The Role of Androgen Receptor as Biomarker in Triple Negative Breast Cancer

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Biotechnology / Microbiology

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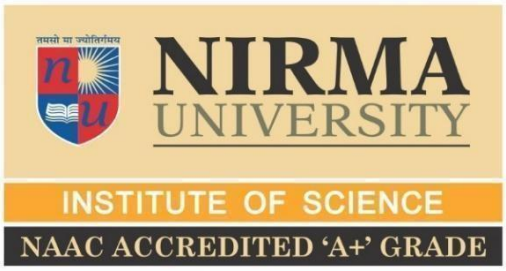

Institute of Science,

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Date: 28/04/2023

Place: Ahmedabad

NIRMA UNIVERSITY

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NAAC ACCREDITED 'A' GRADE

CERTIFICATE

This is to certify that the thesis entitled **"The Role of Androgen Receptor as Biomarker in Triple Negative Breast Cancer"** submitted to the Institute of Science, Nirma University in partial fulfilment of the requirement for the award of the degree of M.Sc. in Biotechnology/Microbiology, is a record research work carried out by Nidhi Shukla (21MMB028), Kedar Soni (21MBT051) and Bhavya Suvarna (21MBT041) under the guidance of Dr. Heena Dave. No part of the thesis has been submitted for any other degree/diploma.

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DECLARATION BY THE CANDIDATE

We hereby declare that the dissertation work entitled **"The Role of Androgen Receptor as Biomarker in Triple Negative Breast Cancer"** submitted to the Institute of Science, Nirma University is a record of the original work done by us under the guidance of **Dr. Heena Dave** and with the support of **Ms. Nirali Shukla, Ms. Deepshikha Rathore** and our lab members. The work incorporated in the thesis has not been submitted for the award of any other degree, diploma associateship and fellowship, title in there or any other university or other institution of higher learning.

We further declare that the material obtained from other sources has been duly acknowledged in the thesis.

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ABBREVIATIONS

- **AR:** Androgen Receptor
- **TNBC:** Triple Negative Breast Cancer
- **ER:** Estrogen Receptor
- **PR:** Progesterone Receptor
- **HER-2:** Human Epidermal Growth Factor Receptor 2
- **LAR:** Luminal Androgen Receptor
- **MRI:** Magnetic Resonance Imaging
- **LCIS:** Lobular Carcinoma In-Situ
- **DCIS:** Ductal Carcinoma In-Situ
- **ILC:** Infiltrating Lobular Carcinoma
- **IDC:** Infiltrating Ductal Carcinoma
- **IHC:** Immunohistochemistry
- **NCCN:** National Comprehensive Cancer Network
- **ESMO:** European Society for Medical Oncology
- **BLIS:** Basal like Immunosuppressed
- **BLIA:** Basal like Immune Activated
- **BL-1:** Basal like 1
- **BL-2:** Basal like 2
- **IM:** Immunomodulatory
- **MSL:** Mesenchymal stem like
- **MES:** Mesenchymal
- **DNA:** Deoxyribonucleic acid
- **EGFR1:** Human Epidermal Growth Factor Receptor 1
- **CK:** Cytokeratins
- **BRCA1:** BReast CAncer gene 1
- **BRCA2:** BReast CAncer gene 2
- **PARP:** Poly ADP-Ribose Polymerases
- **PI3K:** Phosphatidylinositol-3-Kinase
- **TKI:** Tyrosine Kinase Inhibitor

- **ADC:** Antibody Drug Conjugate
- **RAS:** Renin Angiotensin System
- **HR:** Hormone Receptor
- **ICIs:** Immune Checkpoint Inhibitors
- **TIL:** Tumour infiltrating lymphocyte
- **mTOR:** mechanistic Target of Rapamycin
- **LBD:** Ligand Binding Domain
- **NTD:** N-terminal Domain
- **DBD:** DNA-binding Domain
- **AF-1:** Activation Factor 1
- **AF-2:** Activation Factor 2
- **ARE:** Androgen Responsive Elements
- **MAPK:** Mitogen Activated Protein Kinase
- **CYP:** Cytochrome P50
- **DHT:** Dihydrotestosterone
- **HSP:** Heat Shock Protein
- **AR-Vs:** Androgen Receptor splice variants
- **AR-V7:** Androgen Receptor splice variant 7
- **FDA:** Food and Drug Administration
- **CRPC:** Castration Resistant Prostate Cancer
- **BC:** Breast Cancer
- **ADT:** Androgen Deprivation Therapy
- **MCF-7:** Michigan Cancer Foundation
- **MTT:** 3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide
- **MDA-MB:** MD Anderson Metastatic Breast
- **PBS:** Phosphate Buffer Saline
- **FBS:** Fetal Bovine Serum
- **DMSO:** Dimethyl Sulfoxide
- **IP:** Isopropanol
- **DMEM:** Dulbecco's Modified Eagle's Medium
- **EtBr:** Ethidium Bromide

- **TAE:** Tris-Acetate-EDTA
- **c-DNA:** complementary DNA
- **qRT-PCR:** Quantitative Real Time Polymerase Chain Reaction
- **RPM:** Rotations Per Minute
- **RP**: Reverse Primer
- **FP**: Forward Primer
- **GAPDH**: Glyceraldehyde-3-phosphate dehydrogenase

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ABSTRACT

Breast cancer is one of the most common diseases which is prevalent worldwide among women. Currently, there are three main markers that are Estrogen receptor (ER), Progesterone receptor (PR) and Human Epidermal Growth Factor Receptor-2 (HER-2). The presence of any of these three markers will help in deciding the therapeutic line of treatment for curing breast cancer.

TNBC, a subtype of breast cancer, lacks the ER, PR, and HER2 expression. For such kinds of cancer, the options for treatment are fewer and the prognosis is poorer. However, a subtype of TNBC i.e., Luminal Androgen Receptor (LAR) has a significantly higher expression of Androgen receptor (AR) which can be targeted in order to inhibit the cell proliferation occurring as a result of activation of AR signaling pathway. AR-antagonists like Bicalutamide and Enzalutamide can be used against AR.

In our current study, we wanted to analyse the inhibitory effects of AR antagonists on TNBC cells. Therefore, we performed cell viability (MTT) assays and examined the effect of drugs at different concentrations on cells at different time intervals ranging from 24, 48, and 72 hours. On performing the cell viability assay, the IC50 concentration of bicalutamide for cell line MDA-MB-453 was achieved 10.2 μM at time interval of 48 hrs. The IC50 concentration of bicalutamide for the MDA-MB-231 cell line was achieved 10.27 μM at the time interval of 24 hrs. The IC50 concentration of yet another AR antagonist Enzalutamide for the MDA-MB-231 cell line was achieved to be 37.20 μM at the time interval of 72 hrs. We also checked the effect of bicalutamide on the metastatic potential of MDA-MB-453 cells, cells were treated at the concentration of 10 μM and 20 μM for 24 hrs, it was observed that as the concentration increased, bicalutamide was able to inhibit the metastatic characteristics of the cells.

Further, the gene expression of AR was checked by treating the MDA-MB-453 cells with 10 μM of bicalutamide, and significant downregulation of the gene was observed. However, no

significant downregulation of AR-V7 which is a splice variant was observed. MDA-MB-231 cells were also treated with 10 μM bicalutamide nevertheless no significant downregulation of the AR as well as AR-V7 was observed.

INTRODUCTION

Cancer is a condition in which the cells of an individual's body start proliferating uncontrollably and divide rapidly (Schiliro & Firestein, 2021). However, before turning into an aggressive malignant tumour, there takes place a multi-fold aberration at phenotypic and genotypic levels hence termed as a multistep process of carcinogenesis. The steps involve tumour initiation, tumour promotion, malignant conversion, and tumour progression (Sugimura, 1992).

Breast cancer:

Cancer which is developing in the cells and tissues of the breast is known as breast cancer. It is clearly evident in Figure 1, the difference between normal and cancerous breast. Breast cancer invades surrounding healthy tissue and it has the ability to expand from its initial location into the breast ducts or lobules. Both men and women can develop breast cancer, however, it is uncommon in men.

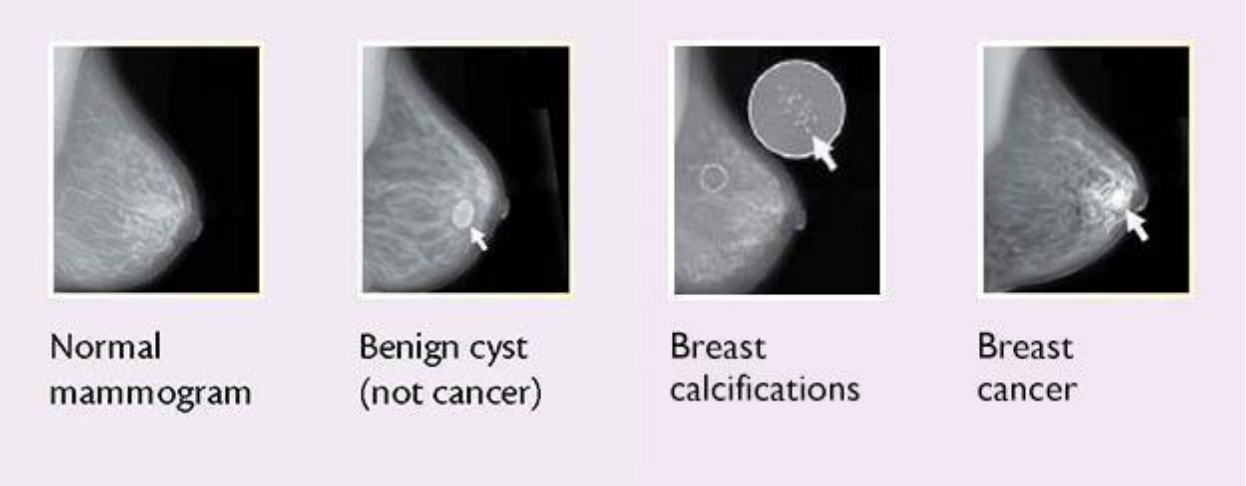

Figure 1: MRI images of Normal breast v/s breast cancer

Ductal carcinoma originates from the lining of the milk ducts and it is the most prevalent type of breast cancer. The breast's lobules (milk glands) are where lobular carcinoma, another type of breast cancer, develops which is mentioned in Figure 2.

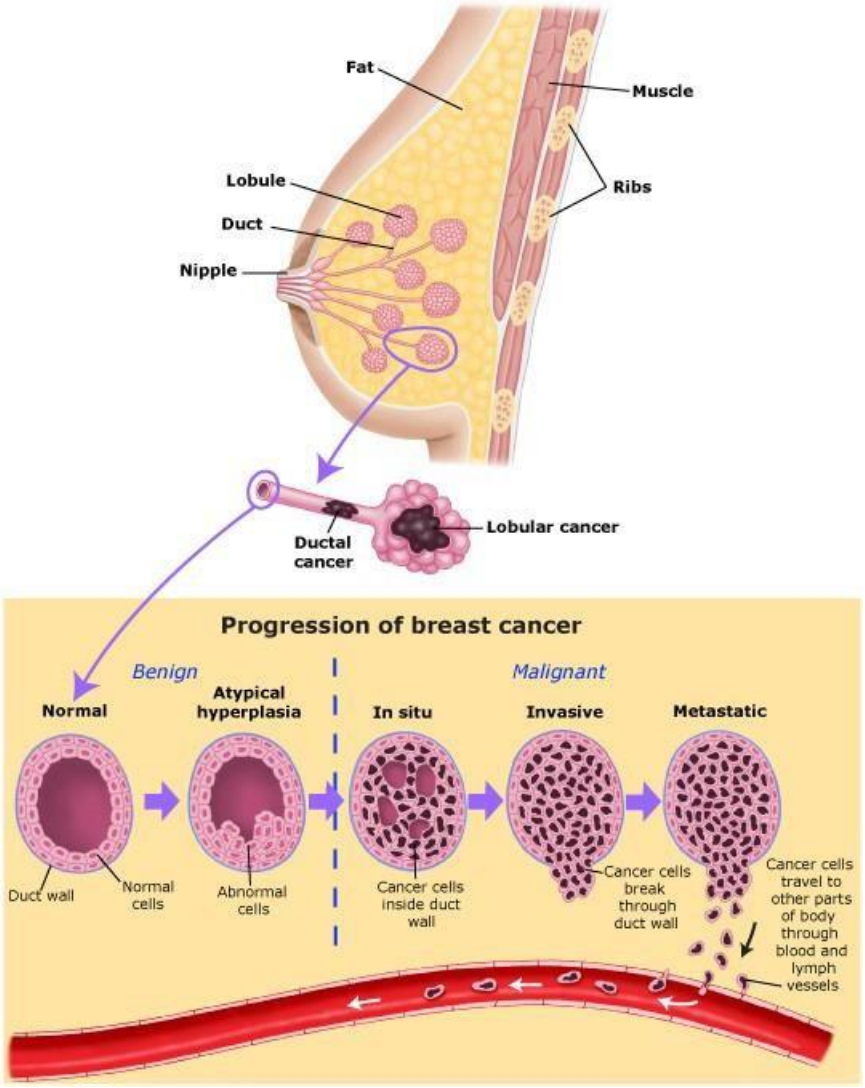

Figure 2: Progression of breast cancer

Incidence of breast cancer:

In accordance to the GLOBOCAN 2020 data, breast cancer is currently the most commonly diagnosed malignancy with an expected 2.3 million recent cases worldwide and it is the fifth cause of cancer-related fatalities (Sung et al., 2021).

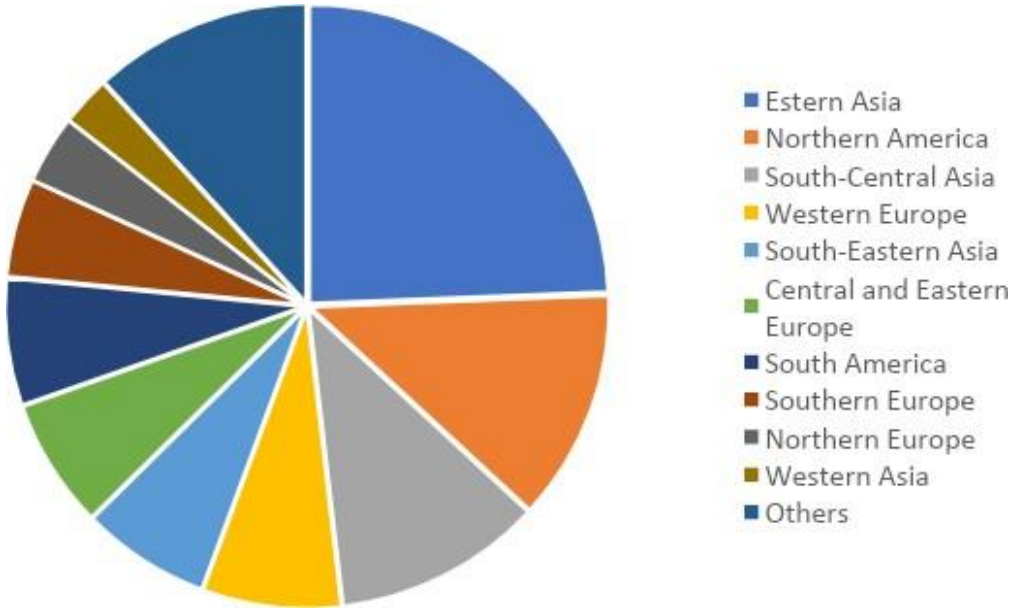

Figure 3: Incidence of breast cancer (Arnold et al., 2022)

Approx 2.3 million recent cases of breast cancer were confirmed all over the world in 2020 and there were also approximately 6,85,000 deaths from this illness, with significant regional variances between different nations and global areas. Although transitioning nations account for asymmetric cases of cancer-related deaths, these nations also have the greatest rates of breast cancer incidence shown in Figure 3 (Arnold et al., 2022).

Different stages of breast cancer:

The tumour size and the extent of metastasis determine the stage of breast cancer. Five stages of breast cancer are shown in Table 1. The higher the number the more is its spread as shown in Figure 4.

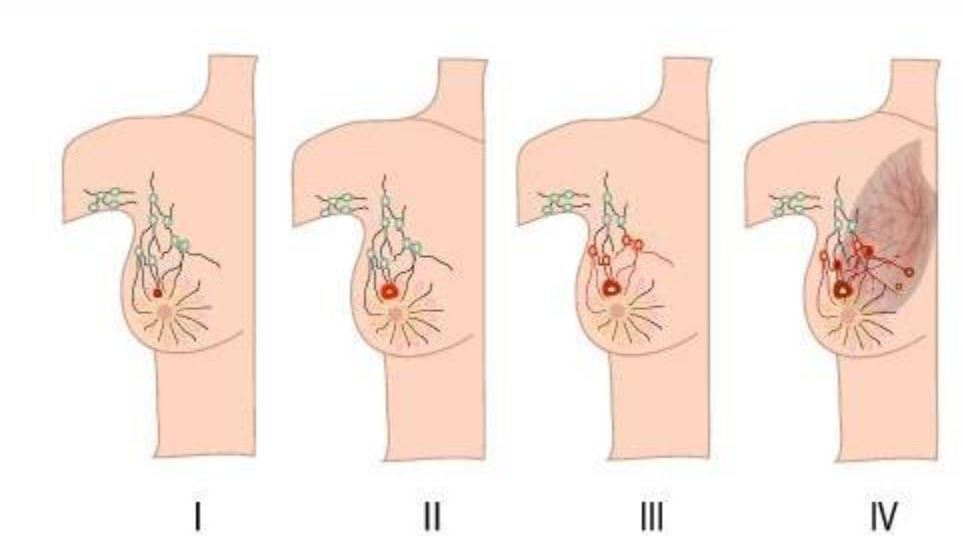

Figure 4: different stages of breast cancer

https://www.facs.org/for-patients/home-skills-for-patients/breast-cancer-surgery/breast-cancer-types/breast-cancer-staging/

Using a stage-based classification system for breast cancer can help us identify the most effective treatment strategy and can help in estimating the prognosis of an individual suffering from breast cancer.

Table 1: Different stages of breast cancer during its progression

Stages of cancer	Sub – stages of cancer	Characteristics
Stage 0		The disease is only in the ducts and lobules of the breast. It has not spread to the surrounding tissue. It is also called noninvasive cancer
Stage I		The disease is invasive. Cancer cells are now in normal breast tissue.
	Stage IA	The tumor is up to 2 centimeters. It has not spread to the lymph nodes
	Stage IB	The tumor is in the breast and is less than 2 cm. Or the tumor is in the lymph nodes of the breast and there is no tumor in the breast tissue.
Stage II		Invasive breast cancer. There are 2 types
	Stage IIA	A tumor may not be found in the breast, but cancer cells have spread to at least 1 to 3 lymph nodes. Stage IIA may show a 2 to 5 cm tumor in the breast with or without spread to the axillary lymph nodes.
	Stage IIB	The tumor is 2 to 5 cm and the disease has spread to 1 to 3 axillary lymph nodes. Or the tumor is larger than 5 cm but has not spread to the axillary lymph nodes
Stage III		Invasive breast cancer. There are 3 types
	Stage IIIA	Spread to more than 4 lymph nodes in the breast or axilla. It has not spread to other parts of the body.
	Stage IIIB	Cause swelling of the breast and may be in up to 9 lymph nodes. Inflammatory breast cancer is considered Stage IIIB
	Stage IIIC	Spread to 10 or more axillary lymph nodes, or nodes above or below the collarbone or breastbone.
Stage IV (metastatic)		The tumor can be any size and the disease has spread to other organs and tissues, such as the bones, lungs, brain, liver, distant lymph nodes, or chest wall

Subtypes of breast cancer:

Women are more likely to be diagnosed with and develop breast cancer globally and it has a wide range of different subtypes shown in Figure 4.

Based on Histological classification:

Histological types of cancer refer to the way cancer cells look under a microscope, and they can be associated with different radiological findings and metastatic patterns. For example, lobular carcinomas are those which arise from the tissue that lines the breast's milk ducts which often have a characteristic appearance on imaging studies and can metastasize to unusual sites, such as the gastrointestinal tract and ovaries. In addition to differences in metastatic patterns, histological types can also have different morphologies or physical characteristics. For example, ductal carcinomas, which arise from the cells that line the milk ducts, often have a distinct pattern of growth, with cancer cells forming into small clusters or tubes. In contrast, lobular carcinomas often have a more diffuse pattern of growth, with cancer cells spreading out in a sheet-like pattern. Overall, understanding the histological type of cancer can provide important information about its behavior, as well as guide treatment decisions. Therefore, it is important for pathologists and radiologists to work together to accurately diagnose and stage cancers based on their histological type and imaging findings. As it is mentioned in Figure 4, it is classified into two major types:

1) Invasive carcinoma 2) Non-Invasive /In-situ carcinoma

Invasive Lobular Carcinoma:

Breast cancer which initiates in the breast glands that produce milk (lobules) can be termed as Invasive lobular Carcinoma. It means that the cancer cells have spread out of the lobule where they first originated and further have the potential to spread to other body parts such as lymph nodes. It is breast cancer's second-most prevalent histologic subtype which represents 5-15% of all invasive breast cancers.

Invasive Ductal Carcinoma:

It is a breast cancer subtype that initiates in the duct lining of the breast and penetrates further from the duct's outside portion to other tissues of the breast is referred to as Invasive Ductal Carcinoma. It can potentially migrate to other sites of the body via the blood and lymphatic system. It makes up around 80% of all cases since it is the most prevalent kind of invasive breast cancer.

In Situ Lobular Carcinoma:

In Situ, Lobular Carcinoma ischaracterizedd by abnormal cells in the breast lobules. This condition rarely progresses to invasive cancer. A person who has lobular carcinoma in situ in one breast is more likely to develop cancer in the other. LCIS is a rare disorder in which abnormal cells are formed in the milk glands of the breast. LCIS is not cancer in itself, but having it indicates that a person is at a higher risk of developing breast cancer. It accounts for 5% to 15% of all invasive breast cancers which is second common histologic type of breast cancer.

In Situ Ductal Carcinoma:

In Situ, Ductal Carcinoma is a situation in which abnormal cells are found in the breast duct lining. In this case, the abnormal cells have not spread beyond the duct to other breast tissues. In some cases, ductal carcinoma in situ can progress to invasive breast cancer and spread to other tissues. It is classified as pre-invasive breast cancer. Though DCIS does not spread outside of the breast, it is considered safe to treat it because if left untreated, some DCIS cells may undergo further development and become invasive in nature. It is a cause for around 20% of all breast cancer cases.

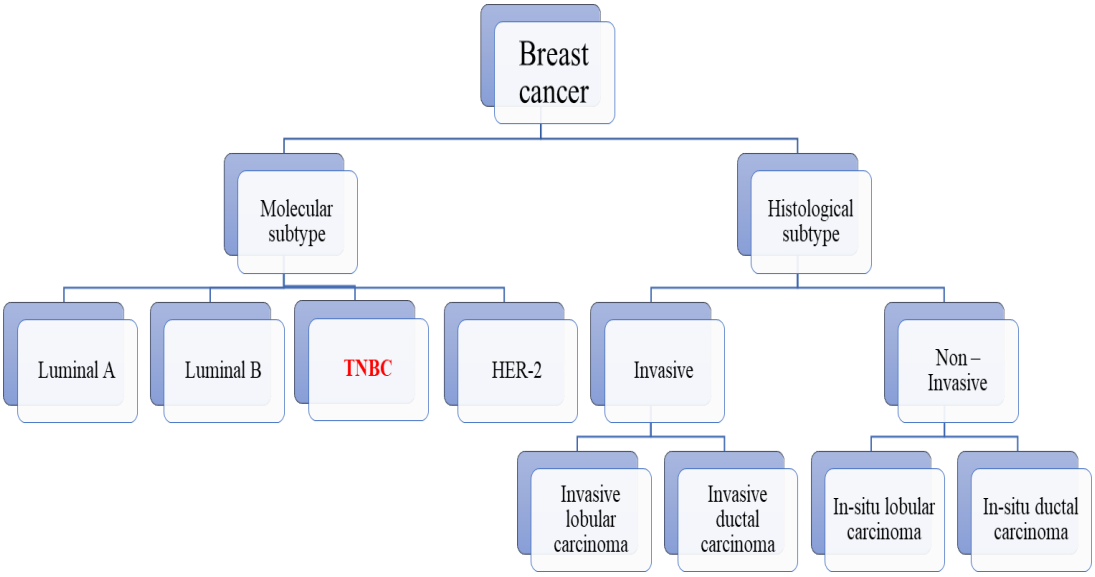

Figure 5: Subtypes of breast cancer

Based on Molecular classification:

The subtypes are classified into four groups based on the immunohistochemical expression of hormone receptors: TNBC (lacks PR, ER, and HER-2 expression), positive for Luminal A, Luminal B, and HER-2 (Shaath et al., 2021). Since most (70% to 75%) the invasive breast carcinoma are characterized by a considerably elevated level of ER, expression of the ER acts as a diagnostic factor (Zhang et al., 2013) (Miah et al., 2019). Since HER2 expression is present in 15–25% of instances, it is crucial to recognizes protein when deciding on the best course of action for individuals with carcinoma of breast (Vaz-Luis et al., 2013). One of the key developments during breast carcinogenesis is HER2 overexpression (Iqbal & Iqbal, 2014). Although it can boost the detection rate by up to 50% to 80%, the identification of HER-2 is particularly helpful in diagnosing metastatic or recurring breast tumours. A further promising method to track the presence or recurrence of breast cancers in real-time is the measurement of HER2 levels in the serum (Krishnamurti et al., 2009). The excessive stimulation of proto-oncogenic signalling pathways brought on by HER2 amplification causes the unchecked development of cancer cells. When HER2 is overexpressed, this condition is linked to worse clinical results. The amount of time a person can live without developing a disease is significantly less when HER2 is overexpressed (Krishnamurti et al., 2009). A cellular antigen called Ki67 is frequently employed as a proliferation marker and can tell us a

lot about how quickly cells divide. The aggressiveness of the cancer, treatment response, and duration to recurrence are all reflected in the proliferative activities as measured by Ki67 (Haroon et al., 2013). The evaluation of proliferative activity using the Ki67 assay is a helpful tool for figuring out how aggressive a cancer is, forecasting how it will respond to treatment, and figuring out when it will return. As such, the measurement of Ki67 levels is an important factor in selecting the most appropriate treatment approach and creating follow-up plans to keep an eye on the return of danger. According to studies, breast cancer cells that express more Ki67 tend to have more aggressive tumour features, a worse prognosis, and shorter survival rates. This shows that Ki67 might be a useful prognostic indicator for those with breast cancer. By monitoring Ki67 levels, doctors can learn more about the likelihood of disease progression and possible treatment results, enabling them to make better treatment decisions and provide better patient care (Hammond et al., 2010). Molecular classification is used as a basis to identify breast cancer. Individuals may gain an advantage from specialized treatments like anti-HER2 and hormonal treatment (Gupta et al., 2018). Out of these main subtypes, luminal tumours are further split into three categories from an immunohistochemistry (IHC) perspective (Gao & Swain, 2018).

Luminal A:

Characteristics of this subtype are low levels of Ki-67, a cell proliferation biomarker, negligible HER2 expression, and PR &/or ER expression. On a clinical level, it displays low-grade characteristics, slow growth, and the best prognosis, which is defined by reduced relapse rates and greater survival rates. Contrary to chemotherapy, hormone treatment, such as tamoxifen or aromatase inhibitors, exhibits a high rate of positive response in luminal A cancers (Higgins & Stearns, 2009). The National Comprehensive Cancer Network of the United States (NCCN) and the European Society for Medical Oncology (ESMO) Guidelines recommended using genomic platforms to determine. Luminal A tumour patients may benefit from adjuvant chemotherapy treatment. These tools can be used to calculate a patient's relapse risk and survival rate (Zhou et al., 2020) (Paluch-Shimon et al., 2020).

Luminal B:

In comparison A type, B type is distinguished by a higher grade and a less favourable prognosis. PR and ER positivity, as well as a high expression of the cell proliferation marker Ki67 (more than 20%), are all present in luminal B malignancies. Due to the moderate to advanced histopathological grade of the majority of luminal B malignancies, hormonal treatment in combination with chemotherapy might prove beneficial. Due to their elevated Ki67 expression, which indicates an increased rate of development, tumors have a poor prognosis than A type (Inic et al., 2014). It makes up roughly 10% to 20% of luminal tumours. In comparison to oestrogen receptors, which are expressed at a somewhat low level, it expresses cell cycle and proliferation genes more strongly. Luminal B tumours are the luminal tumour subtypes having poor prognosis but they can still take advantage of hormone therapy along with chemotherapy. In fact, Luminal B tumours exhibit a better rate of chemotherapeutic response than Luminal A tumours (Lafcı et al., 2023). Luminal B tumours often recur in the bones, but they also have a greater rate of visceral recurrence and a shorter period between the first diagnosis and relapse, which leads to a worse survival rate.

HER2-positive:

10-15% breast cancer cases are of the subtype with HER2 positivity, which is characterized by an elevated level of HER2 protein expression and a lack of expression of ER and PR. Breast tumours that are HER2-positive grow more quickly than luminal tumours and have a better prognosis now that HER2-targeted medicines are available. This subtype of breast cancer spreads rapidly and aggressively as compared to other subtypes. The HER2-positive subtype of breast cancer can be classified into two subgroups depending on the presence of the ER, PR, and the cell proliferation marker Ki-67. The first subgroup, known as luminal HER2, is distinguished by the presence of ER, PR, and HER2, along with Ki-67 expression level between 15 to 30 percent. The second category is referred to as HER2-enriched, and it is composed of individuals who have elevated levels of Ki-67 expression of over 30% and HER2 overexpression without ER or PR expression (Krishnamurti & Silverman, 2014).

Both HER2-positive breast cancer subtypes require specialized treatments that block the HER2/neu protein and have a worse prognosis than luminal tumours. In addition to surgery and targeted chemotherapy, these treatments also comprise trastuzumab, along with Emtasin

(T-DM1), tyrosine kinase inhibitors like lapatinib and neratinib Pertuzumab (Figueroa-Magalhães et al., 2014). Chemotherapy treatments have a high rate of success with them. The most typical location of disease spread is known as bone localization. This cohort experiences visceral relapses more frequently than the original group.

TNBC:

Around 50% of patients with ER-positive breast cancer express the PR, compared to ER-negative individuals who express the receptor significantly less. Breast cancer cells have higher PR and ER expression, and both of these receptors are commonly used as prognostic markers (Nicolini et al., 2018). It has been demonstrated that PR expression levels in breast cancer cells may indicate how the disease is going to progress. High levels of PR expression in particular are linked to a better prognosis for breast cancer patients, including increased survival rates in general, extended recurrence-free survival, and longer time to therapy failure or disease progression. This shows that PR may control the development and response to therapy for breast cancer. Low level of PR expression is frequently associated with more severe disease features, such as a greater probability of disease recurrence and a worse prognosis. In order to forecast illness outcomes and choose the best course of action for breast cancer patients, monitoring PR expression levels may be helpful (Purdie et al., 2014).

TNBC Subtypes:

TNBC has fewer treatment possibilities than other forms of breast cancer, the probability of metastasis and recurrence is high, as well as it has a poor prognosis. The fundamental cause is that specialized endocrine medications and targeted therapies are unsuccessful because ER, PR, and HER2 expression are all negative.

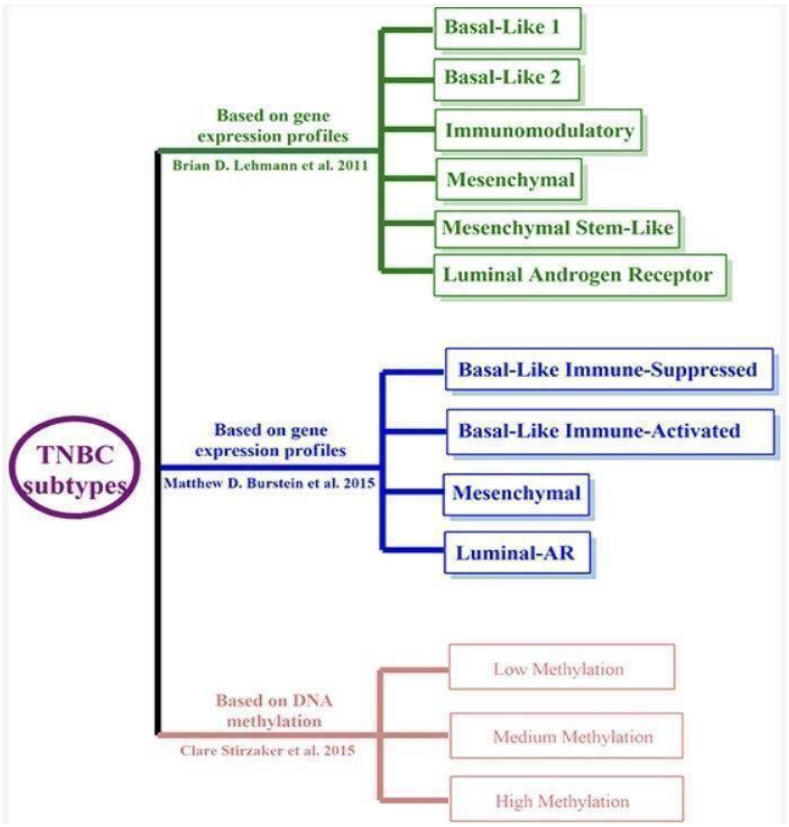

Figure 6: Subtypes of TNBC (Burstein et al., 2015)

Younger patients are more likely to be diagnosed with the TNBC subtype, which attributes to 20% of breast cancer cases, and African-American women in particular. Luminal androgen receptor (LAR) frequency in TNBC, basal-like (BL1 and BL2), claudin-low, mesenchymal (MES), and immunomodulatory (IM) subgroups are all shown in Figure 5 as further divisions of TNBC. Around 50 to 70 percent of cases belong to the basal-like subgroups (BL1 and BL2), whereas the other subgroups are less common (Kumar & Aggarwal, 2016). Additionally, each of these subgroups has unique clinical outcomes shown in Figure 6, behavioural traits, and responses to drug therapy. TNBC accounts for eighty percent of malignancies that lack the genes that suppress tumour i.e., BRCA1 and BRCA2 genes, which is distinguished by an aggressive clinical course (Loibl & Gianni, 2017). Inheritance, color, age, the nursing process, equality, overweight and obesity, all influence the chance of having TNBC (Kumar & Aggarwal, 2016). TNBC is renowned for being aggressive, having a high risk of recurrence early on, and having a tendency to be discovered at an advanced stage.

TNBC shows increased genomic instability, DNA repair gene mutations, and cellular proliferation at high rates. Histology reveals that it is a highly proliferative, heterogeneous neoplasm with limited differentiation. Additionally, there are subgroups of this subtype with a variety of prognoses. Immunohistochemical studies can further categorize TNBC into basal and non-basal subtypes. CK5/6 cytokeratin expression is absent in non-basal TNBC, whereas the expression of EGFR1 and cytokeratins (CK)5/6 are indicators for basal TNBC.

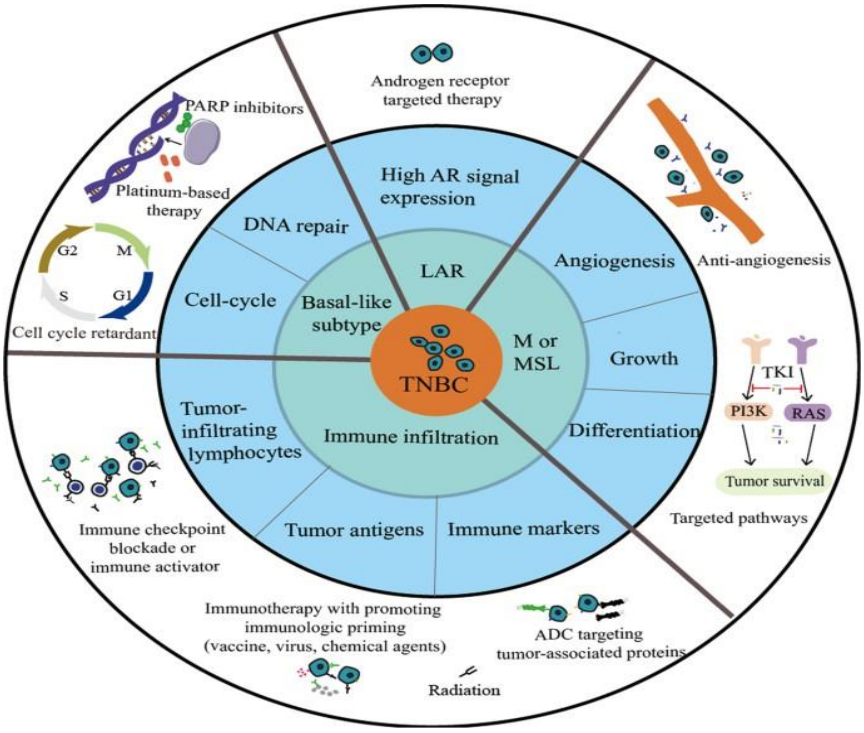

Figure 7: Classification and therapeutic options for TNBC (Li et al., 2022)

In comparison to luminal A subtype, the TNBC subtype shows a higher propensity to form metastases in the brain, liver, and lungs. Nevertheless, metastases in the bone are considerably less likely to form. Patients with TNBC have an 85.5% of 4-year survival rate, which is similar to the 94.2% of 4-year survival rate among individuals without it (Rhee et al., 2008).
TNBC patients experience tumour recurrence for an average of 1.2 years, which is less time as compared to non-TNBC patients. Moreover, the prognosis for TNBC patients with

recurrent breast cancer is typically worse. As compared to other BC subtypes, TNBC carries a greater likelihood of both tumour relapse and mortality. TNBC patients exhibited a ratio of 4.2 in comparison to those with other subtypes, which denotes a 4.2-fold increased chance of cancer recurrence. This shows that in order to lower the risk of recurrence and increase their chances of survival, TNBC patients may need more intensive treatment and vigilant monitoring (Mersin et al., 2008). The 5-year survival rates for various breast cancer types vary. Triple-positive breast cancer has a 91% 5-year rate of survival. On the other hand, TNBC and cancers that are HR-positive/HER2-negative had survival rates of 81% and 94%, accordingly. Hence TNBC have a lesser chance of surviving at the 5-year milestone than those whose tumours are HR-positive and HER2-negative.

Current Therapies for TNBC:

The current therapies for TNBC are shown below in Figure 8, which are helpful in treating TNBC patients.

Figure 8: Current treatment for TNBC

Immunotherapy:

The introduction of immunotherapy has given patients with TNBC new hope. Immune checkpoint inhibitors (ICIs) have the potential to offer patients with TNBC considerable benefits by opening a new therapeutic option that wasn't previously available. This indicates

a positive advancement in the realm of TNBC treatment and could benefit patient outcomes. TNBC is currently being treated with novel immunotherapy techniques, such as adoptive cell therapy and oncolytic viruses. These strategies entail attacking and eliminating TNBC cells with oncolytic viruses or T cells that specifically target proteins like TIL metastasis and carcinoembryonic antigen. These innovative approaches provide encouraging new choices for TNBC treatment and could enhance patient outcomes. The aforementioned phrases illustrate the potential of immunotherapy as a novel approach for treating TNBC, but they also highlight the need for more study and development before these treatments may be employed extensively in the clinic. One such prospective tactic that can improve anti-cancer immunity is the creation of a breast cancer vaccine. Possible adverse reactions associated with cancer vaccines could be caused by an immune system reaction that is misguided and attacks normal cells that also express targeted proteins. A promising strategy for enhancing results for TNBC patients is the use of combination treatments. We may be able to provide a treatment for this aggressive type of breast cancer, or at the very least greatly increase survival rates, by figuring out the most efficient immunotherapy combinations.

Chemotherapy:

Additionally, there isn't a test to decide if chemotherapy must be administered. Instead, therapy options are determined by a variety of variables, including the tumour's grade and stage as well as if it lacks a hormone receptor or overproduces the protein known as HER2. It may be possible to find individuals who have ER-positive, HER2-negative, and node-negative cancers of the breast whose outlook is so excellent that chemotherapy isn't likely to be beneficial using the findings of the 21 gene testing (Oncotype DX). Chemotherapies come in a variety of forms and are frequently administered in tandem or sequentially in the adjuvant situation. The treatment plan which works most suitable for you is going to be determined by your oncologist. In addition, if the tumour is advanced, your medical oncologist may suggest giving chemotherapy prior to surgery.

Targeted therapy:

TNBC is a complicated illness that has a dismal prognosis and infrequently used targeted therapies. Investigating cutting-edge, specific treatment approaches is essential. In TNBC individuals who have BRCA1/2 mutations, blocking PARP has so far demonstrated

considerable effectiveness. For those suffering from TNBC with the wild-type BRCA1/2, the combination of PARP inhibitors and DNA-damaging chemotherapy is extremely hopeful (Li and others, 2021). Examples include the TNBC's blocking of poly (ADP-ribose) polymerases, suppression of EGFR, and suppression of PI3K/Akt/mTOR pathway.

Surgical Reception:

If the initial TNBC tumour is sufficiently tiny to be excised surgically, surgery to preserve the breast or a surgical mastectomy may be performed after checking the lymph nodes for cancer. If the lymph nodes are confirmed to have malignancy, or if the tumour is broad, radiotherapy may be administered after surgery.

Radiotherapy:

Cancer of the breast can be effectively treated with radiation treatment practically at any stage. An excellent method to lower your risk for cancer of the breast coming back after surgery is radiation therapy. Additionally, it is frequently used to relieve signs associated with cancer that has metastasized from the breast to various body parts. The use of radiation followed by a lumpectomy is frequently referred to as breast conservation therapy. This form of treatment is equally as successful as a mastectomy, which involves removing all of the breast tissue. Radiation from an external beam given to the entire breast, also known as whole-breast radiation exposure, is one of the most often used forms of radiation treatment following the removal of a lump. In some of the initial stages, carcinomas of the breast, limited breast irradiation, or radiation treatment can be a choice. This method exposes the region where the tumour was excised to either internal or external radiation.

Other systemic treatments:

Some women with "triple negative" cancers will benefit from immunotherapy, which combats cancer by activating its own defenses. The administration of a drug known as a PARP inhibitor after additional treatment can be advantageous for women with either BRCA1 or BRCA2 mutations in their genes who have cancer that is HER2 negative but have high-risk characteristics.

Luminal Androgen Receptor:

The AR expression separates LAR from other breast cancer subtypes and has a potential impact on available treatments and patient's rate of survival. 15–20% of TNBC is represented by the LAR subgroup. It is characterized by the expression of AR proteins and androgen-induced promotion of growth in vitro that can be prevented by adopting strategies that specifically target the AR. In accordance with studies, LAR malignancies have a distinct gene expression pattern from other TNBC subgroups (Thompson and others, 2022). The LAR subtype has been identified by widespread metastasis in local lymph nodes as well as a propensity or a preference towards bone lesions in situations of distant metastasis. It is important to note that the LAR subtype had a lower rate of pathological full responses to chemotherapeutic (65.6% vs. 21.4%) when contrasted with BL1 TNBC (Echavarria et al., 2018). Approximately 60%–80% of the total breast tumours have AR expression, particularly ER-positive tumours showing the highest prevalence (Asano et al., 2016; Pietri et al., 2016; Bozovic-Spasojevic et al., 2017; Kensler et al., 2019). Moreover, it has been discovered to be less sensitive to chemotherapy and less likely to experience a pathologic full response following neoadjuvant therapy. These traits make LAR a distinct clinical subtype with specific characteristics that must be considered when creating treatment plans for people with this type of breast cancer. AR-positive are defined as malignancies having conflicting information regarding the course and outlook for the illness (Vidula et al., 2019).

Androgen levels in Normal individuals:

Depending on factors like age, gender, health history, and other variables, total androgen levels in men and women under normal circumstances can differ. The values are indicated in nanograms per decilitre (ng/dL). For men, usual androgen levels are between 270 and 1,070 ng/dL, and for women, 15 to 70 ng/dL.

Androgen Receptor (AR): Structure, Function, and Role in Cancer progression:

AR gene is situated at location Xq11-Xq12 on the long arm of the X chromosome. It generates a protein with a molecular weight of 110 kD and an amino acid count of 919. The DNA binding domain (DBD), N-terminal domain (NTD), and ligand binding domain (LBD), which is connected to DBD via the hinge region, are the three primary functioning domains that make up an AR. While the AF2 (activation function 2) of LBD requires a ligand to function, the AF1 (activation function 1) of NTD is always active. (Tan et al., 2015).

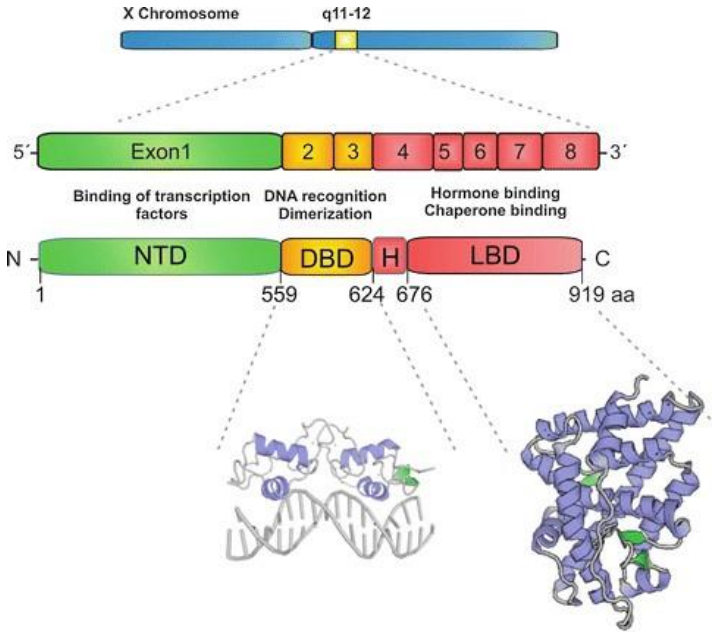

Figure 9: Structure of Androgen receptor (Sutinen et al., 2017)

AR signaling pathway:

AR is a cytoplasmic receptor that upon binding to its Androgen ligand gets translocated into the nucleus and stimulates transcriptional activity of an androgen-responsive element (ARE) (Davey & Grossmann, 2016). The biosynthesis of Androgen is catalyzed by Cytochrome P450 (CYP) enzymes. Testosterone thus formed is converted into 5-alpha

Dihydrotestosterone (DHT) with the help of the 5-alpha reductase enzyme (Naamneh Elzenaty et al., 2022). AR signaling occurs via canonical as well as non-canonical pathways. In the canonical or genomic pathway, testosterone is converted into dihydrotestosterone by the action of 5 alpha reductase enzyme. Initially, AR in its inactivated form is bound to HSP70 and HSP90 in the cytoplasm. Upon binding to DHT, AR is activated and phosphorylation of AR takes place which results in the release of heat shock proteins. The ligand receptor complex thus translocates into the nucleus and binds to DNA which recruits coactivators and acts of Androgen Responsive Elements (ARE). Thus, transcriptional activities will lead to cell growth and proliferation (Heinlein & Chang, 2002). In the non-genomic pathway, AR signaling occurs in the absence of Androgen via the activation of other pathways such as Src Kinase, JAK/STAT, PI3K, etc and eventually leads to cell proliferation (Eder et al., 2001).

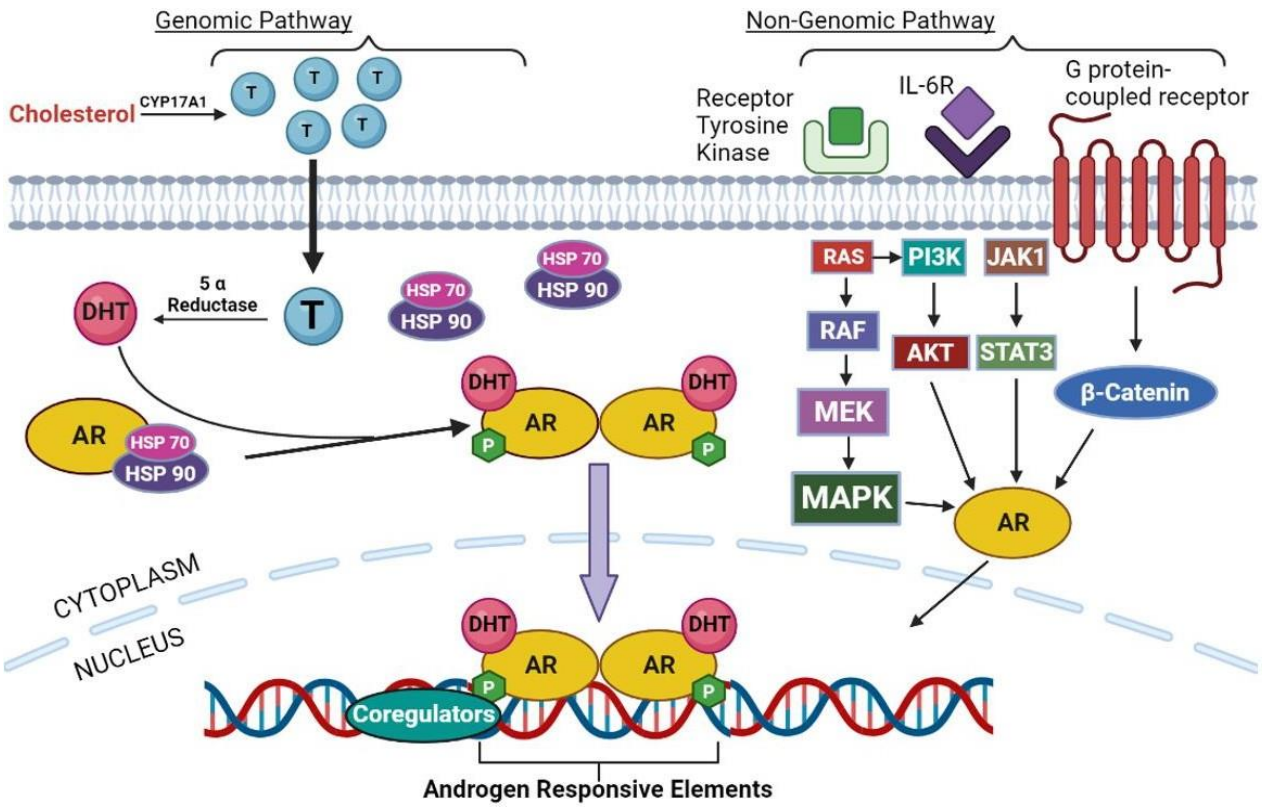

Figure 10: Androgen Receptor (AR) Signalling Pathway

DHT:

Testosterone is reduced which leads to the production of DHT. 5alpha-reductase has recently been found to have two isoenzymes. The predominant form in sebaceous glands is Type 1 and can be found in the majority of bodily tissues where 5alpha-reductase is produced. The main isoenzyme which is present in genital tissues, including the prostate, is Type 2, 5 alpha-reductase. Male pattern baldness and benign prostatic hyperplasia have both been treated with finasteride, a 5-alpha-reductase inhibitor because as compared to the Type 1 isoenzyme, it has a much lower affinity, its main impact at clinical levels is the inhibition of Type 2 5alpha-reductase. DHT is being suppressed due to finasteride by as much as 85–90% in the prostate and by about 70% in serum (Swerdloff et al., 2017).

AR Splice variants:

The isoforms of the AR mRNA which are abnormally spliced are known as AR variants (AR-Vs) which typically produce shortened AR proteins. The NH2-terminal transactivation domain (NTD) and DNA-binding domain (DBD) are the major domains that all AR-Vs and wild-type full-length ARs (AR-FL) have in common. The stringent androgen-dependent control of AR is ensured by the existence of a functional and intact ligand-binding domain (LBD). However, the LBD and other variable COOH-terminal domain components are absent in AR-Vs (Kallio et al., 2018). Exons 2 or 3 may be replaced by a cryptic exon in AR-Vs, which typically have exons 1-3, and exons 4–8 may be alternately included or excluded, resulting in a loss in the LBD. LBD, which is the therapeutic target of all currently available AR-targeting medicines, is absent from the majority of AR-Vs (Kallio et al., 2018).

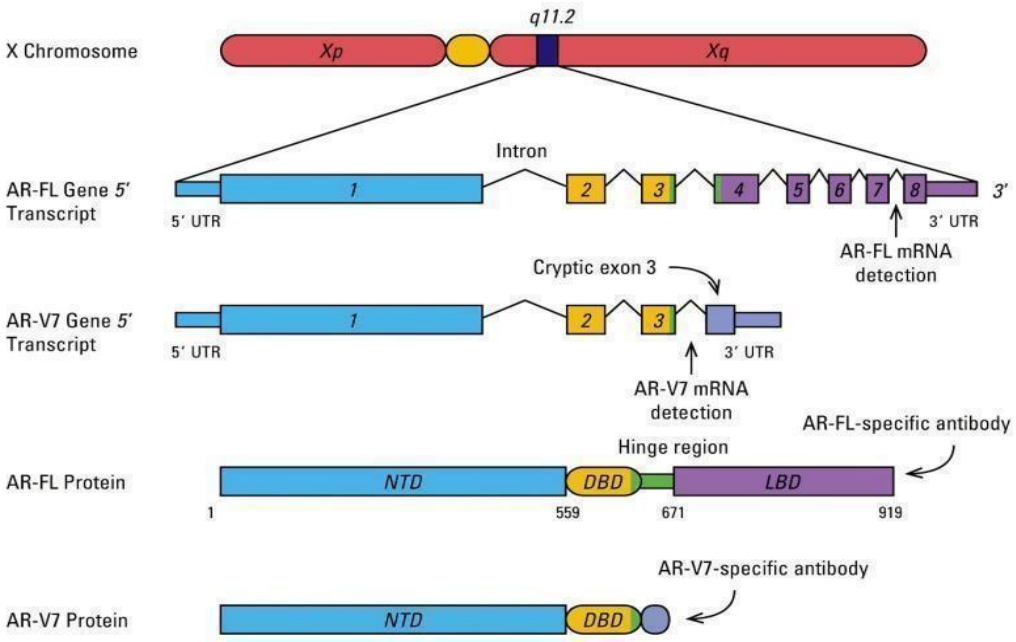

Figure 11: Transcript structures for full-length AR and splice variant AR-V7 (Aurilio et al., 2020)

The AR splice variants that do not have LBD were first isolated from prostate cancer cell lines that originated from one patient and have since been found in both benign and malignant human prostate tissue, with the highest levels found in castration-resistant prostate cancer (CRPC), which is in its late stages (Watson et al., 2010). There have been 22 distinct AR-Vs described so far (Kallio et al., 2018). In CRPC, new anti-AR treatment resistance mechanisms are linked with the presence of AR-Vs. There is evidence that AR-Vs, which are frequently found in patients with primary breast cancers, encourage the proliferation of aberrant cells and aid in the emergence of androgen deprivation therapy resistance (ADT). These AR-Vs may be derived via 2 mechanisms: Genomic rearrangement and/or alternate pre-mRNA splicing (Watson et al., 2015). The most prevalent variant that is mainly overexpressed is AR-V7 (exons 1/2/3/CE3), which has been found to be present in >50% of all breast cancers at the mRNA level and in a small percentage of ER-negative tumours at the protein level. Around half of the BCs evaluated had AR-V7, with ER-negative instances that were HER2-enriched showing the highest expression (Ferguson et al., 2022).

AR Antagonists:

AR antagonists are drugs that bind specifically to AR and inhibit its activity. Antiandrogens and testosterone blockers are a class of medications that stop androgens like testosterone and dihydrotestosterone (DHT) from exerting their biological effects in the body. These medications are often referred to as AR antagonists. They work by reducing or suppressing the production of androgen as well as blocking the AR. Antiandrogens are classified into steroidal and nonsteroidal antiandrogens based on their chemical compositions (Akakura et al., 1998). Contrary to nonsteroidal AR antagonists, which are not steroids and have a structurally different structure, steroidal AR antagonists are related to steroid hormones like progesterone and testosterone structurally. Due to their structural resemblance to other steroid hormones, steroidal AR antagonists frequently have unintended hormonal effects. Contrarily, nonsteroidal AR antagonists are hormonally inert and very selective for the AR (Singh et al., 2000).

Table 2: Classification of AR antagonists

AR antagonists	
Steroidal	**Non-Steroidal**
Cyproterone acetate	Bicalutamide
Megestrol acetate	Enzalutamide
Chlormadinone acetate	Flutamide

Spironolactone	Nilutamide
Oxendolone	Topilutamide
Osaterone acetate	Apalutamide

Mode of action of Bicalutamide & Enzalutamide:

Both Bicalutamide and Enzalutamide, FDA-approved medications for the treatment of prostate cancer, are anti-androgens or AR antagonists (Beer et al., 2014). Trials are being conducted to evaluate their efficacy for treating the LAR subtype of TNBC. Both these drugs have diverse modes of action against the proliferation of abnormal cells the and progression of cancer. Bicalutamide prevents DHT from binding to androgen receptor via competitive inhibition due to which there will be blockage of androgen action and hence cancer cell proliferation will not take place (Tran et al., 2009).

Enzalutamide targets multiple points in the signaling pathway of androgen receptors like it stops the binding of testosterone to its receptor hence blocking the conformational change induced by the ligand-receptor complex. It also reduces the translocation of the Androgen receptor into the nucleus hence AR will remain majorly in the cytoplasm. Also, it can block the AR from binding to DNA and will interfere with coactivator recruitment. Eventually, there will be a decline in cancer cell growth and increased apoptosis will lead to decreased breast tumour volume (Patel et al., 2014).

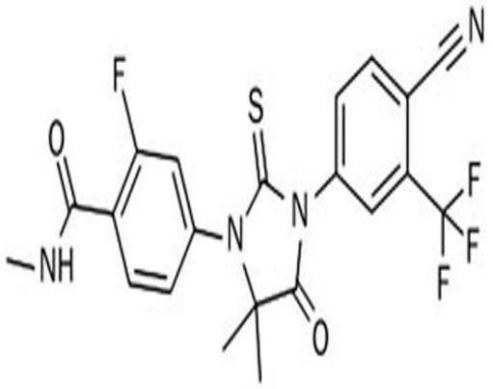

Figure 12: Structure of Enzalutamide (Tan et al., 2015)

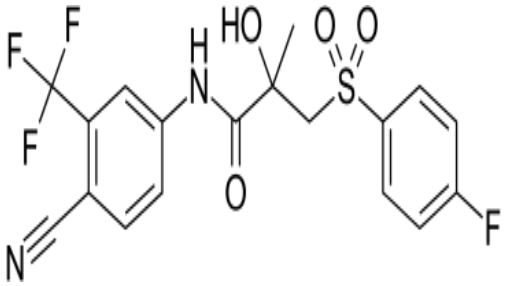

Figure 13: Structure of Bicalutamide (Tan et al., 2015)

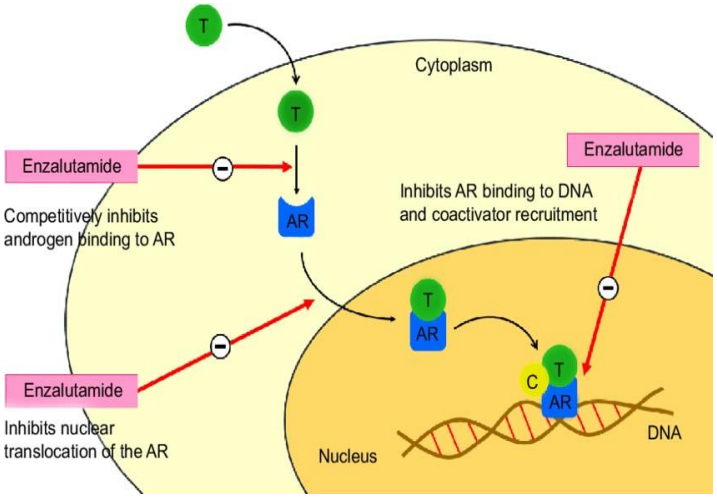

Figure 14: Mode of Action of Enzalutamide (Rodriguez-Vida et al., 2015)

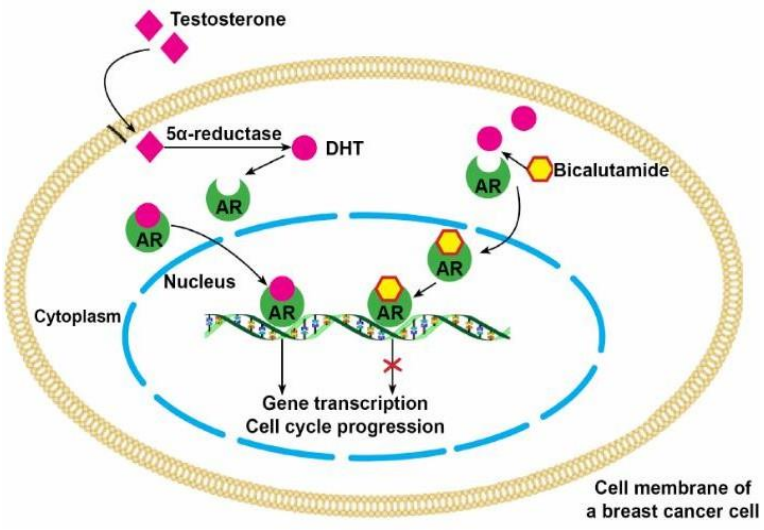

Figure 15: Mode of Action of Bicalutamide (Nguyen et al., 2018)

AIM

- To examine the efficacy of AR antagonists and determine the role of AR in TNBC.

OBJECTIVES

- To check the cell viability in TNBC cell lines when treated with AR antagonists (Bicalutamide and Enzalutamide).
- To examine the genotypic expression of AR and its variant AR-V7 on treatment with AR antagonists.

MATERIALS AND METHODS

Cell lines used:

MDA-MB-453 (M.D. Anderson - Metastatic Breast - 453)

- It expresses the androgen receptor and is 'triple-negative' in terms of the expression of ER, PR and HER-2/neu protein (Vranic et al., 2011).

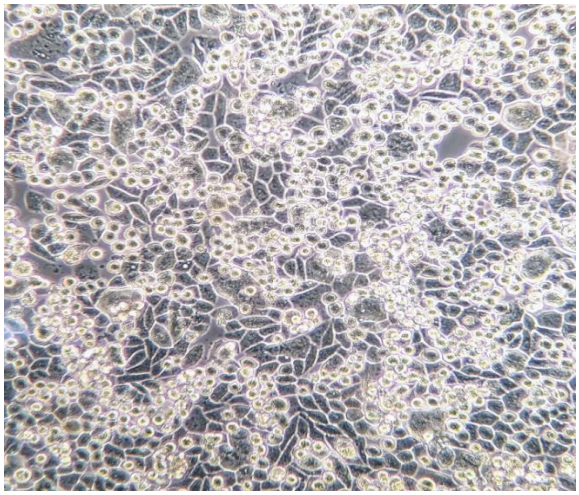

Figure 16: MDA-MB-453 (20X Magnification)

MDA-MB-231

- It is a TNBC cell line that does not express ER, PR, or Her-2. The Dulbecco's Modified Eagle Medium (DMEM)-based complete growth medium was used to cultivate and grow the cells as a monolayer.

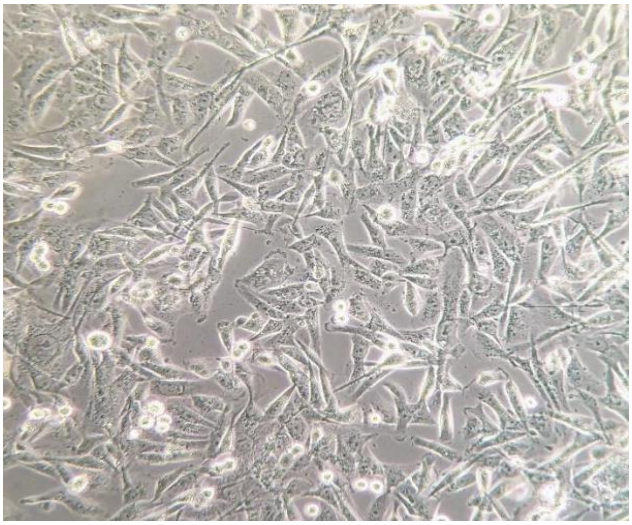

Figure 17: MDA-MB-231 (20X Magnification)

MCF-7 (ER+/PR+)

- A cell line from human breast cancer cells that expresses glucocorticoid, progesterone, and estrogen receptors.

Tissue culture flasks (#25, #75) were used to cultivate all three cell lines at 37 °C, in humid environment, in a 5% CO2 incubator.

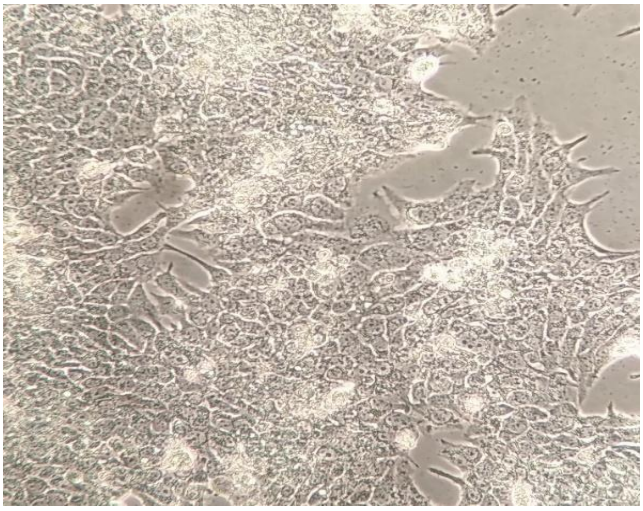

Figure 18: MCF-7 (20X Magnification)

Complete Growth Medium:

- DMEM + 10 % FBS + 1% Antibiotic/Antimycotic.

Drugs Treatments:

- Bicalutamide
- Enzalutamide

1). Revival of cells:

Materials

1). Cryovial containing cell line

2). Complete growth medium

3). 15 ml Centrifuge Tube

4). Tissue culture flask

5). Sterile Serological Pipettes

6). Isopropanol

7). Tissue paper roll

Equipment

- Water bath
- Bio-Safety Cabinet
- Co2 Incubator
- Centrifuge

Procedure:

1. Cells were brought from -80 °C storage facility and were immediately placed in the water bath at 37 °C.
2. Tissue culture flask was labelled properly along with mentioning the passage number.
3. Complete growth medium was added into cryovial dropwise in order to prevent cells from shock.

4. A 15 ml centrifuge tube containing 3–4 ml of full growth media was used to transfer the cell suspension from the cryovial.
5. Run a centrifuge at 1,000 rpm for five minutes.
6. The pellet was resuspended in approximately 1 ml of complete growth media after discarding the supernatant.
7. Re-suspended cell suspension was added to a T25 tissue culture flask along with 4–5 ml of growth media.
8. Flask was swirled gently and was incubated in a C02 incubator at 37 °C.
9. We observed the cells regularly to check confluency and to avoid contamination.
10. We changed the media on alternative days.

2). Subculture:

Materials

1). Complete media

2). 1X PBS

3). Trypsin

4). IP

5). Tissue roll

6). T-25 Flask

Equipment

- Pipette
- Incubator
- Water bath
- Centrifuge
- Inverted phase contrast microscope
- Haemocytometer

Procedure:

1. Use an inverted microscope to examine the culture in order to determine its level of confluency and to ensure that it is free from any type of contamination.
2. Remove the used media.
3. Use 1X PBS to wash the cell monolayer.
4. Trypsinize the flask.
5. Employ centrifugation to pellet down the cells. (at 1000 rpm for 5 mins)
6. The supernatant was discarded and the pellet was resuspended in 1 ml complete media.
7. Next, transfer this into a T-25 flask.

3). Cryopreservation:

Materials

1). Cryo vials

2). Freezing medium

3). Mr. Frosty

4). Ice

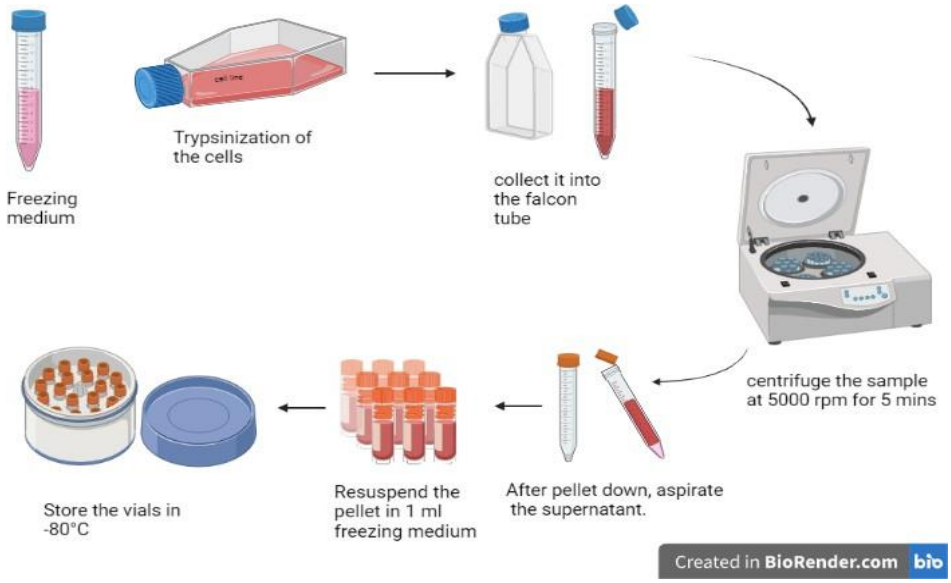

Figure 19: Cryopreservation protocol

Procedure:

1. To dislodge the cells from the flask, trypsin was utilised.
2. Centrifugation was carried out for 5 minutes at 1,000 rpm.
3. The pellet was resuspended in a freezing medium at this point.
4. Aliquot into vials for cryogenic storage. The freezing process was started within 5 minutes after the vials were placed on wet ice or in a 4°C refrigerator.
5. Freeze the cells at a rate of 1°C per minute. A programmable cooler can be used for this, or the vials can be put in an insulated box and then placed in a freezer that is between -70°C and -90°C before being moved to liquid nitrogen storage.

4). Cell counting (Trypan blue):

Materials

1). Sterile Eppendorf tubes (1.5 ml)

2). Trypan Blue

3). Neubauer's Chamber (Haemocytometer)

Procedure:

1. 1:1 solution was prepared containing suspension cells and Trypan blue dye.
2. 10 µL from the solution was loaded in a haemocytometer.
3. Cell viability was calculated using the following formula:

% Viability = Total no. of viable cells (Unstained) / Total no. of cells (Viable +Dead) X 100

5). Cell Viability Assay (MTT):

Materials

1). MTT Dye

2). DMSO

3). Drug treatment

4). 96-Well Cell Culture Plates

5). Complete growth medium

6). Sterile Serological Pipettes

Procedure:

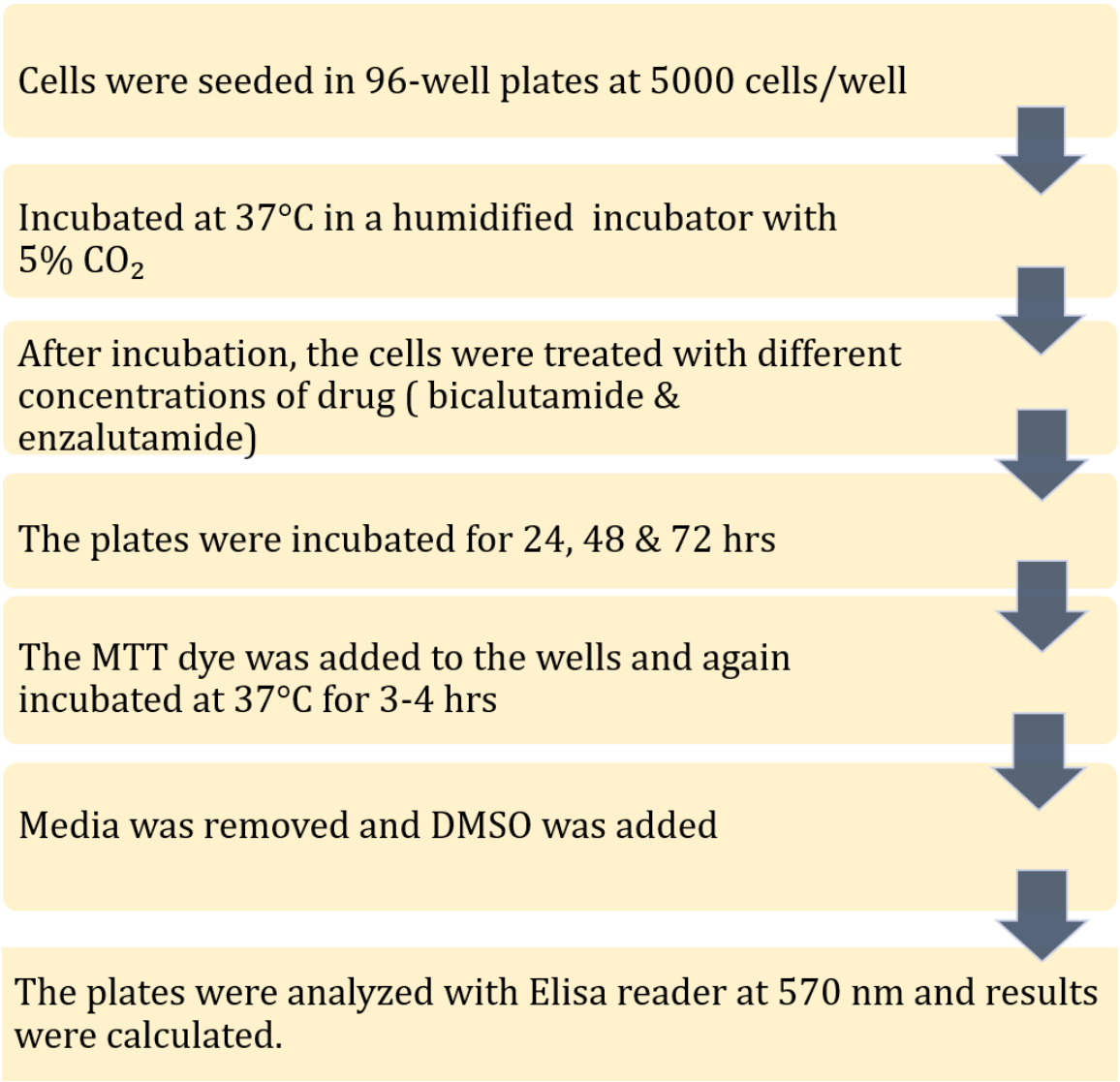

Figure 20: Steps involved in MTT assay

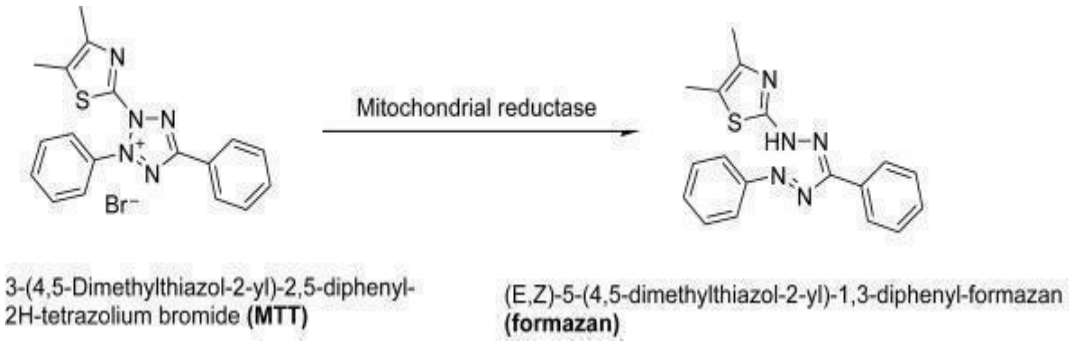

Figure 21: Reduction of MTT-to-MTT formazan (Kumar et al., 2018)

6). Growth Curve Analysis:

Material

1). 6 well plates

2). Trypsin

3). Trypan blue

4). Complete Media

5). 1X PBS

Procedure:

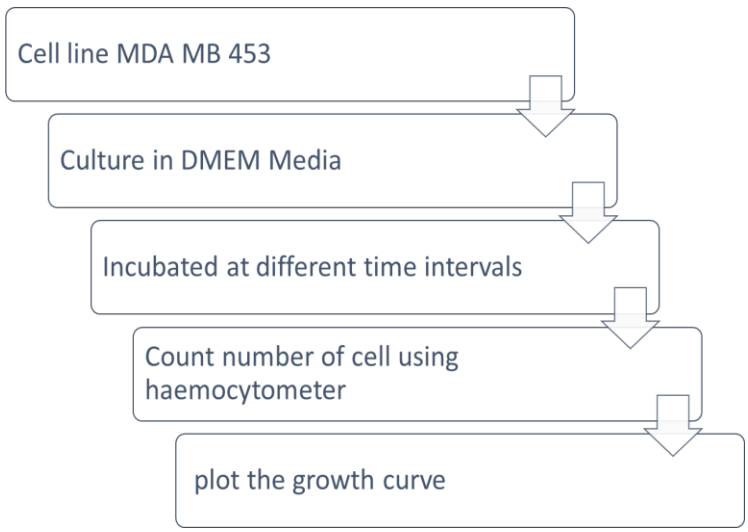

Figure 22: Growth Curve Protocol

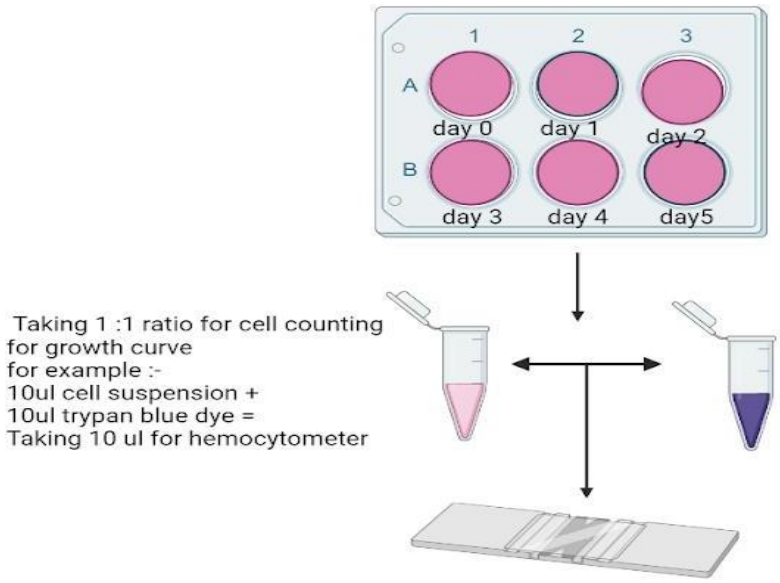

Figure 23: Growth Curve Cell Counting

8). RNA Isolation:

Materials

1). Qiazol lysis reagent

2). Chloroform

3). Isopropanol

4). 75% Ethanol

Procedure:

1. Cells were harvested and stored in 800 µL of Qiazol lysis reagent in -80 °C.
2. Frozen lysate was thawed at room temperature.
3. 200 µL of chloroform was added for 1 ml of lysate. However, we can adjust volume based on lysate volume.
4. Vigorous shaking was done for 15 seconds and lysate was kept at room temperature for 2-3 minutes.
5. Centrifuge at 12,000x g for 15 minutes at 4 °C.

6. Upper aqueous phase was transferred to a new tube. It is important to avoid disturbing the interphase.
7. After the addition of 0.5 ml of isopropanol, vortexing was done in order to mix it thoroughly.
8. Incubate for 10 minutes at room temperature.
9. Centrifuge at 12,000x g for 10 minutes at 4 ℃.
10. Careful aspiration was done, and the supernatant was discarded.
11. 1 ml of 75% ethanol was added.
12. Centrifuge at 7,500x g for 5 minutes at 4 ℃.
13. Supernatant was removed completely and the RNA pellet was kept to air dry.
14. RNA was redissolved in the appropriate volume of RNase-free water.
15. RNA was stored at a -20 ℃ freezer for further use.

9). Quantification using Nanodrop:

Figure 24: Thermo Scientific Nanodrop Lite

Materials

1). Dry laboratory wipe

2). Pipette 0.2-20 µL

3). Blanking buffer

Procedure:

1. From the home screen, select the required application (DNA or RNA).
2. Set a blank by pipetting 1-2 µl of the blanking buffer onto the bottom pedestal, lower the arm, and press the Blank button as directed on the screen.
3. After the measurement is finished, lift the arm and use a dry lab wipe to clean the buffer from the upper and lower pedestals.
4. Press Blank while lowering the arm and pipetting a fresh aliquot of blanking buffer onto the bottom pedestal.
5. After the measurement is finished, lift the arm and use a dry lab wipe to clean the buffer from the upper and lower pedestals.
6. Pipette 1-2 µl of sample onto the bottom pedestal, lower the arm and click measure to take a reading of the sample.
7. The instrument is prepared to measure the next sample after being cleaned with a dry lab wipe on both the top and lower pedestals.

10). Gel Electrophoresis:

Materials

1). 50X TAE Buffer

2). 1.2% Agarose

3). Tracking dye (Bromophenol blue)

4). Ethidium bromide (EtBr)

5). Electrophoresis unit

Procedure:

1. We prepared 1.2% Agarose in 1X TAE buffer. Use a microwave oven for proper dissolving.

2. Allowed to cool it and then added 2 µL/100 ml of EtBr from 10 mg/ml stock. Mix it properly.
3. Set up an electrophoresis unit.
4. Pour gel in the gel caster with a comb. (Avoid bubbles in gel)
5. Allow to solidify.
6. Remove the comb gently.
7. We kept the gel cast in a reservoir tank having 1X TAE buffer.
8. We loaded the prepared sample into the well. (8 µL sample + 2 µL tracking dye)
9. Switch on the unit. (Run on 50 V)
10. Allow ¾ run of tracking dye then stop the run.
11. Take the gel out of the unit and observe under UV.

11). cDNA Synthesis:

Materials

1). PCR Tubes

2). Pipette 100-1000 µL + Tips

3). 70% Isopropanol

4). Revert-Aid First strand cDNA synthesis kit (ThermoFisher #K1621)

Procedure:

➔ After thawing, mix & briefly centrifuge kit components. Store on ice.

➔ Add the below components into Maxiamp PCR tubes (Flat cap tube).

Table 3: Components of cDNA synthesis

NAME	QUANTITY
Nuclease free water	11 µL
PRIMER-Random Hexamer	1 µL
Template RNA	1 µL
5X Reaction buffer	4 µL
Ribolock Rnase inhibitor(20µ/µl)	1 µL
10mM dNTP mix	2 µL
Revert Aid M-MULV-RT	1 µL
Total volume	20 µL

➔ Mix it properly by short spin & amp; incubate for 5 minutes at 25°C.

➔ Give 60 minutes incubation at 42°C.

➔ Terminate the reaction by heating at 70°C for 5 minutes.

11). KAPA SYBR® FAST qPCR Protocol:

Materials

1). Primers details:

GAPDH_FP: TGGAAGGACTCATGACCACA

GAPDH_RP: TTC AGC TCA GGG ATG ACT

AR_FP: AAT TGT CCA TCT TGT CGT CTT CGG

AR_RP: GCC TCT CCT TCC TCC TGT AGT TTC

AR-V7_FP: TGTCACTATGGAGCTCTCACATGTGG

AR-V7_RP: CTG TGG ATC AGC TAC TAC CTT CAG CTC

2). Pipette 100 µL 1000 µL + Tips

3). PCR tubes

4). KAPA SYBR® FAST qPCR Kit

Procedure:

➔ Thaw 2x KAPA SYBR Green RT-PCR Master Mix, template RNA, primers,

QN ROX Reference Dye and RNase-Free water. Mix the individual solutions.

➔ Prepare a reaction mix according to the Table given below

Table 4: Components of Real-time PCR

Component	**20 µl rxn1**
PCR-grade water	6.8 µL
2X KAPA SYBR® FAST qPCR Master Mix[2] Universal	10 µL
10 µM Forward Primer	0.4 µL
10 µM Reverse Primer	0.4 µL
Template cDNA[3]	2 µL
50X ROX Low	0.4 µL

➔ Add template cDNA (≤100 ng/per reaction) to the individual PCR tubes or wells containing the reaction mix.

➔ Program the Real-Time cycler according to the given table.

Table 5: Real-Time PCR cycling conditions

Step	**Time**	**Temperature**	**Ramp rate**
Real-time Heat activation	2 min	95 °C	Maximal/fast mode
2-STEP CYCLING:			
Denaturation	3 sec	95 °C	Maximal/fast mode

Combined annealing/extension	10 sec	60 °C	Maximal/fast mode
Number of cycles	40		
Melting Curve Analysis			

➔ Place the PCR tubes or plates in the real-time cycler and start the cycler program.

➔ Perform a melting curve analysis of the PCR products.

RESULTS

1). Growth Curve:

By performing the growth curve in MDA-MB-453 we were able to know the doubling time of the cell line and its rate of proliferation.

Cells were seeded in a 6-well plate with a seeding density of 5×10^3 cells per well and incubated at different time intervals up to 120 hrs. Later, cells were trypsinized for counting and were stained with Trypan-blue dye. Cell counting was done by Haemocytometer.

Day 1 Calculation for reference	
Upper Left = 4	Upper Right = 2
Lower Left = 6	Lower Right = 7

Total = UL + UR + LL + LR /4

$=19/4 \times$dilution factor

$= 4.75\times 2 \times 10^4$

$= 9.5 \times 10^4$

This equation was used to find out the other day's reading which helped us to analyse the growth of cells.

Table 6: Results of Day 1 to Day 5

Day 1 (24 hr)	9.5×10^4
Day 2 (48hr)	14.5×10^4
Day 3 (72hr)	19×10^4
Day 4 (96hr)	25×10^4
Day 5 (120hr)	33.5×10^4

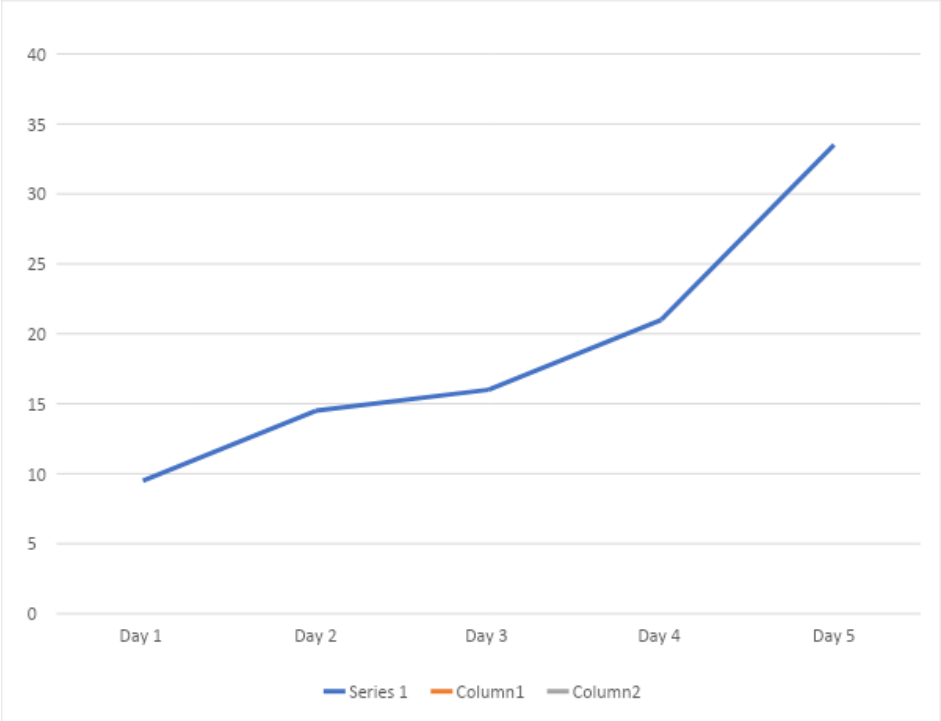

Figure 25: Cell proliferation ratio and the Days of observation

Table 6 shows that MDA-MB-453 cells were observed at different time intervals, and the rate of cell proliferation gradually increased. The cells doubled after 50 to 60 hours which can be seen in **Figure 25**.

2). MTT Assay:

MTT assay was carried out to check the efficacy of AR antagonists on the cell lines as described in the materials and methods section. MTT is a colorimetric assay to check the cell viability by using this assay we can check the cell proliferation after the drug treatment, it is based on the ability of NADPH-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye (MTT) to its insoluble formazan (purple) (Kumar et al., 2018). After performing the experiments, we found the IC50 concentration for the drugs. IC50 concentration indicates the amount of drug needed to inhibit a biological process or the rate of cell proliferation by half. It provides a measure of the potency of antagonists (Bicalutamide and Enzalutamide).

Table 7: Cell lines treated with different drug concentrations and DHT

Antagonists	MDA-MB-453	MDA-MB-231	MCF-7
Bicalutamide (µM)	1, 5, 10, 15, 20	1, 5, 10, 15, 20	1, 5, 10, 15, 20
Enzalutamide (µM)	-	1, 5, 10, 15, 20, 25, 30, 35, 40	1, 5, 10, 15, 20

Agonist			
DHT (To check the cell proliferation rate after adding the ligand with the antagonist)	10 nM	-	-

The cells were seeded in 96 well plates with the seeding density of 5 x 10^3 cells per well and treated with different concentrations of drug for different time intervals i.e., for 24, 48, and 72 hrs.

The Effect of Bicalutamide on the growth of MDA-MB-231 cell line

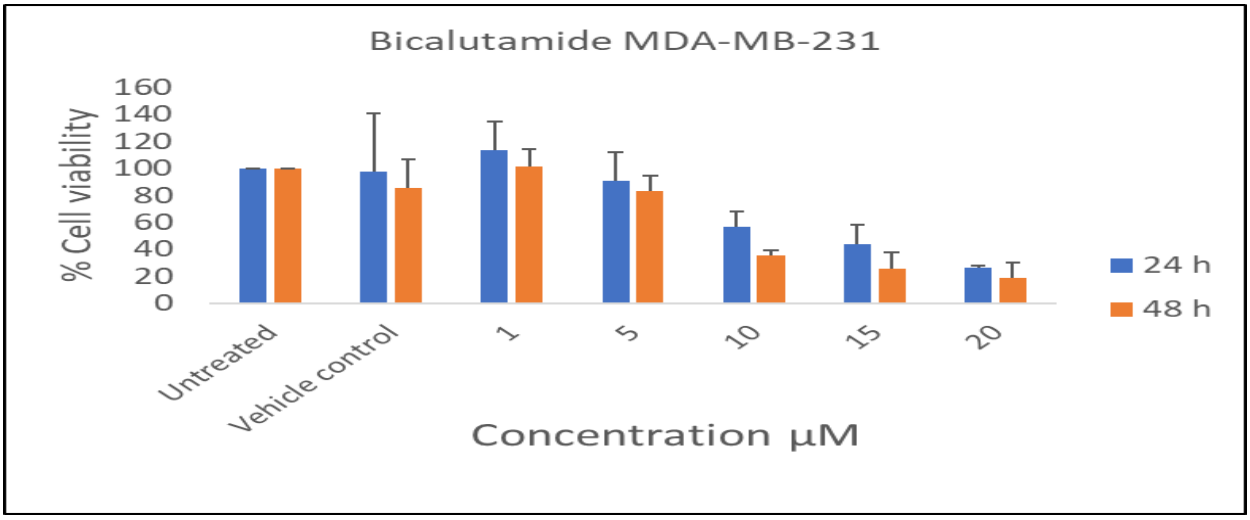

Figure 26: MDA-MB-231 cells were treated with different concentrations of Bicalutamide as described in Table 7 for 24 and 48 hrs to examine the cell viability*:* After 24 hrs, it was visible that the cells treated with 10, 15 and 20 µM drug concentrations showed a notable inhibitory effect which is represented in **Figure 26.**

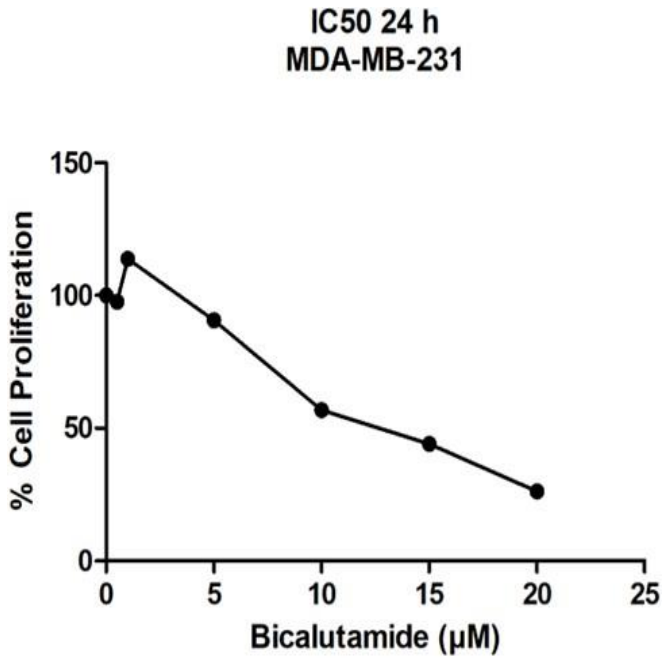

Figure 27: The viability of MDA-MB-231 cells at 24 hrs was determined using the nonlinear regression curve

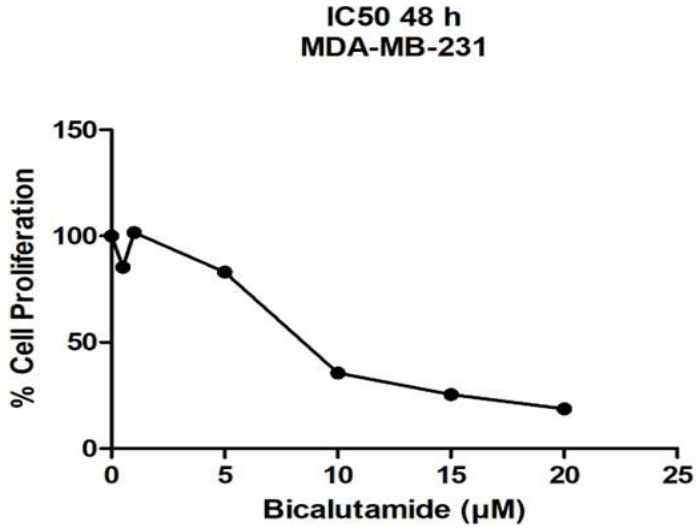

Figure 28: The viability of MDA-MB-231 cells at 48 hrs was determined using the nonlinear regression curve

In **Figure 27**, an assessment of the cytotoxic impact of Bicalutamide on MDA-MB-231 cells was made by performing an MTT assay. The calculation was made by comparing the proportion of viable treated cells to the vehicle control. As the concentration of bicalutamide increased, MDA-MB-231 cell survival decreased. After 24 hours of incubation of MDA-MB-231 cells, the IC50 concentration achieved was 10.27 μM.

As seen in **Figure 28**, an MTT assay was performed to measure the cytotoxicity of Bicalutamide on MDA-MB-231 cells and the percentage of viable cells was calculated on comparison to the vehicle control. After 48 hours of incubation, the IC50 concentration of Bicalutamide for MDA-MB-231 cells was 7.38 μM.

The effect of Enzalutamide on the growth of MDA-MB-231 cell line

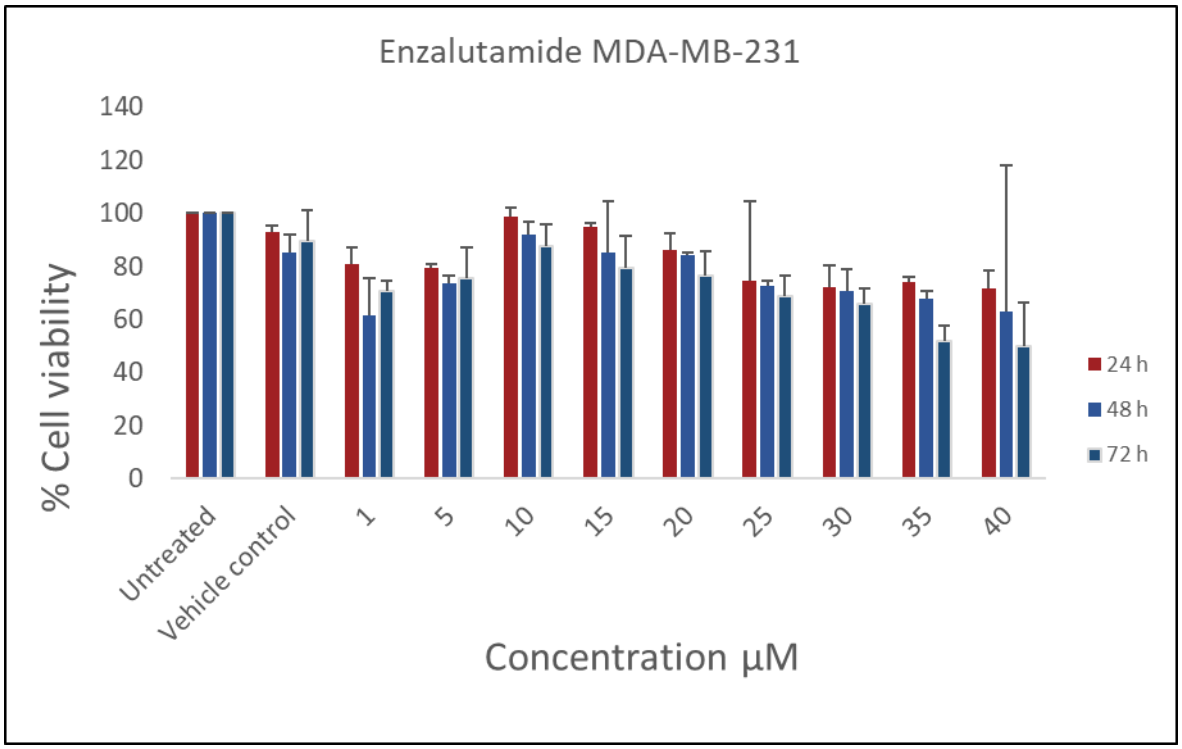

Figure 29: MDA-MB-231 cells were treated with different concentrations of Enzalutamide as described in Table 7 for 24, 48, and 72 hrs to test the cell viability

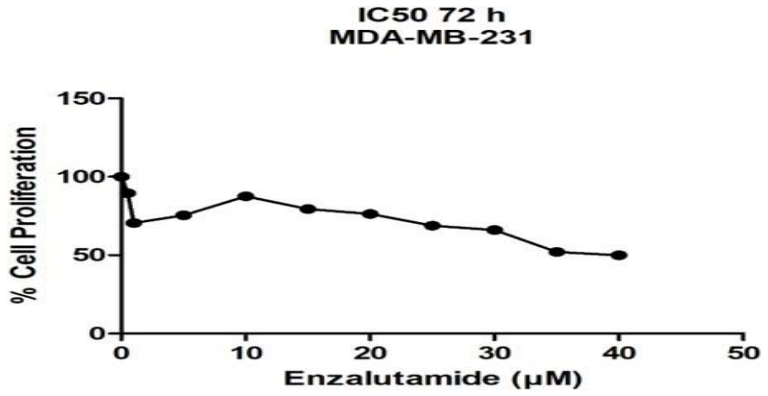

Figure 30: The growth curve was used to calculate the IC50 concentration of enzalutamide through the cell viability assay:

The MTT assay was used to measure cell viability. The results are shown in Figure 29 as a percentage of control in the mean SD of triplicates. The IC50 was measured in MDA-MB-231 at 37.20 µM after 72 hours.

The effect of bicalutamide on the growth of MDA-MB-453 cells

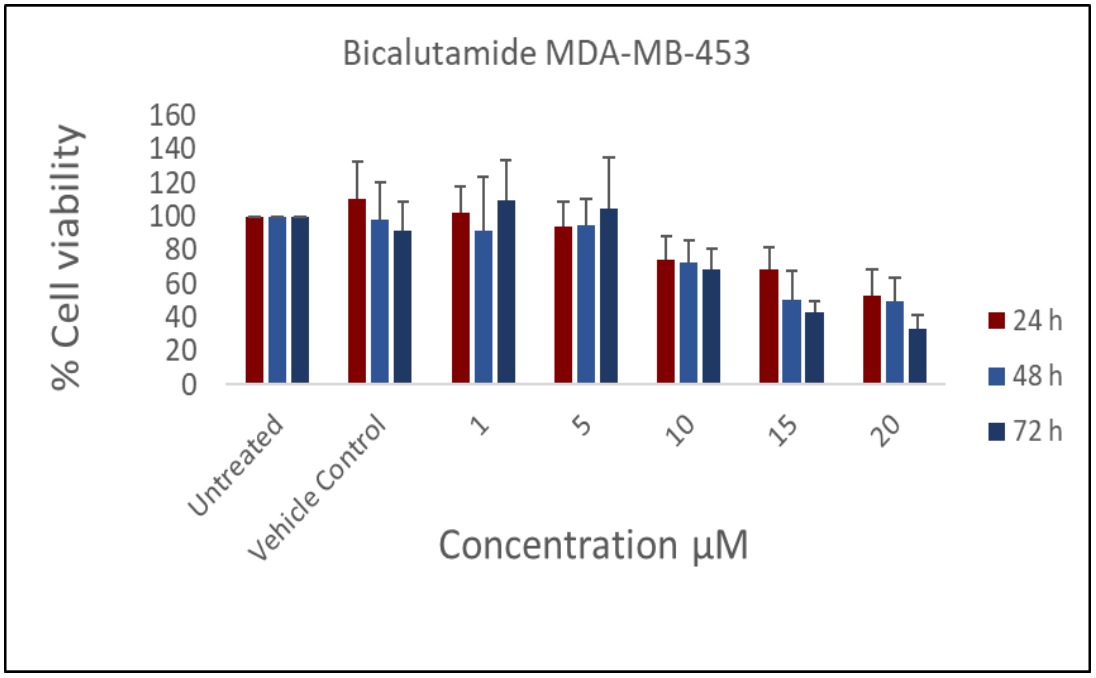

Figure 31: MDA-MB-453 cells were treated with different concentrations as described in the table of bicalutamide for 24h,48h and 72 h to examine the cell viability.

After 24 hrs of the treatment, it is visible that in 5, 10, 15, and 20 µM drug conc treated cells are given a notable inhibitory effect which is shown in **Figure 31.** and in 48 hrs the inhibitory effect was more observable, especially in 5,10,15 and 20 µM drug conc treated cells. and in 72 hrs the inhibitory effect was the most observable especially in 15 and 20 µM/mL drug conc treated cells.

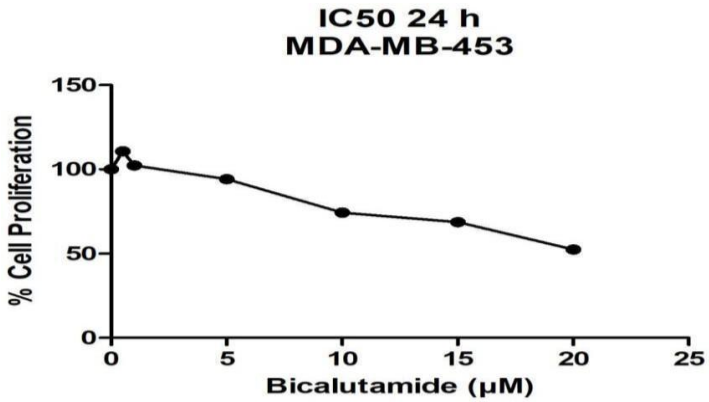

Figure 32: The cell viability assay was determined using the nonlinear regression curves for 24 hrs.

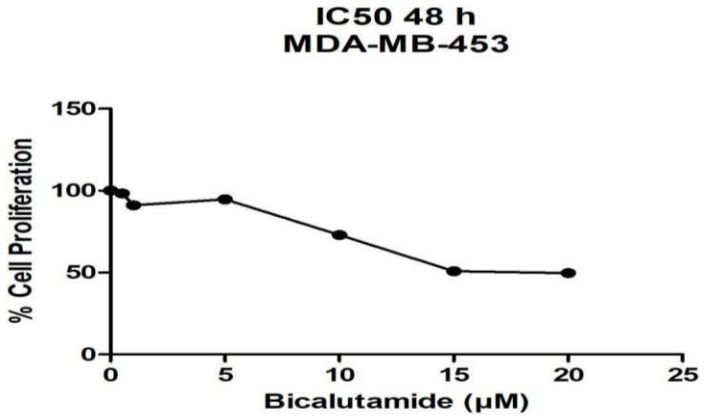

Figure 33: The cell viability assay was determined using the nonlinear regression curve for 48 hrs

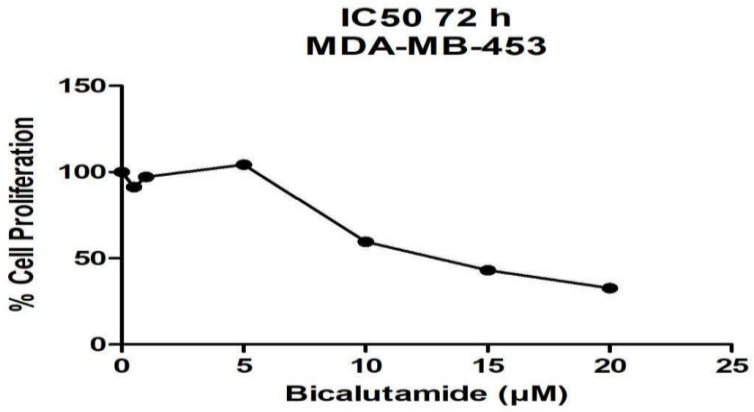

Figure 34: The cell viability assay was determined using the nonlinear regression curves for 72 hrs.

In **Figure 32**, an MTT assay was used to determine the drug's toxic effect on MDA-MB-453 cells. The proportion of treated cells was calculated that were still viable compared to the vehicle control. The capacity of MDA-MB-453 cells to survive declined as the concentration of bicalutamide grew; during 24 hours of incubation, the IC50 was 19.29 g/mL.

In **Figure 33**, using an MTT assay, the drug's cytotoxic impact on MDA-MB-453 cells was assessed. Calculated was the proportion of treated cells that were still viable compared to the vehicle control. The capacity of MDA-MB-453 cells to survive declined as bicalutamide concentration increased; at 48 h of incubation, the IC50 was 10.20 g/mL.

In Figure **34**, Using an MTT assay, the drug's cytotoxic impact on MDA-MB-453 cells was assessed. The proportion of treated cells was calculated that were still viable compared to the vehicle control. The capacity of MDA-MB-453 cells to survive declined as the concentration of bicalutamide increased; at 72 hours of incubation, the IC50 was 9.3 g/mL.

In the MDA-MB-453 cells line the cell viability is half at the concentration of 19.29uM for 24 hrs., respectively, 10.2 µM for 48 hrs and 9.3 µM for 72 hrs. as it is shown in figures **32,33**

and **34.** indicated that the increase in time and a minimal amount of the drug concentration have given cell inhibitory effects.

Cell viability on treatment with DHT and Bicalutamide

DHT plays a major role in the genomic pathway of AR biosynthesis so to check the drug inhibitory mechanism DHT was added with the drug bicalutamide at different concentrations for the cell viability.

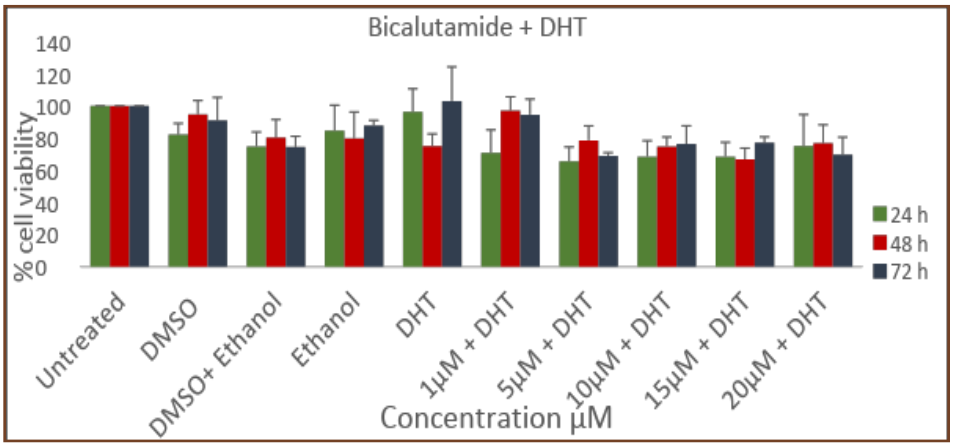

Figure 35: Cell viability on treatment with DHT and Bicalutamide

By the addition of 10nM DHT the cell proliferation was increased in comparison to control cells that are untreated but after the addition of bicalutamide at different concentrations for different time intervals in the presence of 10 nM DHT the cell proliferation seemed to be decreased showing its AR- antagonist effect targeting the canonical/genomic pathway. However, the inhibitory effects of the bicalutamide and enzalutamide were also observed in the cells in the absence of DHT which leads our attention to study the non-canonical/ non-genomic pathway via which the AR activity is inhibited as observed from the above results.

Usually, it is seen that the inhibition in cell proliferation is visible at 24hrs with a concentration of 10.27 μM but the combination of the drug (bicalutamide) and ligand (DHT) will enhance the cell viability and overcoming the drug's effect as it is shown in **figure 35.**

The effect of bicalutamide on the growth of MCF-7 cells

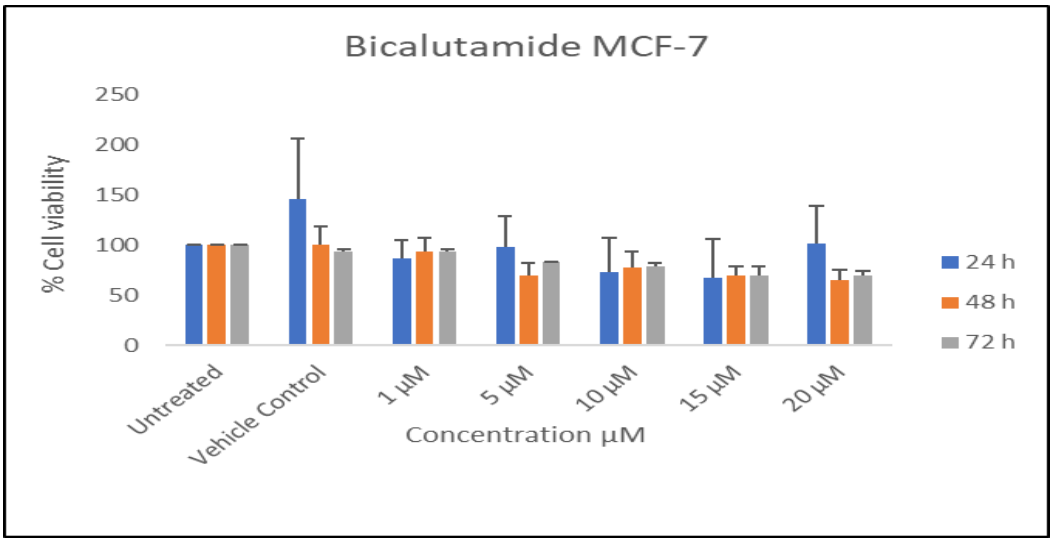

Figure 36: MCF-7 cells were treated with different concentrations bicalutamide for 24h,48h and 72 h to examine the cell viability.

The effect of Enzalutamide on the growth of MCF-7 cells

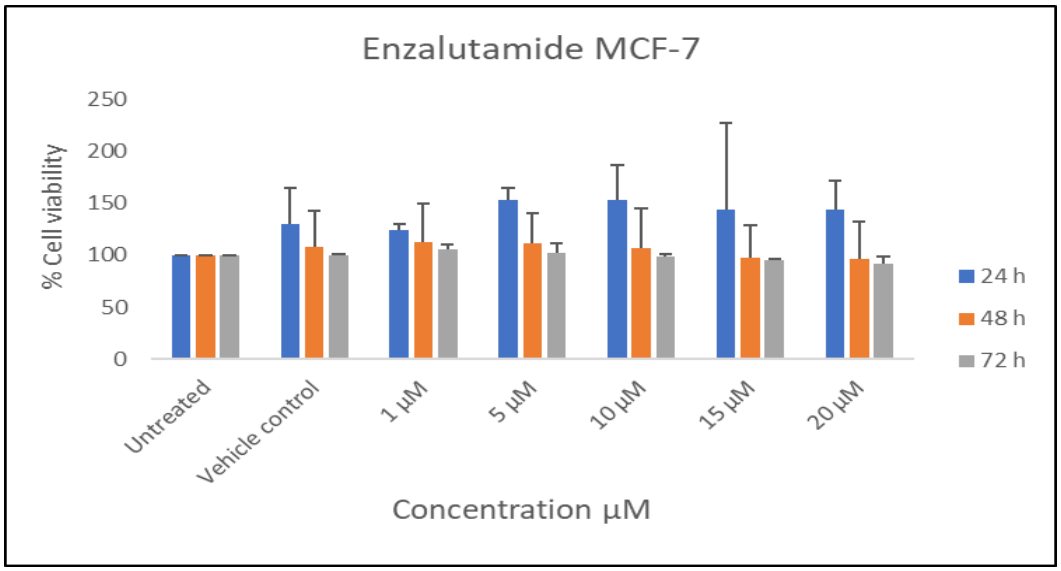

Figure 37: MCF -7 cells were treated with different concentrations as described in the table of enzalutamide for 24,48 and 72 h to examine the cell viability.

In MCF7 the cell proliferation is not inhibited by bicalutamide and enzalutamide as seen in **figure 36** and **figure 37.**

3) Cell scratch/ wound healing assay:

A six-well plate of MDA-MB-453 cells was used as the treatment site, and the IC50 values from the prior MTT studies were used. After 24 hours, a complete media was added, and a straight line had been scratched across the plate using a pipette tip featuring a 20 μL micropipette tip.

After 24 hours, images of the scratched cells were captured under a microscope using various concentrations. Figure 38 shows the analysis of the cell's ability to migrate in relation to the healed area of the scratch at various concentrations.

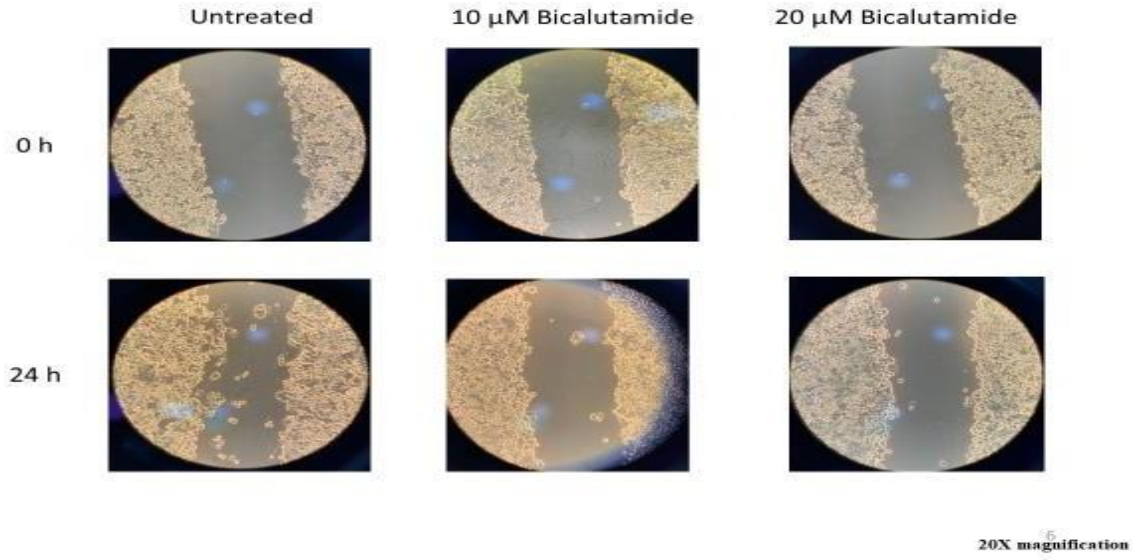

Figure 38: Effect of Bicalutamide on the migration of MDA-MB-453 cells

The representative images from in vitro wound healing tests showing how 10 and 20 μM concentrations of bicalutamide caused MDA-MB-453 cells to migrate. Following the scratch or wound, treated and untreated cells were present in varying concentrations. after 24 hours, the treated and untreated cells migrate. n=1

4) qRT-PCR:

Expression levels of AR in bicalutamide-treated cell lines

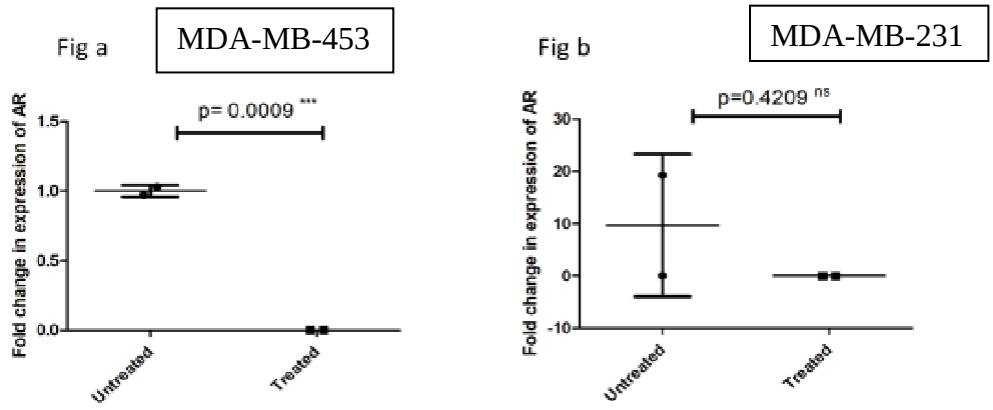

Figure 39: **Expression level of AR in Bicalutamide treated cell lines**

a. Scatter plot for the relative gene expression of AR in MDA-MB-453 cells untreated and treated with bicalutamide

b. Relative gene expression of AR in MDA-MB-231 cells untreated and treated with bicalutamide. N=3; ***$p<0.001$, ns = $p>0.05$

Expression levels of AR-V7 in bicalutamide-treated cell lines

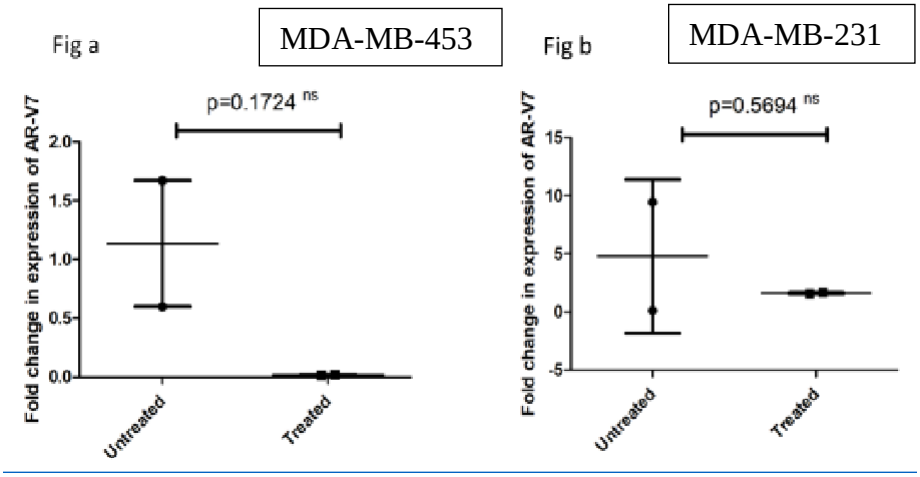

Figure 40: **Expression level of AR-V7 in Bicalutamide treated cell lines**

a. Scatter plot of the relative gene expression of AR-V7 in MDA-MB-453 untreated and treated with Bicalutamide

b. Relative gene expression of AR-V7 in MDA-MB-231 untreated and treated with bicalutamide. N=3;, ns = $p>0.05$

DISCUSSION

TNBC is an aggressive form of breast cancer and it is a targetless subtype because it lacks the expression of ER, PR, and HER-2. Thus, it is difficult to treat patients suffering from TNBC. However, it has been studied and found that there is an expression of AR in TNBC and this finding opened up many doors in the perspective of developing advanced treatments in the future against TNBC subtype. There are various drugs available in the market which have diverse modes of action such as inhibiting the AR signaling pathway by competitively blocking ligand binding to its receptor, inhibiting AR nuclear translocation, and inhibiting AR binding to DNA. Among various AR antagonists currently available, bicalutamide and enzalutamide are FDA-approved drugs used for the treatment of prostate cancer and they have been proven to show higher efficacy and prominent inhibition of proliferation of cancerous cells. (Khadela et al., 2023)

To check the efficacy of enzalutamide and bicalutamide, we performed cell viability assay through MTT assay on the different cell lines such as MDA-MB-231, which is an epithelial, human breast cancer cell line it is a model for more aggressive, hormone– independent breast cancer , MDA-MB-453 which is androgen receptor positive and 'triple negative' in respect to estrogen receptor α, progesterone receptor and the Her-2/neu protein expression and MCF -7 which is a human breast cancer cell line with estrogen, progesterone and glucocorticoid receptor. When MDA-MB-231 cells were treated with bicalutamide, the different concentrations of IC 50 values obtained were 10.27 µM at 24 hrs time intervals and at 48hrs, the inhibitory concentration obtained was 7.38 µM. According to the literature, after 24 hours of exposure, the IC50 obtained was 11.5 µM, and after 48 hours of exposure the IC50 value obtained was at around 7.28 µM (Kong et al.,2020). When MDA-MB-453 cells were treated with bicalutamide, the different concentrations of IC50 values obtained were 19.29 µM after 24 hrs of treatment, 10.2 µM after 48 hrs of treatment, and 9.2 µM after 72 hrs of treatment. Enzalutamide inhibitory concentration in the MDA-MB-231 cell line was observed after 72 hrs which was around 40 µM and in the literature, Enzalutamide drug's IC50 concentration was obtained at 41.12 40 µM (Caiazza et al., 2016)

During initial experiments, we performed an MTT assay by giving drug treatment to cell lines without adding DHT in the 96-well plate. As discussed earlier, DHT is an AR agonist and it activates the AR signaling pathway whereas our drugs are AR antagonists, ideally, there should be merely any inhibition of cell proliferation in the absence of DHT as the AR signaling pathway could not have been activated. However, we obtained an IC-50 concentration of drugs and there was significant cellular cytotoxicity and apoptosis. Hence, we can predict that non-genomic pathways may also be involved and the AR signaling could be occurring in the absence of AR (AR independent). We can also predict that our drugs are efficient in inhibiting AR signaling via non-genomic pathways. (Pietri et al., 2016)

We added 10 nM DHT along with the drug treatment to check the cell proliferation rate of our cell lines in the presence or absence of AR agonists. It was observed that DHT in the absence of drug treatment was responsible for rapid cell proliferation however on treatment with drugs, cell proliferation via DHT was inhibited. Based on our previously described role of DHT in the AR signaling pathway i.e., when DHT is added, it binds to AR and activates the signaling pathway leading to rapid cell proliferation.

Also, Enzalutamide is an AR-specific drug and it should only show its activity in the presence of DHT. However, we found that enzalutamide showed its inhibitory effects in the absence of DHT too which is a matter of research and further investigation needs to be done. (Saad, 2013). Based on our findings, experiments can also be performed to know and better understand exactly which non-canonical pathways could be targeted by bicalutamide and enzalutamide. Since we performed all cell viability experiments by adding either enzalutamide or bicalutamide alone, further experiments can be carried out by adding either the combination of both drugs or by adding bicalutamide in combination with some other drug having different modes of action.

Literature demonstrated that AR-V7 expression confers resistance to bicalutamide and that downregulation of AR-V7 expression resensitizes bicalutamide and enzalutamide resistance cells to the drug treatment (Zhang et al., 2020) On comparing AR gene expression in MDA-

MB-453 and MDA-MB-231 cell lines, there was a significant down-regulation of AR gene expression in MDA-MB-453 cells after treating them with bicalutamide. This decrease in AR expression led us to better understand the role of AR in the presence or absence of drug treatment (Masiello et al., 2002)

CONCLUSION

Our results have shown that the drugs enzalutamide and bicalutamide can inhibit the rate of cell proliferation and migration in the AR-positive TNBC cell line. Our experiments have helped us to better understand the complexities in the AR signaling pathway. We obtained results that showed significant downregulation of the AR gene in the bicalutamide-treated MDA-MB-453 cell line. However, we did not obtain any significant downregulation of AR-V7 due to drug treatment. Finally, as AR is expressed in several different types of cancer, targeting with other androgen, antagonists may have wide applications in cancer treatment.

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