Influence of Antiestrogen on TGF-β Signalling in Hormone Responsive Breast Cancer

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ABBREVIATIONS

BSA: Bovine Serum Albumin

$CuSO_4$: Copper Sulphate

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

ELISA: Enzyme Linked Immunosorbant Assay

EtBr: Ethidium Bromide

FBS: Fetal Bovine Serum

HCL: Hydrochloric Acid

MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide

NaCl: Sodium Chloride

Na_2CO_3: Sodium Carbonate

NaK: Sodium Potassium Tartrate

PBS: Phosphate Buffer Saline

Peni-Strep: Penicillin-Streptomycin

PMSF: Phenyl Methane Sulfonyl Fluride

TMX: Tamoxifen

4-OH TMX: 4 -Hydroxy Tamoxifen

TGF-β: Transforming Growth Factor-β

Trypsin-EDTA: Trypsin Ethylene Diamine Tetra Acetic Acid

E_2: 17 β- Estradiol

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

SAMD-4: SMA (sma gene for small body size) + MAD (Mothers against decapentaplegic homolog-4)

ABSTRACT

Breast cancer is a heterogeneous disease and involves multiple steps. It is controlled by many hormones, growth factors and cytokines. Currently, there are three important markers in which Estrogen receptor (ER), Progesterone receptor (PR) are hormone receptors and its presence is being used as diagnostic and predictive tool for therapeutic selection. Approximately 72.2% of breast cancers are hormone receptor positive. Estrogen receptor (ER) and Progesterone receptor (PR) positive patients were given hormonal treatment antiestrogen (Tamoxifen). The current problem in hormonal therapy is its resistance or non-responsiveness. The possible reason for mechanism of resistance and/or non-responsiveness is unknown. Third predictive marker is Epidermal Growth Factor Receptor-2 (HER-2) and Herceptin, a monoclonal antibody treatment is being given as therapy for HER-2 positive patients. Moreover, there are many markers being investigated in which Transforming Growth Factor β (TGF-β) is one of the surrogate markers. It is a pluripotent cytokine and known for its dual role in breast tumorigenesis. It has been reported that TGF-Beta is increased during antiestrogen treatment and growth inhibitory effect of Antiestrogen is mediated by activation of TGF-Beta. However, the mechanism is yet to be explored. It has been hypothesized that the possible reason of activation of TGF-Beta signaling will be either through alternative signaling of ER which is responsible to activate TGF-β during Antiestrogen treatment. In our study we estimate the TGF-β1, TGF-β2, SMAD-3 and SMAD-4 in hormone receptor positive (T47D) breast cancer cells during antiestrogen treatment using ELISA.

INTRODUCTION

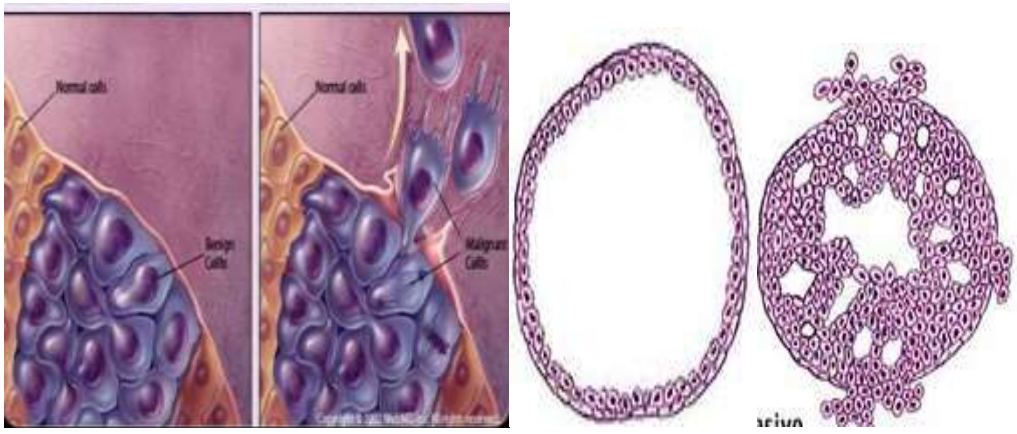
Cancer:

It is heterogeneous disease in which abnormal cell grow and proliferate in uncontrolled manner. When this mechanism disrupted then a mass of tissue form called tumor. Cancerous cells are spread to other parts of the body via the lymphatic system and bloodstream, lodging secondary deposits in a distinct region known as **metastases.** (British Medical Association (BMA), 1997; Walter, 1977; Wells, 2001) Metastasis occurs when normal cell control mechanisms become disrupted (Corner, 2001).

1.1.1 Types of tumor: Benign and Malignant.

a) **Benign:** Tumor cells stay together and surrounded by containing membrane and restricted to local area and do not spread to other parts of the body. It is a non-malignant/ non-cancerous tumor or benign tumor.

b) **Malignant:** Malignant tumor that grows indefinitely and can spreads to other parts of the body creating secondary deposits (metastasis).Cancer cells can invade and damage tissues and organs near the tumor. Cancer cells can break away from a malignant tumor and enter the lymphatic system or the bloodstream and spread to other parts of the body. It has ability to grow rapidly, uncontrollably, and independently from the tissue where it started.



Benign **Malignant** **Normal** **Invasive**

Figure 1: (1) The Genetics of Cancer, (2) Breast Cancer Advance cancer treatment (Beijing Puhua International Hospital).

1.1.2 Examples of Malignant Tumor:

A) **Carcinomas:** Carcinomas are the most common type of cancer which originates from the epithelium. Epithelium is the lining cells of an organ. Common sites of carcinomas are the skin, mouth, lung, breast, stomach, colon and uterus.

B) **Sarcomas:** It is a cancer of connective and supportive tissue of all classes. Sarcomas can be found anywhere in the body and they often form metastasis in the lungs.

1.2 BREAST CANCER:

Breast cancer is a complex and heterogeneous disease that starts in the cells of the breast and metastasize to distant parts of the body.

1.2.1 BREAST CANCER INCIDENCE:

Breast cancer is the commonest cancer in urban Indian female and second common in rural Indian female. Indian Council of Medical Research (ICMR) registry has reported assumed in 2016 total number of newcancer cases is expected to be around 14.5 lakh and the is expected to reach nearly 17.3 lakh new cases in 2020. An annual incidence of approximately 1, 44,000 new cases of breast cancers in India. Breast cancer has ranked number one cancer among Indian females with age adjusted rate as high as 25.8 per 100,000 women and mortality 12.7 per 100,000 women.(ICMR,2017)

1.2.2 Normal breast:

Female breast is made up of lobules (milk-producing glands), ducts (tubes that carry the milk from the lobules to the nipple), and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) (Breast cancer, American cancer society).

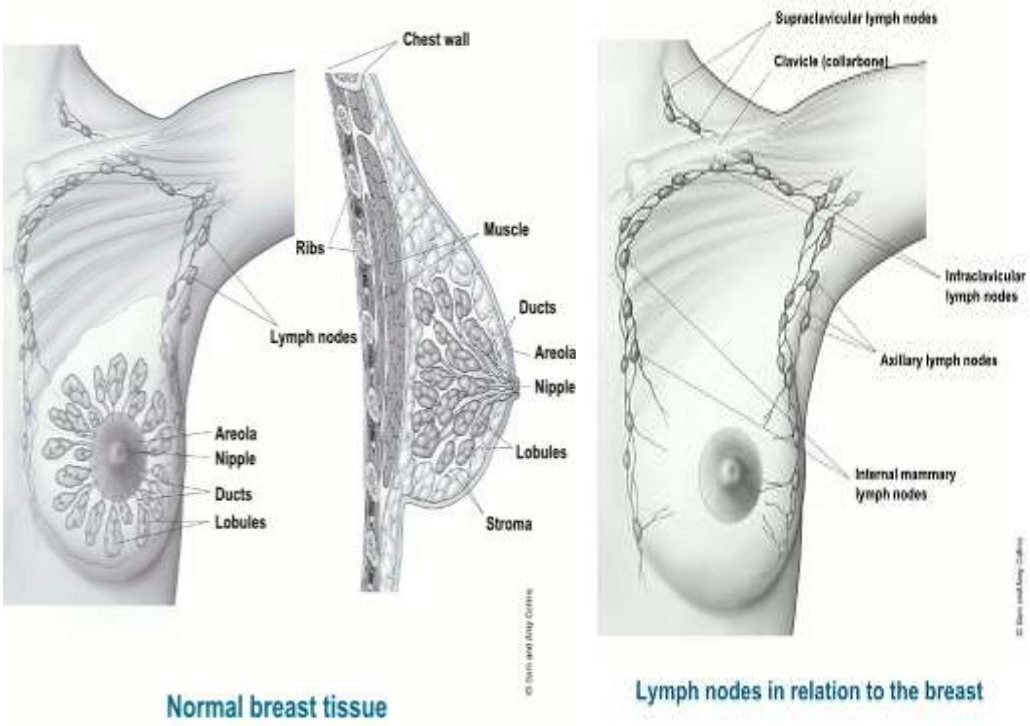


Figure 2: Breast cancer, American cancer society

1.2.3 Types of breast cancers:

There are two types of breast cancer.

(A) Ductal carcinoma in situ: Ductal carcinoma in situ is non-invasive breast cancer. In DCIS the cells have not spread through the walls of the ducts into the surrounding breast tissue. DCIS is known as a pre-cancer but in some cases it become invasive cancers.

(B) Lobular carcinoma in situ (LCIS): In lobular carcinoma in situ (LCIS) cells grow in the lobules of the milk-producing glands of the breast, but they do not grow through the wall of the lobules. This is not a pre-cancer or true cancer.

(C) Invasive Ductal Carcinoma (IDC): Invasive ductal carcinoma (IDC) starts in a milk duct of the breast, it breaks through the wall of the duct and grows into the fatty tissue of the breast. Out of 10, 8 are invasive breast cancers are infiltrating ductal carcinomas

(D) Invasive lobular carcinoma: Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules).It can also spread to other parts of the body. Out of 10 invasive breast cancers about 1 is Invasive lobular Carcinoma (ILC).

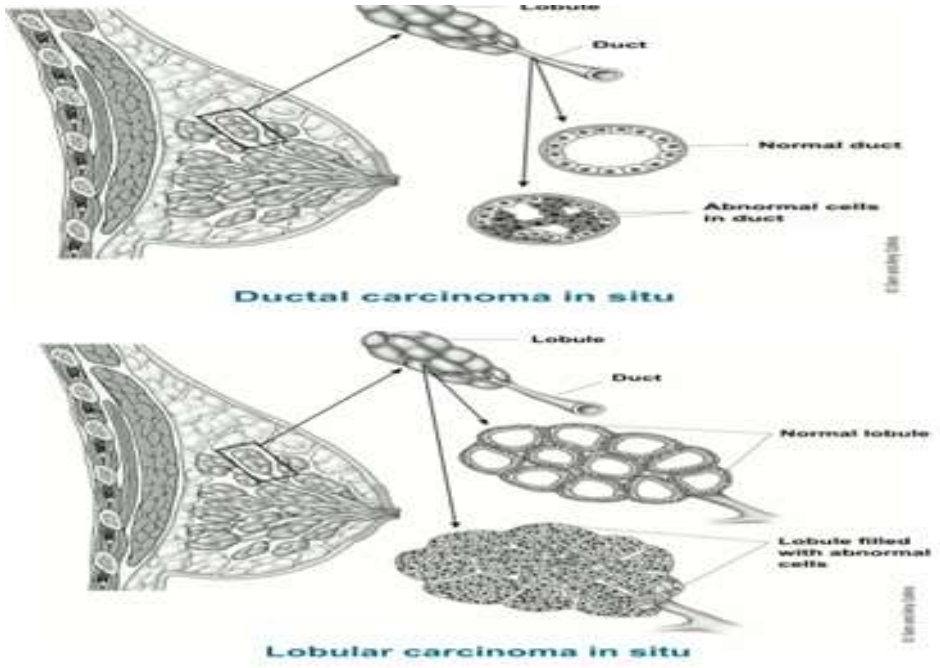


Figure 3: Breast cancer, American cancer society

2.1 Molecular classification of Breast Cancer: There are mainly five molecular subtypes of breast cancer that are based on the genes a cancer expresses: (Molecular subtypes of breast cancer, Breast cancer .ORG)

- **Luminal A:** Luminal A is hormone-receptor positive (Estrogen-receptor and/or Progesterone-receptor positive), HER2 negative which helps to control fast growing cancer cells. Luminal cancers are low-grade, slow growing and have the best prognosis.
- **Luminal B:** Luminal B is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive) and either HER2 positive or HER2 negative. Luminal B cancers grow faster than luminal A cancers and their prognosis is slightly poorer**.**
- **Triple-negative/basal-like:** Triple negative/ Basal like is hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and HER2 negative breast cancer. This type of cancer is more common in women with *BRCA1* gene mutations.

- **HER2-enriched:** HER 2 enriched breast cancer is hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and HER2 positive. HER2-enriched cancers grow faster than luminal cancers and have a poorer prognosis, but they treated with targeted therapies aimed at the HER2 protein. Herceptin (trastuzumab), Perjeta (pertuzumab), and Kadcyla (T-DM1 or ado-trastuzumab emtansine) drugs are used to treat this cancer.
- **Normal-like :** Normal breast cancer is like luminal A disease, but normal-like breast cancer has a good prognosis, its prognosis is slightly poorer than luminal A cancer's prognosis.

2.2 Molecular classification:

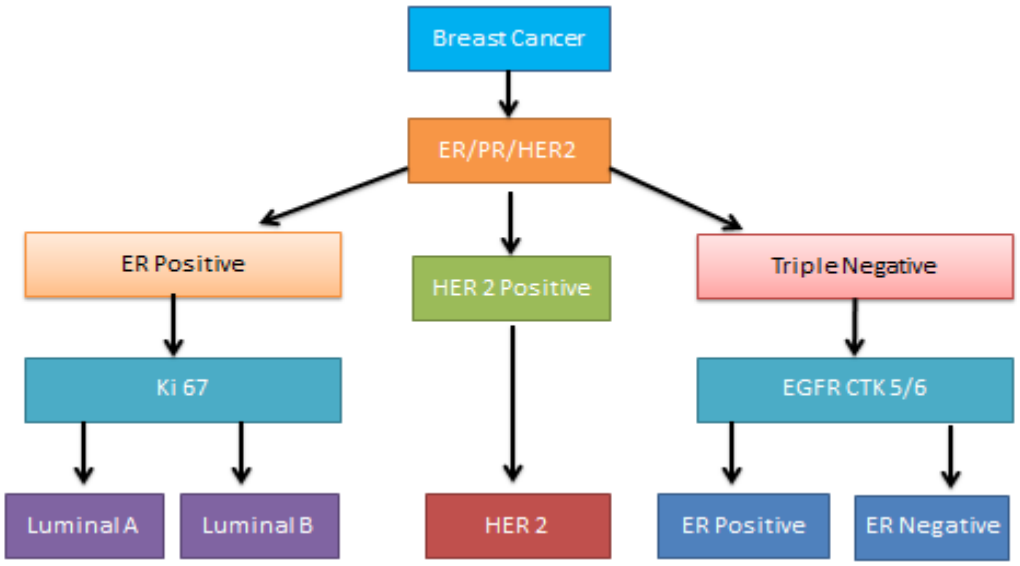


Figure 4: Tumor heterogeneity in breast cancer, concepts and tools

2.3 Treatment of breast cancer:

Four types of standard treatment are used:

- **Surgery:** Surgery is the removal of the part of breast at the cancer site.

a) **Lumpectomy**: Removal of a tumor and a small amount of normal tissue around it.

b) **Partial mastectomy**: Removal of part of the breast that has cancer and normal tissue around and also called a segmental mastectomy.

c) **Total mastectomy**: Removal of the whole breast that has cancer called a simple mastectomy.

- **Chemotherapy:** Chemotherapy involves drug or medicine which has effect on whole body where it kills fast growing cells. Chemotherapy is given using needle, tube, tablets.
- **Radiation therapy:** In radiotherapy x-rays are used to kill cancer cells which are remain after surgery. It is given to the area which needs to be treat. Radiotherapy is painless but has some side effect.
- **Hormonal therapy:** Hormonal or Endocrine therapy includes drug which is given to the patients to stop the growth of cancer cells. Estrogen is the hormone which increases during cancer development and to lower the amount of estrogen in the body or to stop entry of estrogen into cancer cells. Tamoxifen which is a Antiestrogen stops the entry of estrogen hormone into the breast cancer cells and stop their growth. It can be used to treat at any age of life in women

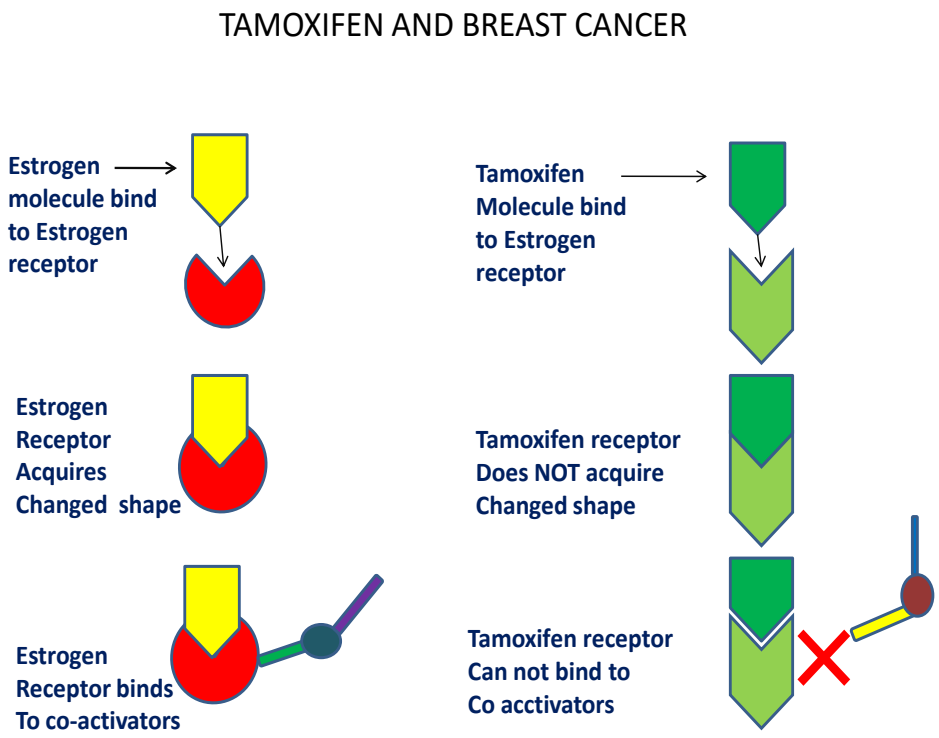


Figure 5: Estrogen receptor drug discovery : National cancer institute

2.4 Molecular marker:

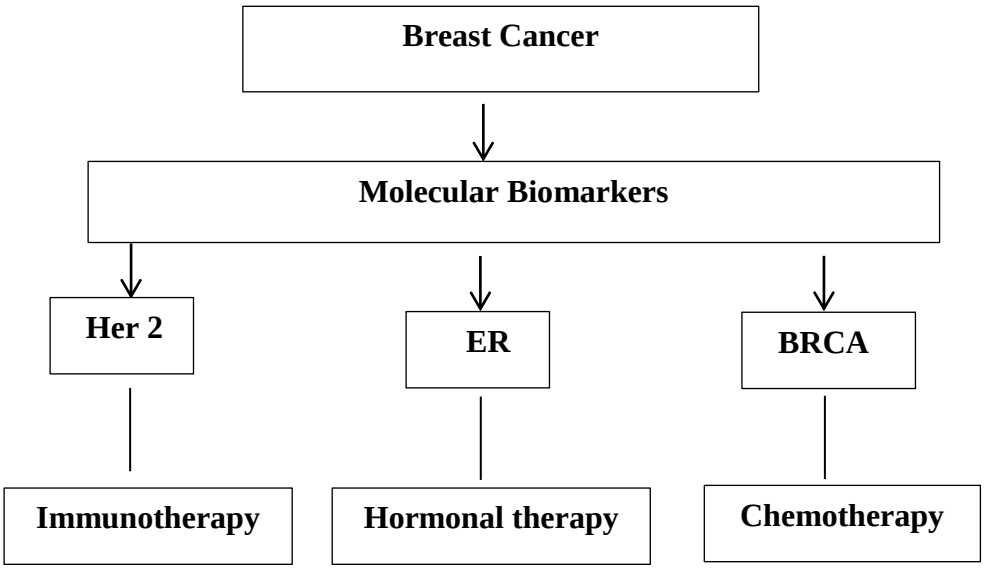


Figure 6: Schematic representation of current molecular biomarkers used in clinical management of breast cancer (Braunwald et al., 2001).

2.4.1 HER2/neu: HER2 gene amplification or protein over-expression is associated with poor prognosis and good clinical outcome receiving systemic chemotherapy treatment.HER2 gene is also known as ERBB2 (Erb-B2 receptor tyrosine kinase 2) gene and are receptor on breast cells. It helps in control of cell growth, divides, and repairs itself. During breast cancer development the HER2 receptor overexpress which is responsible for uncontrolled growth and division of breast cells**.**

2.4.2 Estrogen receptor (ER): ER is the most important and prevalent biomarker for breast cancer classification. It is a modulator of growth and differentiation of cells (Warner and at al 1999). Estrogen function by using two intracellular receptors, ERα and ERβ (Kuiper and at al 1996) which are hormone -dependent transcriptional regulators.ER is also responsible for induction and growth of breast cancer. ER stimulates the secretion of growth factors from the stroma and these induces the epithelial cells to proliferate (Wiensen at al 1999).In breast cancer cells loss of ERα is accompanied by an increase in growth factors and growth factor receptors (Clarke at al 1996 and Nicholson at al 1999).

2.4.3 Progesterone receptor (PR): Progesterone receptors (PRs) are ligand-activated transcription factor members of the steroid hormone (SR) subfamily of nuclear receptor. There are two isoform (A and B) which are formed by alternate transcriptional start site. PR- B (full length receptor) and PR-A (N terminally truncated version; lack of first 164 amino acids which are present in PR- B).Both PR-A and PR-B are acts as a homo and heterodimers and can bind to DNA at progesterone response element (Tsai SY at al1988). Functions of PR are highly influenced by input from peptide growth factor- initiated signal transduction pathway (Lange CA 2008). PRs function as regulators of transcription and signal transduction pathway**.**

2.5 Estrogen Receptor Pathway and Antiestrogen Treatment:

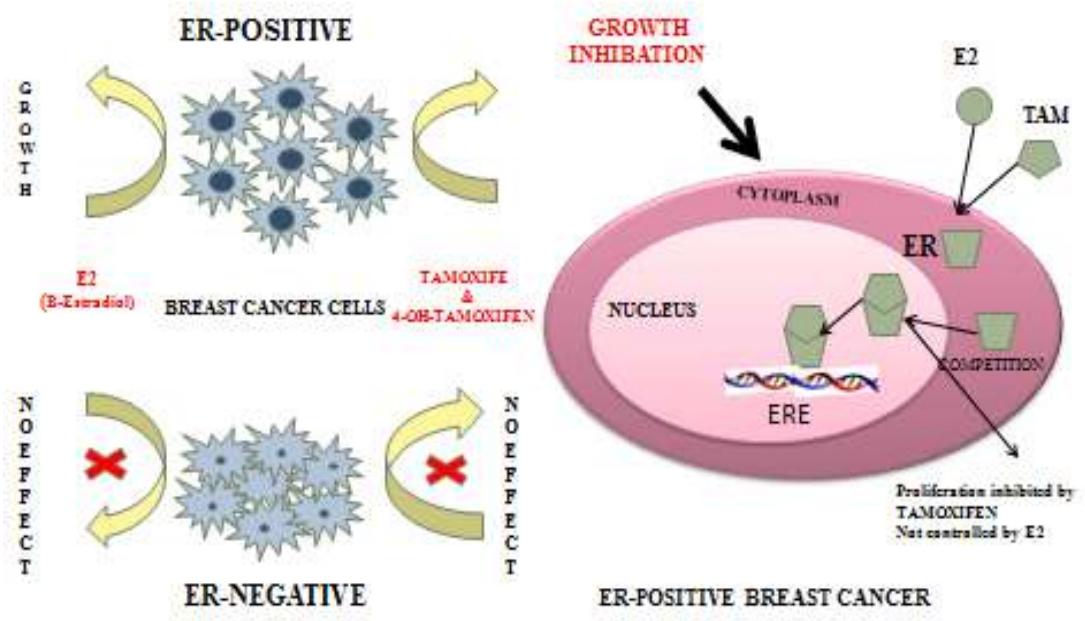
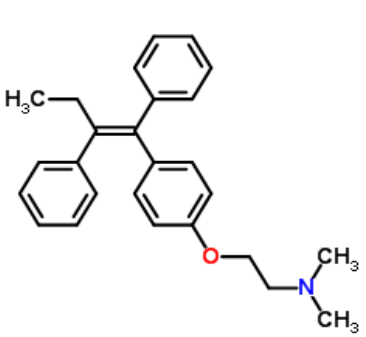


Figure 7: Stope , Matthias B., et al. "Estrogen receptor α attenuates transforming growth factor-β signaling in breast cancer cells independent from agonistic and antagonistic ligands."

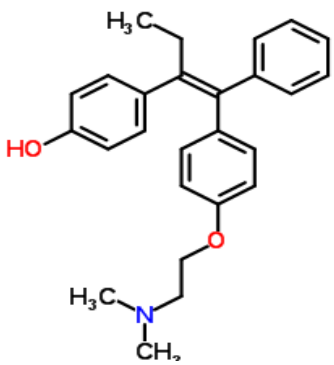
In Estrogen receptor pathway, estrogen binds to the structurally and functionally distinct ERs. There are two isoforms of ERs: ERα and ERβ (C. Thomas and J.-A. Gustafsson at al 2011). ERα is the major subtype in the mammary epithelium and plays a critical role in mammary gland biology and in breast cancer progression (M.warner at al 1999 and S.C. Hawitt at al 2000). ERα involves an N-terminal AF1 domain, a DNA-binding domain, and a C-terminal ligand-binding region that contains

an AF2 domain. When estrogen bind to ERα then ligand-activated ERα translocate to the nucleus where it binds to the responsive element in the target gene promoter and stimulates gene transcription (N.J. mckenna at al 1999 and D. P. McDonnell at al 2002).Multiprotein complexes containing co-regulators assemble in response to hormone binding and activate ER-mediated transcription (N. J. McKenna at al 1999). ERα transcriptional outcome is regulated by dynamic chromatin modifications of the histone tails and the ligand-bound ERα facilitates these modifications via co-regulator recruitment (T. N. Collingwood at al 1999). It is generally accepted that some of the diverse functions of E2 depend on differential recruitment of co-regulators to the E2-ER complex (J. M. Hall at al 2005). ER-coregulatory proteins have potential to be differential expressed in malignant tumors and that their functions may be altered, leading to tumor progression (B. W. O'Malley at al 2006). Collectively, these emerging findings implicate the role of the ERα-coregulator-associated activities/functions in breast cancer metastasis.

During hormonal therapy the Antiestrogen (Tamoxifen and 4-OH-TAM) blocks estrogen receptors in breast cancer cells. It is also known as selective estrogen receptor modulator (SERM).In hormone receptor positive breast cancer tamoxifen lower the chances of relapse**.**



Structure of Tamoxifen



Structure of4-oh Tamoxifen

Figure 8: ChemSpider (Search and Share chemistry)

2.6 Current problems in breast cancer management: Tamoxifen is a drug which is currently used for prevention to breast cancer. Tamoxifen is competitive inhibitors of estrogen action at the tumor ER. However long term

adjuvant tamoxifen therapy must be continuous because failure of tamoxifen to block the tumor ER will result in recurrence. Evolution of acquired resistance to tamoxifen treatment and tumor use tamoxifen treatment or estrogen to maintain tumor growth. There are three possible mechanisms for drug resistance to tamoxifen.

2.6.1 Metabolic Resistance: Metabolic activation of tamoxifen occurs via demethylation of N-demethyltamoxifen and subsequently transformation to the hydroxy metabolite endoxifen (Stearns Vat al.2003 and Jin Y at al 2005). There are wide variations in the CYP2D6 enzyme in population that can influence drug metabolism. If tamoxifen is a prodrug and needs to be converted to endoxifen to achieve maximal antitumor activity at the tumor ER, then these same patients may have severe hot flushes. The selective serotonin reuptake inhibitors (SSRI) have found to be of value to treat hot flushes. The wide spread use of temoxifen as a long- term adjuvant therapy, especially in pre-menoposal patients has naturally increased SSRI use. SSRI such as flupxetine and paroxetine are potent inhibitors of the CYP_2D6 enzyme (Borges S 2006).

2.6.2 Intrinsic Resistance: A proportion of ER positive tumors are intrinsically resistance to tamoxifen therapy. Matastatic breast cancer that is ER and PR positive is approximately 80% responsive to antihormonal therapy (Bloom ND at al 1980). Tumor cell drug resistance to tamoxifen develops very quickly in athymic mice with HER 2/ neu engineered MCF 7 cells compared with the natural process of more than 6 months (Gottardis MM at al 1988). Tamoxifen acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER 2/ neu-, ER positive, PR negative tumors that have an increases in co activator SRC3 (AIB1) levels (Shou J at al 2004).Intrinsic tamoxifen resistance is associated with HER 2/neu-, ER positive tumors that have an increase in coactivator SRC3 (AIB 1) levels (Osborne CK at al 2003).

2.6.3 Acquired Resistance: Treatment of athymic mice implanted with ER positive, PR positive MCF 7 tumor with continuous tamoxifen will eventually develop tamoxifen stimulated tumors that will grow in response to either tamoxifen or estradiol (Gottardis MM at al 1988). Tumor growth is prevented in the absence of a stimulatory signal transduction pathway. Patients with ER positive tumors and treated for 5 years with tamoxifen continue to be responsive to subsequent treatment with 5

years of aromatase inhibitors letrozole (Goss PE at al 2007) slow development of acquired resistance by the breast cancer micro metastases during 5 years of tamoxifen so that these patients respond to a non-cross resistant therapy that prevents tumor growth by blocking the ability of the patient to synthesize estrogen.

2.7 Transforming growth factor-β: TGF--β family of cytokines are ubiquitous, multifunctional and essential to survival. It plays important roles in growth and development, inflammation and repair and host immunity. Mammalian TGF-β isoforms (TGF-β1, β2 and β3) are secreted as latent precursors and have multiple cells surface receptors of which at least two mediate. TGF-β superfamily has five TGF-β isoforms. In mammals three TGF-β isoforms are present. Heterodimers may also form. Some bioactive TGF-β's have atypical molecular weights. These atypical molecules dimer functionally from the TGF-β1 isoforms and may therefore offer valuable insight into the structure-function relationships of the TGF-β isoforms**.(** Moore-Smith, Lakisha, and Boris Pasche. "TGFBR1 signaling and breast cancer." *Journal of mammary gland biology and neoplasia*)

2.7.1 TGF-β signalling pathway:

Transforming Growth Factor-β (TGF-β) is one of the most commonly altered cellular signaling pathways in human cancer (Akhurst RJ at al 2004). There are three types of cell surface receptors involved in TGF-β signaling: TGF-β receptor 1, 2 and 3, all of which are dual specificity kinases (Shi Y at al 2003). TGF-β1 (TGFB1), 2 (TGFB2) and 3 (TGFB3) ligands all bind to the same receptors but with varying affinities. TGFBR2 is a homodimeric receptor that binds to TGF-β ligand and then forms a heterotetramer with TGFBR1. Auto phosphorylation of TGFBR2 causes phosphorylation of TGFBR1 to initiate intracellular signaling. The canonical TGF-β signaling pathway is mediated through SMAD proteins. The receptor-regulated SMAD (RSMADS) are activated through phosphorylation and direct interaction with TGFBR1. The RSMADs consist of SMAD1, 2, 3, 5 and 8, with SMAD2 and 3 being directly involved in the TGF-β signaling pathway. Co-mediator SMADs (Co-SMADs) bind to the rSMAD complex and mediate entry into the nucleus to act as transcription factors. This class of SMADs includes SMAD4 in the TGF-β family signaling cascades. The third group of SMAD proteins inhibits this pathway and these proteins are known as inhibitory-SMADs (i-SMADs). SMAD6 and SMAD7 are members of this class and act to regulate TGF-β signaling and expression levels.

Further investigation into TGF- β signaling through these non- SMAD pathways may help to develop a better understanding of the role of TGF-β in pathological conditions such as cancer and fibrosis. (Moore-Smith, Lakisha, and Boris Pasche. "TGFBR1 signaling and breast cancer." Journal of mammary gland biology and neoplasia)

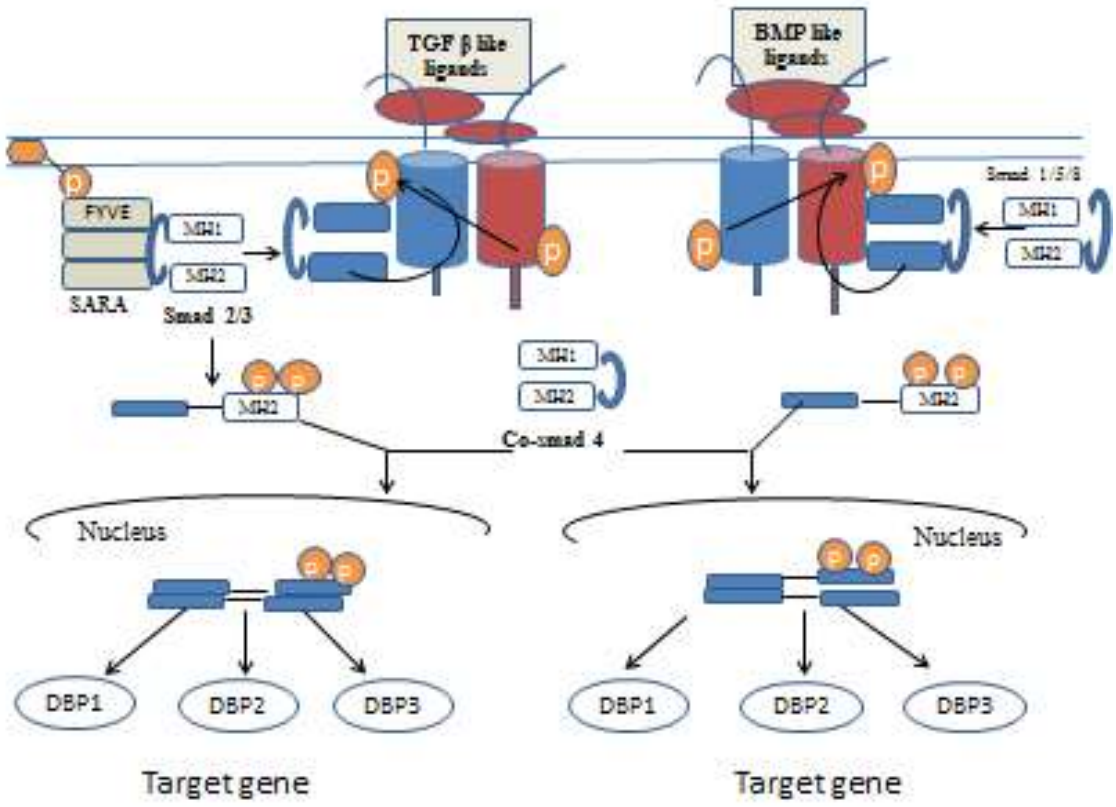


Figure 9: Signal Transduction by the TGF-β Superfamily

2.7.3 Dual role of TGF-β in breast carcinogenesis: Transforming growth factor-β (TGF- β) has dual role in breast cancer. Initially it acts as tumor suppressor and later it acts as a tumor inducer. During early tumor outgrowth, elevated TGFβ is tumor suppressive, whereas at later stages there is a switch towards malignant conversion and progression (Cui W at al 1996, Derynck R at al 2001]. Consistent with the response to TGFβ evolving from growth inhibition to tumor progression during advanced malignancy, the majority of breast tumors, including their metastases, are positive for nuclear phosphorylated Smad2, indicating an actively signaling TGFβ pathway. (Xie W at al 2002 and Kang Y at al 2005).Loss of TGFβ growth inhibition and increased expression of TGFβ have been associated with malignant conversion and progression in breast. (Derynck R at al 2001 and Schmierer B at al 2007) TGFβ growth response is abrogated by changes in the profile of other active signaling

networks or the relative availability of transcriptional co-repressors or co-activators that bind to and modulate the canonical Smad pathway. Estrogens also appear to negatively regulate TGFβ signaling in breast cancer (Kleuser B at al 2008) and there is evidence that many pathway components may be epigenetically regulated during critical transitions in malignant progression (Hinshelwood RA at al 2007).

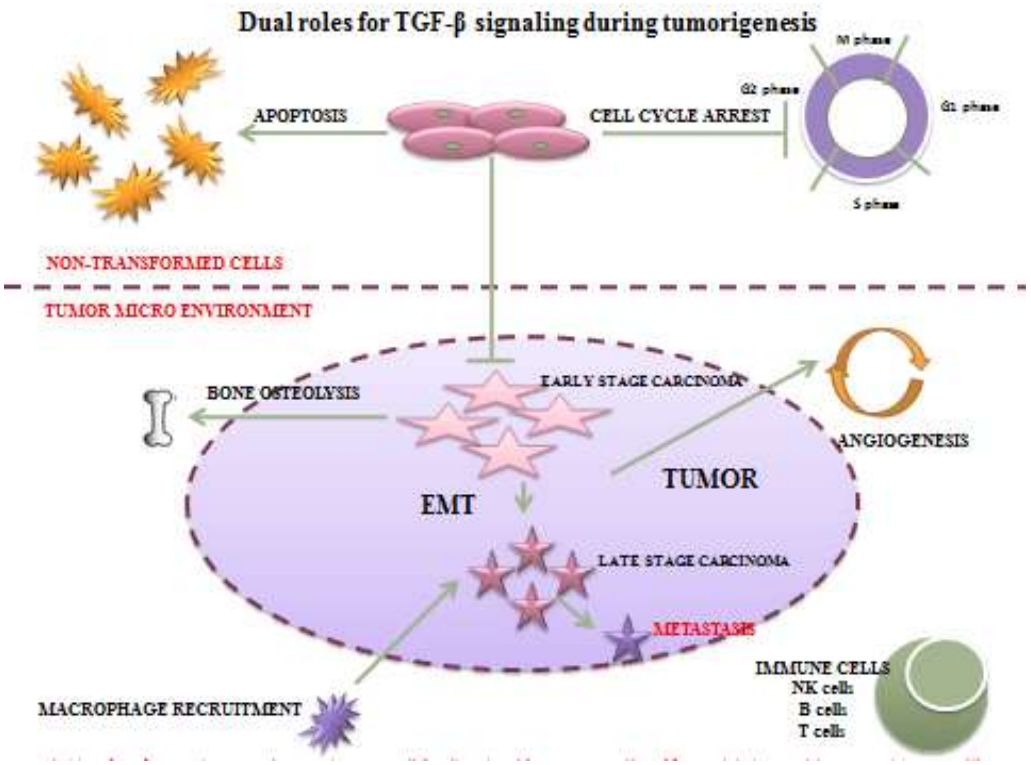


Figure 10: Connolly, Erin C., Julia Freimuth, and Rosemary J. Akhurst. "Complexities of TGF-beta targeted cancer therapy." *Int J Biol Sci* 8.7 (2012): 964-978.

2.8 Crosstalk between Estrogen- and TGFβ Signaling Pathways in Breast Cancer Cells: ERα- expressing luminal cells not often express proliferation markers is fascinating and revealing that ERα mainly functions to conserve and support the differentiated state. The relationship between proliferation, ERα and TGF-β signaling, co-localization of phosphorylated Smads and nuclear ERα which indicated co-regulation of the pathways and suggested that TGF-β could act to restrict ERα mediated proliferation (Ewan KB at al 2005). The proliferation rate of mammary gland cells was significantly increased in a mouse model of heterozygous TGF-β1 expression, and proliferation marker Ki67 expressing cells frequently co-expressed ERα.Estrogen receptor activation has been reported to inhibit transcriptional activity of TGF-β reporter assays by up to 60% (Matsuda T at al 2001,

Ito I at al 2007). It inhibits TGF-β—induced cell migration (Malek D at al 2006). TGF-β treatment increased more than two fold the expression of 956 genes whereas estrogen treatment reduced the expression of 683 genes of these (Ito I at al 2007), indicating that ERα is a major modifier of TGF-β signaling cascade. Activin and ER signaling mutually suppress each other (Burdette JE at al 2007). Overexpression of Snail transcription factor, which drives EMT, in ERα-expressing MCF-7 cells led to down regulation of ERα and increased expression of TGF-β signaling components (Dhasarathy A at al 2007). It has been reported that inhibition of breast cancer cell growth by antiestrogens TAM is mediated by TGF-β (Buck MB at al 2004). Treatment of MCF-7 cells with antiestrogen leads to increased secretion of active forms of TGF-β1 and TGF-β2 as well as increased expression of TGF-β type II receptor (Chen H at al 1996 and Buck MB at al 2006). These antiestrogen activities are mediated through p38 MAPK (Buck MB at al 2006). The expression levels of the type II receptor in ER-negative tumors correlate with more aggressive tumors (Buck MB at al 2006). TGF-β is also a potent immune surveillance factor. An important feature of TGF-β is to regulate immune responses. Interestingly, the TGF-β response signature correlated with ER-negative tumors and poor prognosis. Complete absence of TGF-β signaling correlated with ER-positive and lymph node-positive tumors and poor prognosis increased expression of chemokines driving there cruitment of the myeloid suppressor cells (Bierie B at al 2009). Antiestrogens may induce immunosuppression in the tumor microenvironment in a manner that depends on TGF-β signaling (Joffroy CM at al 2010).

Hypothesis

In hormone receptor positive breast cancer the antiestrogens is given which function through Classical ER pathway. However the resistance or non-responsiveness of antiestrogen is still unexplored. The antiestrogen resistance or non-responsiveness might be possible due to an alternative non-classical ER pathway. Estrogen receptor is a prognostic marker for primary invasive breast cancer and indicator for individual hormonal therapy (Harris L at al 2007).Antiestrogen block the estrogen-stimulated tumor growth and show effective treatment and prevention of ER-positive breast cancer(Harris L at al 2007). The multifunctional TGF-β acts as growth inhibitor and tumor promoter (Roberts AB at al 2003).There is a correlation between TGF-β receptor II and reduction in overall survival in estrogen receptor negative breast cancer patients(Buck MB at al 2004).This correlation indicate the cross talk between ERα and TGF- β pathway in breast cancer cells. The growth inhibition effect of ERα might be dependent on smad and AP1 transcription family. There is significant effect on upregulation of c-fos protein by E2 is due to transient ERα expression (Van der Burg B, 1989). Thus, determination of TGF- β1, TGF- β2, Smad 3 and Smad 4 level during antiestrogen treatment can justify cross talk between ER pathway and TGF-β pathway. In our current study we are targeting TGF-β as a surrogate biomarker for ER^+/PR^+ tumor during antiestrogen treatment by exploring the possible cross talk between ER pathway and TGF-β.

Objectives

The main objective of our study is to determine the cytotoxicity effect of the Antiestrogen on estrogen and progesterone (ER+ve, PR+ve) receptor positive breast cancer cell and to estimate the levels of TGF β molecules in it during antiestrogen treatment to elucidate the mechanism of crosstalk between the TGF β pathway and ER signalling pathway. In order to estimate the crosstalk, the entire signalling mediators will be determined during the current study (TGF-β1, TGF-β2, SMAD-3 and SMAD-4)

MATERIALS AND METHODS

3.1 Establishment and Maintenance of Human Breast Ductal carcinoma Cell Line (T-47D)

3.2 Estimation of Cytotoxic effect of Antiestrogen (TMX and 4-OH TMX) by MTT Assay

3.3 Determination of TGF-Beta 1 level by Enzyme Linked Immunosorbant Assay

3.4 Statistical Analysis

3.1 Establishment and Maintenance of Human Breast cancer Cell line (T47D):

In our study, human breast Ductal carcinoma cell line T-47D hormone positive (ER^+, PR^+) was used for the determination of TGF-Beta 1 molecule. The cell line T47D (P#6) was received from Central university of Gujarat, Gandhinagar as a gift, and it was originally obtained from NCCS, Pune India. The cells were cultured as a monolayer in Complete Growth Medium consisting Dulbecco's Modified Eagle Medium High Glucose (DMEM, Catalog#AL111) supplemented with 10% Heat Activated Fetal bovine serum (Catalog#RM112), 1% Antibiotic solution (penicillin-streptomycin, HIMEDIA Catalog#A007),0.1mM MEM Non-essential amino acid(Catalog#ACL006) . Cell line was maintained in culture flask in 5% CO_2 incubator at 37°C in a humidified environment in cell culture laboratory.

Primary steps of cell culture maintenance:

3.1.1 Thawing and Propagating **of** cells

3.1.2 Sub-culturing cells

3.1.3 Harvesting of cells for cryopreservation

3.1.4. Cryopreservation

3.1.1 Thawing and Propagating of cells

A. Thawing

Materials

Cryo vial containing T-47D cell line (TARSONS Catalog#523021)

Complete growth medium (DMEM + 10% (v/v) FBS+1%P/S)

15 ml Centrifuge Tube Conical Bottom (ABDOS #P10402)

Sterile Serological Pipettes (SPL Life sciences #91010)

70% Ethanol

Tissue paper roll

Equipment

Water bath

BSL Laminar flow chamber (ESCO Class-ll BSC)

Co_2 Incubator (Air-Jacketed DHD Automatic CO2 Incubator NU-5510/E/G)

Cooling Centrifuge (Thermo Scientific SORVALL ST 16R Centrifuge)

Method

Complete growth medium incubated in a water bath at 37°C followed by labelling of flask. Then retrived the frozen vial of T47D cells from -80°C storage facility. Heat shock was given to the T47-D cells containing vials in a water bath at 37°C. Placed the vial to a BSL Laminar flow chamber (ESCO Class-ll BSC).

B. Propagating cells

Method

We added 9 mL of complete growth medium to a 15 mL conical centrifuge tube to propagate the cells then we added 1 ml cell suspension from T47D cells from cryo vial. Resuspended the cells by pipetting up and down.Centrifuged at 1100 RPM for 7 minutes. Gently decant the medium and added 4 mL of complete growth medium. Transferred cell suspension in the flask and placed in Co_2 incubator at 37°C. Then observed the flask to checked confluency, cell condition, and morphology.changed media after alternative days and when needed.

3.1.2 Sub-culturing cells:

Reagents and Materials

0.25% (w/v) Trypsin EDTA (HIMEDIA, 1689649)

1x Phosphate buffer saline (PBS)

Complete growth medium (DMEM +10% (v/v) FBS+1%P/S)

Method

Removed Complete growth medium from the flask and rinsed the cells with 1XPhosphate buffer saline (PBS).Then 1 mL of 0.25% (w/v) Trypsin EDTA (HIMEDIA, 1689649) solution was added to flask and Incubated the flask in incubator at 37°C for 5 minutes and Observe the cell detachment using inverted microscope. Adding complete growth medium neutralize the Trypsin-EDTA cell suspension. Gently pipette up and down the cells carefully until it become single cell suspension. Suspension was centrifuged at 1100RPM and 4°C for 7 minutes. Discarded supernatant and pellet was used for other procedures like cell count, cell viability, MTT, Growth curve analysis.

Cell count and viability:

Sterile eppendorf tubes (1.5)

Trypan blue stain (Himedia TCL005)

Hemocytometer

Using microfuge tube prepare mixture of PBS, Trypan Blue, cell suspension was prepared. From this 10µl of mixture was taken and observed under microscope using Haemocytometer. Then count the number of viable and dead cells. Remaining cell suspension then transferred to new culture flask containing complete growth medium and incubated in Co_2 incubator.

3.1.3 Cryopreservation: (Freezing)

Materials

DMSO (Dimethyl Sulfoxide) (Catalog # D8418, Sigma)

Cryo vials

Cryomedium (10%DMSO+90% Fetal Calf Serum)

Method

Approximately 80-90% of confluent flask of T47D cell trypsinized and centrifuged then supernatant was discarded. Remaining cell Pellet transferred into cryovials containing 1 mL cryomedium. The cryovial was freezed at -80°C or liquid nitrogen storage facility.

3.1.4 Determination of Population Doubling Time by Growth Curve analysis:

When the cells reached to approximately 90% confluency, trypsinize it then count cells. Growth Curve experiment was performed to determine the population doubling

time for 5 days with a seeding density of 1×10^4 cells/flask and incubated in CO_2 incubator. Every day, trypsinized the flask and cell count was done. Change the media of culture flask on 3rd day. Cell counting analysis was done in Microsoft excel and the plot was generated using graph pad prism to derive Population Doubling Time.

3.2 Estimation of Cytotoxic effect of Antiestrogen by MTT Assay:

3.2.1 Chemical preparation of Antiestrogen Drug

In our experimental study we selected two antiestrogen drugs (1) TMX and (2) 4-OH TMX (metabolite of Tamoxifen). Stock solution for both the drugs were prepared using absolute alcohol at 10^{-2}M concentration for TAM and 10^{-3}M concentration for 4-OH TAM. Working solutions prepared by using complete growth medium (10-6M to 10-10M). Solutions were stored in -20°C.

Cell proliferation (MTT) assay:

Materials:

Round bottom 96 well plates

TMX (Sigma#5648)

4-OH TMX (Sigma#H6278)

Absolute Ethanol

MTT Solution (Himedia # RM 1131 1G)

DMSO solution

Method: MTT Assay was performed for three days. On

Day1, when the cells reached to approximately 90% confluency, trypsinize it then count cells.100µl cell suspension containing cell density of 1×10^4 cells/well was added in each well of a 96 well plate. The plate was incubated overnight in CO2 incubator.

On Day2, Prepared different dilutions (from 10^{-6} to 10^{-10}) of both TMX and 4-OH TMX in complete growth medium and added to the respective wells and incubated overnight in CO_2 incubator.

On 3rdday, MTT Solution (1mg/ml) was prepared using sterile PBS and using 0.2µm syringe filter, filter it. 50µl of MTT solution was added in each well and plate was incubated for 3 hours at 37°C in CO2 incubator. After 3 hour removed media from each well and 150 µl DMSO solution was added in plate per well. After incubation for 20 minutes, absorbance was measured at 570nm using ELISA reader. The graph was plotted for Absorbance verses Concentration of drug. The Cell Survival Rate and cytotoxicity of drug concentration were established using Graph Pad Prism6 software.

$$\text{Cell Survival Rate} = \frac{(A_{sample} - A_{Blank})}{(A_{control} - A_{Blank})} x100$$

$$\text{Cytotoxicity (\%)} = \frac{(A_{control} - A_{Blank}) - (A_{sample} - A_{Blank})}{(A_{control} - A_{Blank})} x100$$

3.3 Growth inhibition of T47-D cells during Antiestrogen treatment

In this experiment, 6-well plates were seeded with a seeding density of 1×10^4 cells/ml in duplicate with complete growth medium. Second day treat cells with optimum cytotoxic dose of antiestrogen [TMX : 10^{-10} and 4OH-TMX: 10^{-10}]. Every day, each group of cells were trypsinized and cell count was done. The results were plotted as Mean ± SEM in graph. To determine the antiestrogen drug effects, two different sets of experimental design was implemented. Cytotoxic effect of optimum doses of TAM and 4-OH TAM on hormone receptor positive breast cancer cells.

3.3.1 Short term drug exposure assay: To determine single dose effect of antiestrogen drugs on T47D cells.

(A) Short Term Inhibition of Cell Growth:

T47-D cells were seeded in 6 well plate with a cell density (1×10^4 cells/ml) in complete growth medium and the cells were allowed to grow in CO_2 incubator. After

24 hours of incubation, antiestrogens (TAM 10^{-10} and 4-0H-TAM 10^{-10}) were added. 6 well plate was put inside the CO_2 Incubator for 5 days and then the cells were trypsinized and the cell count was done.

3.3 Determination of Phenotypic expression of growth factors and Signaling mediator by Enzyme Linked Immunosorbent Assay (ELISA) :

3.3.1 Extraction of cytoplasmic and nuclear proteins :

Lysis buffer

1X Cold PBS

Nuclear lysis buffer

Method:

After Trypsinization, the cell suspension was centrifuged at 1100 rpm at 4°C for 7 min. Then 1 ml of ice cold lysis buffer was added to cell pellet and dissolved it. Incubate it on ice for 30-60 minutes (vortex 2-3 times in between).Centrifuged the cell lysate for 15 minutes at 12000 rpm at 4°C.Supernatant was used for cytoplasmic Protein Estimation. Supernatant contain cytoplasmic protein, which is removed carefully, pellet was taken and resuspended in 62 μL nuclear lysis buffer and incubated on ice for 5 minutes. Cell lysate was centrifuged at 17000 rpm at 4°C for 10 minutes. Supernatant was used for nuclear protein estimation.

3.3.2 Estimation of Total protein by Folin Lowry method Materials:

Materials:

Test tubes

Test tube stands

Pipette

Glass road

Spectrophotometer

Protein sample

Bovine serum albumin (BSA)

Pipette tips

Reagents:

Reagent A: 2% $Na_2 Co_2$ in 0.1 N NaOH

Reagent B: 1% Na-K Tartarate in D/W

Reagent C: 0.5% CuSo4

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

Reagent II: 1:1 (Folin-Phenol [2 N]: Distilled water)

BSA Standards: Five different standards (Ranging from 0.2 to 1.0 mg/ml)

Method

- First, BSA working solution (Ranging from 0.2 to 1.0mg/ml), Blank and Sample were prepared in distilled water. Then 4.5 mL of (**Reagent I**: 48 ml of A, 1 ml of B, 1 ml of C) was added and incubated for 10 minutes at room temperature. Then 0.5 mL of **Reagent II** 1:1 (Folin-Phenol [2 N]: water) added and incubated in dark for 30 minutes. Absorbance measured at 570 nm and the graph was plotted against standard BSA protein. The protein concentration of T47-D cell sample was determined. The final protein concentration was calculated as mg/ml.

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

3.3.3 Estimation of phenotypic expression of TGF-β1 by sandwich ELISA Reagents:

- Primary (coating) Antibody: Anti-TGF-β 1,2,3 Mouse Monoclonal Antibody (Catalog# MAB1855, R&D systems)Secondary (Detection)Antibody: Anti h-TGF β; (Catalog# AF-101 NA, R&D system)
- Labelling Antibody : Alkaline Phosphatase labelled Rabbit- anti-Goat IgG (H+L) (Catalog# 151-24-06, Kirkegaard &Perry Laboratories)
- Peptide: h-TGF-β1 (Catalog# 100B, R&D systems)

Method:

- DAY-1: On first day 100 µl primary antibody was added to each well in round bottom ELISA plate. Plate was covered and incubated overnight 4°C.
- DAY-2: Inverted the plate to remove antibody. Then 250 µl of blocking buffer was added to each well and incubated for 2 hours at room temperature. Washed the plate with 250 µl washing buffer (3 times).100 µl sample, standards and blank (PBS or D/W) was added to each well. Plate was covered and incubated overnight 4°C.
- DAY-3: On 3rd day Plate was decanted and washed 3 times with washing buffer(250 µl).Then 100 µl of detection antibody (diluted in Binding buffer II) was added to each well (final concentration 5µg/ml).Incubated the plate on shaker at room temperature

for 1 hour. Again washed plate 3 times with wash buffer. Alkaline Phosphate labelled Goat anti-chicken IgG Antibody (diluted in Binding buffer I) with final concentration of 500 ng/ml was added in100 µl/well. Incubated the plate at room temperature for 1 hour. Absorbance was measured at 450 nm in ELISA reader (Reading taken after 30 and 60 minutes of incubation).

3.3.4 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 system)
- Secondary (Detection) Antibody: Anti h-TGF-β2; polyclonal Rabbit IgG (Catalog#Ab-12-NA, R&D Systems)
- Labeling Antibody :HRP conjugate Goat anti-rabbit IgG (Catalog# HP03,GeNei)
- Peptide: p-TGF-β paracrine platelet derived (R&D system# 102-B2).

Method

DAY-1: On 1st day primary (coating) antibody (100µl/well) (Final concentration 10µg/ml Diluted in sterile PBS) was added to each well in round bottom ELISA plate. Plate was covered and incubated overnight 4°C.

DAY-2: On 2nd day inverted the plate to remove antibody and 250 µl blocking buffer was added. Incubated the plate at room temperature for 2 hours. Washed plate with 250 µl of washing buffer (3 times).All the sample standard (Prepared in 4mM HCL+ 01% BSA) added to nuclease free water. Solution filtered from 0.2 µM filter and blank (PBS or D/W) 100 µl added to each well. plate was covered and incubated overnight 4°C.

DAY-3: On 3rd day plate was decanted and washed 3 times with washing buffer 250 µl/well. 100 µl of detection antibody (diluted in binding buffer II) was added to each well (final concentration 5µl/ml).Incubated the plate on shaker at room temperature for 1 hour. washed 3 times with wash buffer. HRP labelled Goat anti-Rabbit IgG (Dilution 1:1000) was added (100 µl/well).Antibody diluted in 2% BSA in PBS Plate was incubated at room temperature for 1 hour. Washed the plate Twice with wash buffer and once with PBS (200 µl/well).TMB –H_2O_2 substrate 100µl/well was added. The optical density was measured at 650 nm. 100 µl of stop solution (0.16 M H_2So_4

prepared in D/W) was added after 15 minutes of incubation and absorbance was measured at 450nm.

3.3.5 Estimation of Phenotypic expression of smad-3 by Direct ELISA method

Reagent

- Primary Antibody :Smad2/3 (FL- 425) rabbit polyclonal IgG Antibody (Catalog#SC-8332, SCBT)
- Labeling Antibody: HRP conjugate Goat anti-rabbit IgG (Catalog#HP03, GeNei)
- Peptide :smad-3 Peptide (catalog#204884,Abcam)

Method

- DAY-1: Round bottom ELISA plate was coated with (100µl/well) respectively blanks (PBS or distilled water),Smad-3 standard peptide (Prepared in Binding Buffer I) and Protein sample (Diluted in sterile PBS).Plate was covered and incubated overnight at 4°C.
- DAY-2: On 2nd day plate was inverted to remove the content. Then 250 µl of blocking buffer was added and incubated at room temperature for 2 hours. The plate was washed with 250 µl washing buffer (3 times).Primary Antibody (100 µl/well) was added and plate was incubated overnight at 4°C.
- DAY-3: On 3rd day inverted the plate and washed 3 times with wash buffer (250 µl/well).HRP labelled Goat anti- Rabbit IgG (Dilution 1:1000) 100 µl/well was added. Antibody diluted in 2% BSA in PBS. Plate was washed twice with wash buffer and once with PBS (200µl/well). TMB-H_2O_2 substrate 100 µl/well was added and the optical density was measured at 650 nm.100 µl of stop solution (0.16 M H_2So_4 prepared in D/W) was added after 15 minutes of incubation and absorbance was measured at 450 nm.

3.3.6 Estimation of Phenotypic expression of smad-4 by Direct ELISA method

Reagent

- Primary Antibody :Smad 4 (FL- 425) rabbit polyclonal IgG Antibody (Catalog#SC-8332, SCBT)
- Labeling Antibody: HRP conjugate Goat anti-rabbit IgG (Catalog#HP03, GeNei)
- Peptide :smad-3 Peptide (catalog#204884,Abcam)

Method

- DAY-1: Round bottom ELISA plate was coated with (100µl/well) respectively blanks (PBS or distilled water), Smad-3 standard peptide (Prepared in Binding Buffer I) and Protein sample (Diluted in sterile PBS).Plate was covered and incubated overnight at 4°C.
- DAY-2: On 2nd day plate was inverted to remove the content. Then 250 µl of blocking buffer was added and incubated at room temperature for 2 hours. The plate was washed with 250 µl washing buffer (3 times).Primary Antibody (100 µl/well) was added and plate was incubated overnight at 4°C.
- DAY-3: On 3rd day inverted the plate and washed 3 times with wash buffer (250 µl/well).HRP labelled Goat anti- Rabbit IgG (Dilution 1:1000) 100 µl/well was added. Antibody diluted in 2% BSA in PBS. Plate was washed twice with wash buffer and once with PBS (200µl/well). TMB-H_2O_2 substrate 100 µl/well was added and the optical density was measured at 650 nm.100 µl of stop solution (0.16 M H_2So_4 prepared in D/W) was added after 15 minutes of incubation and absorbance was measured at 450 nm.

3.4 Statistical Analysis:

Statistical difference was assessed using two-sided Student's T test and Chi-square test. Graphs were plotted using Graph pad Prism and Sigma plot software.

Result and Discussion

In our present study we have determined the effect of antiestrogen on Hormone Receptor Positive (ER^+/PR^+) Breast Cancer cell line T47D. The optimum dose response and the effect of antiestrogen (TAM and 4-OH TAM) were determined by MTT assay. The phenotypic expression level of growth factors (TGF-β1 and TGF-β2) and its signalling mediators (smad 3 and smad 4) were determined by ELISA to explain the cross talk between the ER signalling pathway and TGF- β signaling pathway.

4.1 Establishment and maintenance of the Human breast cancer cell line T47D:

For our current study we have selected T47D cells, the main objective is to evaluate the antiestrogen treatment in hormone receptor positive breast cancer and to identify the crosstalk between ER pathway and TGF- β pathway. For establishment and maintenance of T47D breast cancer cells the primary steps are retrieving, passaging, freezing. ATCC guidelines was used for cell count and preservation which are mentioned in materials and methods.

4.2 Cell characteristics of T47D Cells:

T47D cells were regularly observed after 24 hours. As the cells confluency increases the cytoplasmic projection also increases. Cells morphology changes with time were observed and shown in figure:

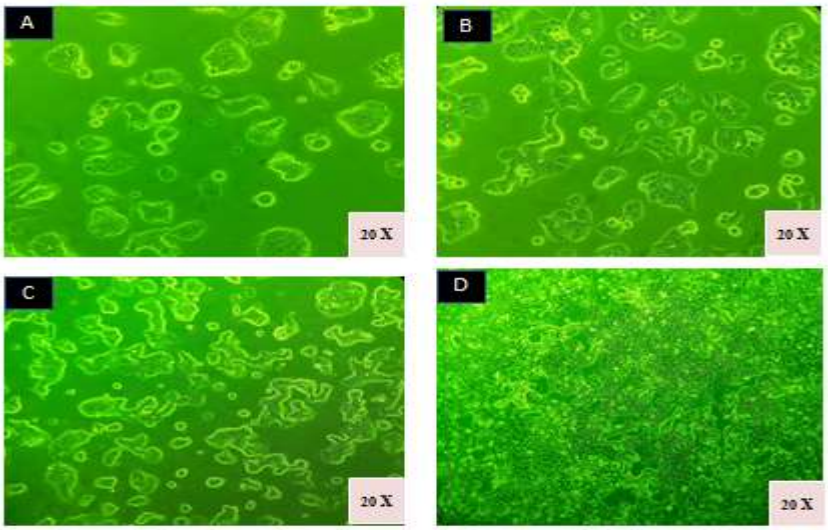


Figure 11: Cell morphology at different confluency

Cells were grown in T25 flask using complete growth medium. With increase in cell confluency the increased cytoplasmic projection and changes in cellular morphology were observed. In Figure A-20% cell confluency observed with absence of cytoplasmic projection. Some cells appear small and large with different morphology. In Figure-B, 40% confluency was observed with irregular shape and grows in clump. Figure-C shows 60-70% confluency of cells. Most of cells are attached with each other via cytoplasmic projection. Figure-D shows 95% confluency or over confluency in which complete surface of flask is covered.

Cell Viability Assay: Cell viability assay is based on dye exclusion test to determine the viable cell number. The principle of this assay is that the intact membrane of live cells excludes dyes like Trypan Blue. In live cells the intact mitochondrial membrane which have potential to produce ATP and via ATP dependent pathway removes dye out. While the ATP is not produce by dead cells and they are unable to remove dye out of the cells. Hence, the dead cells retain the dye and seem blue.

4.3 Determination of effective Dose of TAM and 4-OH TAM:

MTT Assay was performed to determine the optimum cytotoxic doses of TAM and 4-OH-TAM .The significant decrease in absorbance was observed in antiestrogen treated cells in comparison to untreated cells. There are six different concentration (10-6 to 10-10M) were selected according to (Karami-tehrani & Salami, 2003)

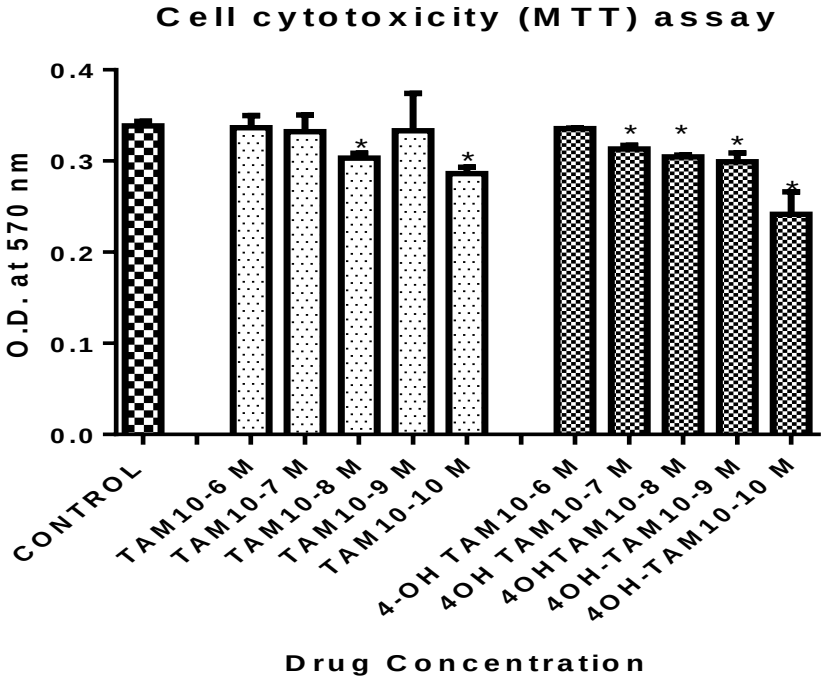


Figure 12: Optimum dose determination by Cell Cytotoxicity (MTT) Assay: Result indicates that optimum cytotoxic dose for TAM is 10^{-10} M (p =0.0132) and for 4-OH TAM is 10^{-10} (p =0.0322). (*p≤ 0.5),**p≤0.01,***p≤0.001)

Out of five different dose concentration (10^{-6} M to 10^{-10} M), the significant reduction in growth was seen in two doses of TAM: 10^{-10} M (p =0.0132) and 4-OH TAM is 10^{-10} (p =0.0322) showed significant reduction in absorbance (*p≤ 0.5).

Cell proliferation kinetics were plotted in two different manners.

1. The relative cytotoxicity exhibits the effectiveness of drug response.
2. The cell survival rate shows the actual amount of cells that survived after drug exposure. Both are inversely proportional to each other.

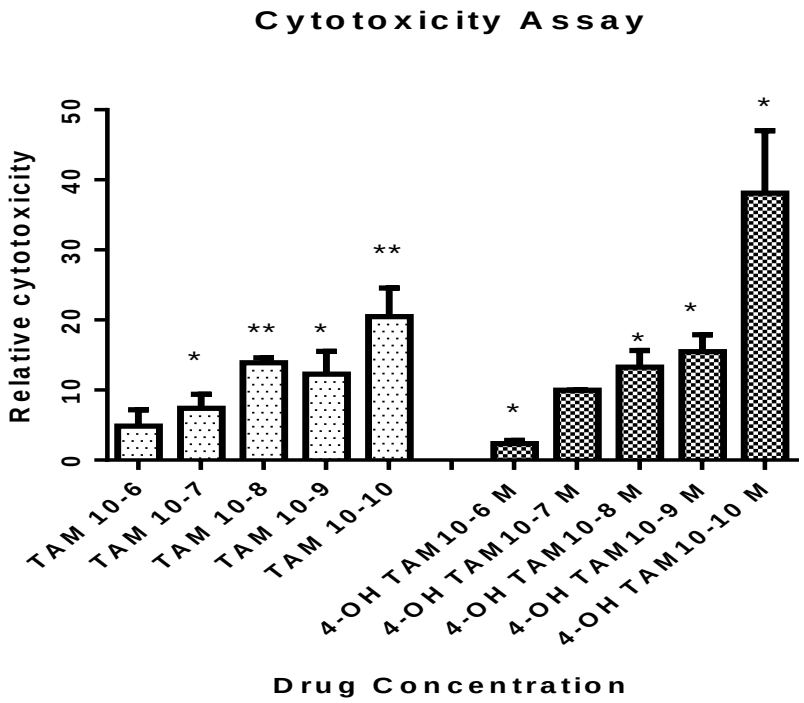


Figure 13: Relative cytotoxicity of TAM and 4-OH -TAM

The optimum relative cytotoxicity observed in TAM: 10^{-10} M (p =0.0193) and 4-OH TAM is 10^{-10} (p =0.0263).

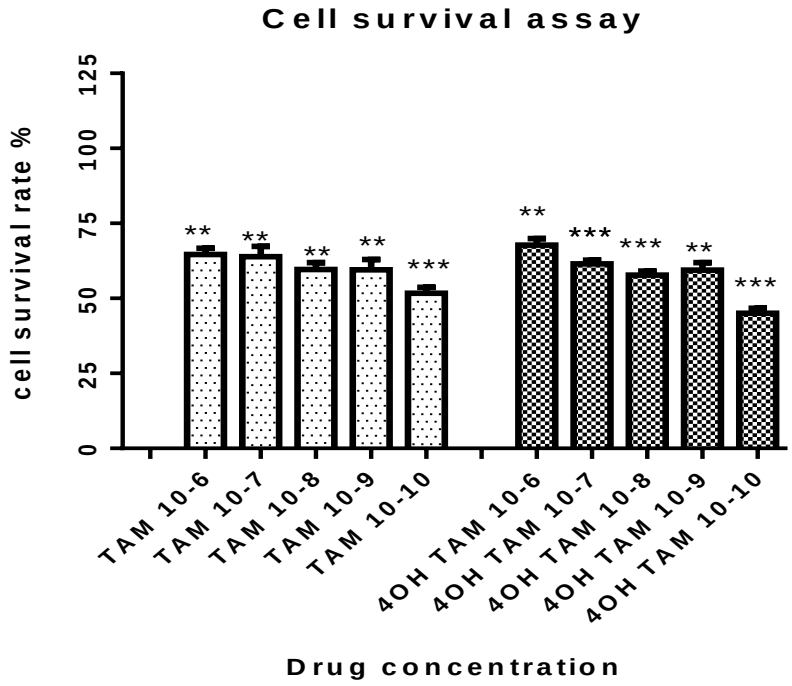


Figure 14: Cell Survival Rate

The significant reduction in cell survival rate was observed in TAM: 10^{-10} M ($p = 0.0008$) and 4-OH TAM is 10^{-10} ($p = 0.0004$).

4.3 Determination of the Population Doubling Time (PDT) and Growth curve analysis:

Growth curve assay was performed using cell density 1 x 10^4 cells/flask for 5 days. After every 24 hours of time interval cell count was performed for 4 days. Population doubling time of T47D cell line determined 24 to 30 hours.

TABLE 1: Growth Curve analysis of T47D cells

TIME (hours)	Untreated
0	1 x 10^4
24	6.8 x 10^4
48	36 x 10^4
72	68 x 10^4

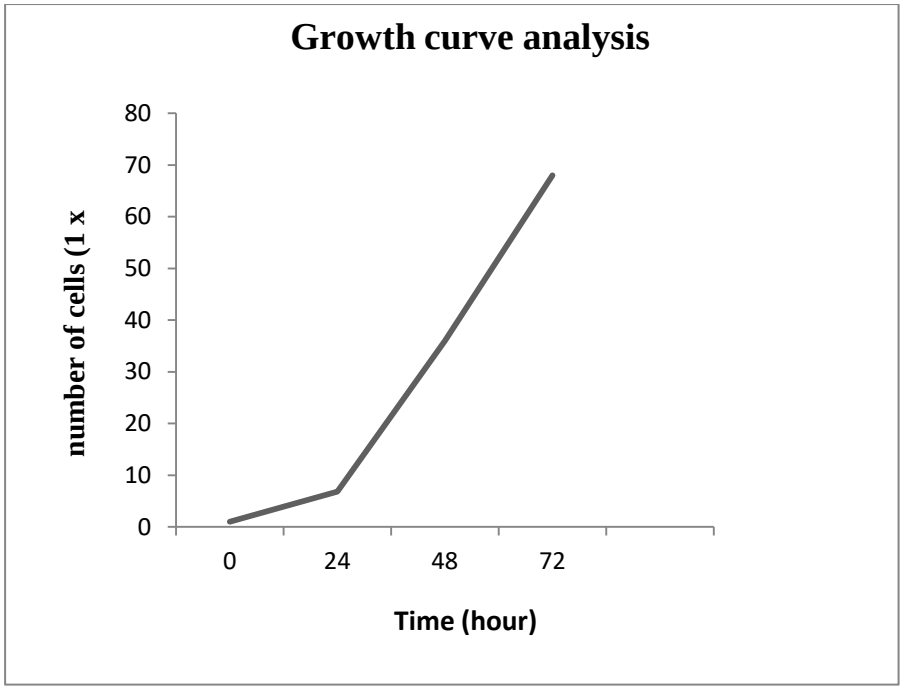


Figure15: Growth curve analysis

4.4 Drug Exposure Assays:

TAM is administered daily (20 mg/day) to breast cancer patients for 3-5 years. However, after long time drug treatment results in failure and/or resistance. The main purpose of our study is to evaluate the differences in cell viability during optimum cytotoxic drug exposure in time dependent manner including short term drug exposure.

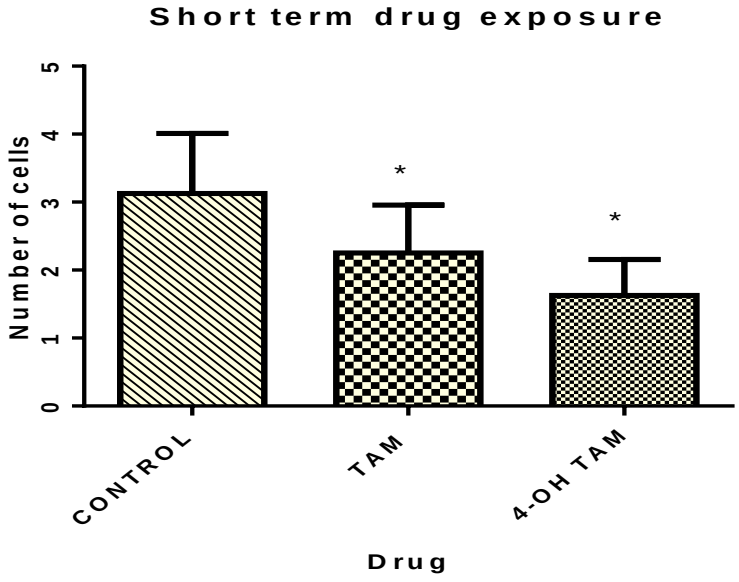


Figure 16: Cell viability during short term drug exposure

Significant reduction in cell viability during TAM (P=0.3884) and 4-oh TAM (P=0.1758) Treatment was observed during short term or single time drug exposure assay.

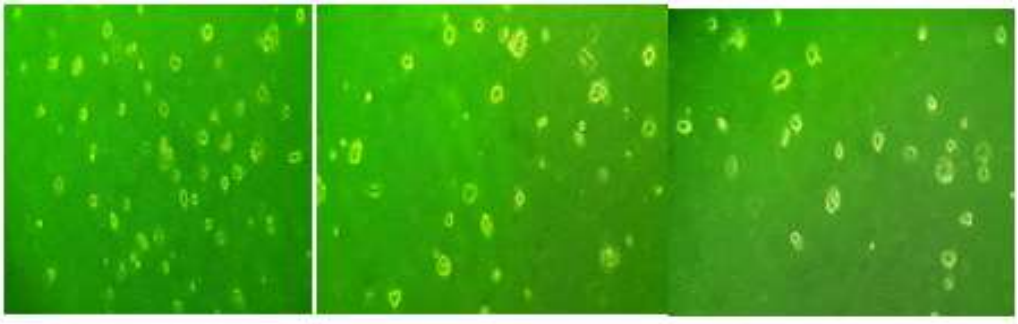


Figure 17: Morphology of T47D cells during short term drug exposure assay:

(A) Untreated cells, (B) TAM treated cells (C) 4-OH-TAM treated cell

4.5 Estimation of Phenotypic expression of growth factors TGF-β1, TGF β-2, Smad-3 and Smad 4 protein by ELISA:

4.5.1 Estimation of TGF- β1 expression by Sandwich ELISA:

4.5.1.1 Total Protein Estimation:

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

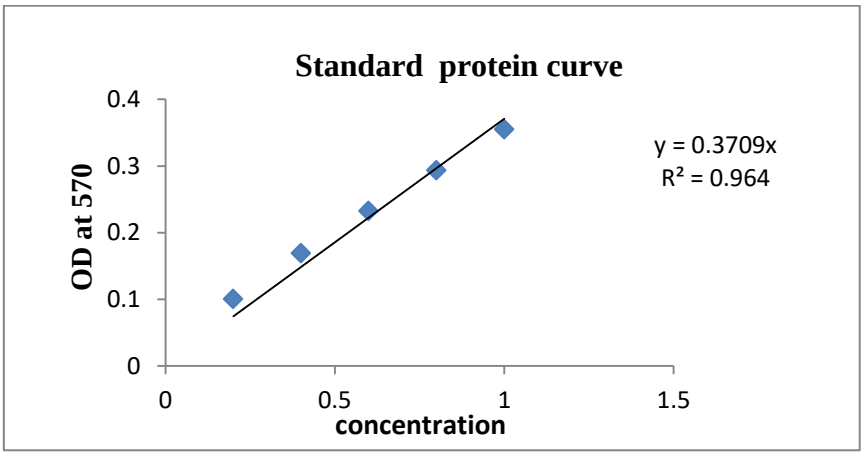


Figure 18: Standard curve of protein (Bovine Serum Albumin)

Standard Curve was plotted using Bovine Serum Albumin. Absorbance was taken at 570 nm in spectrophotometer.

Standard TGF-β1 peptide:

Human Recombinant TGF-β1 peptide was used as standard.

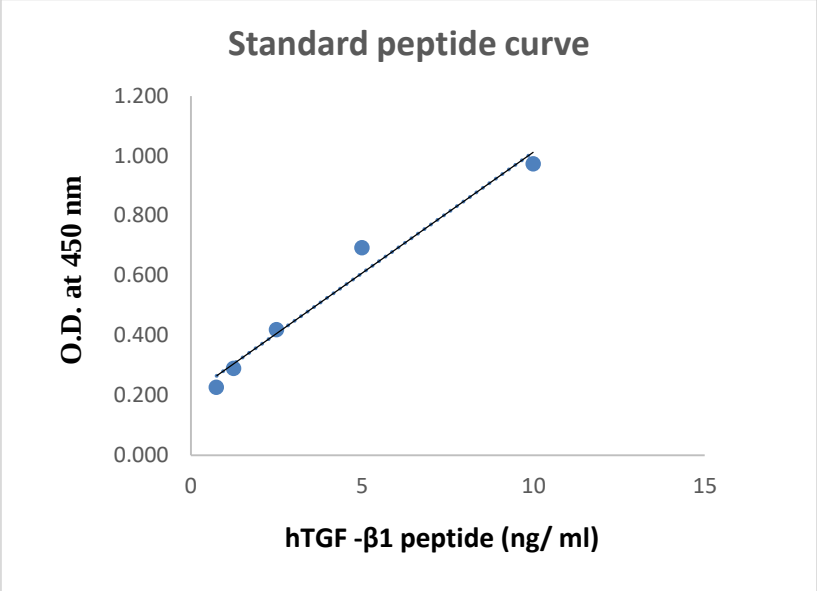


Figure 19: Standard curve for human recombinant TGF- β1 peptide

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

4.5.1.2 Estimation of phenotypic expression of TGF- β1 during short term Drug exposure assay:

Short term drug exposure assay was done in duplicate and the cells were harvested from which total protein was isolated. Total protein was used to determine the level of TGF- β1 during short term drug exposure assay.

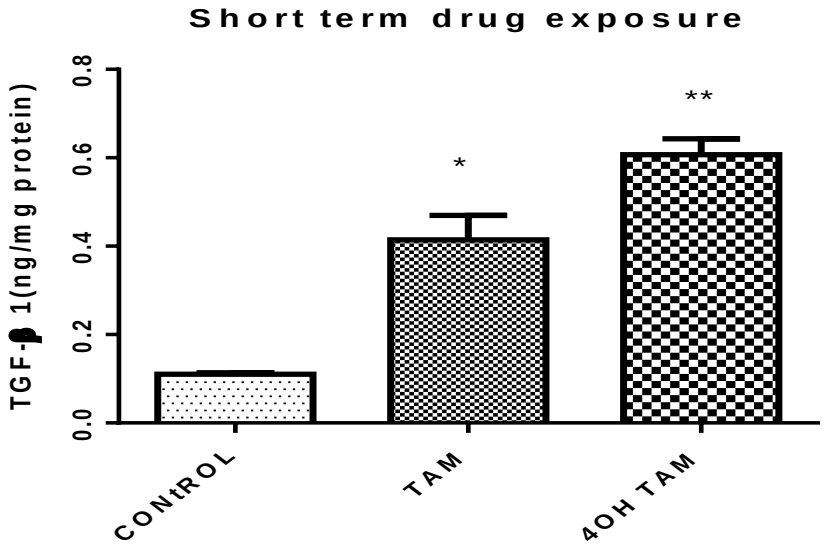


Figure 20: Phenotypic expression of TGF-β 1 during short term drug exposure

There is a significant elevation of TGF-β1 during short term exposure of TAM(0.016375) and 4-OH-TAM(0.002607) compared to untreated cells. The levels of TGF-β1 increases in 4-OH-TAM treated cells compare to TAM treated cells.

Table 2: Level of TGF- β1 during short term drug exposure assay

Treatment	Total protein (mg/ml)	TGF- β1 (ng /mg)	Mean ± SEM
Untreated	7.29	0.1102845	0.1102845 ± 0.002478
TAM	4.48	0.414368	0.414368 ± 0.03932
4-OH -TAM	2.5	0.923216	0.923216 ± 0.025294

4.6 Estimation of TGF-β2 expression by ELISA:

4.6.1 Standard TGF-β2 peptide curve

Human Recombinant TGF-β2 peptide was used as standard.

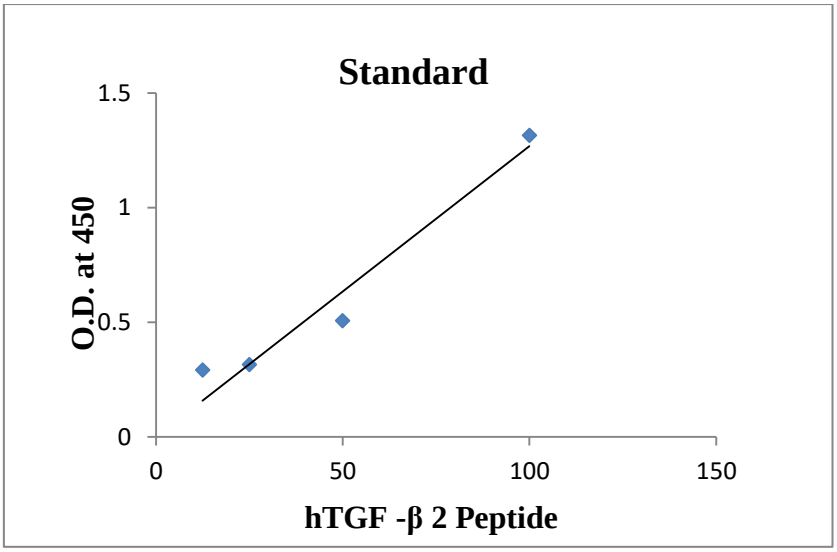


Figure 21: Standard curve for human recombinant TGF-β 2 peptide

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

4.6.2 Estimation of phenotypic expression of TGF-β2 during short term Drug exposure assay:

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

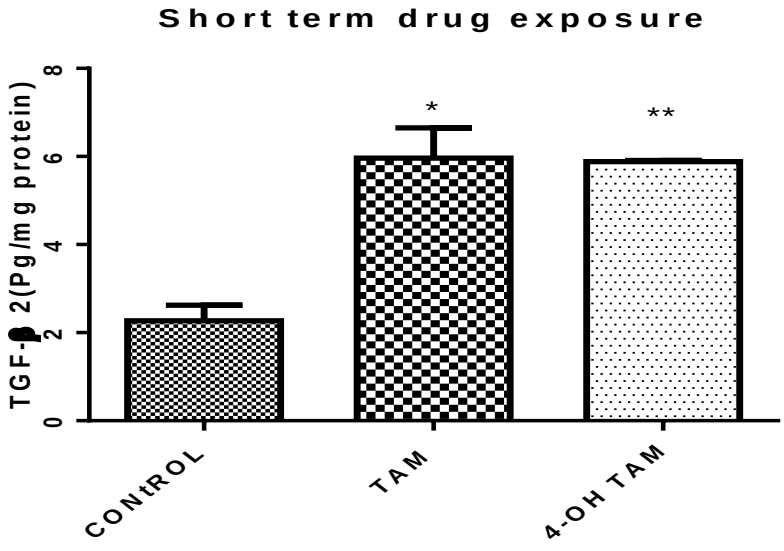


Figure 22: Phenotypic expression of TGF- β2 during short term drug exposure

There is a significant elevation of TGF –β2 during short term exposure of TAM and 4-OH TAM compared to untreated cells. The levels of TGF-β2 increases in TAM and 4-OH-TAM treated cells compared to untreated cells.

TABLE 3 : Level of TGF-β2 during short term drug exposure assay

Treatment	Total Protein (mg/ml)	TGF-β2 (pg/mg)	Mean ± SEM
Untreated	7.290	2.27	2.27 ± 0.25
TAM	4.480	5.96	5.96 ± 0.485
4-OH-TAM	2.500	5.88	5.88 ± 0.02

4.7 Estimation of Smad -3 expression by ELISA:

4.7.1 Standard Smad3 peptide curve

For the sandwich ELISA assay, Smad3 standard peptide was taken in a range of 15 pg to 250 pg and absorbance was taken at 450 nm. This standard curve was used for determination of Smad 3 levels in experiment sample.

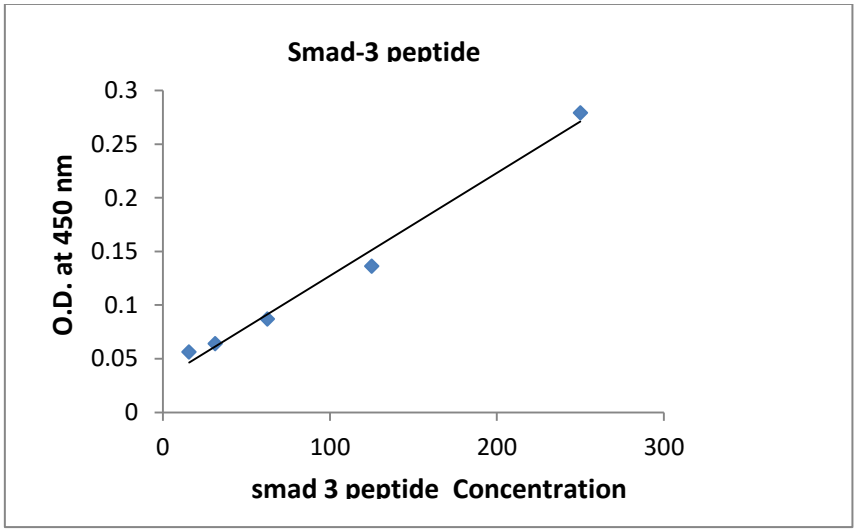


Figure 23: Standard curve for SMAD 3 peptide

4.7.2 Estimation of Phenotypic expression of SMAD3 during Short term Drug exposure assay: Short term drug exposure assay was done in duplicate and the cells

were harvested to extract cytoplasmic as well as nuclear proteins to determine the phenotypic expression

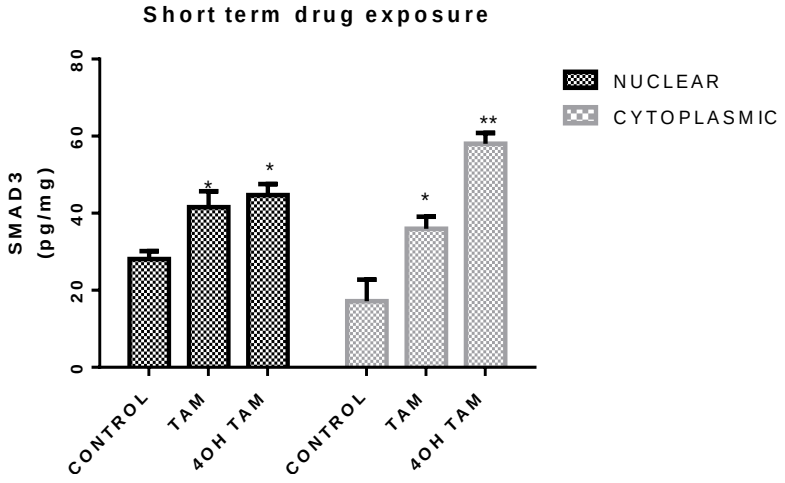


Figure 24: Phenotypic expression of Smad 3 during short term drug exposure

Table 4 : Level of cytoplasmic SMAD 3during short term drug exposure assay			
Treatment	Total cytoplasmic Protein (mg/ml)	Cytoplasmic SMAD3 (pg/mg)	Cytoplasmic SMAD 3 (pg/mg) Mean ± SEM
Untreated	7.29	17.14	17.14 ± 3.97
TAM	4.48	35.93	35.93 ± 2.23
4-OH TAM	2.5	88.00	88.00 ± 2

There is a significance elevation of cytoplasmic SMAD3 in both TAM and 4-OH TAM treated cells compared to control. However there is a elevation of SMAD3 in TAM treated cells (p= 0.054) and 4-OH- TAM (P= 0.011).

Table 5 : Level of Nuclear SMAD 3 during short term drug exposure assay			
Treatment	Total nuclear protein (mg/ml)	Nuclear SMAD 3 (pg/mg)	Nuclear SMAD 3 (pg/mg) Mean ± SEM
Untreated	4.16	28.12	28.12 ± 1.44
TAM	2.9	41.55	41.55 ± 2.93
4-OH- TAM	2.44	46.67	46.67 ± 2.04

4.5.4 Estimation of Phenotypic expression of SMAD 4 during Short term Drug exposure assay:

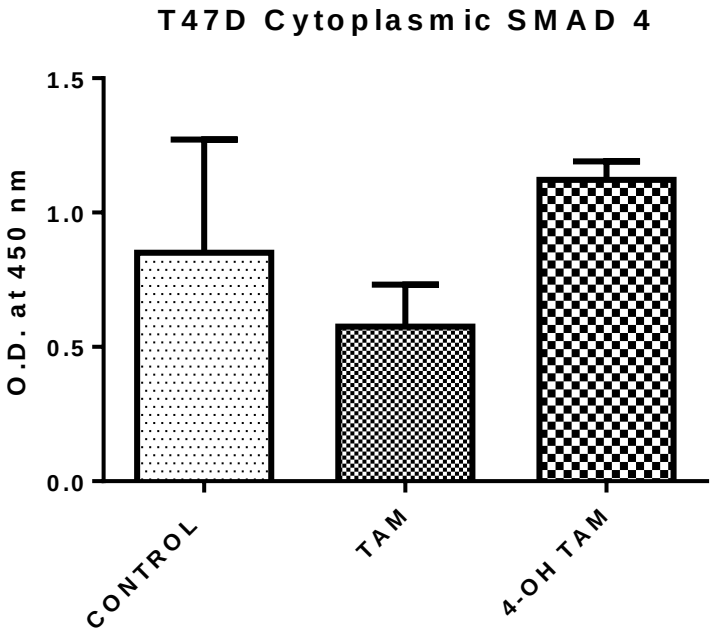


Figure 25: Phenotypic expression of Smad 4 during short term drug exposure

There is a significant inhibition of SMAD-4 in TAM treated cells in comparison to control while elevation is observed in 4-OH –TAM treated cells.

TAM treated cells (p= 0.4792) and 4-OH- TAM (P= 0.4620).

Conclusion

In hormone responsive breast cancer antiestrogen therapy such as Tamoxifen has been successful therapy. We have evaluate the cytotoxic effect of antiestrogen on hormone receptor positive breast cancer cell line T47D.We have determined the phenotypic expression level of growth factors TGF –β1 , TGF –β2 and signaling mediators (Smad 3 , Smad 4) during antiestrogens (TAM and 4-OH- TAM) treatment.

In our study we have evaluated and conclude that:

- The Population Doubling Time (PDT) of T47D cell line is 24 hours_.This population doubling time was repeatedly checked and maintained during the study.
- Antiestrogen (TAM and 4-OH-TAM) have significant cytotoxic effect on T47D cells. the optimum significant cytotoxic effect of TAM was seen at 10^{-10} M and for 4-OH-TAM was seen at 10^{-10} M concentration.
- There was a significant reduction in cell viability during short term drug exposure assay.
- The levels of TGF-β 1 were significantly increased in TAM and 4-OH-TAM treated cells during short term drug exposure assay.
- The level of TGF -β 2 were significant increased in TAM and 4-OH-TAM treated cells during short term drug exposure assay.
- The level of cytoplasmic as well as nuclear smad 3 protein was significantly increase in TAM and 4-OH-TAM treated cells during short term drug exposure assay.
- There was significant reduction of Smad 4 in TAM and increase in 4-OH –TAM treated cells during short term drug exposure assay.

The above results indicate that the elevation of TGF-β-1, TGF-β-2, SMAD-3 molecules during antiestrogen treatment might be the possible reason for antiestrogen resistance and/or non-responsiveness. This elevation of molecules shows the cross talk between the TGF-β and ER signaling pathway. So the TGF- β can be used as a surrogate marker in breast cancer for alternative adjuvant therapy in future.

Appendix

Complete growth Medium

Dulbecco's modified Eagle medium High glucose (catalog #AL111,HIMEDIA)	500ml
Fetal bovine serum (catalog # RM 112 , HIMEDIA)	10%
L-glutamine (catalog # TCL 012)	2mM
MEM –Non essential amino acids (catalog # ACL006, HIMEDIA)	0.1mM
Antibiotic(penicillin-streptomycin) solution (catalog # A007,HIMEDIA)	1%

1x PBS

Nacl	8gm
Kcl	0.2 gm
Na_2HPO_4	1.44gm
KH_2PO_4	0.24gm

Lysis buffer

Triton X100	1% W/V
Tris-Cl (pH-7.4)	50 mM
Nacl	300 mM
EDTA	5mM
Sodium Azide	0.02%
PMSF	1 mM
Leupeptin	2 µg/ml

Nuclear lysis buffer

Tris –base(pH:8.0)	10mM
LiCL/KCL	0.5mM
Glycerol	20%

Binding Buffer 1

Tris –Base (pH:7.4)	100nM
Nacl	150mM
NaN_3	0.02%
Tween 20	0.05%
BAS Fraction V	1mg/ml

Binding Buffer 2

Tris –Base (pH:7.4)	100nM
Nacl	150mM
NaN3	0.02%
Tween 20	0.05%
BAS Fraction V	10mg/ml

Blocking Buffer

Tris- Base (pH:7.4)	100mM
Nacl	150mM
NaN_3	0.02 %
BAS Fraction V	10mg/ml

Wash Buffer

1xPBS	-
Tween 20	0.05%

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