:

MDA-MB-468 cells were observed at regular time interval throughout the study. It was observed that the cells showed increased cytoplasmic projections with increase in cell confluency. The changes in cell morphology are demonstrated in Figure.

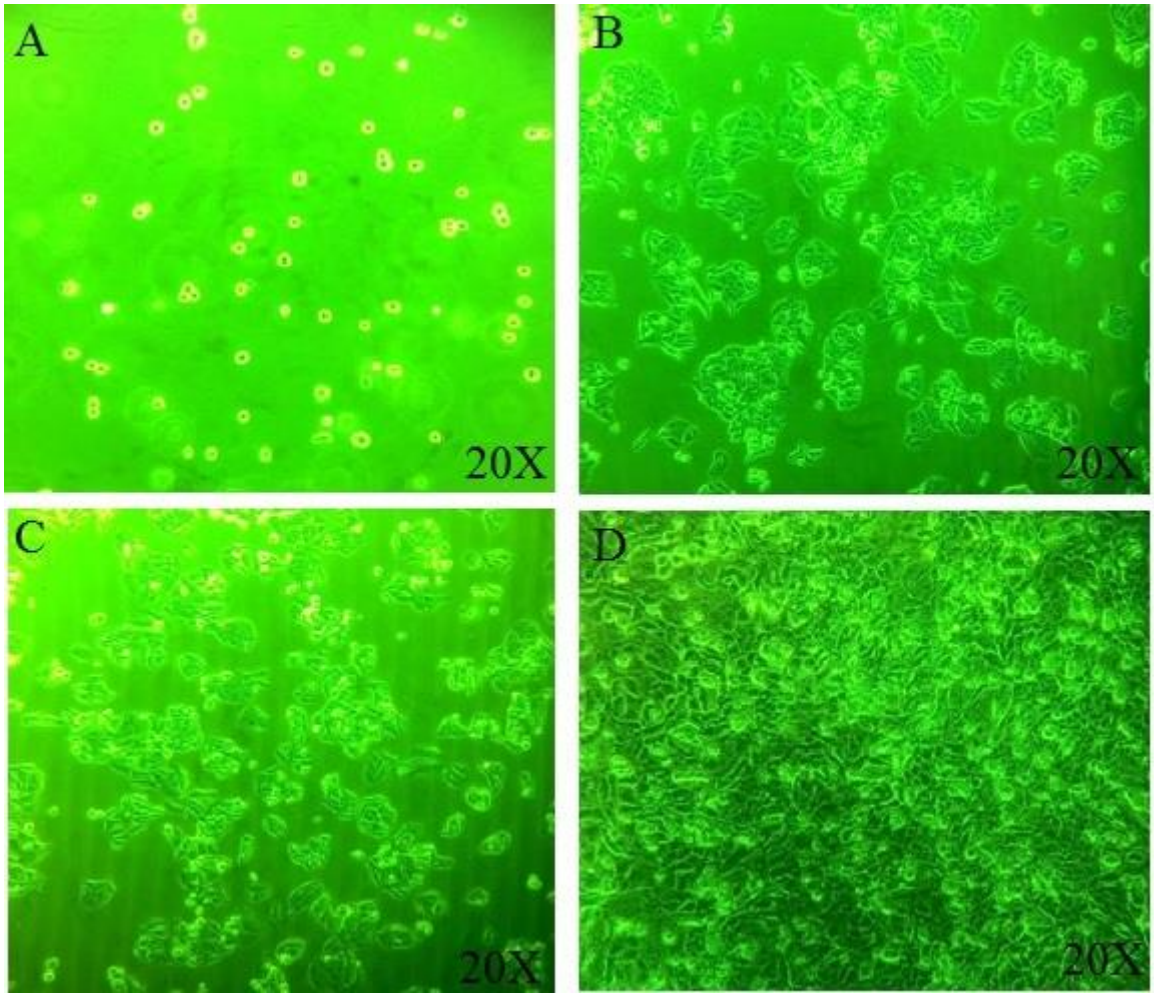

Figure 8: Cell morphology at different confluency of MDA-MB-468: The cells were grown in T25 flask using complete growth medium DMEM. The increased cytoplasmic projections and changes in cellular morphology were observed with increase in cell confluency. Figure A shows 25% cell confluency with absence of cytoplasmic projections. Majority of cells are in oval shape. Figure B shows ~50% confluent cells in which all cells are attached to surface of the flask. Figure C shows 65-70% confluency of cells. Figure D shows over confluent cells in which all the surface of a flask is covered with cells.

4.1.2. Cell Characteristics of MDA-MB-231:

MDA-MB-231 cells were observed at regular time interval throughout the study. It was observed that the cells were of oval shape when seeded and attached to flask surface after 24 h of seeding. The changes in cell morphology are demonstrated in Figure.

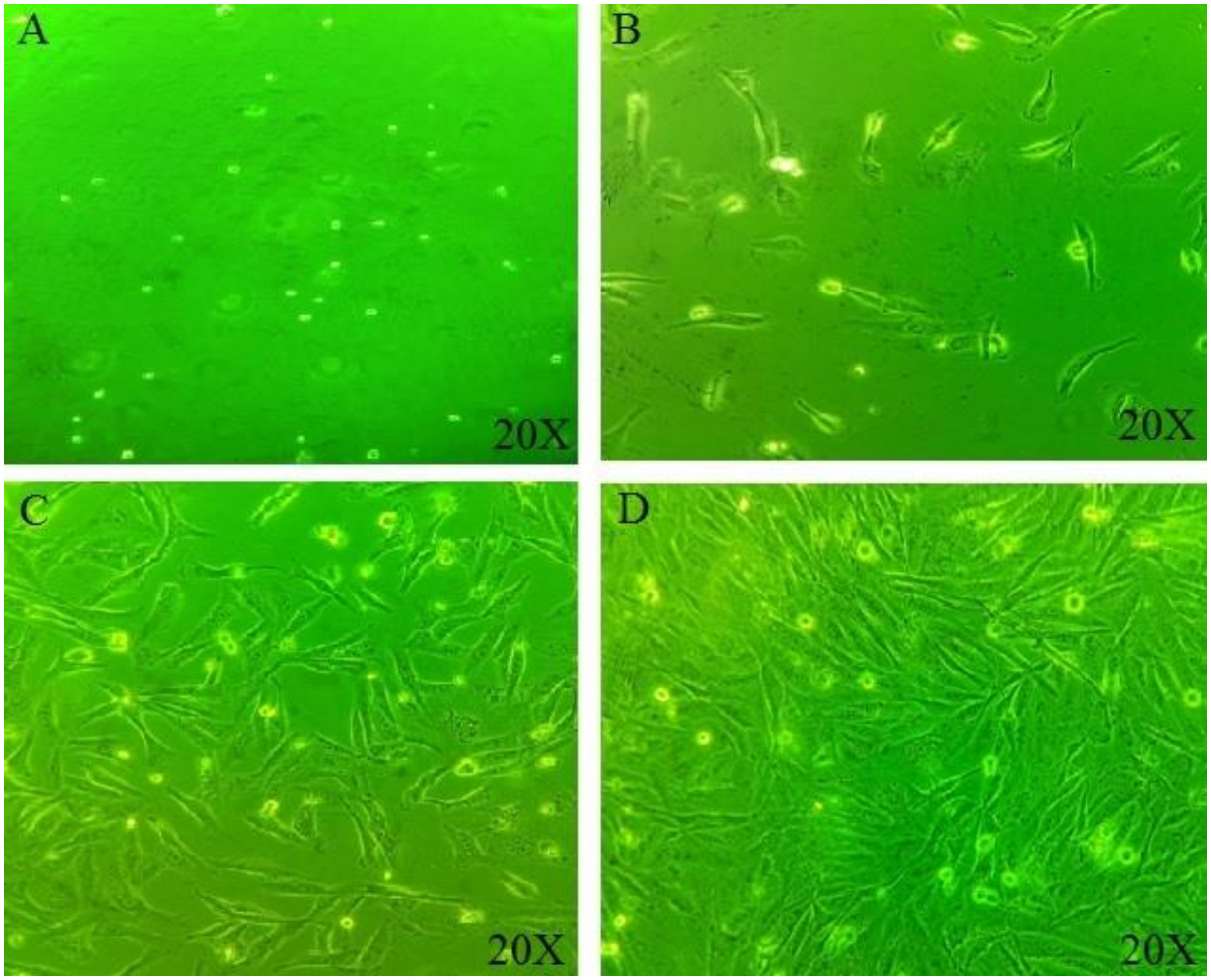

Figure 9: Cell morphology at different confluency of MDA-MB-231: The cells were grown in T25 flask using complete growth medium DMEM. The changes in cellular morphology were observed with increasing cell confluency. Figure A shows ~10% confluent cells in oval shape and in unattached condition. Figure B shows 30% cell confluency with cells having tapered ends and attached to flask surface. Figure C shows 65-70% confluent cells. Figure D shows ~95% cell confluency in which flask surface is completely covered with cells.

4.2. Determination of effective dose of TAM and 4-OH-TAM by MTT assay:

To determine the optimum cytotoxic dose of TAM and 4-OH-TAM, MTT assay was performed. Results clearly indicate that there is significant reduction in absorbance in antiestrogen treated cells as compared to untreated cells. Five different concentrations (10^{-6} M to 10^{-10} M) were selected with reference to the work carried out by Karami-Tehrani and Salami, 2003.

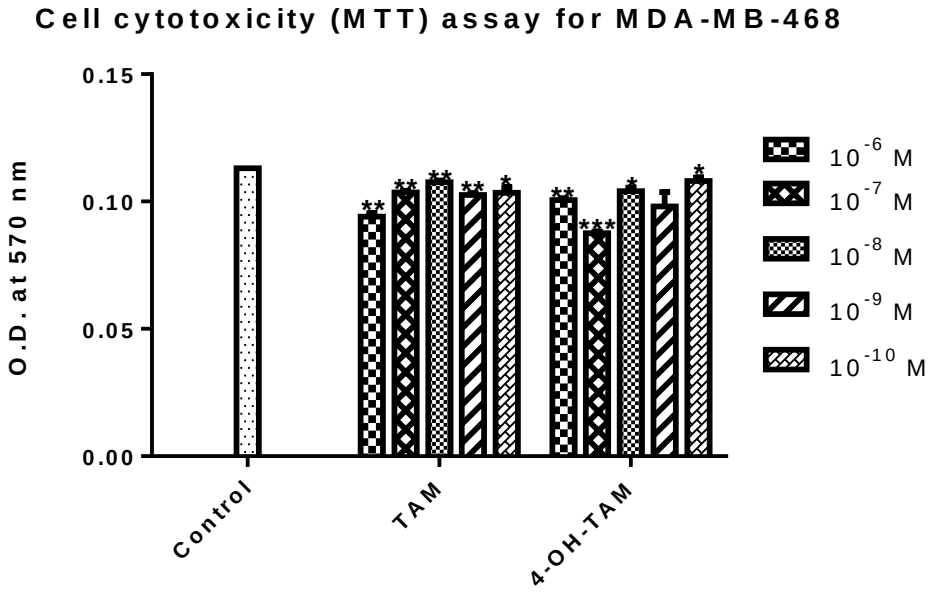

Figure 10: Optimum dose detemination by cell cytotoxicity (MTT) assay for MDA-MB-468: Result of MTT assay indicates that the optimum cytotoxic dose for TAM is 10-6 M (p=0.0028) and for 4-OH-TAM is 10-7 M (p=0.0004).

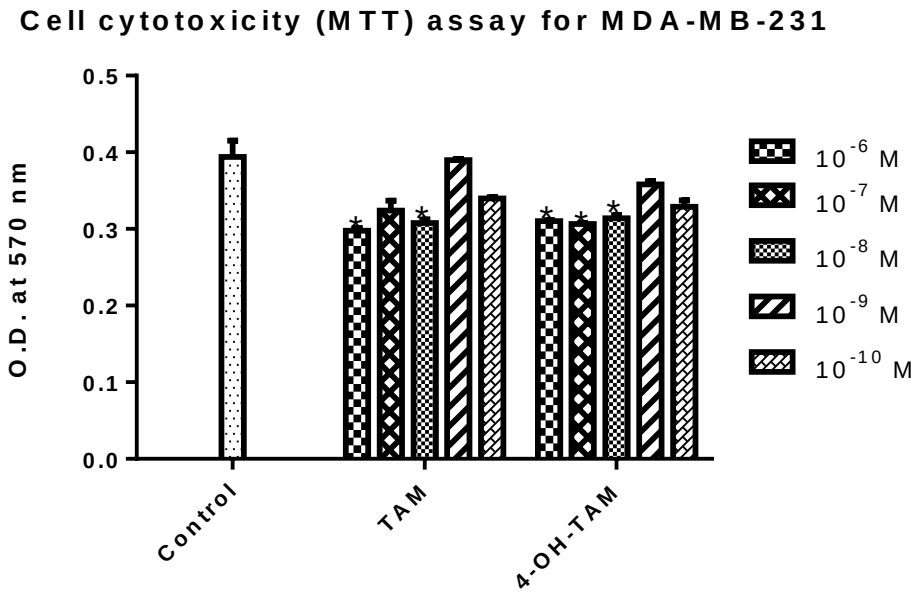

Figure 11: Optimum dose determination by cell cytotoxicity (MTT) assay for MDA-MB-231: Result of MTT assay indicates that the optimum cytotoxic dose for TAM is 10-6 M (p=0.0239) and for 4-OH-TAM is 10-7 M (p=0.0282).

From five different dose concentrations (10^{-6} M to 10^{-10} M), significant growth reduction was seen in two doses of TAM: 10^{-6} M (p=0.0028) and 10^{-7} M (p=0.0028) in MDA-MB-468 cell line as well as 10^{-6} M (p=0.0239) and 10^{-8} M (p=0.0303) in MDA-MB-231 cell line. All five dose concentrations of 4-OH-TAM treated cells showed significant reduction in absorbance ($p<0.05$) in case of MDA-MB-468 but not for MDA-MB-231 cell line. Cell proliferation kinetics were plotted in two different ways: (1) The relative cytotoxicity exhibits the effectiveness of the drug response (Figure 12 and 13) and (2) The cell survival rate shows the actual number of cells that survived after drug exposure (Figure14 and 15). Both parameters are inversely proportional to each other.

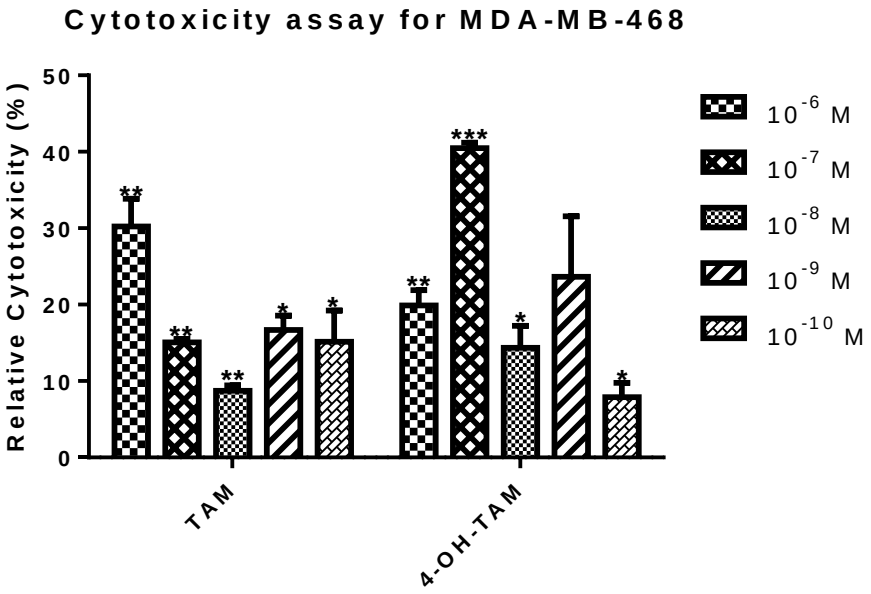

Figure 12: Relative cytotoxicity of TAM and 4-OH-TAM for MDA-MB-468

The optimum relative cytotoxicity was observed in 10^{-6} M concentration (p=0.0070) of TAM and in 10^{-7} M concentration (p=0.0001) of 4-OH-TAM.

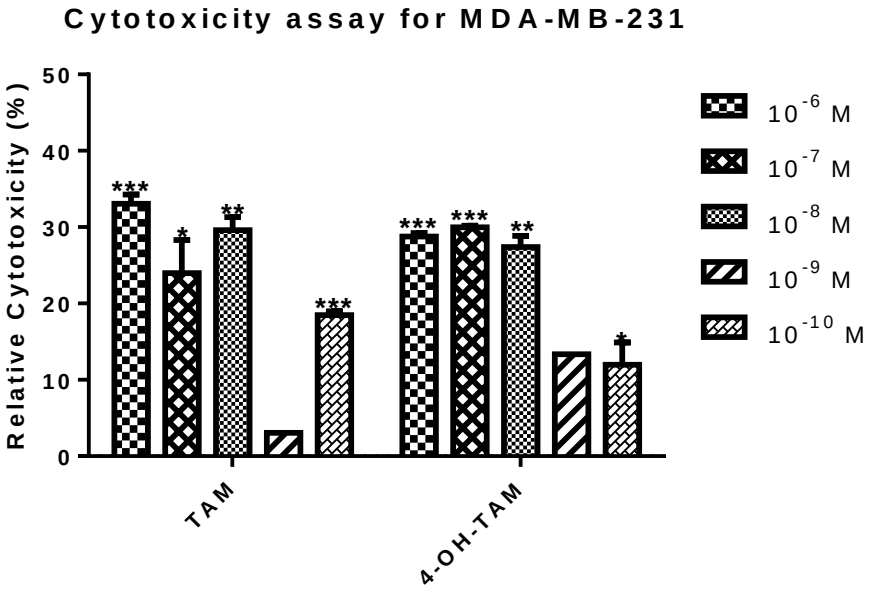

Figure 13: Relative cytotoxicity of TAM and 4-OH-TAM for MDA-MB-231

The optimum relative cytotoxicity was observed in 10^{-6} M concentration (p=0.0007) of TAM and in 10^{-7} M concentration (p=0.0001) of 4-OH-TAM.

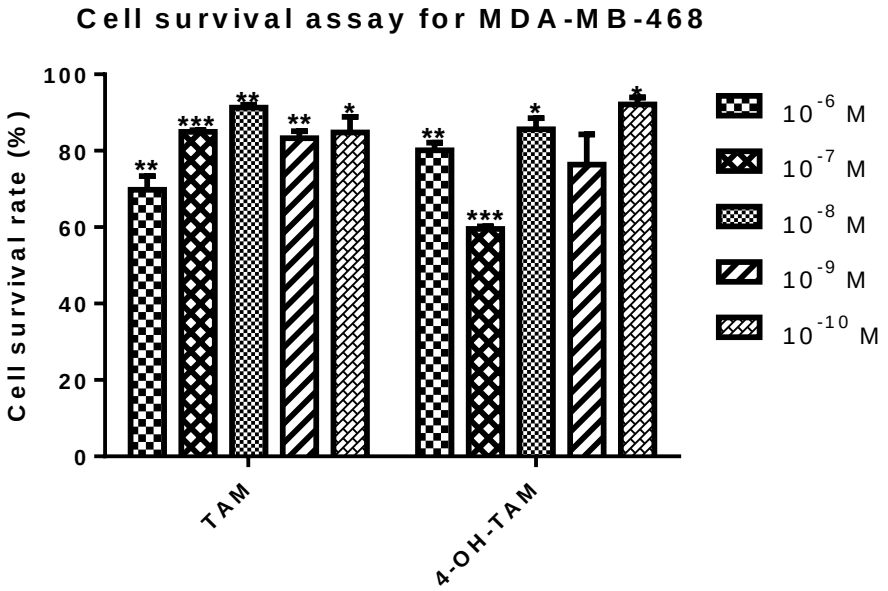

Figure 14: Cell survival rate for MDA-MB-468

The cell survival rate was significantly reduced in 10^{-6} M concentration (p=0.0070) of TAM and 10^{-7} M concentration (p=0.0001) of 4-OH-TAM.

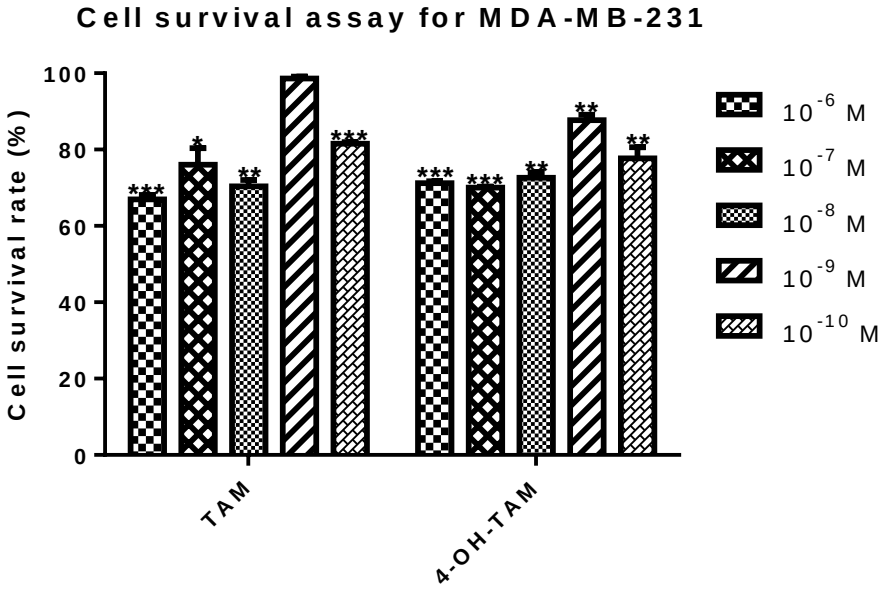

Figure 15: Cell survival rate for MDA-MB-231

The cell survival rate was significantly reduced in 10^{-6} M concentration (p=0.0007) of TAM and 10^{-7} M concentration (p=0.0001) of 4-OH-TAM.

4.3. Determination of Population Doubling Time (PDT) and Growth Curve analysis:

Growth curve experiment was performed with a seeding density of 1 X 10^5 cells/flask for 5 days. Cell count was performed after 24 hr of time interval for 5 consecutive days (Table). Population doubling time of both cell lines MDA-MB-468 and MDA-MB-231 determined were 22 h and 38 h respectively.

Table 3: Growth Curve analysis of MDA-MB-468

Time (hour)	Cell Count
0	1 X 10^5
24	4 X 10^5
48	5 X 10^5

72	7 X 10^5
96	19 X 10^5
120	47 X 10^5

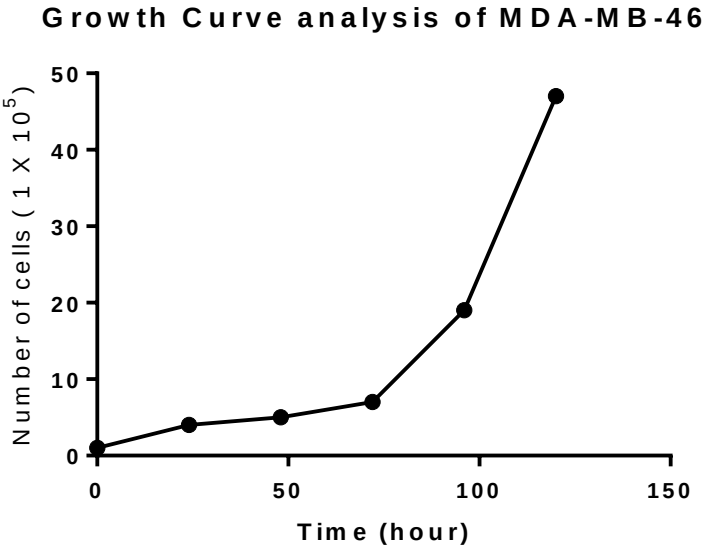

Figure 16: Growth Curve analysis of MDA-MB-468

Table 4: Growth Curve analysis of MDA-MB-231

Time (hour)	Cell Count
0	1 X 10^5
24	5.2 X 10^5
48	9 X 10^5
72	21 X 10^5
96	32 X 10^5
120	42 X 10^5

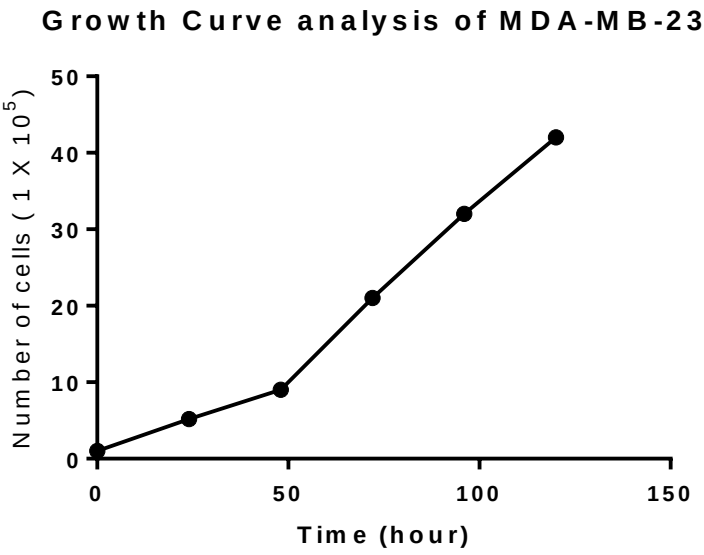

Figure 17: Growth Curve analysis of MDA-MB-231

4.4. Drug exposure assay

Tamoxifen is administered daily (20 mg/day) to breast cancer patients for 3 to 5 years. However, after the loner tenure of 3-5 years, this treatment usually results in failure and/or resistance. The main purpose of the study is to evaluate the differences in cell viability during single dose treatment of antiestrogen drugs.

4.4.1. Growth inhibition during Short Term Drug Exposure Assay for MDA-MB-468

We have assessed the effect of single dose administration of both antiestrogen drugs on cell viability during short period (5 days) of time.

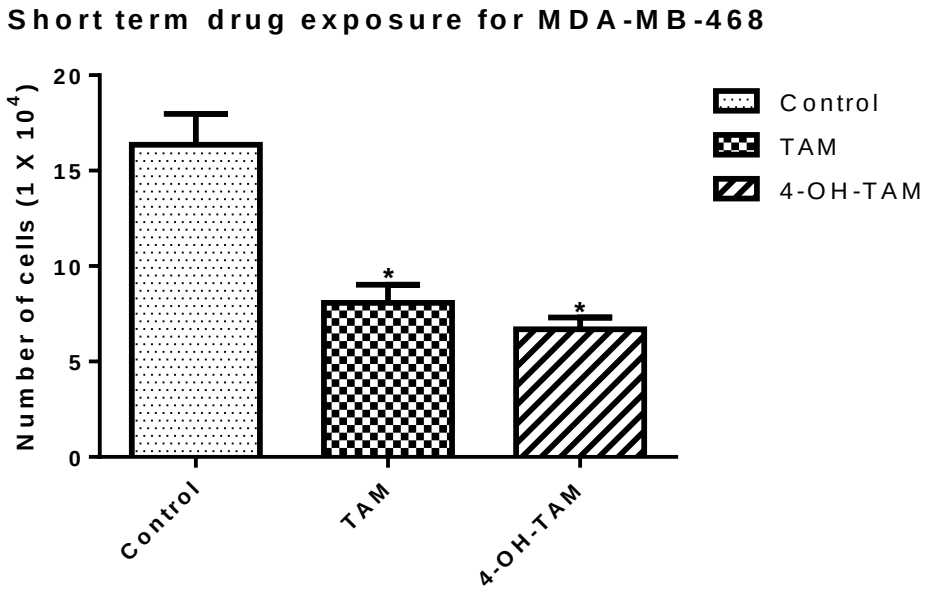

Figure 18: Cell viability during ST drug exposure assay for MDA-MB-468

There was significant reduction in cell viability due to administration of TAM (p=0.0249) and 4-OH-TAM (p=0.0159) during short term drug exposure assay.

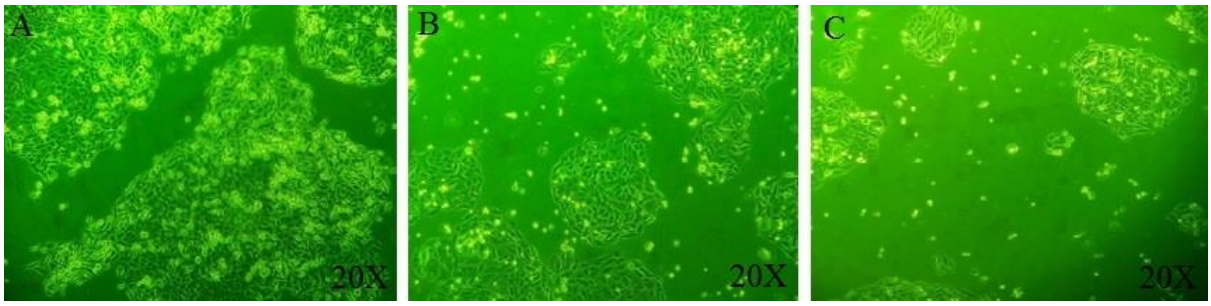

Figure 19: Morphology of MDA-MB-468 cells during Short Term drug exposure assay: (A) Untreated cells, (B) TAM treated cells, (C) 4-OH-TAM treated cells

4.4.2. Growth inhibition during ST Drug Exposure Assay for MDA-MB-231

We have estimated the effect of short term administration of both antiestrogen drugs on cell viability during short period (5 days) of time.

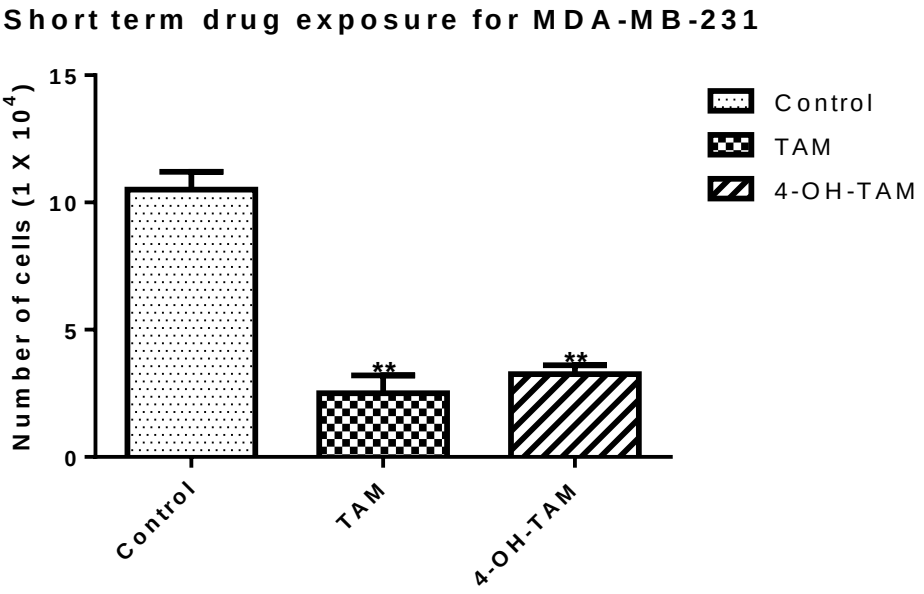

Figure 20: Cell viability during ST drug exposure assay for MDA-MB-231

Cell viability was significantly reduced due to administration of TAM (p=0.0077) and 4-OH-TAM (p=0.0059) during short term drug exposure assay (Figure).

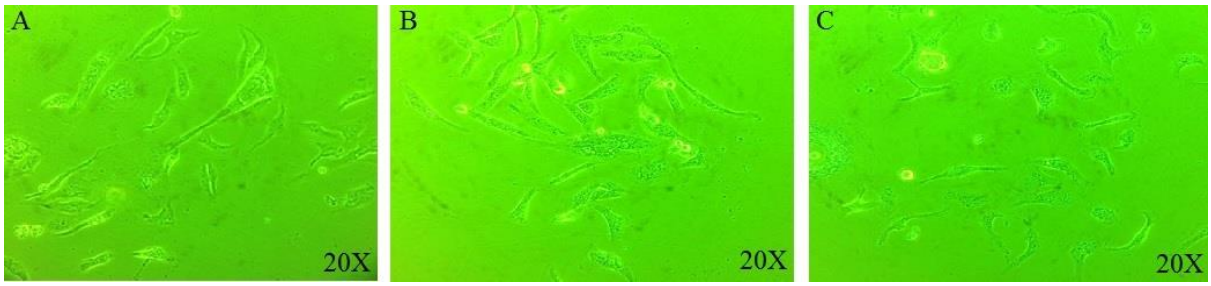

Figure 21: Morphology of MDA-MB-231 cells during Short Term drug exposure assay: (A) Untreated cells, (B) TAM treated cells, (C) 4-OH-TAM treated cells

4.5. Estimation of phenotypic expression of TGF-β1, TGF-β2, SMAD-3 and SMAD-4 protein by ELISA

4.5.1. Total Protein Estimation

Total protein estimation was performed from cell lysate by Folin Lowry method. Bovine Serum Albumin (BSA) was taken as standard. The total protein concentration of the sample was calculated in mg/ml using standard protein curve (Figure). Total protein estimation was done from three different cell lysates [untreated cells, TAM (10^{-6} M) and 4-OH-TAM (10^{-7} M) treated cells] for short term drug exposure assay.

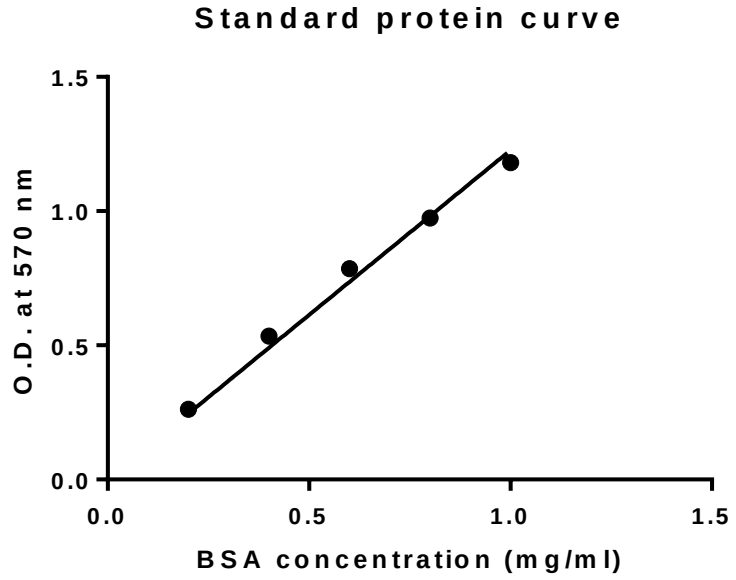

Figure 22: Standard Curve of Protein (Bovine Serum Albumin)

4.5.2. Standard TGF-β1 peptide curve

The human recombinant TGF-β1 peptide was used as standard.

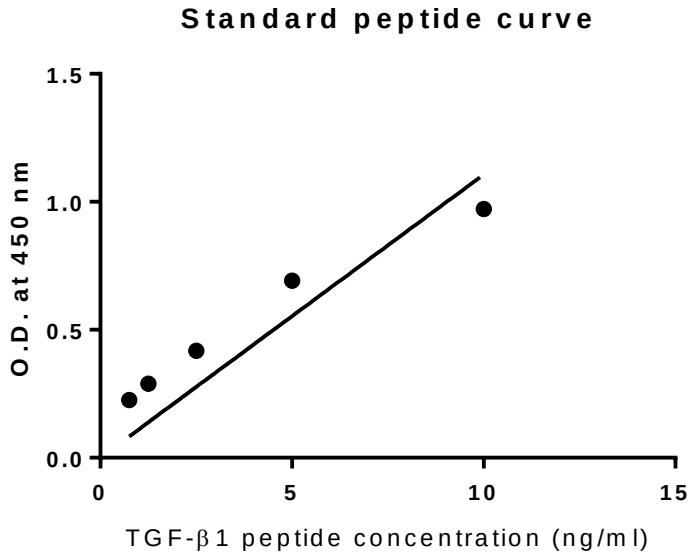

Figure 23: Standard curve for human recombinant TGF-β1 peptide

For Sandwich ELISA, standard hTGF-β1 peptide was taken in a range from 2 to 10 ng/ml and absorbance was taken at 450 nm.

4.5.3. Estimation of phenotypic expression of TGF-β1 during Short Term drug exposure assay

Short term drug exposure assay was performed in duplicates and the cells were harvested to isolate total protein to estimate the levels of TGF-β1 during short term drug exposure assay.

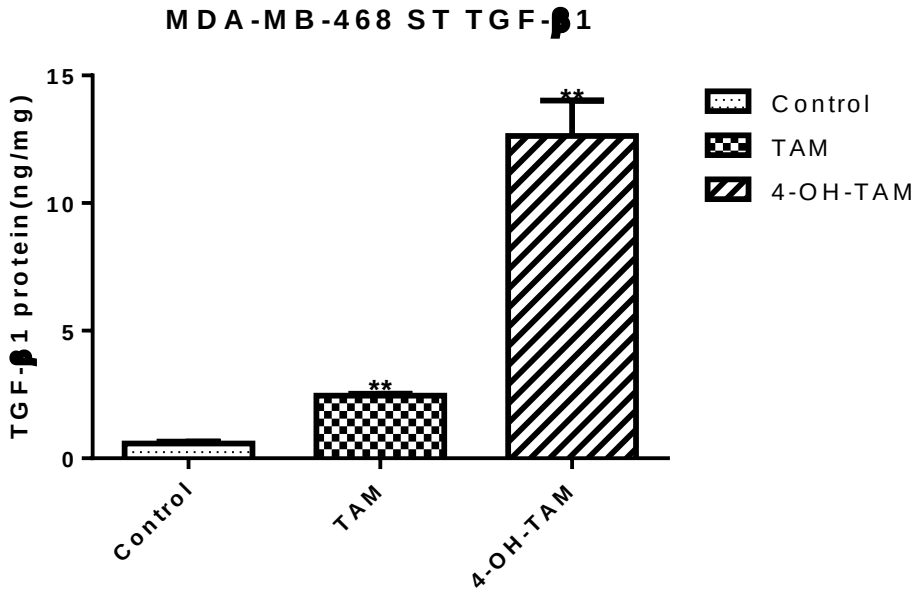

Figure 24: Phenotypic expression of TGF-β1 during Short Term drug exposure for MDA-MB-468

There is a significant elevation of TGF-β1 during short term exposure of both TAM (p=0.0014) and 4-OH-TAM (p=0.0065) as compared to untreated cells. The level of TGF-β1 increases in 4-OH-TAM treated cells as compared to TAM treated cells.

Table 5: Levels of TGF-β1 during Short Term drug exposure assay in MDA-MB-468

Treatment	TGF-β1 (ng/ml)	Total cytoplasmic protein (mg/ml)	TGF-β1 (ng/mg) Mean ± SEM
Control	1.87	3.160	0.58 ± 0.05
TAM	1.76	3.760	2.46 ± 0.05
4-OH-TAM	1.87	4.330	12.64 ± 0.98

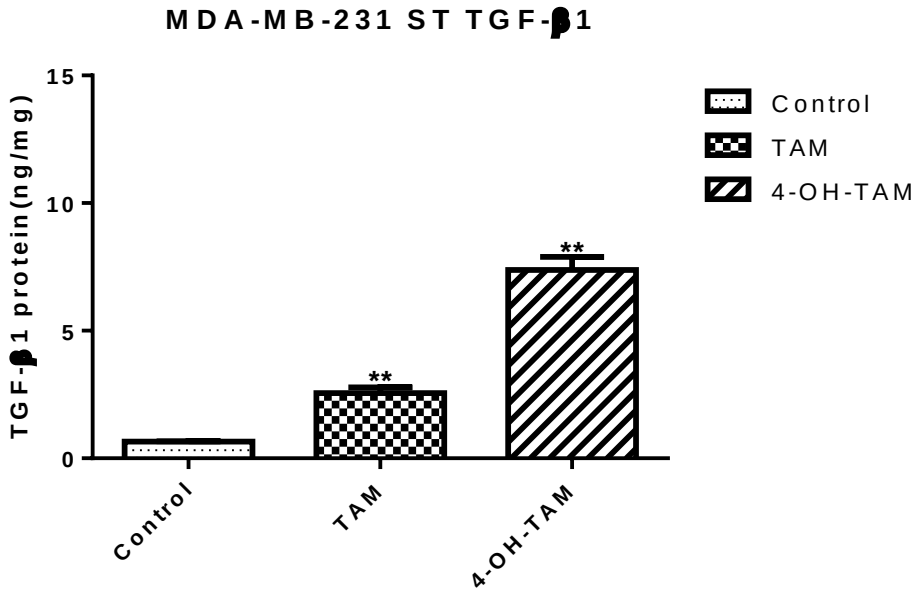

Figure 25: Phenotypic expression of TGF-β1 during Short Term drug exposure for MDA-MB-231

There is a significant elevation of TGF-β1 during short term exposure of both TAM (p=0.0071) and 4-OH-TAM (p=0.0029) as compared to untreated cells. The level of TGF-β1 increases in 4-OH-TAM treated cells as compared to TAM treated cells.

Table 6: Levels of TGF-β1 during Short Term drug exposure assay in MDA-MB-231

Treatment	TGF-β1 (ng/ml)	Total cytoplasmic protein (mg/ml)	TGF-β1 (ng/mg) Mean ± SEM
Control	1.61	2.440	0.66 ± 0.01
TAM	1.58	0.621	2.55 ± 0.16
4-OH-TAM	1.45	0.196	7.38 ± 0.37

4.5.4. Standard TGF-β2 peptide curve

The porcine platelet derived TGF-β2 peptide was used as standard.

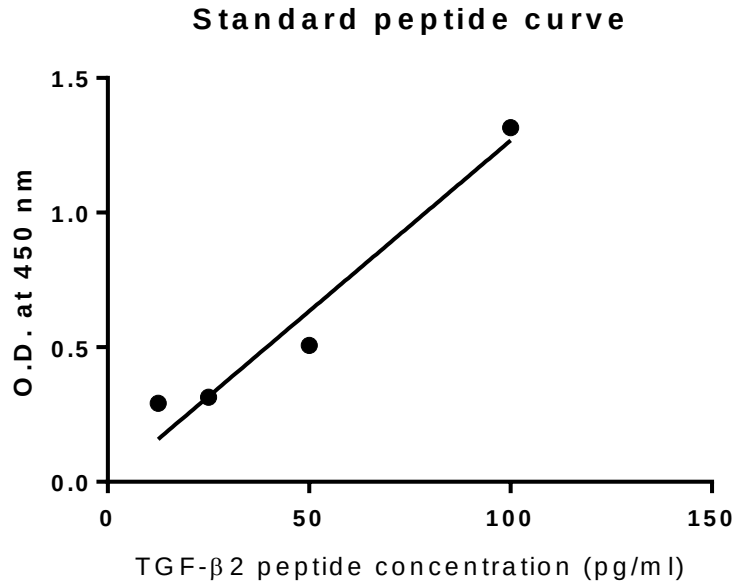

Figure 26: Standard curve for TGF-β2 peptide

For Sandwich ELISA, standard peptide was taken in a range from 10 to 100 pg/ml and absorbance was taken at 450 nm. This standard curve was used for determination of TGF-β2 levels in experimental samples.

4.5.5. Estimation of phenotypic expression of TGF-β2 during Short Term drug exposure assay

Short term drug exposure assay was done in duplicates and the cells were harvested from which total protein was isolated. Total protein was used to determine the levels of TGF-β2 during short term drug exposure assay.

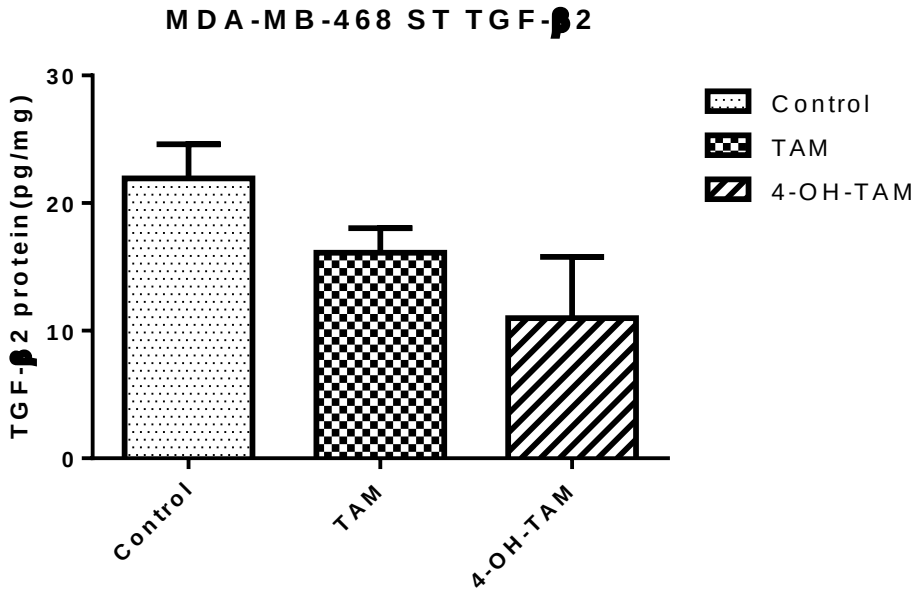

Figure 27: Phenotypic expression of TGF-β2 during Short Term drug exposure for MDA-MB-468

There is a non-significant reduction of TGF-β2 during single dose treatment of TAM and 4-OH-TAM as compared to untreated cells. The level of TGF-β2 decreases in 4-OH-TAM treated cells as compared to TAM treated cells.

Table 7: Levels of TGF-β2 during Short Term drug exposure assay in MDA-MB-468

Treatment	TGF-β2 (pg/ml)	Total cytoplasmic protein (mg/ml)	TGF-β2 (pg/mg) Mean ± SEM
Control	69.33	3.160	21.94 ± 1.90
TAM	60.63	3.760	16.12 ± 1.36
4-OH-TAM	47.54	4.330	10.98 ± 3.40

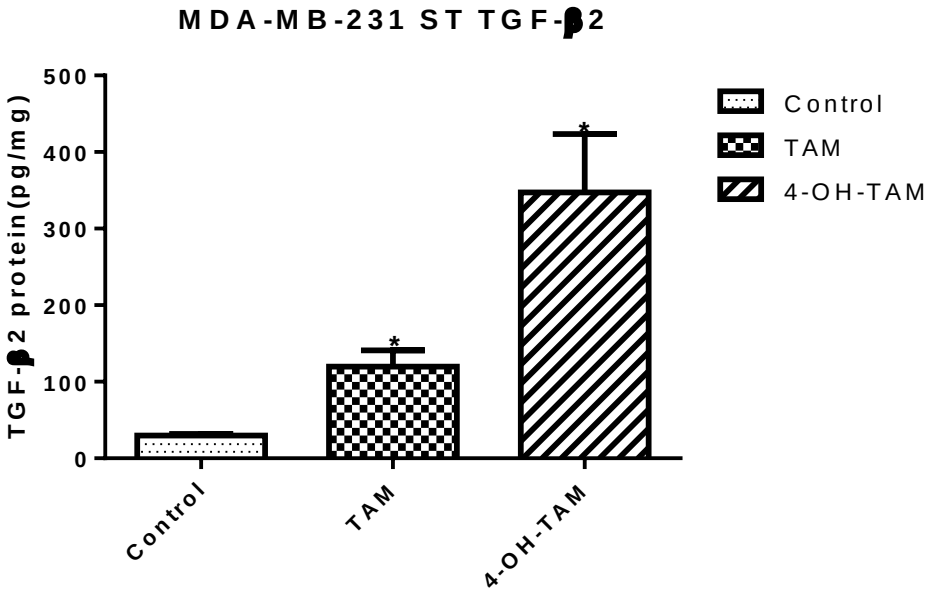

Figure 28: Phenotypic expression of TGF-β2 during Short Term drug exposure for MDA-MB-231

There is a significant elevation of TGF-β2 during short term exposure of both TAM (p=0.0270) and 4-OH-TAM (p=0.0277) as compared to untreated cells. The level of TGF-β2 increases in 4-OH-TAM treated cells as compared to TAM treated cells.

Table 8: Levels of TGF-β2 during ST drug exposure assay in MDA-MB-231

Treatment	TGF-β2 (pg/ml)	Total cytoplasmic protein (mg/ml)	TGF-β2 (pg/mg) Mean ± SEM
Control	72.79	2.440	29.83 ± 1.35
TAM	74.33	0.621	119.69 ± 15.03
4-OH-TAM	68.08	0.196	347.34 ± 53.98

4.5.6. Standard SMAD-3 peptide curve

For Direct ELISA, SMAD-3 standard peptide was taken in a range of 10 to 250 pg/ml and absorbance was taken at 450 nm. This standard curve was used for determination of SMAD-3 levels in experimental samples.

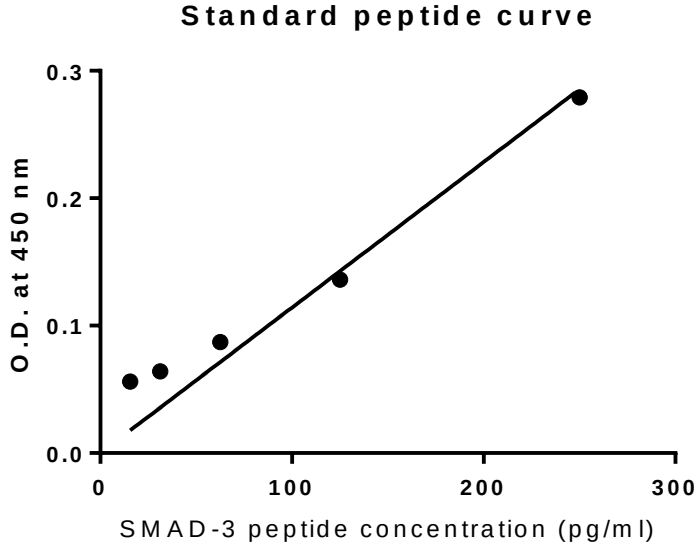

Figure 29: Standard curve for SMAD-3 peptide

4.5.7. Estimation of phenotypic expression of SMAD-3 during Short Term drug exposure assay

Short term drug exposure assay was performed in duplicates and the cells were harvested to extract cytoplasmic as well as nuclear proteins to determine the phenotypic expression of SMAD-3.

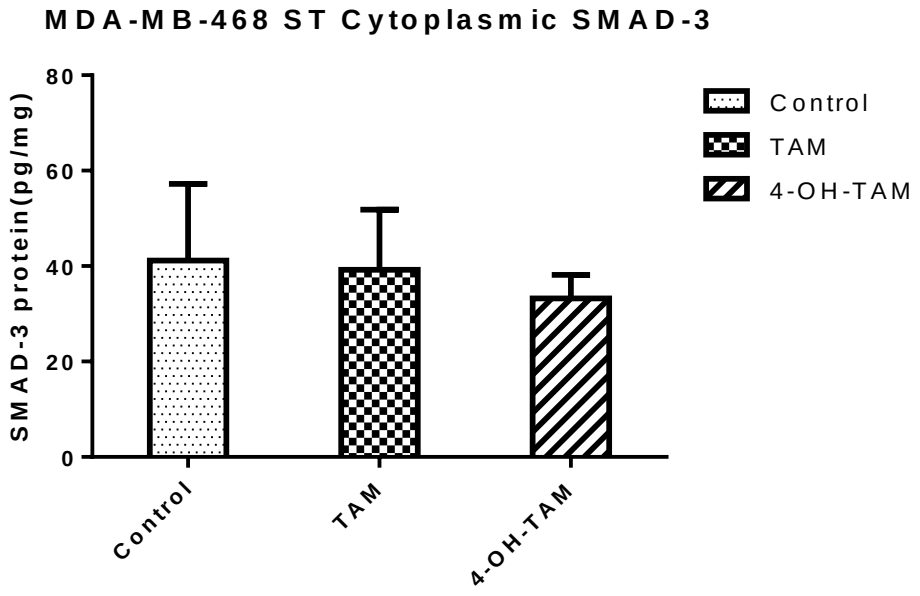

Figure 30: Phenotypic expression of cytoplasmic SMAD-3 during Short Term drug exposure for MDA-MB-468

Table 9: Levels of cytoplasmic SMAD-3 during Short Term drug exposure assay in MDA-MB-468

Treatment	Cytoplasmic SMAD-3 (pg/ml)	Total cytoplasmic protein (mg/ml)	Cytoplasmic SMAD-3 (pg/mg) Mean ± SEM
Control	130	3.160	41.14 ± 11.39
TAM	147.5	3.760	39.23 ± 8.91
4-OH-TAM	144	4.330	33.26 ± 3.47

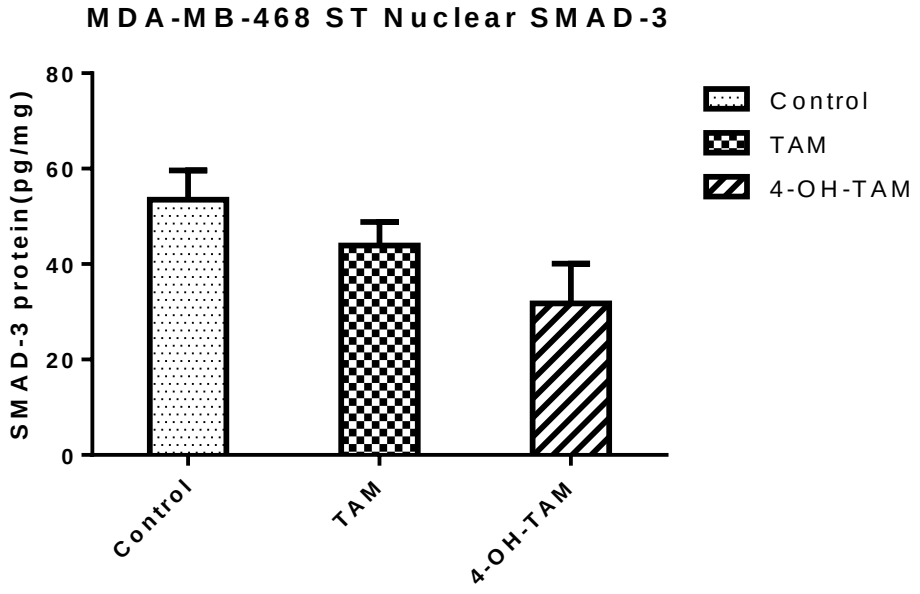

Figure 31: Phenotypic expression of nuclear SMAD-3 during Short Term drug exposure for MDA-MB-468

Table 10: Levels of nuclear SMAD-3 during Short Term drug exposure assay in MDA-MB-468

Treatment	Nuclear SMAD-3 (pg/ml)	Total nuclear protein (mg/ml)	Nuclear SMAD-3 (pg/mg) Mean ± SEM
Control	210	3.923	53.53 ± 4.33
TAM	183.5	4.178	43.92 ± 3.47
4-OH-TAM	154.5	4.859	31.80 ± 5.87

There is a non-significant reduction of both cytoplasmic and nuclear SMAD-3 in TAM as well as 4-OH-TAM treated cells. The level of cytoplasmic as well as nuclear SMAD-3 decreases in 4-OH-TAM treated cells as compared to TAM treated cells.

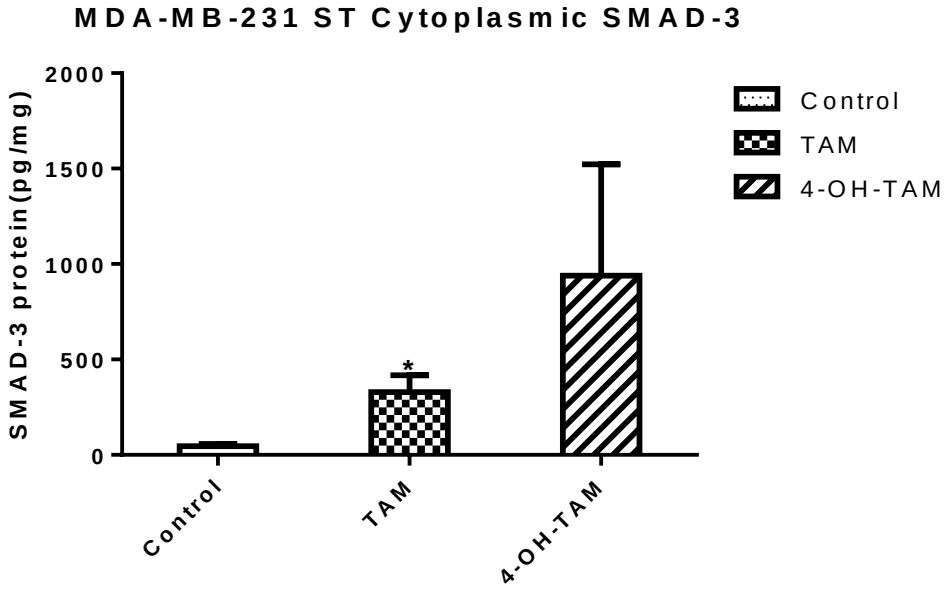

Figure 32: Phenotypic expression of cytoplasmic SMAD-3 during Short Term drug exposure for MDA-MB-231

Table 11: Levels of cytoplasmic SMAD-3 during ST drug exposure assay in MDA-MB-231

Treatment	Cytoplasmic SMAD-3 (pg/ml)	Total cytoplasmic protein (mg/ml)	Cytoplasmic SMAD-3 (pg/mg) Mean ± SEM
Control	112.5	2.440	46.11 ± 7.58
TAM	204.5	0.621	329.31 ± 62.00
4-OH-TAM	184	0.196	938.78 ± 413.27

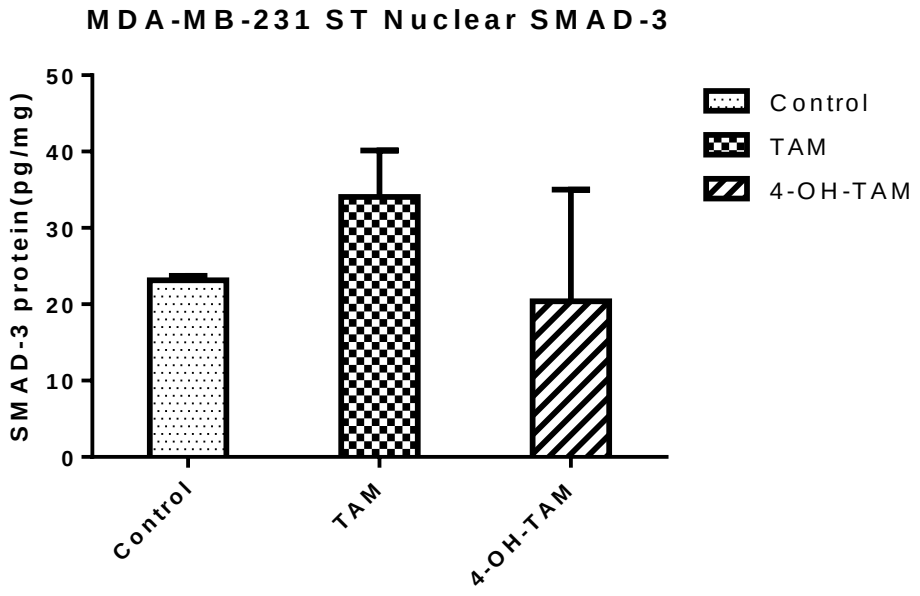

Figure 33: Phenotypic expression of nuclear SMAD-3 during Short Term drug exposure for MDA-MB-231

Table 12: Levels of nuclear SMAD-3 during Short Term drug exposure assay in MDA-MB-231

Treatment	Nuclear SMAD-3 (pg/ml)	Total nuclear protein (mg/ml)	Nuclear SMAD-3 (pg/mg) Mean ± SEM
Control	116	5.008	23.16 ± 0.40
TAM	122.5	3.597	34.06 ± 4.31
4-OH-TAM	71	3.482	20.39 ± 10.34

There is a significant elevation of cytoplasmic SMAD3 in TAM treated cells and non-significant elevation of cytoplasmic SMAD-3 in 4-OH-TAM treated cells as compared to untreated cells during short term drug exposure. In the case of nuclear SMAD-3, there is non-significant increase in TAM treated cells and non-significant decrease in 4-OH-TAM treated cells as compared to control cells.

4.5.8. Determination of relative expression of cytoplasmic SMAD4 during Short Term drug exposure assay

The relative expression of cytoplasmic as well as nuclear SMAD-4 during short term drug exposure assay was determined by measuring absorbance at 450 nm.

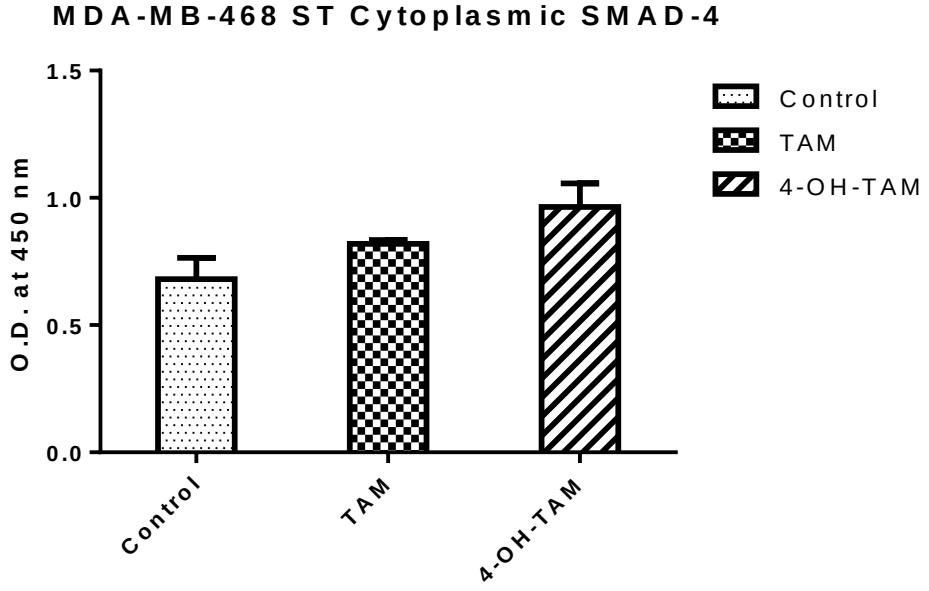

Figure 34: Relative expression of cytoplasmic SMAD-4 during Short Term drug exposure for MDA-MB-468

Table 13: Relative expression of cytoplasmic SMAD-4 during Short Term drug exposure for MDA-MB-468

Treatment	O.D. at 450nm
Control	0.68
TAM	0.82
4-OH-TAM	0.97

4.5.9. Determination of relative expression of nuclear SMAD-4 during Short Term drug exposure assay

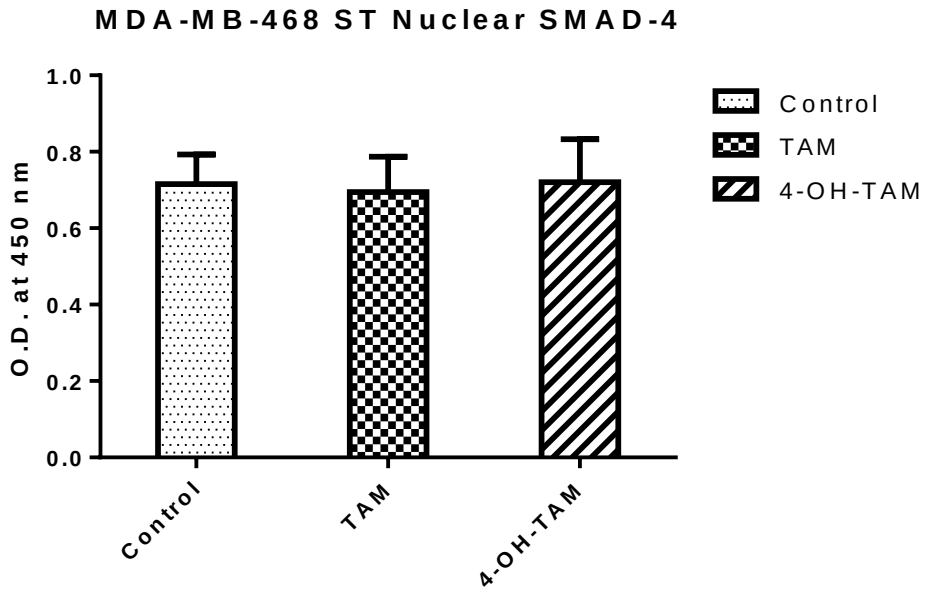

Figure 35: Relative expression of nuclear SMAD-4 during Short Term drug exposure for MDA-MB-468

Table 14: Relative expression of nuclear SMAD-4 during Short Term drug exposure for MDA-MB-468

Treatment	O.D. at 450nm
Control	0.72
TAM	0.70
4-OH-TAM	0.72

There is a slight increase in absorbance of cytoplasmic SMAD-4 in TAM as well as 4-OH-TAM treated cells but the absorbance of nuclear SMAD-4 in TAM and 4-OH-TAM treated cells is nearly the same to the untreated cells.

4.5.10. Determination of relative expression of cytoplasmic SMAD-4 during Short Term drug exposure assay

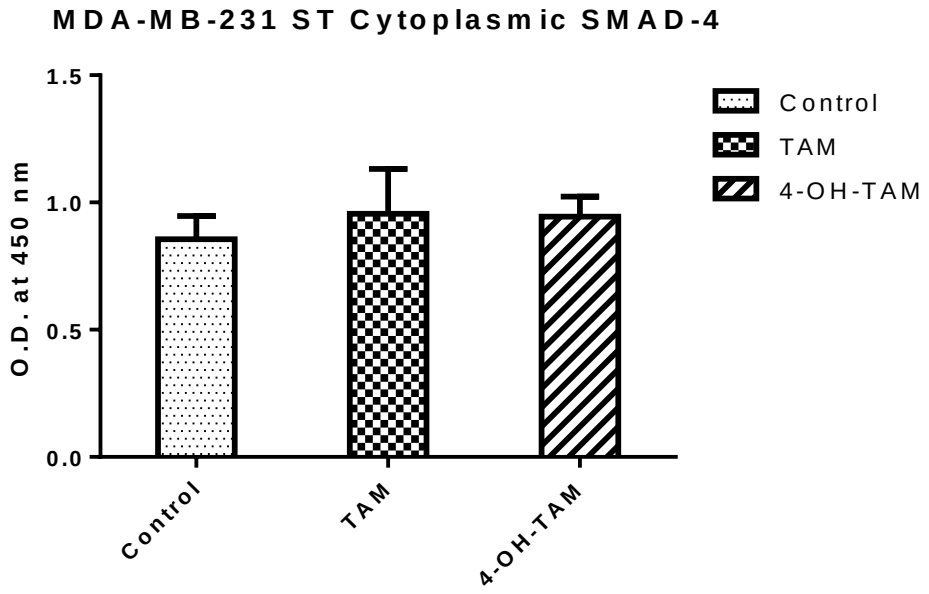

Figure 36: Relative expression of cytoplasmic SMAD-4 during Short Term drug exposure for MDA-MB-231

Table 15: Relative expression of cytoplasmic SMAD-4 during Short Term drug exposure for MDA-MB-231

Treatment	O.D. at 450nm
Control	0.86
TAM	0.96
4-OH-TAM	0.95

4.5.11. Determination of relative expression of nuclear SMAD-4 during Short Term drug exposure assay

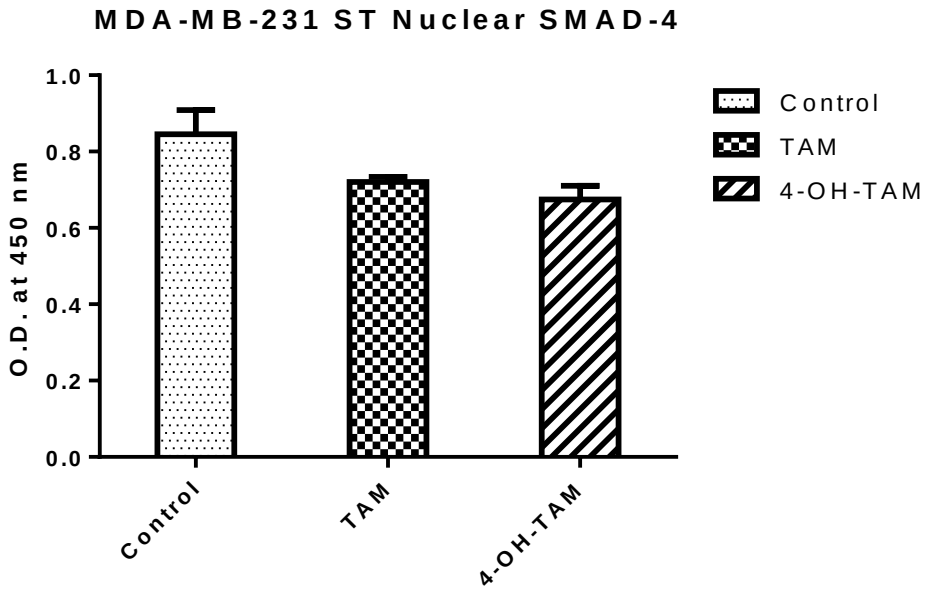

Figure 37: Relative expression of nuclear SMAD-4 during Short Term drug exposure for MDA-MB-231

Table 16: Relative expression of nuclear SMAD-4 during Short Term drug exposure for MDA-MB-231

Treatment	O.D. at 450nm
Control	0.85
TAM	0.72
4-OH-TAM	0.68

There is a little increase in absorbance of cytoplasmic SMAD-4 in TAM as well as 4-OH-TAM treated cells whereas there is non-significant decline in absorbance of nuclear SMAD-4 in TAM and 4-OH-TAM treated cells during short term drug exposure assay.

Conclusion

Antiestrogen therapy such as Tamoxifen has been a successful therapy for hormone responsive breast cancer patients. In our study, we have evaluated the cytotoxic effects of antiestrogen on TNBC cell lines (MDA-MB-468 and MDA-MB-231). Furthermore, we have determined phenotypic levels of Growth Factors (TGF-β1 and TGF-β2) and signaling mediators (SMAD-3 and SMAD-4) during antiestrogen (TAM and 4-OH-TAM) treatment.

The conclusion from our study is as following:

- ✓ The population doubling time of MDA-MB-468 and MDA-MB-231 cell line is 22 h and 38 h respectively.
- ✓ Both the antiestrogen drugs (TAM and 4-OH-TAM) have significant effects on MDA-MB-468 and MDA-MB-231 cells. The significant cytotoxic effect of TAM was noticed at 10^{-6} M and of 4-OH-TAM was noticed at 10^{-7} M for both the cell lines.
- ✓ There was significant decline in cell viability during Short Term drug exposure assay in case of both the cell lines.
- ✓ The levels of TGF-β1 were significantly increased in case of TAM and 4-OH-TAM treatment during ST drug exposure assay for MDA-MB-468 and MDA-MB-231.
- ✓ In MDA-MB-468, the levels of TGF-β2 were non-significantly reduced; on the contrary in MDA-MB-231, significant elevation of TGF-β2 was observed in TAM and 4-OH-TAM treated cells during ST drug exposure assay.
- ✓ There was non-significant diminution observed in case of cytoplasmic as well as nuclear SMAD-3 levels during ST antiestrogen treatment in MDA-MB-468. However, the levels of cytoplasmic and nuclear SMAD-3 were enhanced in TAM and 4-OH-TAM treated cells except for nuclear SMAD-3 level during 4-OH-TAM treatment for MDA-MB-231.
- ✓ Non-significant elevation in absorbance was noticed in cytoplasmic SMAD-4 for both the cell lines whereas the absorbance for nuclear SMAD-4 was non-significantly reduced in antiestrogen treated cells during Short Term drug exposure assay.

Our study indicates that the hormone non-responsive TNBC cells also represent similar cytotoxic effects towards antiestrogen which supports our hypothesis that in presence of antiestrogens, SMAD-4 interacts with ERβ and induces the apoptotic pathway in TNBC cells.

The elevation of TGF-β1 during antiestrogen treatment indicates the activation of non-classical ER pathway through Fos-Jun transcription factors. The auto induction of TGF-β1 transcription is also mediated by AP-1 binding site in deleterious effects. The enhancement of TGF-β1 in TNBC indicates the role of ERβ in activation of alternate pathway. Various studies have demonstrated that the patients in subsidence showed a significant increase in TGF-β2 levels at the initial phase of hormonal therapy (Jonat, Kopp, Schmahl and Knabbe, 1995), followed by a subsequent decrease. Our data also point out the similar pattern of TGF-β2 elevation during Short Term drug exposure in Triple Negative Breast Cancer cells.

Estrogen Receptor physically interacts with SMAD-2/3 but only when ER is in a ligand activated form. This interaction leads to recruitment of ubiquitin ligase, Smurf1 which leads to the degradation of SMAD-3 through ubiquitin-proteasome pathway. Our data indicate significantly increased cytoplasmic SMAD-3 level during single dose treatment of Tamoxifen in MDA-MB-231 cell line which corroborates the crosstalk between ER and TGF-β pathways.

Appendix

Complete Growth Medium

Dulbecco's Modifies Eagle Medium with High Glucose	500 ml
Fetal Bovine Serum	10%
L-Glutamine	2 mM
MEM-Non essential Amino Acids	0.1 mM
Antibiotic (Penicillin-Streptomycin) solution	1%

1X PBS (1000 ml)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
pH	7.4

Cytoplasmic Lysis Buffer

Triton X 100	1% w/v
Tris-Cl (pH 7.4)	50 mM
NaCl	300 mM
EDTA	5 mM
Sodium Azide	0.02%

PMSF	1 mM
Leupeptin	2 µg/ml

Nuclear Lysis Buffer

Tris Base (pH 8.0)	10 mM
KCl	0.5 M
Glycerol	20%

Blocking Buffer

Tris Base (pH 7.4)	100 mM
NaCl	150 mM
NaN_3	0.02%
BSA Fraction V	10 mg/ml

Binding Buffer I

Tris Base (pH 7.4)	100 mM
NaCl	150 mM
NaN_3	0.02%
Tween 20	0.05%
BSA Fraction V	1 mg/ml

Binding Buffer II

Tris Base (pH 7.4)	100 mM
NaCl	150 mM
NaN_3	0.02%
Tween 20	0.05%
BSA Fraction V	10 mg/ml

Washing Buffer

1X PBS	
Tween 20	0.05%

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