NEAR EAST UNIVERSITY

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EFFECT OF ENZALUTAMIDE AND miR-26b ON CHEMORESPONSE OF TRIPLE NEGATIVE BREAST CANCER CELLS TOWARD CHEMOTHERAPY

Ph.D. THESIS

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Nicosia
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Approval

We certify that we have read the thesis submitted by Laila ALSawalha titled "**Effect of Enzalutamide and miR-26b on Chemoresponse of Triple Negative Breast Cancer Cells Toward Chemotherapy"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy in Medical Biochemistry.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of the Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Laila ALSawalha
14/6/2022

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Laila Sawalha

Özet

Enzatulamid ve miR-26b'nin Üçlü Negatif Meme Kanseri Hücrelerinin Kemoterapiye Karşı Duyarlılığına Etkisi

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Haziran, 2022, 100 sayfa

Üçlü negatif meme kanseri (TNBC), heterojen karakteri ve tedavi seçeneklerinin bulunmaması nedeniyle tedavisi zor bir kanser türüdür. Sistemik kemoterapi TNBC tedavisi için kullanılmaktadır. Ancak hastalar genellikle uygulanan tedaviye direnç geliştirmektedir. Androjen reseptörü vasıtasıyla gerçekleşen androjen sinyalinin TNBC hücrelerinde direnç gelişiminden sorumlu olduğu bildirilmiştir. Apoptoza direnç ilaç direncinin temel özelliklerinden biridir. Androjenlerin TNBC'deki rolleri iyi bilinmektedir ve androjen reseptörü (AR) sinyali karsinogenezde gün geçtikçe önemli hale gelmektedir. Bu nedenle androjen sinyal yolağını hedeflemek meme kanseri tedavisinde önemli rol oynamaktadır. Bir AR antagonisti olan enzatulamid erken yanıt oluşturmakta, ancak bu yanıtı ilaç direnci ve tümörün nüksü izlemektedir. Enzalutamidin hücre proliferasyonunu azalttığı ve bazı kanser türlerinde hücre ölümünün uyarılması yoluyla anti-tümör etkileri olduğu gösterilmiştir.

Çalışmamızda enzatulamid ve miR-26b'nin apoptoza ve TNBC hücrelerinin doksorubisine karşı olan duyarlılığına olan etkisi araştırılmıştır. MDA-MB-231 ve MDA-MB-453 hücre hatları artan derişimlerde doksorubisine maruz bırakılmış, IC_{50} dozunu belirlemek için hücre canlılığı incelenmiştir. Hücreler dihidrotestosteron (DHT) veya enzalutamid veya her ikisinin kombinasyonu kullanılarak muamele edilmiş ve hücre canlılığı belirlenmiştir. Aynı zamanda hücrelerin DHT ve enzalutamid varlığında doksorubisine karşı yanıtı araştırılmıştır. Hücreler miR-26b ile transfekte edilmiş ve apoptoz için TUNEL yöntemi ve kaspaz 3/7 tayini

kullanılarak bu hücre hatlarının tedaviye yanıtları incelenmiştir. Ayrıca apoptoz ile ilişkili genlerin ekspresyonlarındaki değişikliğe de qPCR ile bakılmıştır.

Dihidrotestosteron her iki hücre hattında da doksorubisine karşı direnç gelişimine neden olmaktadır. Buna karşılık, enzalutamid hücre canlılığını ve DHT'nin neden olduğu kemorezistansı azaltarak MDA-MB-231'in doksorubisine karşı duyarlılığını arttırmaktadır. TUNEL yöntemi sonuçları enzalutamidin MDA-MB-231 ve MDA-MB 453 hücrelerini doksorubisine duyarlı hale getirdiğini, apoptozu arttırarak DHT'nin neden olduğu kemorezistansın üstesinden geldiğini ortaya koymaktadır. Ayrıca, kaspaz 3/7 aktivitesini de arttırmaktadır. *mcl1* gen ekspresyonunun düzenlenmesi, DHT ve enzalutamidin her iki hücre hattının kemoyanıtı üzerindeki androjenik etkiyi modüle ettiği bir mekanizma gibi görünmektedir. *bcl2* ve *bid* genlerinin düzenlenmesi yalnızca MDA-MB-453 hücreleri için geçerlidir. Bu veriler, enzalutamidin potansiyel bir ortak terapötik yaklaşım olabileceğini düşündürmektedir. miR-26b'nin yalnızca MDA-MB-453 hücrelerinde doksorubisine karşı duyarlılığı arttırdığı, DHT ile oluşmuş olan direncin üstesinden geldiği ve kaspaz 3/7 aktivitesini arttırdığı gözlemlenmiştir. Hücre hatlarının miR-26b ile transfeksiyonu hücre canlılığını etkilemezken MDA-MB-453 hücrelerinde doksorubisine duyarlılığı arttırmaktadır. miR-26b yalnızca MDA-MB-453 hücrelerinde bazı anti-apoptotik genlerin (*mcl1* ve *bcl2*) ekspresyonunu azaltmakta ve *bid* ifadelenmesini arttırmaktadır.

Bulgular enzalutamidin doksorubisin tarafından indüklenen apoptozu artırarak DHT'nin neden olduğu kemorezistansı azaltmada her iki hücre hattında da etkili olduğunu ortaya koymaktadır. Ayrıca miR-26b'nin apoptozu indükleyerek DHT'nin neden olduğu direncin üstesinden gelme potansiyeline sahip olduğunu göstermektedir.

Anahtar Kelimeler: üçlü negatif meme kanseri, enzatulamid, apoptoz, doksorubisin, miR-26b

Abstract

Effect of Enzalutamide and miR-26b on Chemoresponse of Triple Negative Breast Cancer Cells Toward Chemotherapy

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Due to its heterogeneity and lack of therapeutic options, triple-negative breast cancer is challenging to treat. As a result, systemic chemotherapy remains as the treatment of choice for TNBC. Chemoresistance is unfortunately widespread among patients. Androgen signaling via its receptor is essential in breast cancer since it has been demonstrated to provide chemoresistance to TNBC cells. Resistance to apoptosis is one of the essential features of drug resistance. Androgens are well known for their role in TNBC, and therefore, androgen receptor (AR) signaling is becoming critical and an essential contributor to carcinogenesis. Therefore, targeting the androgen signal pathway plays an essential role in breast cancer treatment. The androgen receptor (AR) antagonist, enzalutamide, produces an early response, followed by drug resistance and tumor recur. Enzalutamide has been shown to affect cancer cell viability and has anti-tumour effects by inducing apoptosis in certain types of cancer. We also used miR-26b to understand its contribution to apoptosis and role in sensitizing TNBC toward doxorubicin.

MDA-MB-231 and MDA-MB-453 cell lines were treated with different concentrations of doxorubicin; the cell viability was examined to determine the IC_{50} dose. Cell viability after treatment with DHT or enzalutamide or its combination was investigated. Transfection of cells with miR-26b mimic was followed by qPCR to quantify relative expression. Then the effects of miR-26b on chemoresponse of breast cancer cells were investigated while TUNEL assay technique was used to detect late stages of apoptosis, followed by caspase-glo 3/7 assay. Changes in the expression of apoptosis-related genes were quantified by qPCR.

DHT increases the chemoresistance of both cell lines toward doxorubicin. In contrast, enzalutamide increases the sensitivity of MDA-MB-231 toward doxorubicin by decreasing cell viability and reducing the chemoresistance caused by DHT. TUNEL assay results revealed that enzalutamide sensitizes both cell lines; by increasing apoptosis induced by doxorubicin and overcoming the chemoresistance caused by DHT. Moreover, it increases Casp 3/7 activity. Regulation of the *mcl1* gene expression appears to be a mechanism by which DHT and enzalutamide modulate the androgenic effect on the chemoresponse of both cell lines. The regulation of *bcl2* and *bid* genes may also be relevant in MDA-MB-453 cells only. These findings imply that enzalutamide might be used as a co-therapeutic agent to target cell-specific TNBC. We found that miR-26b mimics increased cell sensitivity toward doxorubicin in MDA-MB-453 cells but not in MDA-MB-231 cells, overcoming the chemoresistance caused by DHT and increasing the caspase 3/7 activity. Transfection of MDA-MB-453 cell lines with miR-26b affects cell viability non significantly and increases chemosensitivity toward doxorubicin, but not in MDA-MB-231 cells. Gene expression of some antiapoptosis genes revealed that miR-26b downregulated *mcl1* and *bcl2* in MDA-MB-453 cells only and increased *bid* expression, thus how miR-26b induces apoptosis in combination with dox through *mcl1*, *bcl2* axis.

Our findings show that enzalutamide can reduce DHT-induced chemoresistance by enhancing apoptosis produced by doxorubicin in both cell lines; also, miR-26b mimic has the potential to overcome resistance caused by DHT through induction of apoptosis.

***Key Words**:* triple negative breast cancer, enzatulamide, apoptosis, doxorubicin, miR-26b

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List of Abbreviations

3′-UTR	3′ Untranslated region
3-HSD	3-Hydroxysteroid dehydrogenase
A4	Androstenedione
ABC	ATP binding cassettes
ADT	Androgen deprivation therapy
AF-1	Activation function 1
AF-2	Activation function 2
ANOVA	Analysis of variance
Apaf-1	Apoptosis activating factor
AR	Androgen receptor
ARA70	Androgen receptor associated protein 70
ARE	Androgen response elements
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bak	BCL2 antagonist/killer 1
Bax	BCL2 associated X
BC	Breast cancer
BCL2	B-cell lymphoma 2
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1
cAMP	Cyclic adenosine-3,5-monophosphate
CBP	CREB-46 binding protein
CBR	Clinical benefit rate
cDNA	Complementary DNA
CHIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukaemia
CREB	cAMP response element-binding protein
CSC	Cancer stem cell
Ct	Threshold cycle
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate

DHT	5α-dihydrotestosterone
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
E2	Estradiol
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FADD	Fas-related death domain
FasL	Fas ligand
FBS	Fetal bovine serum
GPER	G-protein coupled estrogen receptor
GR	Glucocorticoid receptor
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor-like receptor 2
HSD	Hydroxysteroid dehydrogenase
IAP	Inhibitor of apoptosis proteins
IC_{50}	The half-maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
JNK	c-Jun N-terminal kinase
kD	Kilodaltons
LAR	Luminal androgen receptor
LBD	Ligand binding domain
MCL1	Myeloid cell leukemia
MDR	Multidrug resistance
MOMP	Mitochondrial outer membrane permeability
MPA	Medroxyprogesterone acetate
miRNA	Micro ribonucleic acid
mRNAs	Messenger ribonucleic acid
mtOXPHOS	Mitochondrial oxidative phosphorylation
ncRNA	Non-coding RNA
NGFR	Nerve growth factor receptor
NLS	Nuclear localisation signal

NTD	N terminal domain
PARP	Poly ADP ribose polymerase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PR	Progesterone receptor
Pre-MiRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
qPCR	Quantitative real time-polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SHBG	Sex hormone binding globulin
siRNA	Small interfering RNA
TAU	Transactivation units
TFIIF	Transcription factor IIF
TFBP	TATA box-binding protein
TNBC	Triple-negative breast cancer
TRAIL	Tumor necrosis factor-dependent apoptosis-inducing ligand
TRNC	Turkish Republic of North Cyprus
TSS	Transcription start site
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
WHO	World Health Organization
Wnt	Wingless-type
XIAP	X-Linked inhibitor of apoptosis

CHAPTER I

Introduction

Background

Breast cancer is the most frequent cancer in women worldwide (Ferlay et al., 2019). Despite advances in treating the disease, it remains a significant health burden. Approximately two-thirds of breast cancer patients are positive for hormone receptors such as estrogen and progesterone. Both estrogen and progesterone lead to the growth and progression of breast cancer; hence, blocking their synthesis or targeting their receptors has proven to be an effective therapeutic strategy (Matutino et al., 2018). A targeted therapy approach is also taken into consideration to treat another breast cancer type, defined by human epidermal growth factor 2 amplification, termed HER2-enriched. Triple-negative breast cancer (TNBC), on the other hand, is negative for the three receptors. TNBCs account for approximately 10-15% of breast cancers, and compared to other subtypes, they have the lowest 5-year survival rate, owing to the lacking of a therapeutic target and high heterogeneity (Bauer et al., 2007; Barton et al., 2015; Zhu et al., 2015).

Many factors are associated with breast cancer prognosis, such as age, radiation exposure, family history, and lifestyle (Vuong et al., 2014a). Therefore, cancer research has gained attention globally. Despite significant progress in understanding the molecular basis of cancer, there are still some cancers for which there is no definite therapy. Surgery, hormone therapy, anti-angiogenic therapy, immunotherapy, chemotherapy, and radiotherapy are the primary treatment approaches currently being used worldwide.

The significance of sex steroid hormones and their receptors in the development and growth of breast cancer is significant. It is well defined that estrogen and estrogen receptors affect the signaling pathways of breast cancers (Matutino et al., 2018). However, little is known about the involvement of androgen and androgen receptors in breast cancers and the precise processes through which androgen functions. The significance of androgen in breast cancer signaling is hypothesized to vary between estrogen-rich and estrogen-replete environments. Considering these differences, innovative prospects for preventing or rehabilitating breast cancer progression have emerged. Androgens are the essential precursors to estrogens; the enzyme, aromatase, converts testosterone to estradiol and the androstenedione to the estrone. Peripheral androgen precursors produce 50%

testosterone, though ovaries and adrenal glands provide 25%. Unlike estrogens, the levels of circulating androgens do not drop as precipitously after menopause, making androgens the dominant sex hormone in aged women (Labrie et al., 2003). Circulating androgens have been associated to an increased risk of breast cancer in postmenopausal women, suggesting that they might be used as indirect indicators of hormonal signaling in the breast (Risbridger et al., 2010).

MicroRNAs (miRNAs or miRs) are 20–25 nucleotide long, single-stranded non-coding RNAs highly conserved across species (Gao & Liu, 2011). miRNAs' main function is post-transcriptional gene silencing by base-pairing their conserved 5'-heptameric seed sequence with the 3'untranslated region (3'-UTR) of mRNAs (Liu, 2012). It has been shown that each type of tumor has a relatively unique miRNA expression profile and that the same type of miRNA can vary between types of tumors (Sahlberg et al., 2015). Many studies have shown that miRNAs are required for the onset, development, and promotion of numerous human malignancies (Garzon et al., 2009). miRNAs have been identified to have oncogenic and tumour suppressor activities (Sabit et al., 2021). During breast cancer progression, several molecular modifications tend to occur. Differential expression of various miRNAs is one of these modifications. Previous studies have clarified the ability to differentiate different cancer types, diagnoses and prognoses related to miRNA expression (Lu et al., 2005).

Androgen has also been shown to control the expression of particular miRNAs. In a recent study, Ahram and his colleagues revealed that more than two folds had modified the expression of 33 miRNAs in MDA-MB-231 TNBCs (Al-Othman et al., 2020).

Targeting the androgen receptor alone or in combination with other therapies is a viable therapeutic strategy for treating patients with prostate cancer. This suggests that targeting androgens and androgen signaling in other cancer types may also be useful.

Few studies have examined the effect of DHT on the chemoresponse in TNBC. Al-Momany and colleagues reported that DHT induces chemoresistance in MDA-MB-231 cells (Al-Momany et al., 2021). However, the mechanism by which androgen receptor (AR) activation leads to chemoresistance and the effect of enzalutamide in reducing chemoresistance have to be elucidated. This work aims to

understand the mechanistic effect of enzalutamide and the AR-responsive microRNA, miR-26b, in sensitising TNBC cells to doxorubicin.

Statement of the Problem

Diagnostic and characterization challenges make it more difficult to treat TNBCs and their subgroups. This demands the identification of new targets that may also play regulatory roles in breast cancer therapy; till now, it is unknown whether inhibition or stimulation of androgen activity in TNBC patients achieve therapeutic benefits. This study aimed to investigate the effect of enzalutamide on chemoresponse of TNBC and investigate a mechanistic relationship with miR-26b.

Research Questions/Hypotheses

Hypothesis: Dihydrotestosterone, enzalutamide and miR-26b modulate the sensitivity of TNBC cells towards doxorubicin through the apoptosis pathway.

Alternative Hypothesis: Dihydrotestosterone, enzalutamide and miR-26b do not affect the sensitivity of TNBC cells towards doxorubicin through the apoptosis pathway.

The Intended Outcome of the Thesis/Significance

- Study the effect of DHT, enzalutamide and their combinations on the chemoresponse of TNBC toward doxorubicin.
- Examine the effect of DHT, enzalutamide and their combinations on apoptosis in TNBC cells through the alteration of apoptotic protein expression, DNA degradation and caspase 3/7 activity.
- Examine the chemoresponse of TNBC cells transfected with miR-26b mimic or antisense alone or in combinations with DHT and enzalutamide.
- Examine the effect of transfection with miR-26 mimic or anti-sense on doxorubicin-induced apoptosis in TNBC cells through the alteration of apoptotic protein expression, DNA degradation, and caspase3/7 activity.
- The knowledge gained from this project may help to improve novel approaches to breast cancer prevention and treatment.

Limitations

- The effects of DHT, enzalutamide, and miR-26b on the chemosensitivity of other TNBC cell lines besides MDA-MB-231 and MDA-MB-453 should be investigated.
- Further signaling pathways showing the effect of DHT, enzalutamide and miR-26b on TNBC cells should be elucidated.

Definition of Terms

Triple-Negative Breast Cancer: This term refers to breast cancer cells that are differentiated by the absence of three types of receptors on their surface (estrogen, progesterone and HER-2 receptor).

A microRNA is a short, single-stranded, non-coding RNA that functions in RNA silencing and post-transcriptional gene regulation.

Androgens are androgenic steroids that promote the development of sexual organs and secondary sexual traits in males. Androgens are synthesized in the testes and by the adrenal gland in smaller amounts. Female androgens are produced in lower amounts, mainly in the adrenal glands and the ovaries, and they play an essential role in the hormonal regulation that begins with puberty and regulate body functions. Androgens are necessary for the synthesis of estrogen in adult women and for the prevention of bone loss.

Dihydrotestosterone is the most prevalent androgen found in the testes and the most potent androgen *in vivo* and *in vitro*. In breast tissue, testosterone is converted to dihydrotestosterone by 5-α-reductase or to 17-estradiol by aromatase and can act as an androgen or estrogen agonist.

Androgen Receptor is a type of protein that binds to androgens and is present mainly in male reproductive organs and in specific cancer cells that respond to androgen, and in certain types of breast cancer cells.

Androgen Antagonist is a natural or synthetic substance that competes for androgen receptor binding sites and counteracts the effects of testosterone and DHT. In this study, enzalutamide was used as a synthetic androgen antagonist.

Chemoresponse is how cancer cells respond to chemotherapeutic drugs; although many tumors are initially responsive to chemotherapy, they can develop resistance through several pathways over time.

Apoptosis is a biological phenomenon observed in multicellular organisms. It is a highly regulated biochemical and genetic mechanism that plays an important role in proper tissue growth, homeostasis and death. The distinct cell modifications (morphology), dense cytoplasm, tightly packed organelles, cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation, and mRNA degradation are the most characteristic features of apoptosis. Defective apoptotic mechanisms have been linked to a wide range of diseases. Inadequate apoptosis results in uncontrolled cell growth, which is considered one of the hallmarks of cancer.

CHAPTER II

General Information

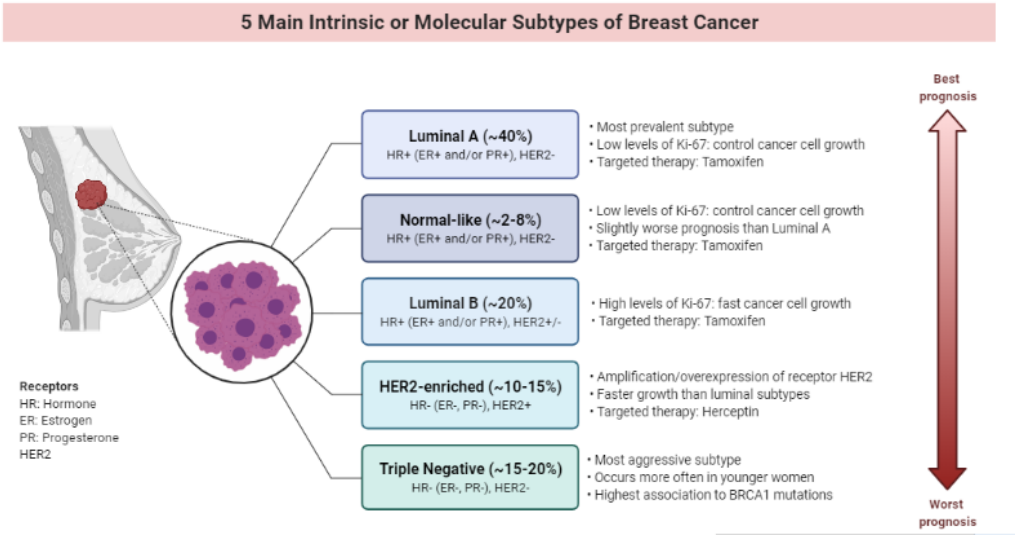
Breast Cancer

Cancer, in the Turkish Republic of Northern Cyprus (TRNC) as well as in the world, is the leading cause of mortality after heart disease, and its incidence is increasing (Pervaiz et al., 2018). Breast cancer incidence among females increases globally. The global cancer percentages are expected to increase by 70% in the next two decades if preventive measures are not implemented; according to the most recent data from the World Health Organization (WHO), 2.3 million women worldwide have breast cancer, causing 685 000 death (Torre et al., 2015).

The genetic heterogeneity of breast cancers is well known. This heterogeneity is based on examining the alterations of gene expression that drive cancer, such as a change in the expression of specific receptors HER2, progesterone receptor, and estrogen receptor (Vuong et al., 2014b). At the molecular level, five significant subtypes of breast cancer have been identified: luminal A and B, HER2 overexpressed, normal-like and triple-negative breast cancers (Prat et al., 2015). Details of the major molecular subtypes of breast cancer are listed in Figure 1.

Figure 1

Molecular Subtypes of Breast Cancer, Determined By Gene Expression Profiling (Prat et al., 2015; Vuong et al., 2014) (Created with biorender.com)



Luminal Subtypes

Luminal subtypes can be divided into two subtypes:

Luminal A is low-grade, develops slowly and has the best prognosis. Luminal A breast cancer has hormone receptors but lacks HER2. It contains low levels of ki-67 protein, which helps to restrain how quickly cancer cells develop (Guedj et al., 2012).

Luminal B is hormone receptor-positive and has a high ki-67 level. The luminal B subtype has a significantly worse prognosis with a high proliferation rate (Provenzano et al., 2018).

HER2-Enriched

The HER-2 receptor is a tyrosine kinase receptor located in the cell membrane and plays a role in the signaling pathways that lead to cell differentiation and proliferation. HER2-enriched cancers grow faster than luminal tumours and have a poorer prognosis. They are negative for both the progesterone and estrogen receptors (Vuong et al., 2014b). They are often treated with targeted therapies for the HER2 protein, such as transtuzumab/deruxtecan (Gompel & Plu-Bureau, 2018).

Triple-Negative

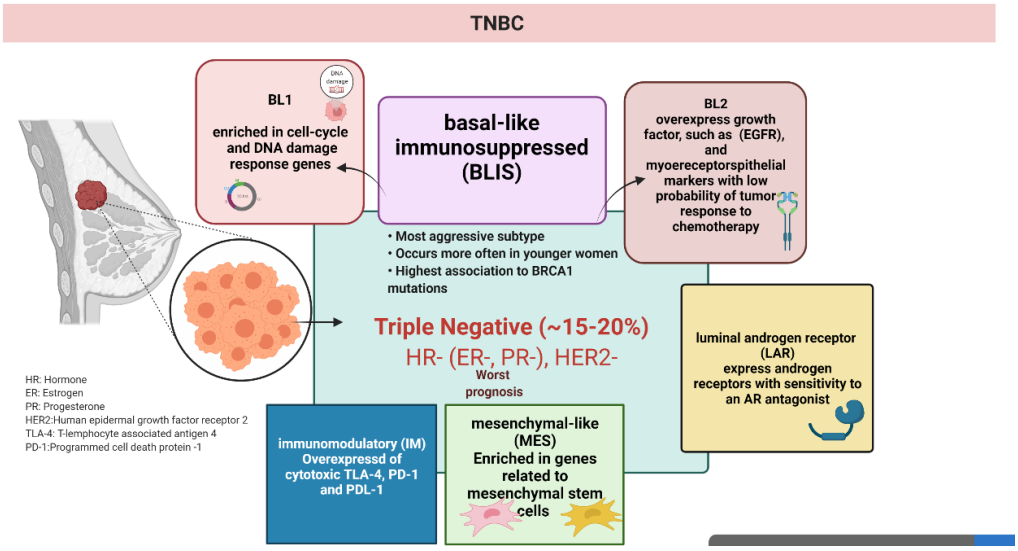
Triple-negative breast cancer is distinguished by the lack of expressions of the hormone receptors, progesterone and estrogen receptor, and the HER2 (Bai et al., 2021). Lack of those receptors results in the ineffectiveness of various hormone therapies, extensive resistance (30–50%) and a poor prognosis (Dent et al., 2007).

The revelation of TNBC heterogeneity and molecular subtyping represent a significant milestone in the treatment of TNBC. In 2011, Lehmann et al. first suggested six molecular subgroups based on 587 TNBC patients from 21 data sets. These molecular subtypes included basal-like types 1 and 2, mesenchymal, mesenchymal stem-like, immunomodulatory, and luminal androgen receptor positive (LAR) (Lehmann et al., 2011). Luminal androgen receptor-positive (LAR) tumors compared to other TNBC subtypes, have more than ten-fold AR expression and are enriched for hormone-regulated pathway genes; genes involved in steroid production, porphyrin metabolism, and androgen/estrogen metabolism were found. A luminal gene expression profile is defined by AR's most highly expressed genes downstream (Doane et al., 2006; Lehmann et al., 2011). The gene expression profile

was significantly linked with the LAR subtype when tumors were chosen based on histological apocrine traits demonstrating that the LAR TNBC group comprises cancers categorized with molecular apocrine histology (Lehmann et al., 2011). Androgen receptor is one such useful marker and target for TNBC treatment. Based on genomic and transcriptome data from 465 Chinese patients, Jiang et al. hypothesized four molecular categories (Jiang et al., 2019); nonetheless, there is no uniformity in the biomarker selection for each subtype (Tang et al., 2014), making it impossible to compare distinct subtypes across investigations. With such variability, lack of focused therapy, decreased chemosensitivity and poor prognosis; it is critical to have distinct biomarkers, which may assist in discriminating different TNBCs and may provide novel therapeutic targets. Figure 2 illustrates TNBC subtypes.

Figure 2

Triple-Negative Breast Cancer Subtypes (created with biorender.com)



Androgen Signaling in Breast Tissue

Androgen Sources and Synthesis in Females

Androgens are steroid hormones generated from cholesterol that are responsible for developing and maintaining sexual characteristic (Gelmann, 2002). Female androgens play vital physiological roles such as regulating mammary gland development and serving as precursors to estrogen production (Ahmad & Kumar,

2011). In women, androgens are primarily responsible for initiating the hormonal cascade during puberty, regulating bodily processes prior to, during, and after menopause, and further have an anabolic function in maintaining the functioning of a variety of other organs, such as the bones, kidneys, liver, and muscles (Liao, 2007).

The five main androgens found in the circulation given in descending order of serum concentration are: dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone, and 5-α-dihydrotestosterone (DHT) (McFarlane et al., 2018)

In females, androgen production occurs mainly in the ovaries and adrenal glands (Figure 3); although it can also occur in peripheral organs via enzymatic conversions, the ovaries and adrenal glands synthesize androgens from cholesterol (Finlay-Schultz & Sartorius, 2015). They are then delivered through the circulation attached to serum sex hormone-binding globulin (SHBG) or albumin to their respective target tissues, exhibiting tissue-specific effects. The enzyme 5-α-reductase can convert testosterone to the more potent form, dihydrotestosterone (DHT), which can bind to the androgen receptor with a 2-fold greater affinity and with a 5-fold lower dissociation rate when compared to testosterone (Cochrane et al., 2014).

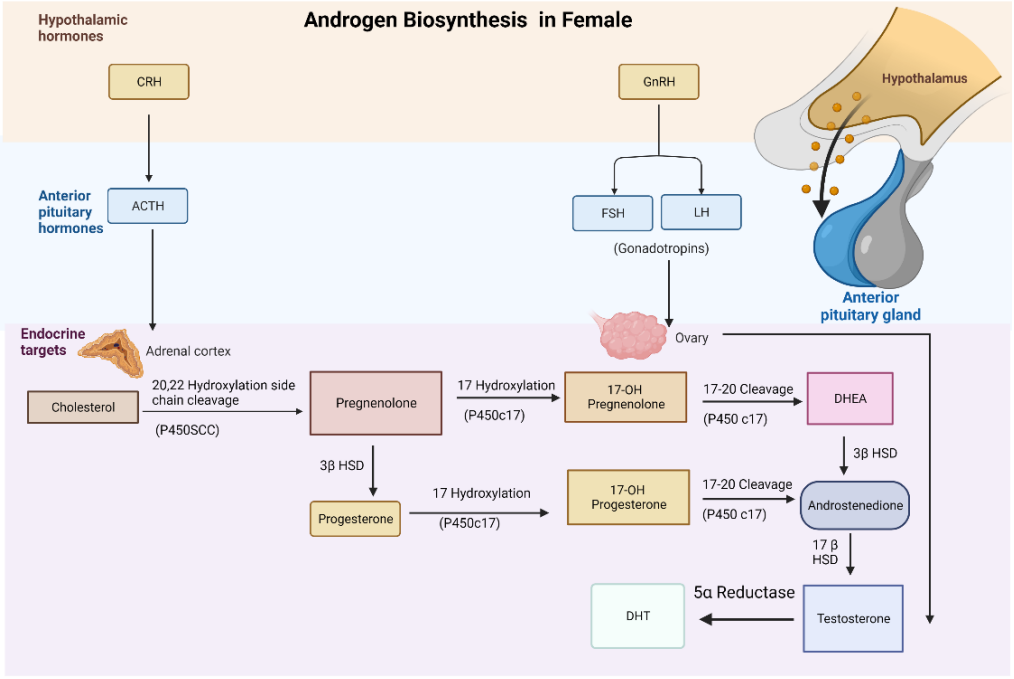
The enzyme 5-α-reductase have two isoforms, 5-R1 and 2-R1 isozymes are NADPH-dependent, membrane-associated enzymes profoundly buried in the lipid bilayer with molecular weights of 29.5 and 28.4 kilodaltons, respectively. They have high numbers of hydrophobic amino acids in their sequences, indicating that they are intrinsic membrane proteins (Langlois et al., 2010; Russell & Wilson, 1994; Suzuki et al., 2001).

In women, about 66 percent of total circulating testosterone is bound to SHBG, and changes in SHBG levels substantially influence free testosterone levels in the blood. SHBG increases by elevated levels of E2 and thyroxine, whereas growth hormone, insulin like growth factor 1 (IGF-1), androgens, prolactin, and obesity inhibit SHBG (Santos & Schulze, 2012). The adrenal glands primarily release the steroid precursors, DHEA and DHEAS, which are metabolized to androstenedione in peripheral tissues by 3-hydroxysteroid dehydrogenase (3-HSD) and eventually to testosterone via 17-HSD. Androgens generated in peripheral tissues mostly exert local (intracrine) activities within the place of synthesis, with just a little quantity secreted into circulation. As a result, serum androgen levels are not always predictive of bioactivity in target tissues (Labrie et al., 2003).

In vivo and *in vitro*, dihydrotestosterone is more potent than testosterone (Hickey et al., 2012). Different studies have shown that in the absence of estrogen, testosterone is converted to estrogen (E2) but is preferentially metabolized into DHT when both hormones are present at normal levels, limiting the E2-induced effects (Labrie et al., 2003). The maintenance of this equilibrium supports the physiological response of the mammary gland based on hormonal requirements and menopausal state (Zhou et al., 2000).

Figure 3

Androgen Synthesis in Females (adapted from (Burger & Johnson, 2005)*)*



Role of Androgen Receptor in Breast Cancer

Androgen Receptor Structure

The androgen receptor (AR) is a member of the nuclear receptor superfamily. The estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR) are all steroid hormone receptors (Porter et al., 2019).

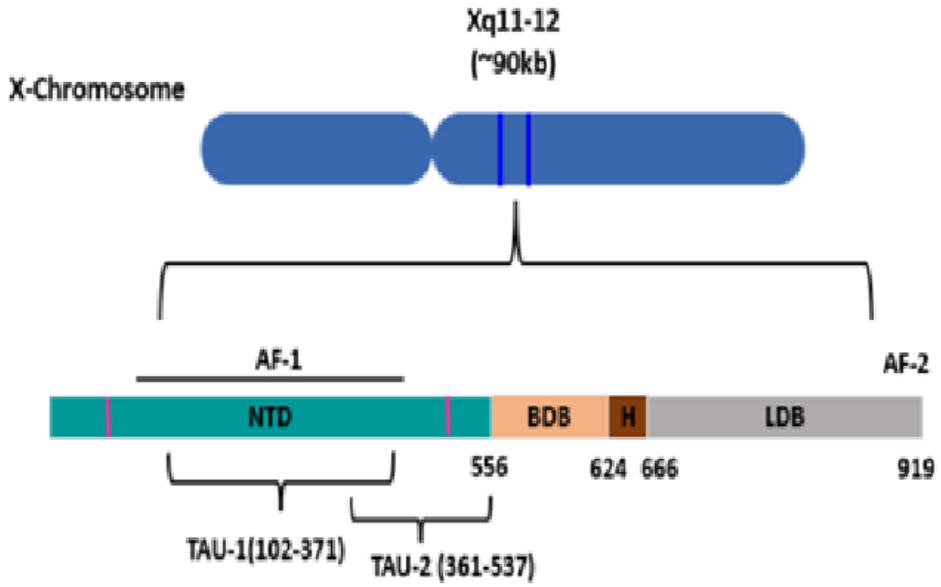
Androgens' biological effect is mediated by binding to the androgen receptor (AR), a ligand-inducible nuclear receptor superfamily transcription factor. The

androgen receptor is encoded by a single copy gene (>90 kb) on the X chromosome at Xq11-12 (Tilley et al., 1989). The androgen receptor coding sequence consists of 8 exons that create a protein of around 917 amino acids with a molecular weight of approximately 98.8 kD, depending on the size of two polymorphic microsatellite regions in exon 1 of the AR gene. AR, like other members of the nuclear receptor superfamily, has a modular structure with three main domains as in Figure 4; the amino-terminal domain (NTD), the DNA binding domain (DBD), and the ligand-binding domain (LBD). The C-terminal of LBD connected to DBD by a flexible hinge region (Brinkmann et al., 1989). Whereas the DBD and LBD have a highly organized canonical structure that is conserved across species, the NTD and hinge regions have undergone substantial divergence over evolution, allowing for varied homeostatic regulation and complexity of AR signaling among organisms (Heemers & Tindall, 2007). The three domains are all necessary for receptor function. The highly conserved DBD binds directly to the promoter and enhancer regions of AR-regulated genes, allowing the NTD and LBD activation to increase the transcription of these genes. The androgen receptor's main activity, known as activation function 1 (AF-1), is present in the NTD. A constitutively active AR is generated when the LBD and AF-1 are no longer connected. It has been demonstrated that the LBD interacts with two motifs in the NTD of androgen receptor, namely 23-FQNLF-27 and 433-WHTLF-473. This results in an NH_2-COOH terminal intra- or intermolecular androgen receptor interaction, which has been proposed to be significant for the transcriptional activity of some, but not all androgen receptor target genes. AF-1 is highly modular, including two transactivation units (TAUs) that function in transcriptional activation, TAU 1 and TAU 5. When activated by basal transcription factors such as TFIIF, the AF-1 domain undergoes induced folding, resulting in a more compact and active configuration that allows for additional coregulator recruitment and transcription (van Steensel et al., 1995; Yu et al., 2020). Furthermore, the NTD contains a variable number of homopolymeric repeats, the most prominent of which is a polyglutamine repeat that varies from 8 to 31 repetitions in normal individuals, with an average length of 20 repeats (Yu et al., 2020). However, amino acid changes in the AF-1 domain have been identified in individuals with androgen insensitivity, highlighting the significance of this area in the full-length AR's activity (Davey & Grossmann, 2016; Heinlein & Chang, 2002).

The DBD of the androgen receptor is a cysteine-rich region that is substantially conserved within steroid hormone receptors (van Steensel et al., 1995). The DBD has two zinc finger motifs, each composed of four cysteine residues containing a zinc ion (Tilley et al., 1989). A conserved amino acid motif (P-Box) within the first zinc finger drives AR binding to androgen response elements (AREs), which are inverted hexameric DNA half-sites separated by three base pairs (e.g. CCAGAACATCAAGAACAC) in target gene regulatory regions. The second zinc finger, which contains the D-box motif, stabilizes the DNA-bound receptor complex and facilitates AR dimerization (Claessens et al., 2008; Davey & Grossmann, 2016; Ueda et al., 2002).

Figure 4

Androgen Receptor (AR) Structure (adapted from (Davey & Grossmann, 2016)*)*



In contrast, the LBD's activation function 2 (AF-2), a hydrophobic surface consisting of helices 3, 4, and 12, is ligand-dependent (Tan et al., 2015). The LBD promotes AR's high-affinity binding to its natural androgenic ligands DHT and testosterone (K_d = 0.2-0.5 nM) and synthetic androgens like R1881 (K_d = 0.6 nM) (Chmelar et al., 2007). AF-2 is crucial in the interaction between AR and coregulators that include the LxxLL motif, and mutation of this area has been

reported to diminish ligand-dependent AR activation significantly (Heemers & Tindall, 2007).

Androgen Receptor Mechanism of Action

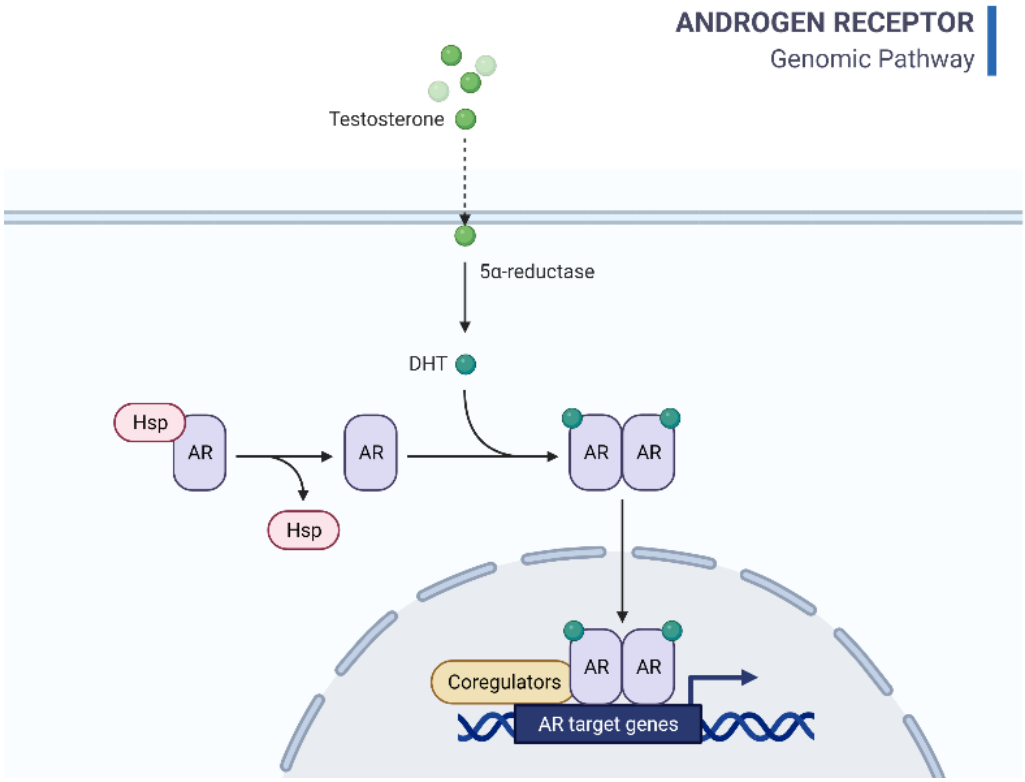
Many factors determine the mechanism of androgen actions. The kind of target cell, the position of the receptor inside cells, and the ligand itself affect the steroid-induced signal's nature (genomic versus non-genomic). The role of AR in the development of breast cancer is complex, where it can perform its actions at the genomic level as a ligand-dependent transcription factor through the regulation of gene expression. It can function in the non-genomic signaling pathways by suppressing G-protein receptor signaling in TNBC (Shen et al., 2018).

Genomic or "Canonical" AR Signaling. The AR is a large multi-protein complex found primarily in the cytoplasm of cells when its ligand is not present. This complex is made up of the receptor protein, heat shock proteins, co-chaperones, and cytoskeletal proteins, which preserve the AR's structural stability and keep it in an inactive state. After ligand binding, AR assumes an active conformation that makes it easier for chaperones to break up, N/C termini to connect, the NLS to be exposed, and AR to go into the nucleus (Davey & Grossmann, 2016). AR binds to AREs in the promoter and enhancer regions more easily after forming a homo-dimer (two AR proteins linked together) in the nucleus (Figure 5). Binding to DNA might not be restricted to loci with traditional AREs. The lack of AREs at 16–22% of AR-DNA binding locations in previous genome-wide profiling studies may indicate that AR is linked to other components bound to DNA (Heinlein & Chang, 2002); it was believed that AR mostly bound to target gene promoters before the discovery of ChIP-seq technology to characterize genome-wide DNA binding patterns. The majority of AR binding sites, however, were more than 10 kb away from the target genes' transcription start site (TSS), according to ChIP-seq analysis (Massie et al., 2007). As a result, AR binding to enhancer sites is the current paradigm. The recruitment of the basic transcription machinery, such as transcription factor TATA box-binding protein (TFBP), is made possible by AR-DNA binding. As well as other regulators like CREB and transcription factor IIF (TFIIF) and other regulators like CREB-46 binding protein (CBP), transcription-activated p300, and AR-binding protein 70 (ARA70) can all be recruited to AR by AR-DNA binding. Enhancer

locations are often far from the gene promoters where AR binds; however, AR interacts with gene promoters at these sites through looping chromatin. Wang and colleagues demonstrated that RNA-polymerase II travels around a large DNA loop created by the enhancers and promoters of AR target genes. The transcription of AR target genes then proceeds via RNA-polymerase II. Free AR is then transferred back to the cytoplasm after completing their transcriptional activity (Wang et al., 2007).

Figure 5

Androgen Receptor Genomic Pathway (created with biorender.com)



Non-Genomic AR Signaling. Non-genomic effect of AR may be mediated by direct binding to a target molecule's specific binding site in the absence of an AR or by activating a specific non-classical intracellular transmembrane receptor, such as the transmembrane G-protein coupled receptor, or by alterations in membrane fluidity transport. The physiologic levels of 17b-estradiol have been shown to raise the uterine cyclic adenosine-3,5-monophosphate (cAMP) content in ovariectomized mice within 15s (Fiocchetti et al., 2012). According to Koenig and colleagues, the mouse kidney cortex responds quickly (within 1 minute) to physiological amounts of testosterone (1–

10 nM) by stimulating endocytosis, amino acid transport, and hexose transport. Ornithine decarboxylase activity and polyamine concentration increased quickly and momentarily in response to testosterone. Ca^{2+} flux and Ca^{2+}-dependent membrane transport were acutely stimulated (Koenig et al., 1983).

Role of Androgens in Triple-Negative Breast Cancer

A substantial proportion of TNBC patients (12-55%) express the androgen receptor (AR) (Guedj et al., 2012; D'Amato et al., 2016). This suggests that AR may have a role in the biology of TNBC, including progression and response to treatment. According to preclinical findings, AR-positive TNBCs may rely on AR signaling to proliferate (Tran et al., 2009). In addition, TNBCs that express AR have worse chemotherapeutic sensitivity and lower complete remission rates following neoadjuvant treatment (Guedj et al., 2012). MDA-MB-453, which was demonstrated to be growth-stimulated by DHT is the most widely used model of molecular apocrine breast cancer. The idea that AR signaling may play an oncogenic role in molecular apocrine breast cancers is supported by subsequent studies using this cell line *in vitro* (Doane et al., 2006; Naderi & Hughes-Davies, 2008; Cochrane et al., 2014); and *in vivo* (Cochrane et al., 2014; Feng et al., 2017). Similar to this, it has been demonstrated that androgens promote the *in vitro* proliferation of several, less popular cancer cell line models, such as HCC202, SUM190, and SUM185PE (Naderi & Hughes-Davies, 2008). Contrary to these results, research by Wang et al. revealed that DHT inhibits MDA-MB-453 cell proliferation, whereas AR knockdown by siRNA increases cell growth (Wang et al., 2013; Feng et al., 2017); these results on proliferation may differ from those of the others ascribed to their different experimental setups that might influence AR activity, such as the circumstances of cell culture (especially the nature of the medium and fetal bovine serum type), the kind of AR ligand utilized, the timing, treatment dose regimens. However, owing to inadequate technique, Wang et al. reported that it is difficult to pinpoint the causes of this disparity (Wang et al., 2013).

Divergent proliferative effects of androgens on various AR-positive breast cancer cell line models likely reflect the disease's fundamental heterogeneity and imply that not all patients would benefit from decreased AR activity treatments. The inconsistent outcomes of clinical studies using androgen deprivation treatment (ADT) in women with molecular apocrine breast tumors provide more evidence

(Bonnefoi et al., 2016; Traina et al., 2018). These clinical investigations had mixed findings; while 25% of patients demonstrated clinical improvement, including some lasting responses, most patients did not (Jiang et al., 2016). Additionally, in these trials, AR expression did not predict the outcome.

A recent study found that treatment of TNBC with DHT increases breast cancer cell growth while inhibiting apoptosis in vitro (Zhu et al., 2016). Similarly, reactivated AR improves the viability of MDA-MB-231 xenografts in vivo. On the other hand, bicalutamide, the AR antagonist, induced apoptosis and inhibited breast cancer progression (Dong & Alahari, 2020). Furthermore, DHT-dependent AR activation is implicated in regulating cell cycle genes such as p21, p73 and cyclin D1. Some of these genes are tumour suppressors, such as cyclin-dependent kinases (CDK) p21, which prevents cell division by inducing cell cycle arrest, and downregulation of p21 is common in breast cancer (Bachman et al., 2004). AR has an inhibitory impact on p73 and p21, and there is a decrease in protein levels after DHT treatment. Furthermore, AR inhibition causes an increase in p73 and p21 levels in vivo and in vitro, resulting in a decrease in tumor volume (Zhu et al., 2016).

Therefore, AR may serve as a therapeutic target. Anti-androgen treatment with enzalutamide, an AR antagonist, has shown modest clinical efficacy in phase II clinical studies (Traina et al., 2018). Several preclinical and early clinical investigations have demonstrated that androgen receptors can be a targeted therapy in AR-positive TNBC (Lee et al., 2020).

It is unknown whether inhibition or stimulation of androgen activity in TNBC patients achieves therapeutic benefits. The contradiction in the role of androgen in TNBC between in vitro study and the clinical outcome comes from the disparity between these molecular biology investigations and the findings of immunohistochemistry and a few cell culture research (Tarulli et al., 2014; D'Amato et al., 2016; Anestis et al., 2020). A previous study by Shen and colleagues revealed that G-protein coupled estrogen receptor (GPER) expression was found to be reduced by DHT-induced TNBC cell growth (Shen et al., 2018). DHT-stimulated proliferation was reduced by G-1, a GPER agonist. Furthermore, in patient samples and in vitro, a negative connection between androgen and GPER was demonstrated in MDA-MB-231 cells. The role of androgen was investigated by Ahram et al., and it was found that androgen plays a critical role in TNBC metastasis and chemoresistance (Ahram et al., 2021). DHT treatment induces a mesenchymal

phenotype in LAR MDA-MB-453 cancerous cells. After a brief exposure of cells to DHT, a long-term nuclear translocation of AR was detected. Wnt signaling was identified to have a role in DHT-induced morphological changes. The mesenchymal transition of cells might be triggered by glycogen synthase kinase-3 inhibitor (Ahram et al., 2021).

Targeting Androgen Receptor as a Therapy

In 1958, non-tissue-selective androgens like fluoxymesterone and danazol were used to treat metastatic breast cancer, but severe side effects such as hirsutism, hoarseness, and baldness led to their discontinuation (Kennedy, 1958). Following the introduction of tamoxifen and aromatase inhibitors, medroxyprogesterone acetate (MPA), which was used to treat metastatic breast cancer in the early 1990s, lost its utility. While MPA was used to treat metastatic breast cancer, a study to determine its prognostic value revealed that patients who responded had much higher AR levels than those who did not; the clinical benefit rate (CBR) of fluoxymesterone, in comparison, was 33%, independent of the degree of AR expression in patients with ER-positive metastatic breast cancer who had progressed while receiving modern hormonal treatment. Breast cancer could be treated using selective AR modulators. Treatments of the MDA-MB-231 cell line with enobosarm inhibited the paracrine factors that promote metastasis, including interleukin 6 and matrix metalloproteinase 13, as well as subsequent migration and invasion. Enobosarm and GSK2849466 are two selective AR modulators undergoing clinical studies (Masiello et al., 2002). Several medications, such as AR inhibitors and CYP17A inhibitors, are included in the AR antagonists and they are frequently used to treat prostate cancer. In both preclinical and clinical research, several AR antagonists are now being studied in breast cancer.

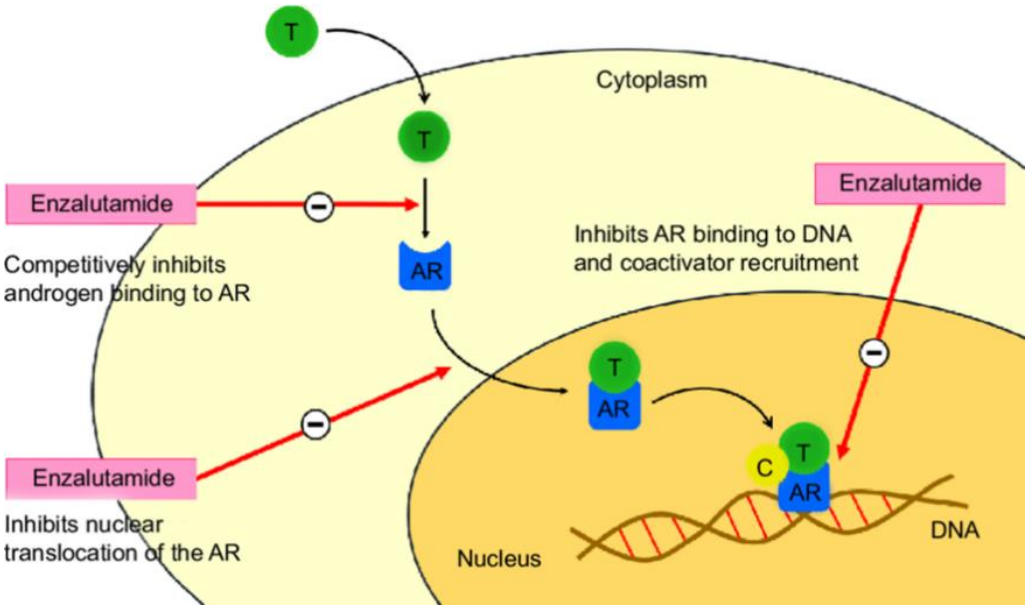
Androgens antagonists compete for androgen receptor binding sites and counteract the effects of testosterone and DHT. These substances prevent androgens from exerting their biological effects on sensitive tissues. A few drugs with a steroidal structure are employed as androgen receptor antagonists. The earliest nonsteroidal, antiandrogens were substituted with toluidine like bicalutamide, flutamide, and nilutamide (Masiello et al., 2002). However, due to significant side effects, cyproterone acetate, oxendolone, and spironolactone have restricted

applicability (Liao, 2007). The non-steroidal first-generation AR antagonist called bicalutamide prevents the DNA-binding domain from attaching to the androgen-response region. There were no measurable responses in the phase 2 clinical study of bicalutamide for breast cancer; however, resistance to bicalutamide is caused by inherited mutations in the ligand-binding region of AR or a rise in AR protein concentration (Osguthorpe & Hagler, 2011).

Enzalutamide (MDV3100) is an AR signaling antagonist with a five-fold higher affinity for androgen receptors than bicalutamide. It inhibits the nuclear translocation of AR, and lacks the agonist activity that bicalutamide had at therapeutic doses (Ni et al., 2011) (Figure 6). Enzalutamide can potentially reverse the effect of androgen by acting as a tumor suppressor in AR-positive TNBC (Zhu et al., 2016). The underlying molecular processes for these modifications have been addressed, illustrating that androgen can activate the several signaling pathways linked to DHT's anti-apoptotic activity (Santer et al., 2015; Nedeljković & Damjanović, 2019).

Figure 6

Androgen Receptor Antagonist Enzalutamide: Mechanism of Action (Rodriguez-Vida et al., 2015)



There are other types of AR antagonists in development. A selective AR down regulator called AZD-3514 inhibits nuclear translocation of AR and lowers AR levels (Rodriguez-Vida et al., 2015). EPI-001 is an another inhibitor targeting the AF-1 region, which is responsible for most of AR's transcriptional activity and inhibits the N-terminal domain of AR. Antisense oligonucleotides are also being developed, which use a locked structure to increase the binding affinity for mRNA and cut oligonucleotide length. Full-length AR and several AR splice variants are downregulated by EZN-4176 (AR-V7, AR-V12, AR-V13, and AR-V14). Abiraterone acetate, orteronel (TAK- 700), and VT-464 (Viamet), among other CYP17A inhibitors, are now being studied for breast cancer. By inhibiting 17-hydroxylase or 17,20-lyase activity, these medications prevent the synthesis of androgens. (Provenzano et al., 2018).

Drug Resistance

Despite a favourable initial response to chemotherapy, it is generally documented that TNBC is an aggressive group of breast cancer subtype. It is also well recognized that individuals with persistent TNBC after neoadjuvant treatment have a worse prognosis than those with non-TNBC (Lyons & Traina, 2017). Importantly, there is no recommended standard chemotherapy for these individuals and the regimens utilized are often determined by tumor size, lymph node status, tumor stage, overall performance status and the presence or absence of medical co-morbidities (Nedeljković & Damjanović, 2019). In the lack of ER, PR, and HER-2 receptors, endocrine therapy such as aromatase inhibitors like tamoxifen and HER-2 specific therapies such as trastuzumab and Lapatinib are ineffective in women with TNBC (Matutino et al., 2018). TNBC patients appear highly chemosensitive to anthracyclines and taxanes, used as a routine therapy for high-risk patients, such as those with node-positive disease. Despite this, there is a lower overall survival rate (Balaji et al., 2016).

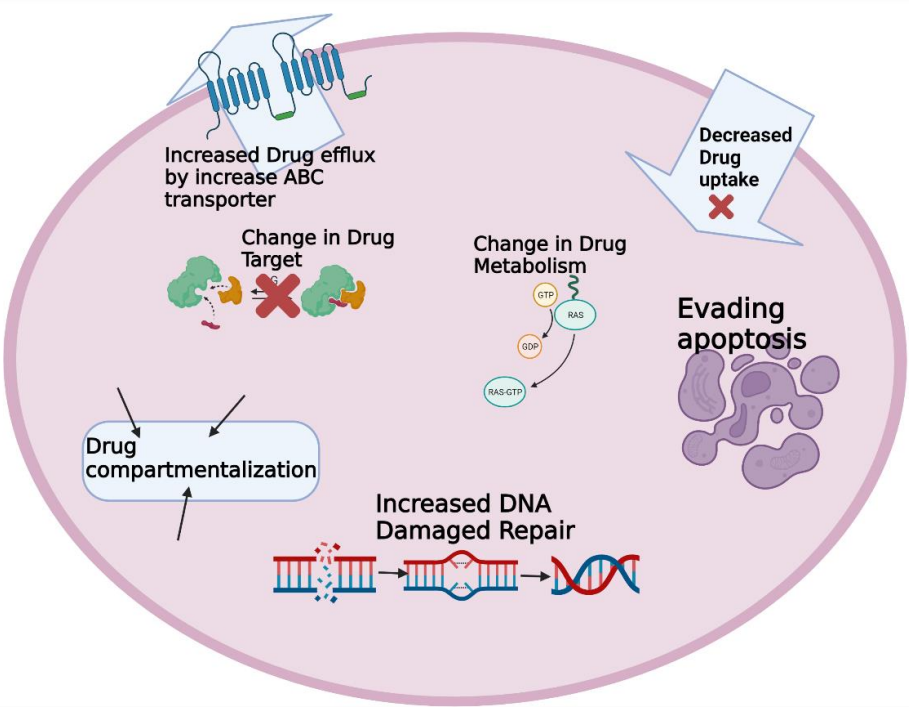
Chemotherapy resistance is a serious barrier in the successful breast cancer treatment. Although many cancers respond initially to chemotherapy, they can acquire resistance through various mechanisms over time (Housman et al., 2014). These mechanisms include DNA mutations and metabolic changes that improve drug inactivation and degradation of the drug, drug efflux, modification of drug target,

DNA damage repair, increased EMT and cell death inhibition (Figure 7) (Vasan et al., 2019).

Cancer cells survive chemotherapy via evading apoptosis. Apoptosis is a highly regulated cell death mechanism with particular biochemical and genetic aspects that play a crucial role in the homeostasis and development in normal tissues (Hassan et al., 2014). The molecular steps in apoptosis can be divided into three stages: 1) initiation of caspases by an apoptosis-inducing agent, 2) activation of caspases by a signal transduction cascade, and 3) proteolytic cleavage of cellular components. Many death and survival genes are involved in apoptosis and are regulated by extracellular factors. The apoptosis pathway in mammalian cells is regulated through two distinct pathways: the extrinsic and the intrinsic pathways. Those two pathways are regulated by a group of enzymes called caspases (cysteine-aspartic acid proteases) that play an essential role in apoptosis. Activation of the caspase enzyme family causes some cellular changes such as condensation of chromatin, fragmentation of DNA, swelling of the membranes and shrinkage of the cell (Sun & Peng, 2009).

Figure 7

Mechanisms of Drug Resistance in Breast Cancer (created with biorender.com adapted from Vasan et al., 2019)



The extrinsic pathway starts by the binding of extracellular ligands to cell surface receptors such as nerve growth factor receptor (NGFR) and TNF-dependent apoptosis-inducing ligand (TRAIL), also known as death receptors (Figure 8). After binding to the receptor, the Fas-related death domain (FADD) and the death-inducing signaling complex (DISC) consisting of procaspase-8 and -10 are formed. This complex activates caspase 8/10 within itself, and as a result, caspase-3 decomposes, becomes active, and initiates the apoptosis process (Carneiro & El-Deiry, 2020).

The intrinsic pathway, also known as the mitochondrial pathway, is induced by stress signals including ionizing radiation, cytotoxic agents and the absence of growth factors. They cause increased mitochondrial outer membrane permeability (MOMP) and transcriptional or post-transcriptional activation of BH3-only pro-apoptotic B-cell leukaemia/lymphoma (BCL-2) family proteins. Mitochondrial permeability is the crucial step in the apoptosis cascade and is mediated by BCL-2 family proteins. The permeability of mitochondria allows apoptotic proteins such as cytochrome c and the second caspase activator (Smac) to be released from the intermembrane space to the cytosol (Brusselmans et al., 2005; Rogers et al., 2019; Carneiro & El-Deiry, 2020).

The combination of cytochrome c and apoptotic protease activator factor-1 (Apaf-1) activates caspase-9, resulting in caspase-3, -6, and -7 activation (Sun & Peng, 2009). The inhibitor of apoptosis proteins (IAP) inactivates both intrinsic and extrinsic pathways by inhibiting caspase activity. This mechanism is the last protective measure against apoptosis. In the intrinsic pathway, the death signal can also be activated by c-Jun N-terminal kinase (JNK), for which Bcl-xL is phosphorylated at serine 62, and its anti-apoptotic effect is reduced. Both the intrinsic and extrinsic pathways of apoptosis are impaired in cancer cells, and escape from apoptosis is considered one of the main features of cancer cells (Simões- Wüst et al., 2002; Van Meer et al., 2009; Ma et al., 2013; Baig et al., 2016; Carneiro & El-Deiry, 2020; Dong & Alahari, 2020).

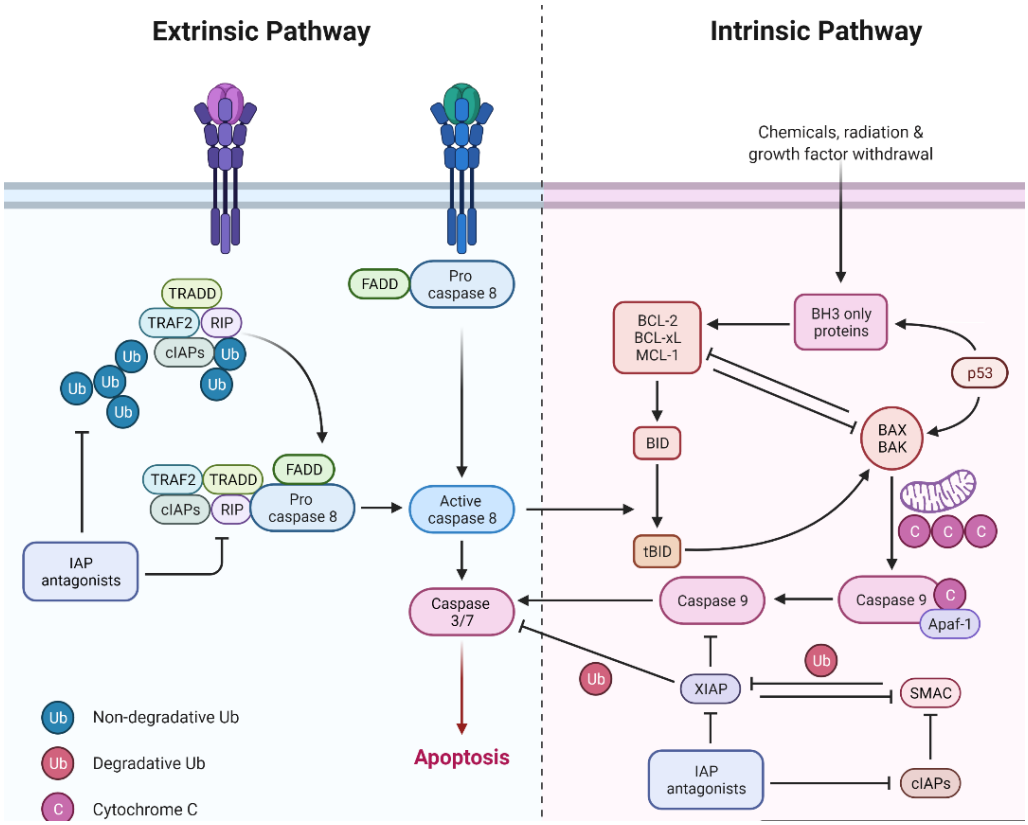
Although several studies imply that chemotherapeutic treatments kill breast cancer cells by inducing apoptosis, there are several critical implications for the future of breast cancer chemotherapy. The efficiency of a wide range of unrelated chemotherapeutic medications can be altered by metabolic changes that render cells more or less vulnerable to apoptosis. Changes that reduce cancer cells' capacity to activate the apoptotic machinery may play a role in chemoresistance to a wide range

of drugs. To fully assess the significance of this approach in creating more efficient chemotherapy, complete knowledge of the apoptotic signal transduction machinery would be required (Sun & Peng, 2009).

Resistance to chemotherapeutics such as cyclophosphamide, doxorubicin, methotrexate, fluorouracil and tubulin inhibitors has been associated with changes in genes controlling apoptosis (p53, caspase-3, BCL-2, BCL-xl) (Aas et al., 1996; Végran et al., 2006).

Figure 8

Apoptosis: Extrinsic and Intrinsic Pathways (created with biorender.com)



A previous study showed that MYC and MCL1 synchronize to keep chemotherapy-resistant cancer stem cells (CSCs) alive in TNBC. MYC and MCL1 boosted mitochondrial oxidative phosphorylation (mtOXPHOS) and the production of reactive oxygen species (ROS), all of which are necessary for cancer cell progression (Hutchinson et al., 2018).

Disruptions in the regulation of the intrinsic pathway in cancer are caused mainly by the anti-apoptotic BCL-2 protein family (BCL-2, BCL-XL and MCL-1) and pro-apoptotic molecules (BAX, BAK, BID, BIM, BIK, NOXA and PUMA)

(Pilling & Hwang, 2019; Shahar & Larisch, 2020). Rather than the expression level of a specific molecule, the imbalance of the anti-apoptotic/pro-apoptotic protein ratio determines cell survival and sensitivity to apoptotic stimuli (Hutchinson et al., 2018).

Anti-apoptotic BCL-2 and BCL-XL proteins are highly expressed in glioma cancers; the high expression level of MCL-1 in glioma cancer is proportional to the resistance to BCL-2 inhibitor (Wang et al., 2017a). Downregulation of BCL-2 and upregulation of BAX induces apoptosis in TNBC MDA-MB-231 cells (Inao et al., 2018). Therefore, inhibition of the mitochondrial death pathway is among the targeted cancer treatment approaches.

Several studies in breast cancer have demonstrated that one mechanism of anticancer drug therapy in sensitising breast cancer cells to apoptosis is increasing the expression of death receptor ligands such as Fas ligand (FasL) (Shahar & Larisch, 2020).

microRNA

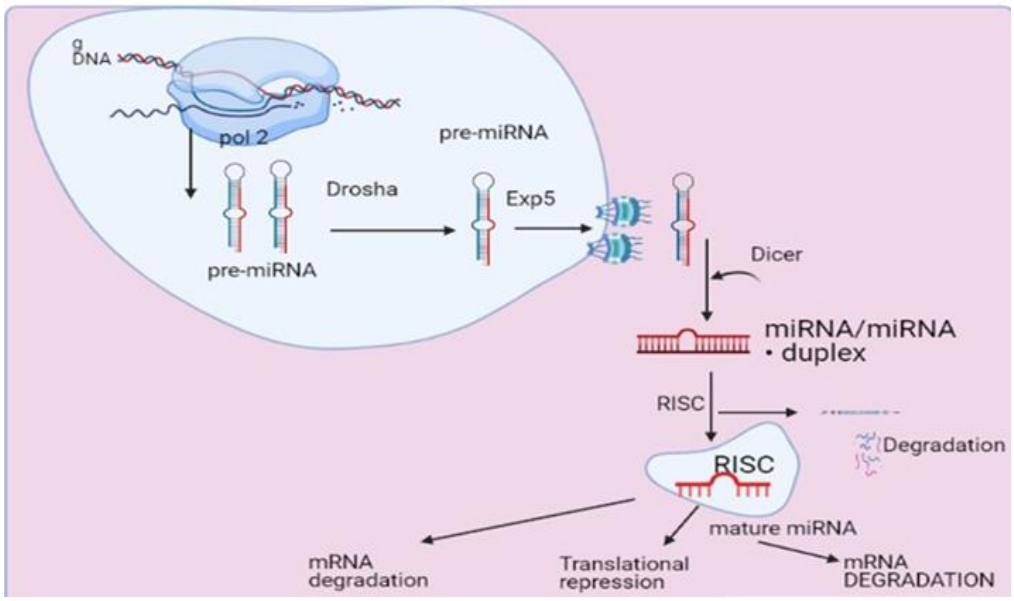
The history of miRNA discovery goes back to 1993. Short non-coding RNA molecules known as microRNAs (miRNAs) were first identified in the *Caenorhabditis elegans*, Lin-4, is the first miRNA found in *C. elegans* (Lee et al., 1993). microRNAs are evolutionary conserved, short non-coding RNAs (ncRNAs) of roughly 22 nucleotides (Garzon et al., 2009). So far, around four thousand mature miRNAs have been discovered and sequenced in the human genome (Cantini et al., 2017). They have a role in various biological processes and regulate protein translation by binding to target mRNAs. miRNAs are associated with cell growth, migration, invasion and drug sensitivity (Lu et al., 2005; Rasool et al., 2016; Nagesh et al., 2018; Li et al., 2019; Al-Othman et al., 2020; Sadakierska-Chudy, 2020). In tumor cells, miRNA expression varies, and it acts as either an oncogene or tumor suppressor. For example, miR-223, miR-122 and miR-26 are liver tumor suppressors, while miR-130b, miR-221 and miR-222 are oncogenic (Williams, 2008; Liu, 2012; Egeland et al., 2015; Luo et al., 2015). Other miRNAs influence the fate of cancer cells by controlling their self-renewal. For example, miR-34 inhibits human pancreatic cancer by regulating Notch and BCL-2 gene expression (Hosseinahli et al., 2018), and miRNA-122 suppresses hepatocellular carcinoma (HCC) by targeting various oncogenes (El-Halawany et al., 2015).

There are three main steps in miRNA biogenesis (Figure 9). First, RNA polymerase begins to transcribe the segment, which may be found either within genes or in genes resulting in a lengthy stretch of mRNA known as primary miRNA (pri-miRNA). The next step is to shorten pri-miRNA by DROSHA and DGCR8 to create a precursor miRNA (pre-miRNA), which is 70 bases long. The final step includes exporting the pre-miRNA to the cytoplasm (via exportin 5), where Dicer catalyzes the breakdown of pre-miRNA into 22-base parts and separates the duplex into two single strands: a mature strand and a degraded strand.

The mature strand forms the RNA-induced silencing complex (RISC) with AGO-2 and R2D2. The RISC may either degrade or slow down the translation rate of the target mRNA. (Iqbal et al., 2019). The target mRNA can also be sequestered from the translation machinery and transported to the cytoplasmic bodies, where untranslated mRNAs await degradation. Although each miRNA's seed region is entirely complementary to its target mRNA, the full-length miRNA does not have to match with its target mRNA. Instead, a single miRNA needs just 2-8 nucleotides to recognize its target and accomplish its activity (Jay et al., 2007).

Figure 9

miRNA Biogenesis (adapted from Garzon et al., 2009)



miRNA Role in Breast Cancer

The significance of miRNA in cancer was initially investigated in patients with chronic lymphocytic leukaemia (CLL). A particular region on chromosome 13 (specifically 13q14) was identified to have no tumor suppressor gene, but two types of miRNAs, miR-15a and miR-16a, which are the target of 13q14 deletions in CLL; the mutations are likely to have a role in the inactivation of their expression (Calin et al., 2004).

miRNAs have dual roles in breast cancer; for example, some miRNAs have tumor suppression activity that dysregulation has been documented in virtually all breast cancers and can affect all phases of carcinogenesis. Piasecka and colleagues suggested that increase in the levels of miR-203 inhibits cell proliferation and migration (Piasecka et al., 2018). This reduces tumor invasion *in vitro* via suppressing the zinc finger transcriptional repressor (SNAI2). This discovery suggests that the SNAI2 and miR-203 regulatory loop is critical in epithelial-to-mesenchymal transition (EMT) and tumor metastasis (Cheung et al., 2015). miR-200c inhibits cellular proliferation by targeting the XIAP apoptosis inhibitor gene (Damiano et al., 2017). D'Ippolito & Iorio reported let-7 miRNA family to have tumor suppressor effect in breast cancer, and a few members can enhance tamoxifen sensitivity in TNBC cells *in vitro* by interrupting the ER signaling pathway (D'Ippolito & Iorio, 2013).

Several miRNAs act as oncomiRs in TNBC, including miR-9, miR-21, miR-93, miR-181a/b, miR-182, miR-221, miR-21,miR-182 and miR-155, all of which coordinate and control the whole carcinogenesis process (Sabit et al., 2021).

miRNA and Androgens in Breast Cancer

A few groups have investigated how androgens influence miRNA expression in breast cancer. Nakano et al. used a PCR microarray to reveal that five miRNAs were dysregulated in MCF-7 cells (Nakano et al., 2013). Lyu et al. discovered that four miRNAs, let-7a, b, c and d, were upregulated in androgen-treated MDA-MB-453 cells whereas the remaining seven miRNAs were downregulated (Lyu et al., 2014). Further research by Ahram and colleagues has discovered several types of miRNAs of which their expression was affected by androgens. By using PCR arrays, differential expression of 20 miRNAs in MDA-MB-453 cells upon androgen

treatment was reported. Surprisingly, only three microRNAs, let-7a, b and d, were shown to be downregulated (Ahram et al., 2017). The same group also investigated the role of androgens in the control of miRNA expression in MCF-7 and T47D cells (Ahram et al., 2017). However, none of the alterations was similar to those reported by Nakano et al. (Nakano et al., 2013). In a recent study on MDA-MB-231 cells using PCR arrays, a differential change in the expression of 84 miRNA following treatment with DHT was investigated. They revealed that the expression of 33 miRNAs increased by more than two folds, including miR-328-3p, which increased by 13 folds. Also, several miRNAs were affected by androgens, including miR-26b-3p and miR-26a-3p; they were up-regulated upon androgen treatment (Al-Othman et al., 2018).

miRNA and Drug Resistance in Cancer

Several microRNAs have been linked to drug resistance in TNBC (Ma et al., 2010). Chemotherapy resistance appears to be modulated by particular miRNAs. Climent et al. postulated that the deletion of chromosome 11q, which carries the miR-125b gene, may be connected to the increased sensitivity of breast cancer patients treated with anthracycline. This finding showed a link between miRNA dysregulation and cancer therapy resistance (Climent et al., 2007).

miRNAs have a function in modulating drug resistance mediated by multidrug resistance/ P-glycoprotein (MDR1/P-gp) (Zhu et al., 2008). Compared to their parental cell lines A2780 and KB-3-1, expressions of miR-27a, miR-451, miR-99a, miR-100 and miR-125b1 were elevated in human MDR ovarian cancer cell line A2780DX5 and human cervical carcinoma cell line KB-V1. P-gp and MDR1 levels were reduced in A2780DX5 cells after transfection with miR-27a. The mimics of miR-27a, on the other hand, boosted MDR1 expression in the parental A2780 cells (Zhu et al., 2008). A recent study by Wang et al. on TNBC revealed that tamoxifen enhances the chemosensitivity of mesenchymal TNBC cells by upregulation of miR-200c, reversing their EMT-like behavior. The EMT-reversal effect is the result of the demethylation of its promoter (Wang et al., 2017).

A different study on miR-26b suggested its role as a tumor suppressor due to re-sensitizing cancer cells to chemotherapy. Zhao et al., by using gastric cancer cell lines BGC823 and SGC7901, revealed that the miR-26b suppresses the CDC6 gene and therefore decreases the proliferation of cells, resulting in sensitizing gastric

cancer to paclitaxel chemotherapy (Zhao et al., 2019). Another study on colorectal cancer cells found that miR-26b boosted sensitivity to 5-FU *in vitro* and improved the efficacy of drug in inhibiting tumor development *in vivo*. miR-26b suppressed tumors via decreasing P-gp production. Furthermore, this study revealed an inverse relationship between miR-26b and P-gp expression (Wang et al., 2018). In hepatocellular carcinoma (HCC) cell lines, miR-26b was found to sensitize HCC cells to doxorubicin-induced apoptosis by targeting the NF-κB and decreasing the expression of TAK1 and TAB3 (Zhao et al., 2014). Up to our knowledge, no previous study investigated the link between miRNA26 and chemoresistance in TNBC.

CHAPTER III

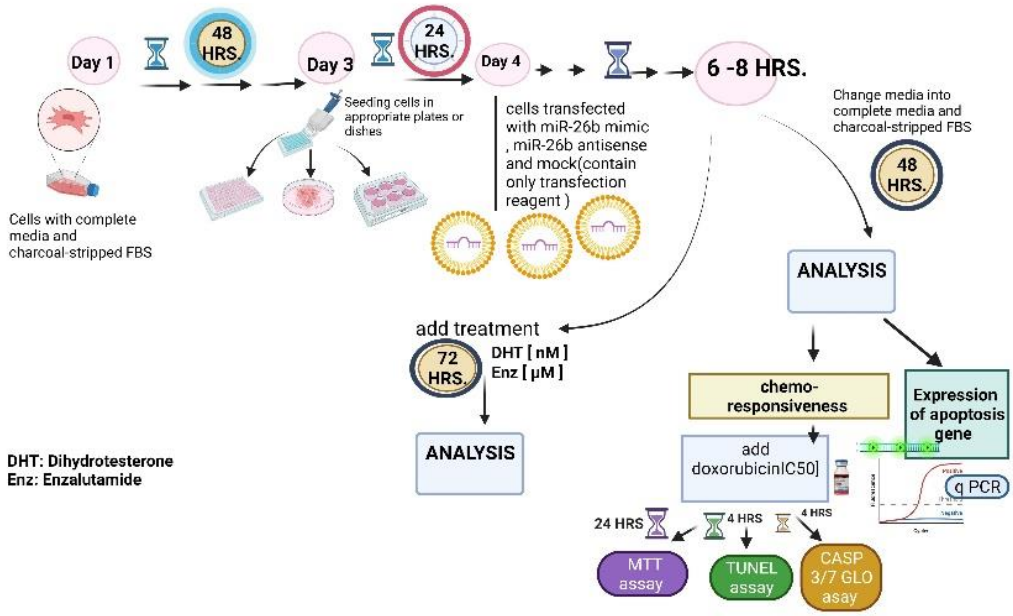
Materials and Methods

Experimental Design

The experimental design is illustrated in Figure 10. Briefly, culture media fortified with 10% charcoal-stripped FBS were used as optimum media for cell growth for two days. After that, cells were given DHT and/or enzalutamide for three days. The cells were then treated for the given time with doxorubicin with or without DHT and/or enzalutamide before the experiment. To assess the effect of miR-26b, the cells were transfected with miR-26b mimic, or antisense, then treated with DHT and/or enzalutamide for three days. Then, the cells were treated with doxorubicin with or without DHT and/or Enz for the indicated period before performing the assays.

Figure 10

Experiment Workflow



Experiment Model

MDA-MB-231 and MDA-MB-453 cells were obtained from Prof. Ahram's laboratory and used in this study. The cells were grown in DMEM high glucose cell culture media and Leibovitz's L-15 media (Capricon Scientific, Ebsdorfergrund,

Germany). 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, and 10% (v/v) fetal bovine serum were added to the medium. All media reagents were purchased from Biowest (Nuaillé, France). The cell lines were incubated for 24 hours in media supplemented with fetal bovine serum in a humidified incubator at 37ºC with 5% CO_2 for MDA-MB-231 cells and without CO_2 for MDA-MB-453 cells. Before applying different treatments, 10% of charcoal-stripped FBS (Biowest, Cat. No. S181F-500) was used. The word "control" refers to cells which only contain culture media instead of any other treatment.

Preparation of Solutions

Preparation of Dihydrotestosterone

10 mM stock DHT solution was prepared by dissolving 2.9 mg DHT (Tokyo Chemical Industry, Cat. No. A0462) in 1 mL of DMSO (ACS Grade, Bioworld, Cat. No. 40470005-1). The stock solution (10 mM) was then diluted in DMSO to prepare 10 and 100 µM DHT solutions which were kept at -20ºC and utilized within three months of preparation.

Preparation of Enzalutamide

Different concentrations (2 mM, 4 mM, 8 mM and 16 mM) of enzalutamide were prepared by diluting a 100 mM stock solution of enzalutamide (Cayman Company, MDV 3100) with DMSO and stored at -20ºC until used. For treating cells with DHT ± enzalutamide, the final concentration of compounds was achieved by adding to media at a ratio of 1:1000.

Preparation of Doxorubicin

Different concentrations of (0.5 mM, 1 mM and 2 mM) of doxorubicin (Sigma-Aldrich, MO, USA, D1515) were prepared and stored at -4ºC until used. For treating cells with doxorubicin combined with DHT and enzalutamide, the final concentration of compounds was achieved by adding to media at a ratio of 1:1000.

Preparation of Culture Medium for Cell Lines

The DMEM/F-12 medium solution was supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 1% glutamine. By withdrawing numerous times with a pipette, a homogeneous dispersion was established. The prepared culture media was stored at 4^{o}C. The substances used and their volumes are given in Table1. Furthermore, cryopreservation media preparation is shown in Table 2.

Table 1

Culture Media and its Components

Solutions	Percent	Volume
Culture Media	1X	440 ml
Fetal Bovine Serum (FBS)	10%	50 ml
Penicillin / Streptomycin	1%	5 ml
L-Glutamine	1%	5 ml
Total volume		500 ml

Table 2

Cryopreservation Media Preparation

Solutions	Percent	Volume
Culture Media	1X	8.4 ml
Fetal Bovine Serum (FBS)	10%	1 ml
Penicillin / Streptomycin	1%	100 µl
DMSO	5%	500 µl
Total volume		10 ml

Methods

Optimization and Cell Passage

MDA-MB-231 was cultivated and multiplied in the laboratory conditions (DMEM medium with l-Glutamine, pH 7.4). For MDA-MB- 453, the base medium was ATCC-formulated Leibovitz's L-15 Medium.

The cell lines were incubated at 37°C with 5% CO_2 /or without CO_2 in a medium containing 10% (v/v) fetal bovine serum or 10% of charcoal-stripped FBS

(Biowest, Cat. No. S181F-500) in the case of preparing cells for treatments. The media is supplemented with 1% glutamine and 1% penicillin/streptomycin. ATCC and Sigma were used as a reference for culture conditions and requirements specific to cell lines. The doubling time of cells was determined, and optimization studies were performed in our laboratory conditions. The duplicated cells were frozen in at least five vials at -86°C after each passage and stored in the Prof. Ahram's Laboratory at the University of Jordan.

Cell Counting

The hemocytometer chamber and cover slip were cleaned with alcohol to determine the cell count. The coverslip was dried and held in place. 10 µL of collected cells were put on the hemacytometer with 0.4% trypan blue stain (Lonza, Cat. No. 17-942E) in a 1:1 ratio. DMIL Leica inverted microscope with a 10X objective was used to count the cells (Leica Microsystems, Wetzlar, Germany). The counting chamber contains 16 medium squares, each with 25 small squares (9 divided in half). The following formula was used to count the cells:

$$\mathbf{N \times 10^4 \times df = cells\ /mL}$$

N: number of the cells in one of the large square;

Df: dilution factor

Cell Viability

Cell viability was determined using an MTT assay. MDA-MB- 231 and MDA-MB- 453 cells were added to 96-well plates at 5000 cells per well. The cells were allowed to recover and retain their morphology for 24 hours. After the waiting period, the cells were exposed to different concentrations of DHT (0, 10, and 100 nM) in combinations with different concentrations of enzalutamide (8 and 16 µM). After 72 hours of incubation, different concentrations of doxorubicin (Dox) were added to wells and cells were incubated for 24 hours. Cell viability was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, M5655) in a concentration of 5 mg/ml of PBS.The solution was filtered through a 0.2 µm filter and added to cell culture media in a ratio of 1:10, and to culture well being assayed for 3 to 4 hrs at 37°C. In all the experiments, one set of wells, having only cells and medium, but no drug was taken as the control. After that,

the medium with MTT solution was removed, and the converted dye was solubilized with acidic isopropanol and DMSO (1:1). The absorbance of dissolved crystals were measured at 570 nm using the Synergy HTX Multi-Mode Reader (BioTeck, Canada) and Gen5TM Microplate Reader and Imager Software (BioTeck, Canada) (Al-Othman et al., 2018).

Transfection

RNAifectin™ Transfection Reagent (abm, Cat. No. G073) was used for transfection according to the manufacturer's instructions. Cells were incubated for 6 hr in a serum-free media containing RNAifectin™ with 50 nM of either of miR-26b-mimic (Qiagen, Cat. No. MS00003234), miR-26b-3p antisense (Qiagen, Cat. No. MIN0000083), or Silencer™ sc-siRNA (Thermo Fisher, Cat. No. 4390843) as a negative control. Then, the media was changed with new appropriate media supplemented with 10% of charcoal-stripped FBS in addition to treatments and incubated for three days (Ahram et al., 2017)

Gene Expression Analysis

RNA Isolation, cDNA Synthesis and Quantitative PCR Analysis

To analyse the expression of miR-26b, *bid*, *bcl2* and *mcl1* mRNA, cells were seeded in 6-well plates. As mentioned earlier, before seeding, cells were grown in media supplemented with 10% of charcoal-stripped FBS for 48 hrs. Cells were then seeded and incubated for 24 hrs before treatment.

The total mRNA was extracted from the cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany, Cat. No. 217004), following the manufacturer's instructions. The QuantiTect Reverse Transcription Kit (Qiagen, 204143) synthesized cDNA for the *bid*, *bcl2* and *mcl1* mRNA analyses. Nucleic acid concentrations and purity were assessed using the NanoDropTM 2000 Spectrophotometer (Thermo Fisher, Scientific, Waltham, MA, USA, ND-2000). Quantitative PCR (qPCR) was performed using the Quanti-Tect SYBR® Green PCR Kit (Qiagen, 204143). Relative mRNA expression was quantified using the 7500 Fast Real-Time PCR instrument Applied Biosystems™ (CA, USA). The relative expression of the target gene was quantified and normalized to that of the reference β-actin genes using the livak method (Livak & Schmittgen, 2001).

cDNA samples of miRNA from treated MDA-MB-231, and MDA-MB-453 cells were synthesised using, miScript II RT Kit (Qiagen. Cat. No. 218161) ,

transfected RNA samples for both cell lines used in this study were diluted to be 1 µg RNA/ 12 µl dH2O. Then, the following reverse-transcription reaction master mix was prepared as of enough volume for all samples as in Table 3:

Table 3

Reverse Transcription Reaction Components

Component	Volume per reaction
5x miScript HiSpec Buffer	4 µl
10X miScript nucleic mix	2 µl
miScript Reverse Transcriptase mix	2 µl
RNase-free water + RNA Template for each sample(depends on conc measured for each sample)	12 µl
Total volume	20 µl

For each sample, 12 µl of diluted RNA template were mixed with 8 µl of the reverse-transcription master mix that prepared until the final volume reached 20 µl for each reaction. Tubes were incubated for 5 minutes at 95˚C and 60 minutes at 37˚C and then placed on ice to inactivate miScript Reverse Transcriptase Mix until they were utilized. Samples were stored at -20˚C until used.

In qPCR, to detect changes in miR-26b level upon treatment and/or transfection. To do so, Hs_miR-26b miScript Primer Assay (Qiagen, Cat. No. MS00081472), which targets mature hsa-miR-26b was used. RNU6 (RNA, U6 Small Nuclear 1) was used as the reference gene and detected by Hs_RNU6-2_11 miScript Primer Assay (Qiagen, Cat. No. MS00033740). PCR reactions were performed in regular 0.1 ml PCR strip tubes using QuantiTect SYBR® Green PCR Kit (Qiagen, Cat. No. 204143). The reaction contained 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2 µl of 10x miScript Universal Primer, 2 µl of 10x miScript Primer Assay (Hs_miR-26b, miScript Primer Assay or RNU6), and the concentrations of cDNA were 3ng per PCR reactions.

The following qPCR conditions were used: 95oC for 15 min then 40 cycles of 94oC for 15 sec, 55oC for 60 sec and 72oC for 30 sec. The following forward (F) and reverse (R) primers were used in this study:

- *Bcl2* (human):

Forward primer: 5' TTCTTTGAGTTCGGTGGGGT 3’

Reverse primer: 5’ GCTGAAACTCCCTTAGCCCT 3’

- *Mcl1* (human):

Forward primer: 5' CCAAGAAAGCTGCATCGAACCAT 3’

Reverse primer: 5’ CAGCACATTCCTGATGCCACCT 3’

- *Bid* (human):

Forward primer: 5' TGGGACACTGTGAACCAGGAGT 3’

Reverse primer: 5’ GAGGAAGCCAAACACCAGTAGG 3’

- *β-actin* (human):

Forward primer: 5' CTGTGGCATCCACGAAACTA 3’

Reverse primer: 5’ CGCTCAGGAGGAGCAATG 3’

Caspase 3/7 Glo Assay

In a white-walled multiwell luminometer cell culture plate (Thermo Fisher Scientific, Waltham, Massachusetts, United States), cells were seeded at 5000 cells per well. The cells were treated with DHT and/or enzalutamide for 72 hours, then doxorubicin was added for 4 hours. The activity of caspase 3/7 was evaluated using Promega Caspase 3/7 Glo Assays (Promega Corporation, WI, USA, Cat. No. G8091) according to manufacturers' instructions. The Synergy HTX Multi-Mode Reader (BioTek Instruments, Canada) and Gen5TM Microplate Reader and Imager Software were used to measure luminescence (BioTek Instruments) (Sundquist et al., 2006).

TUNEL Assay

DNA degradation was assessed using the DeadEnd™ Fluorometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) System (Promega, Madison, USA, Cat. No. TB235) according to the manufacturer's instructions. Briefly, cells were seeded at a density of 10000 cells/well in 15×1-mm, type I collagen-coated glass coverslips and placed in 12-well plates for 24 hrs. After incubating with the different treatments, the cells were fixed in 4%

paraformaldehyde for 20 min before permeabilization with 0.5% Triton X-100. The TUNEL reaction mixture was applied to the cells, followed by ProLong Gold Antifade Mountant with DAPI (Thermo Fisher, P36962). The Zeiss Axio Imager 2 Fluorescent Microscope was used to examine the cells and the images were analyzed using Zen 2012, Blue Edition (Zeiss, Germany). For quantitative analysis the TUNEL labeled nuclei were analyzed using ImageJ software (Schneider, et al., 2012).

CHAPTER IV

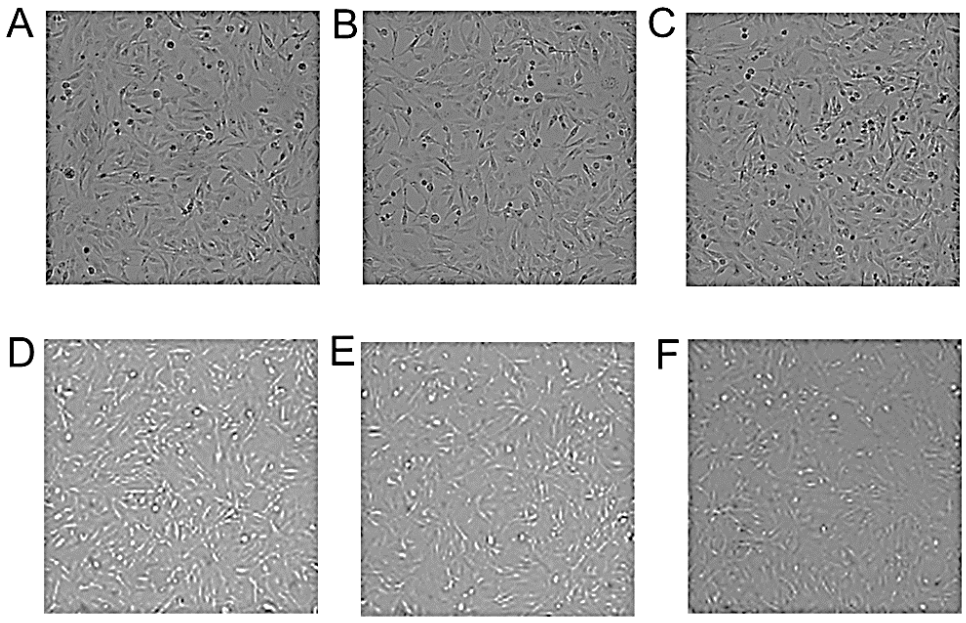
Results

The Effect of Dihydrotestosterone and Enzalutamide on Cell Morphology

To investigate the influence of DHT on cell morphology, cells were microscopically examined after three days of treatment with 16 µM enzalutamide with or without 10 nm and 100 nm DHT in a medium supplemented with 10% charcoal-stripped FBS (Figure 11). Present microscopic investigation indicated no significant effect of DHT and enzalutamide on MDA-MB-231 cell morphology: that the spindle-like shape of cells was not altered in the presence of DHT alone or in combination with enzalutamide. On the other hand, DHT affects MDA-MB-453 cell morphology that is originally spheroid shape; when DHT was added, cell stacking was decreased, and cells began to take on an extended, fibroblast-like morphology after three days of DHT treatment (Figure 12).

Figure 11

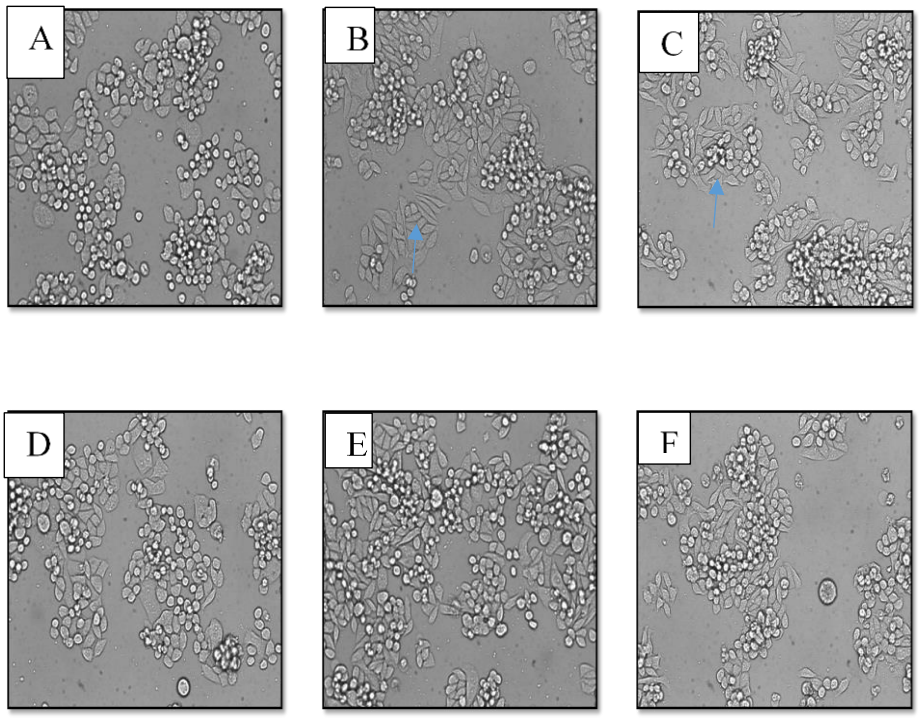
The Effect of DHT Treatment on the Morphology of MDA-MB-231 Cells



(A) MDA-MB-231 control cells (B) Cells treated with 10 nM DHT (C) Cells treated with 100 nM DHT (D) Cells treated with 16 µM enzalutamide (E) Cells treated with 16 µM enzalutamide in combination with 10 nM DHT (F) Cells treated with 16 µM enzalutamide in combination with 100 nM DHT.

Figure 12

The Effect of DHT Treatment on the Morphology of MDA-MB-453 Cells



(A) MDA-MB-453 control cells (B) Cells treated with 10 nM DHT (C) Cells treated with 100 nM DHT (D) Cells treated with 16 µM enzalutamide (E) Cells treated with 16 µM enzalutamide in combination with 10 nM DHT (F) Cells treated with 16 µM enzalutamide in combination with 100 nM DHT.

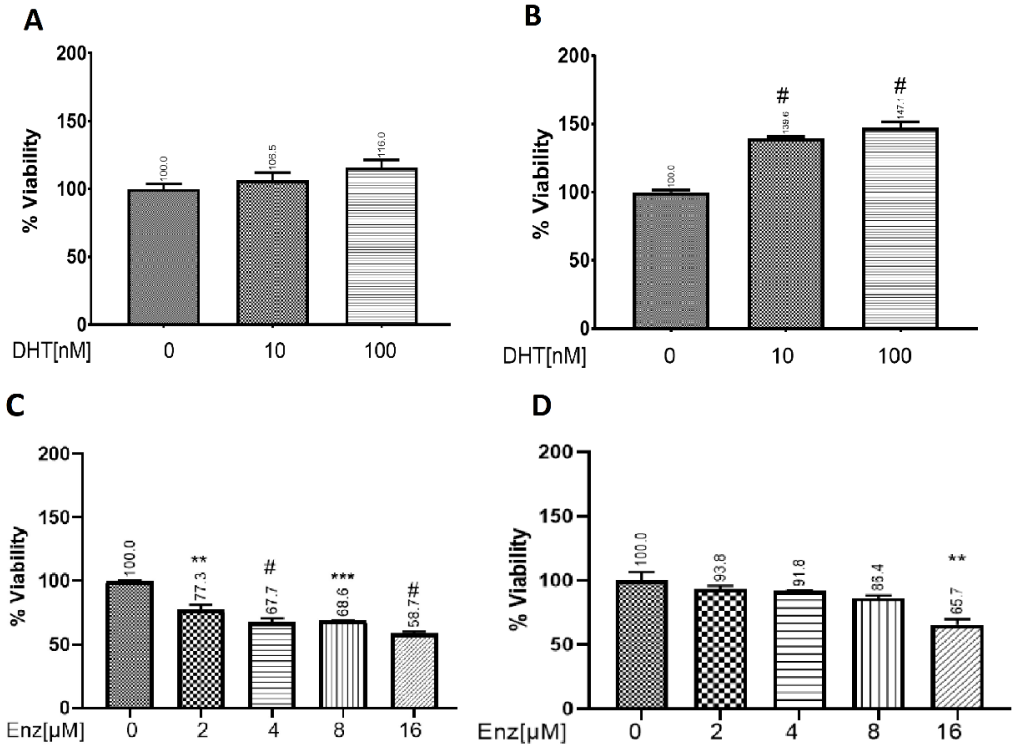
The Effect of Dihydrotestosterone and Enzalutamide on the Proliferation of MDA-MB-231 and MDA-MB-453 Cells

To assess the effect of DHT and enzalutamide on the growth of cells, the cells were treated with different concentrations of both DHT and enzalutamide for three days. DHT had no significant effect on the proliferation of MDA-MB-231 cells (Figure 13A). In contrast, it significantly induced the growth of MDA-MB-453 cells in a dose-dependent manner, increasing the cell number by 30 and 50% at 10 and 100 nM DHT, respectively (Figure 13B). The sensitivity of cells to enzalutamide was variable, though. Enzalutamide reduced the growth of MDA-MB-231 cells in a dose-dependent manner, decreasing cell numbers by 20% at 2 µM up to 40% at 16 µM (Figure 13C). On the other hand, the inhibitory effect of enzalutamide on MDA-MB-

453 cells was only significantly apparent at 16 µM, which reduced cell growth by 34% (Figure 13D).

Figure 13

The Effect of DHT and Enzalutamide on the Proliferation of MDA-MB-231 and MDA-MB-453 Cells



*(A and C) MDA-MB-231 cells, (B and D) MDA-MB-453 cells. All presented concentrations are final concentrations. p-values are indicated as **< 0.01 and ***<0.001, # <0.0001 compared with control (cells treated with 0.1% DMSO). Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

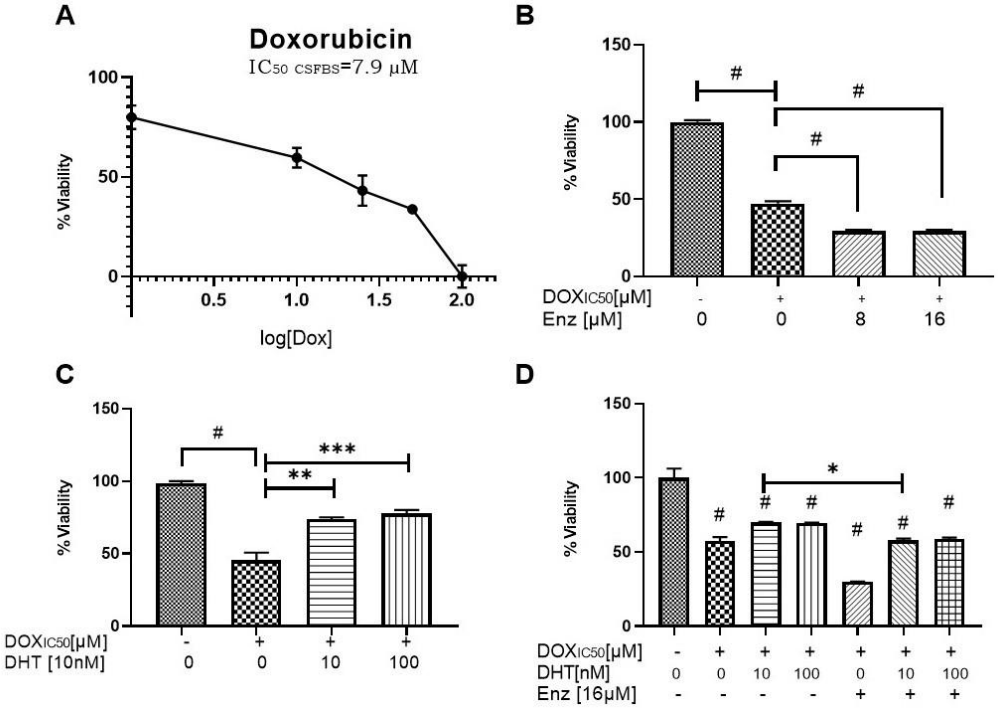
Chemoresponse of MDA-MB-231 and MDA-MB-453 Cells Toward Doxorubicin in Combination with Dihydrotestosterone and Enzatulamide

The influence of DHT and enzalutamide on the chemoresponse of both cell lines toward doxorubicin was then investigated. The half-maximal inhibitory concentrations (IC_{50}) of doxorubicin on both cell lines were first determined and then each cell line was treated with this concentration in the presence of DHT and/or enzalutamide. As for MDA-MB-231 cells, the IC_{50} of doxorubicin was determined to be 7.9 µM (Figure 14A). Adding 8 or 16 µM enzalutamide to the cells treated with

doxorubicin further reduced cell viability (Figure 14B). On the other hand, DHT increased cell viability at 10 and 100 nM (Figure 14C). Enzalutamide, however, reduced the effect of DHT on the chemoresistance of cells at 10 nM DHT, but not at 100 nM (Figure 14D).

Figure 14

DHT and Enzalutamide Affect the Chemoresponse of MDA-MB-231 Cells toward Doxorubicin

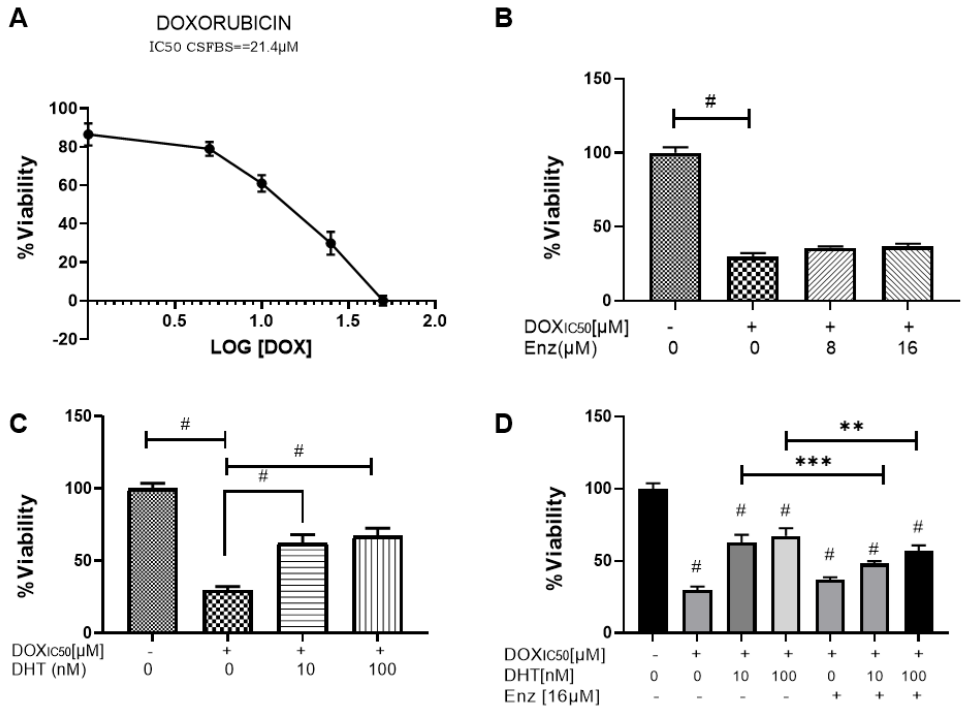


*(A) Dose-response curve for MDA-MB-231 cells treated with doxorubicin (DOX) for 24 hrs. (B) Effect of Enz on chemosensitivity (C) Effect of DHT on chemosensitivity (D) Effect of the combination of DHT and Enz on chemosensitivity. p-values are indicated as NS, Not Significant, *<0.05, **< 0.01, and ***<0.001 compared with the indicated treatment, and # <0.0001 versus the vehicle-treated sample. Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

The IC_{50} of doxorubicin on MDA-MB-453 cells was calculated to be 21.4 µM (Figure 15A). Enzalutamide did not change the chemoresponse of cells toward doxorubicin at either 8 or 16 µM (Figure 15B). On the other hand, treatment of cells with either 10 or 100 nM DHT increased their chemoresistance (Figure 15C). Furthermore, the addition of enzalutamide reduced the DHT-induced chemo-resistance toward doxorubicin at 10 and 100 nM (Figure 15D).

Figure 15

The Effect of DHT and Enzalutamide on ChemoResponse of MDA-MB-453 Cells Toward Doxorubicin



*(A) Dose-response curve for MDA-MB-453 cells treated with doxorubicin (DOX) for 24 hrs. (B) Effect of Enz on chemosensitivity. (C) Effect of DHT on chemosensitivity. (D) Effect of the combination of DHT and Enz on chemosensitivity. p-values are indicated as **< 0.01 and ***<0.001 compared with the indicated treatment, and # <0.0001 compared with the vehicle-treated sample. Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

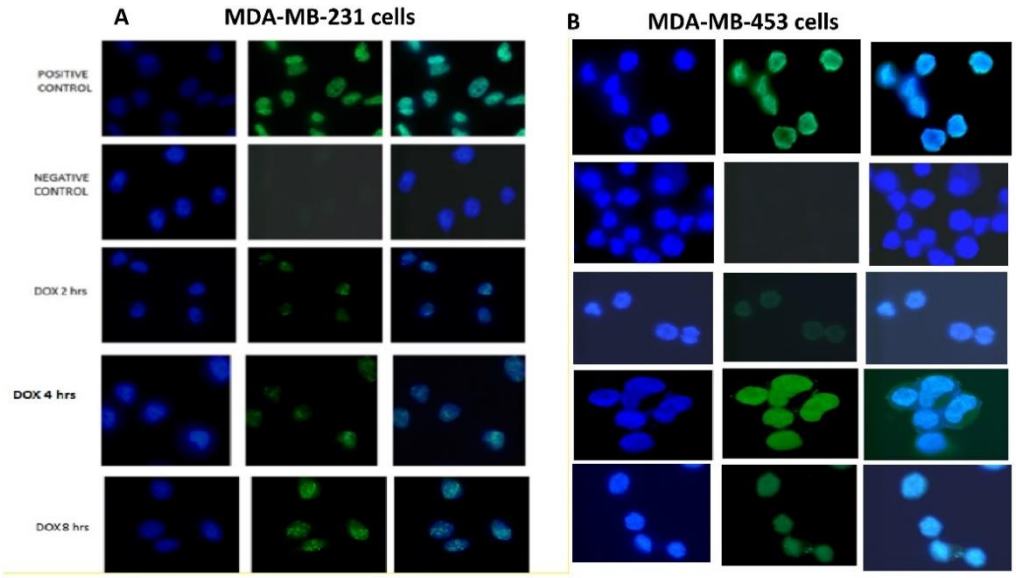
The Effect of Dihydrotestosterone and Enzalutamide on the Late Stages of Doxorubicin-Induced Apoptosis

In order to determine whether DHT increases the chemoresistance of MDA-MB-231 and MDA-MB-453 cells toward doxorubicin via suppressing apoptosis, two assays were performed. The first assay was the TUNEL assay, which would illustrate the extent of DNA cleavage at the final stages of apoptosis. Treatment of the cells with DNase was used as a positive control and treatment of cells with doxorubicin for 4 hours was optimal to observe differences in DNA cleavage among the different treatments. As shown in Figure 16, doxorubicin induced DNA cleavage in MDA-

MB-231 cells but adding 10 nM DHT to cells blocked this induction. On the other hand, enzalutamide (16 µM) induced a strong signal of DNA cleavage, which increased by three folds when combined with doxorubicin. The addition of DHT by itself or in combination with enzalutamide to doxorubicin-treated cells reduced the signal intensity indicating a lower incidence of DNA nicking (Figure 17A). Similar results were observed for MDA-MB-453 cells, except that enzalutamide had a weaker effect on DNA cleavage compared to MDA-MB-231 cells (Figure 17B)

Figure 16

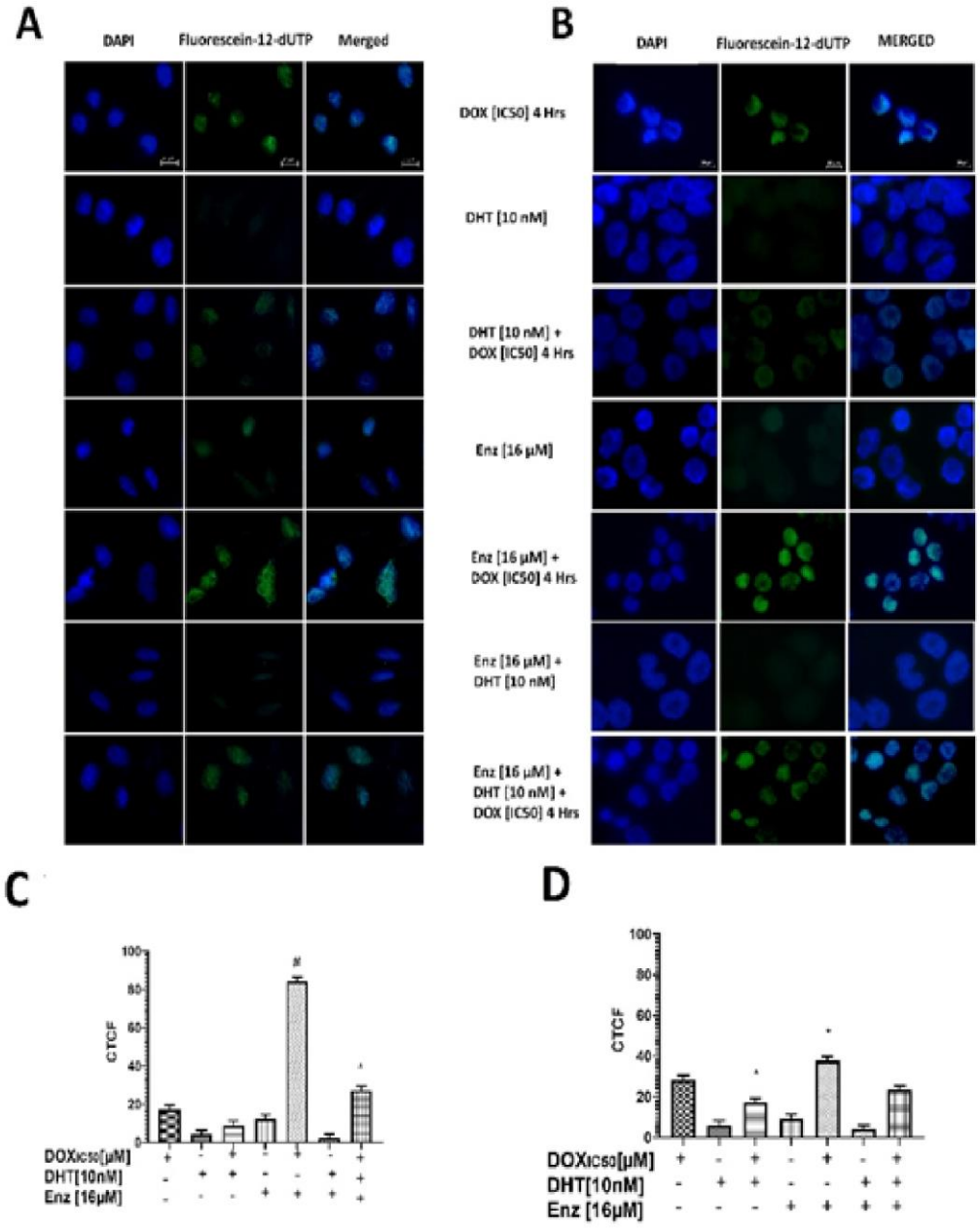
TUNEL Assay Results for MDA-MB-231 (A) and MDA-MB-453 Cells (B)



The nuclei were visualized by staining with DAPI (blue), and DNA nicking was visualized by fluorescein-12-dUTP (Green). Cells were treated with doxorubicin (DOX) at different time intervals 2, 4, 8 hrs.

Figure 17

TUNEL Assay Results for the Effect of DHT and Enzalutamide on DNA Degradation Induced by Treatment of MDA-MB-231 Cells (A and C) and MDA-MB-453 Cells (B and D) with Doxorubicin (DOX)



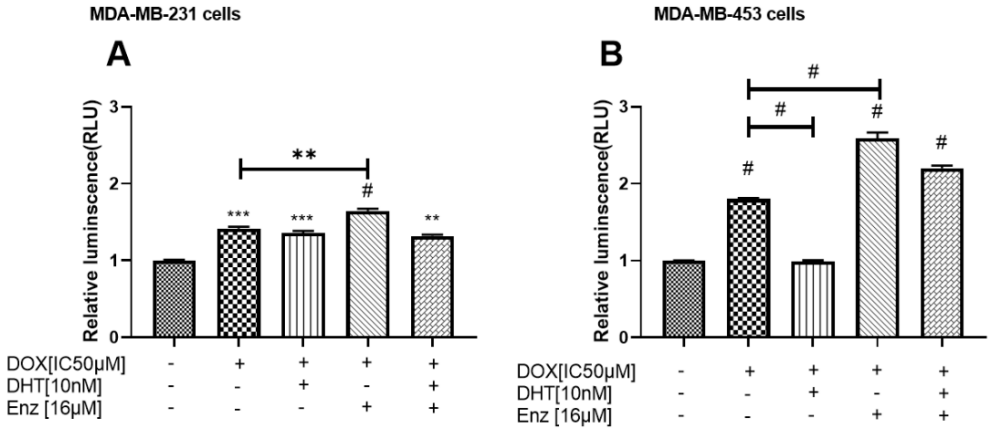
*The nuclei were visualized by staining with DAPI (blue), and DNA nicking was visualized by fluorescein-12-dUTP (Green). Scale bar 10µm. p-values are indicated as *<0.05 and # <0.00001 compared with cells treated with DOX (IC_{50}). Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

The Effect of Dihydrotestosterone and Enzalutamide on the Caspase 3/7 Activity

The activity of caspase 3/7 in cells was then measured under different treatments to further assess the effect of DHT and enzalutamide on doxorubicin-induced apoptosis. The activity of caspase 3/7 was found to be elevated in both cell lines upon exposing cells with doxorubicin (Figure 18A and B). Prior treatment of MDA-MB-231 cells with DHT did not reduce the enzyme activity, whereas it blocked the enzyme activity in MDA-MB-453 cells. In both cell lines, enzalutamide further increased the activity of caspase 3/7 when combined with doxorubicin relative to the treatments with doxorubicin alone. In addition, there was no difference in enzyme activity in cells treated with doxorubicin, DHT and enzalutamide relative to the combination of doxorubicin and enzalutamide.

Figure 18

The Effect of DHT and Enzalutamide on the Activity of Caspase 3/7 in the Presence or Absence of Doxorubicin in MDA-MB-231 and MDA-MB-453 Cells



*P-value is indicated as **< 0.01 and ***<0.001 compared with the indicated treatment and # <0.0001 versus vehicle-treated sample. Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

The Effect of Dihydrotestosterone and Enzalutamide on the Expression of the Apoptosis-Related Genes

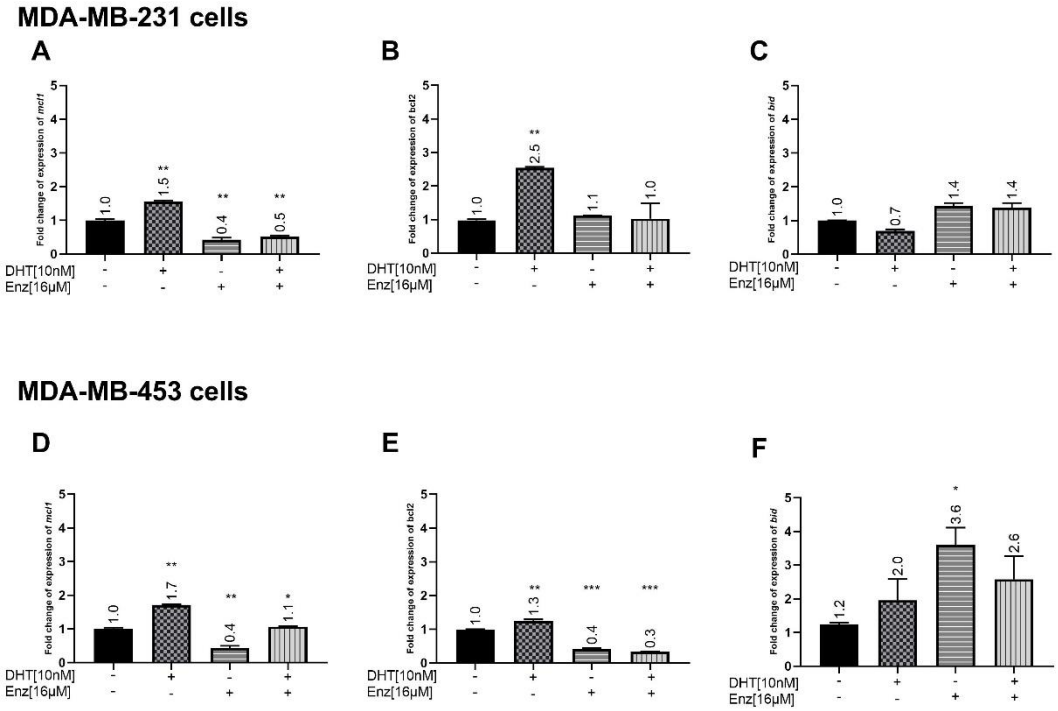
The effect of DHT and enzalutamide on doxorubicin induced apoptosis suggests that they may act via altering the expression of apoptosis-regulatory genes. Therefore, three genes were investigated: the anti-apoptotic *bcl2* and *mcl1* genes and

the pro-apoptotic *bid* gene. The cells were treated with 10 nM DHT or 16 µM enzalutamide for three days, and gene expression was assessed by qPCR. DHT significantly increased the expression of *bcl2* by 2.5 folds in MDA-MB-231 cells (Figure 19A). Although the same gene expression in DHT-treated MDA-MB-453 cells was significant, it was modest (1.3 fold). DHT also significantly increased the expression of *mcl1* by 1.5 and 1.7 folds in MDA-MB-453 and MDA-MB-231 cells, respectively. The *bid* expression was found to be unaffected by DHT treatment in both cell lines.

On the other hand, enzalutamide significantly reduced the expression of *mcl1* by 0.5 fold in both MDA-MB-231 and MDA-MB-453 cells (Figure 19A and D) and decreased the expression of *bcl2* in MDA-MB-453 cells only by 0.4 fold (Figure 19E). As for the *bid* expression, enzalutamide increased it by 3.6 folds in MDA-MB-453 cells but not MDA-MB-231 cells.

Figure 19

The Effect of DHT and Enzalutamide on the Expression of mcl1 (A and D), bcl2 (B and E) and bid (C and F) in MDA-MB-231 and MDA-MB-453 Cells



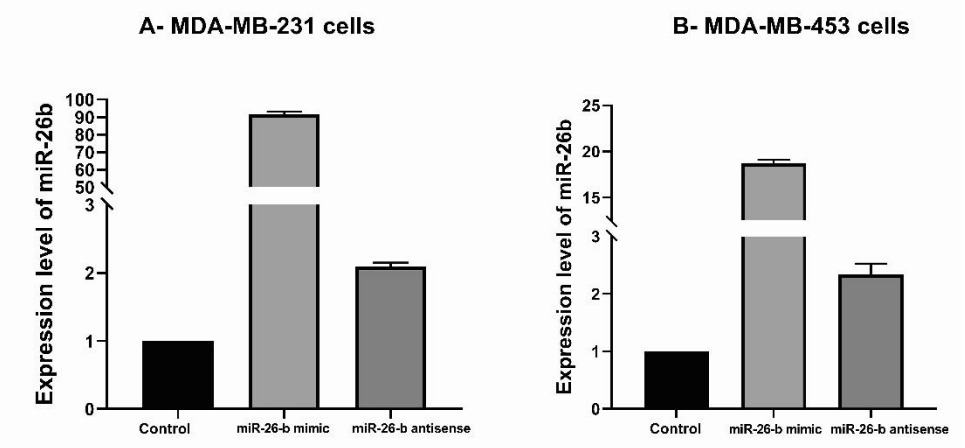
*p-values are indicated as *<0.05, **< 0.01, and ***<0.001 compared with the vehicle-treated sample. Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

Determination of the Effectiveness of miR-26b Transfection

To ensure the transfection efficiency of miR-26b on both cell lines tested, a qPCR was used to check the expression levels of miR-26b. Briefly, cells were transfected with miR-26-b mimic and anti-sense. Cells not transfected only containing transfection reagent were used as the control. When cells were transfected with miR-26b mimic, the expression increased by 90 fold compared to the control (mock) in MDA-MB-231 cells as in Figure 20; while the expression of miR-26b mimic in MDA-MB-453 cells increased by 20 fold. In contrast, transfection with the anti-sense did not affect the miR-26b in both cell lines tested.

Figure 20

The Effect of Transfection on the Expression Level of miR-26b in MDA-MB-231 MDA-MB-453 Cells

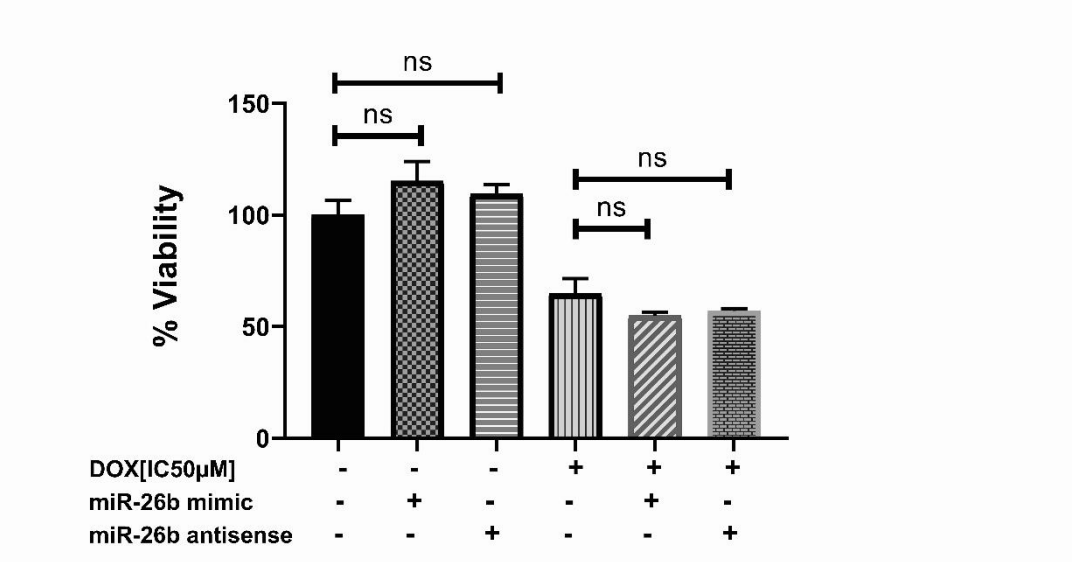


The Effect of miR-26b Transfection on the Proliferation and Chemosensitivity of MDA-MB-231 Cells

To investigate the effect of miR-26b transfection on the chemosensitivity of MDA-MB-231 cells, cells were transfected with miR-26b mimic and its anti-sense. Cells only contained transfection reagent were used as control (mock). As shown in Figure 21, transfection of MDA-MB-231 cells with miR-26b mimic or anti-sense does not affect cell proliferation compared to the control.

Figure 21

The Effect of miR-26b on Chemoresponse of MDA-MB-231 Cells to Doxorubicin



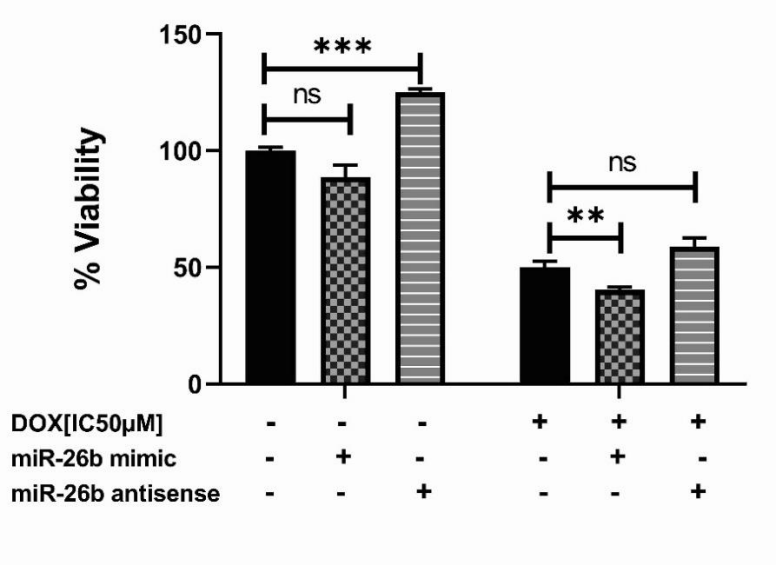
Results were normalized to control cells (0.01% DMSO treated cells) and analyzed by one-way ANOVA

The Effect of miR-26b Transfection on the Proliferation and Chemosensitivity of MDA-MB-453 Cells

To investigate the effect of miR-26b transfection on the chemosensitivity of MDA-MB-453 cells, cells were transfected with miR-26b mimic and anti-sense. Cells only contained transfection reagent were used as control. As shown in Figure 22, transfection of MDA-MB-453 cells with miR-26b mimic decreases cell viability in a non-significant manner (Figure 22) but increases cell sensitivity toward doxorubicin compared to control.

Figure 22

The Effect of miR-26b on Chemoresponse of MDA-MB-453 Cells to Doxorubicin

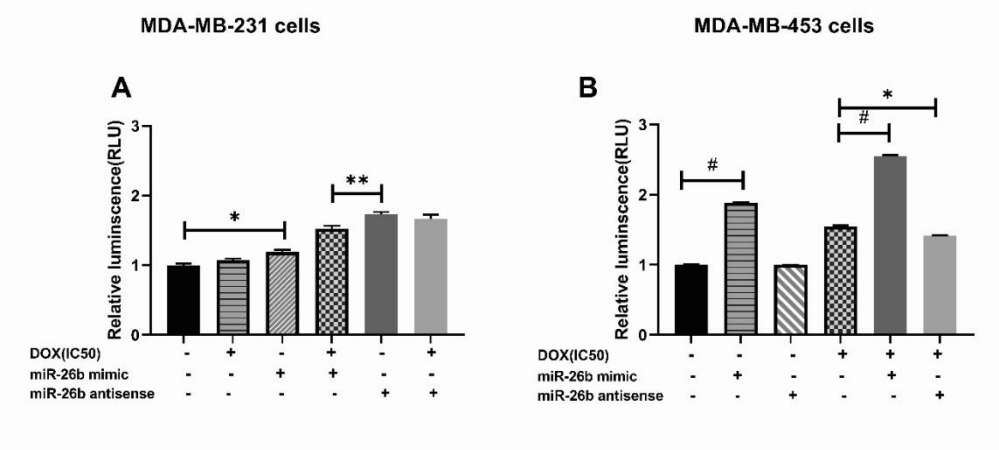


The Effect of miR-26b Mimic on the Caspase 3/7 Activity in MDA-MB-231 and MDA-MB-453 Cells

In order to investigate the effect of miR-26b on the caspase 3/7 activity in the presence of doxorubicin, a caspase 3/7 Glo assay was done to ensure the apoptotic behaviour of miR-26b mimic and anti-sense (Figure 23). miR-26b mimic increased the activity of caspase 3/7 significantly. Also, it increased the activity of caspase 3/7 in the presence of doxorubicin.

Figure 23

The Effect of miR-26b Mimic on the Caspase 3/7 Activity in MDA-MB-231 and MDA-MB-453 Cells

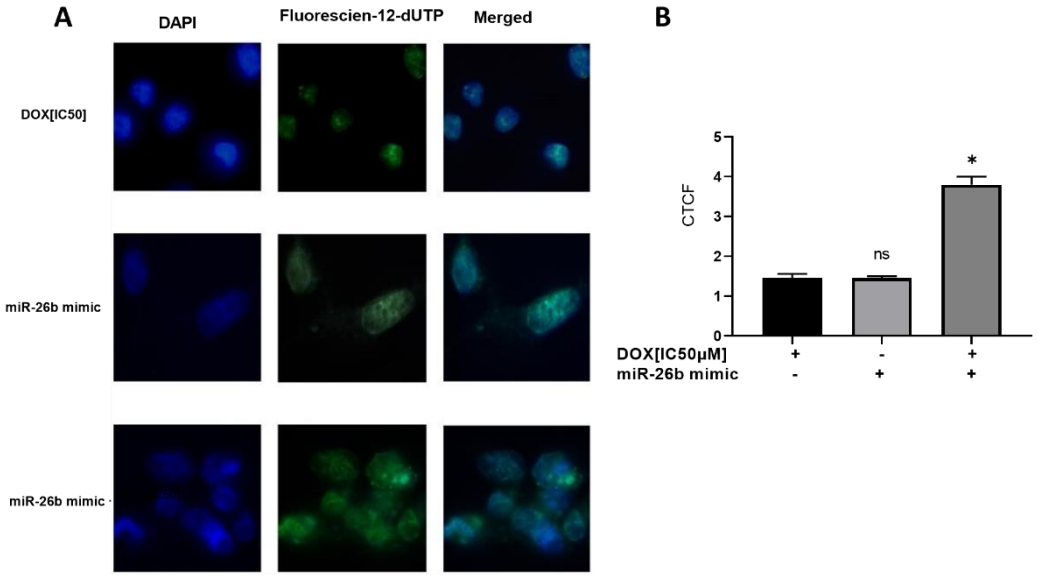


The Effect of miR-26b Mimic on the Late Stages of Doxorubicin-Induced Apoptosis

In order to determine if miR-26b mimic increases the chemoresistance of MDA-MB-231 and MDA-MB-453 cells toward doxorubicin via apoptosis induction, two assays were performed. The first assay was the TUNEL assay. Treatment of the cells with DNase was used as a positive control, and treatment of cells with doxorubicin for 4 hours was optimal to observe differences in DNA cleavage among the different treatments. As shown previously in Figure 14, doxorubicin induced DNA cleavage in both cell lines. Cells that were transfected with miR-26b mimic induce a strong signal of DNA cleavage when combined with doxorubicin in MDA-MB-231 cells (Figure 24). Similar results were observed for MDA-MB-453 cells (Figure 25).

Figure 24

The Effect of miR-26b Mimic on DNA Degradation Detected by TUNEL Assay in MDA-MB-231

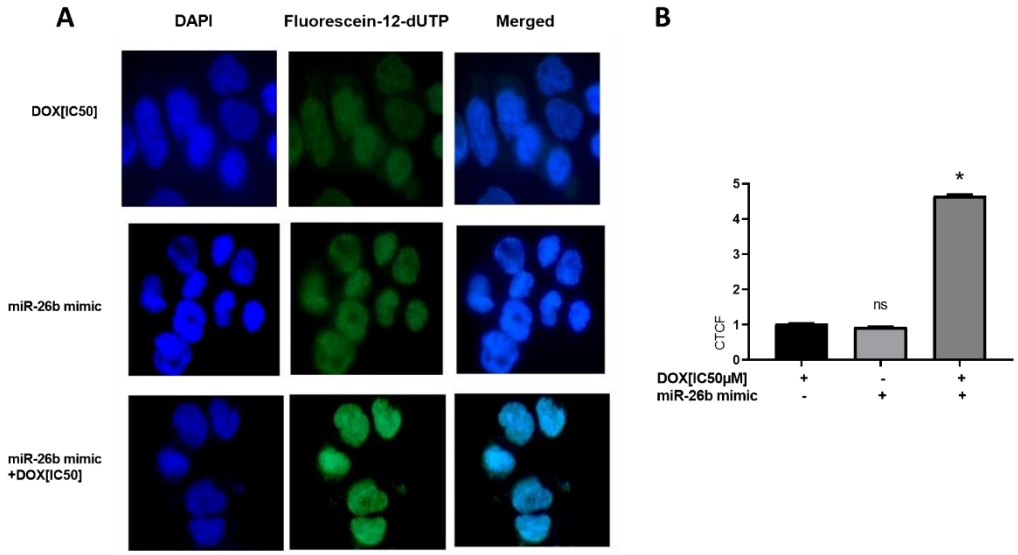


*A) The nuclei were visualized by staining with DAPI (blue), and DNA nicking was visualized by fluorescein-12-dUTP (Green). Scale bar 10μm B) Quantitative results of TUNEL analysis of doxorubicin (DOX) in combination with miR-26b mimic. p-values are indicated as as ns, Not Significant *<0.05*

compared with cells treated with DOX (IC50). Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.

Figure 25

The Effect of miR-26b Mimic on DNA Degradation Detected by TUNEL Assay in MDA-MB-453 Cells



*A) The nuclei were visualized by staining with DAPI (blue), and DNA nicking was visualized by fluorescein-12-dUTP (Green). Scale bar 10µm.B)Quantitative results of TUNEL analysis of doxorubicin (DOX) in combination with miR-26b mimic, p-values are indicated as as ns, Not Significant and *<0.05 compared with cells treated with DOX (IC50). Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

The Effect of miR-26b Mimic on the Expression of the Apoptosis-Regulatory Genes

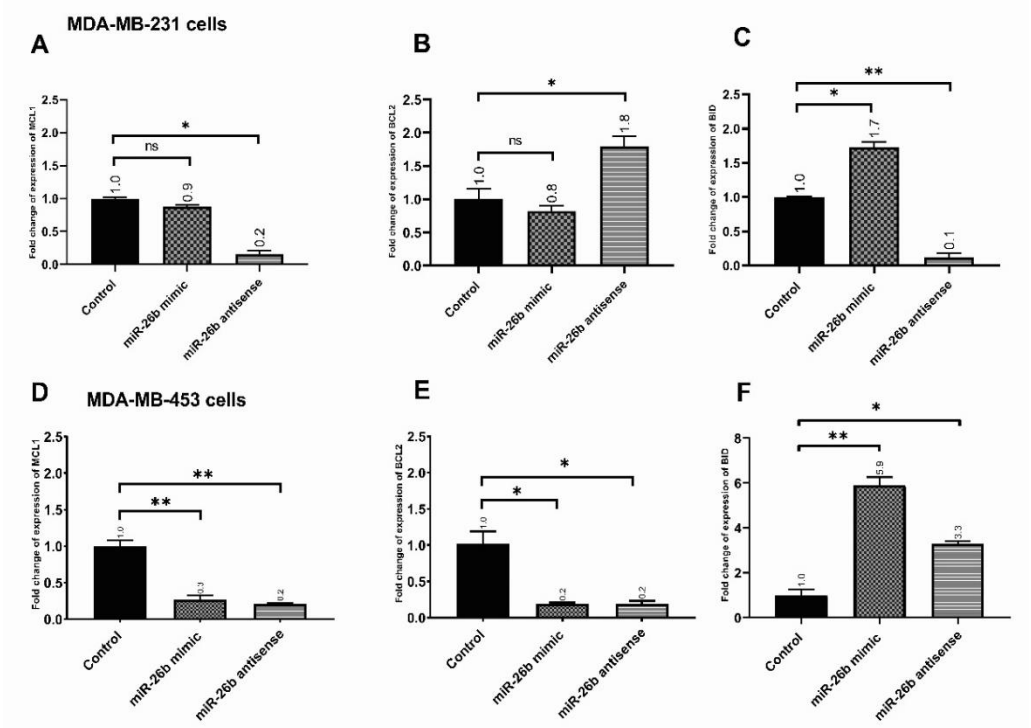
To assess the effect of miR-26b mimic on apoptosis, anti-apoptotic *bcl2* and *mcl1* genes and the pro-apoptotic *bid* gene were investigated by qPCR. We found that the miR-26b mimic decreased the expression of *mcl1* and *bcl2* in MDA-MB-453 cells only. miR-26b mimic increased *bid* expression in MDA-MB-453 and MDA-MB-231 cells (Figure 26).

The Effect of Dihydrotestosterone and Enzalutamide on miR-26b Levels in MDA-MB-231 and MDA-MB-453 Cells

The role of DHT on the expression level of miR-26b in MDA-MB-231 and MDA-MB-453 cells was investigated using qPCR after treating cells with 10 nM or 100 nM DHT (16 µM) enzalutamide and a combination of DHT with enzalutamide for three days in media supplemented with 10% charcoal-stripped FBS. Results showed that DHT increased the expression of miR-26b significantly by 3.1 fold in MDA-MB-231 and by 2.6 fold in MDA-MB-453 cells; also, enzalutamide increased the expression of miR-26b in both cell lines by 2.8 and 1.6 fold (Figure 27).

Figure 26

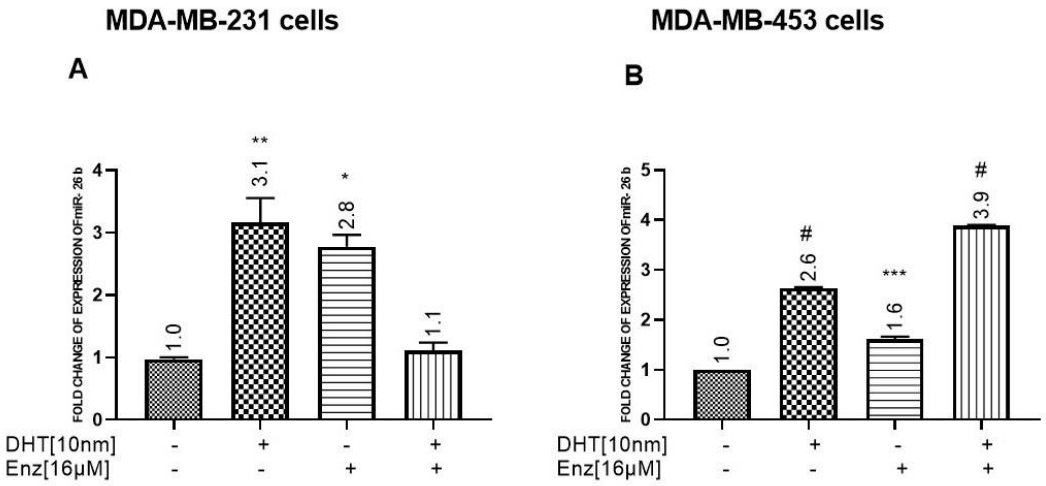
The Effect of miR-26b Mimic on the Expression of mcl1 (A and D), bcl2 (B and E) and bid (C and F) in MDA-MB-231 and MDA-MB-453 cells



*p-values are indicated as (ns) non significant, *<0.05, **< 0.01, and ***<0.001 compared with the vehicle-treated sample. Data points are presented as mean ± SD of three replicates. Experiments were repeated at least three times.*

Figure 27

The Effect of DHT, Enzalutamide and its Combination on the Expression Level of miR-26b



CHAPTER V

Discussion

The androgenic pathway is proposed to be an alternative target of therapy of breast cancers, including TNBCs, that are resistant to hormonal therapy (Lyons & Traina, 2017). Therefore, it is crucial to better understand the biological roles of this pathway on breast cancer subtypes, particularly TNBCs. Previous studies have demonstrated the acquisition of chemoresistance against paclitaxel, doxorubicin and cyclophosphamide in breast cancer cells (Simões-Wüst et al., 2002). This study reported a similar phenomenon but in association with the AR pathway, where AR activation confers chemoresistance to MDA-MB-231 cells toward doxorubicin (Al-Momany et al., 2021). Herein, this is true in MDA-MB-231 cells but also in another TNBC model system, specifically the LAR MDA-MB-453 cells. Importantly, this can be reversed by inhibiting AR with enzalutamide. Another important finding to note is the differential molecular regulation of these two cell lines, which represent different subtypes of TNBC.

Chemoresistance can develop through various processes, one of which is drug efflux by ATP-binding cassette (ABC) transporters. However, this does not seem to be a mechanism by which DHT induces chemoresistance in MDA-MB-231 cells (Al-Momany et al., 2021) . Another mechanism of increasing drug resistance is via EMT. In fact, it is becoming increasingly clear that chemoresistance is commonly associated with EMT in various cancers, including breast cancer (Huang et al., 2015) . The relationship between EMT and chemoresistance was proposed by Sommers et al. who discovered EMT in adriamycin-resistant MCF-7 cells and a vinblastine-resistant ZR-75-B cell line (Sommers et al., 1992). Adriamycin-resistant MCF-7 cells had more vimentin expression and deficient development of desmosomes and tight junctions, a hallmark feature in EMT (Sommers et al., 1992). Notably, not all drug-resistant MCF-7 cells displayed EMT characteristics, suggesting that EMT provides a selective growth advantage in the presence of chemotherapy among the heterogenic cancer cell populations. Among the signaling pathways that promote the EMT phenotype and contribute to drug resistance were the Wnt and hedgehog pathways where the overexpression of Wnt3 activated the Wnt/β-catenin signaling pathway promoting EMT and leading to trastuzumab resistance in HER2-overexpressing breast cancer cells (Wu et al., 2012). Recently, Ahram et al. reported that treatment of the MDA-MB-453 cells with DHT induced

partial EMT suggesting the contribution of EMT to the DHT-induced chemoresistance of these cells. However, the later study found that after 72 or 144 hours of DHT treatment, the expression of Slug, an EMT inducer, increased (Ahram et al., 2021). The upregulation of Slug is associated with the acquisition of chemoresistance in breast cancer cells (Kim & Ko, 2021; Liu et al., 2014; Tan et al., 2019; Zhou et al., 2020). Interestingly, no changes in the expression of EMT-regulatory genes in MDA-MB-231 cells were found (Ahram et al., 2021). Taking into consideration that the inhibition of AR has an opposite effect of DHT on doxorubicin-induced apoptosis and the apoptosis regulatory factors (Mehta et al., 2015), the mechanism of evading apoptosis by modulating the expression and activity of pro- and anti-apoptotic proteins seems to be the most plausible mechanism of inducing chemoresistance by DHT.

Caspase-3/7 activation, a component of the downstream apoptosis pathway, is required for the process of apoptosis through the degradation of essential proteins necessary for cellular survival and function, leading to cell death (Lamkanfi & Kanneganti, 2010; Virág et al., 2013). Treating breast cancer cells with doxorubicin induces the activities of these enzymes, and DHT counteracts that. These findings are consistent with those of Rokhlin et al., who reported that DHT inhibited apoptosis in prostate cancer cells in a dose-dependent manner through caspase inhibition (Rokhlin et al., 2005). Furthermore, another study revealed that DHT has a protective effect on glial cells by blocking apoptosis through the inactivation of caspase 3 (Yao et al., 2016).

In this study, enzalutamide has the opposite effect of DHT in both breast cancer cell lines tested. In addition, the observation that enzalutamide reduces the effect of DHT parallels the previous findings of Shanker et al. They illustrated that treatment of prostate cancer cells with enzalutamide in combination with GSK126, an inhibitor of enhancer of zeste homolog 2 (EZH2), resulted in caspase activation leading to the cleavage of poly ADP ribose polymerase (PARP-1) and consequently, apoptosis (Shankar et al., 2020). Based on these observations, it can be concluded that DHT reverses the chemotherapeutic drug-induced apoptosis by blocking the caspase 3/7 activity, and this can be reversed by enzalutamide.

The anti-apoptotic MCL1 is a primary factor of drug resistance to a large variety of cancer therapies where its overexpression increases resistance to multiple chemotherapeutic drugs in solid tumors and hematological malignancies (Wuillème-

Toumi et al., 2005; Santer et al., 2015; Pan et al., 2016; Bai et al., 2021; Shimizu et al., 2021). In fact, MCL1 has more significant apoptosis-inhibitory effects relative to other BCL2 family members (Oyesanya et al., 2012). The *mcl1* gene appears to be under the regulation of AR in both cell lines, and induction of its expression by DHT could explain a mechanism by which DHT induces chemoresistance. It is noteworthy that the up-regulation of MCLI by DHT favours cell survival and drug resistance since MCL1, in cooperation with MYC, promotes drug resistance in TNBC by increasing mitochondrial oxidative phosphorylation and expanding cancer stem cells (Hutchinson et al., 2018). DHT may also induce chemoresistance via regulating the expression of other genes, such as *bcl2*. However, *bcl2* was found to be up-regulated in MDA-MB-453 cells, only highlighting the cell-specific actions of DHT. Other genes were identified in MDA-MB-453 cells to be DHT-dependent, including the pro-apoptotic *bik*, *bok*, and *enog* genes, whose DHT-induced down-regulation reduces drug-induced apoptosis (Mehta et al., 2015). DHT has been shown to increase resistance and inhibit drug-induced apoptosis by inhibiting the expression of the pro-apoptotic *bax* gene in glial cells (Bing et al., 2015).

Interestingly, enzalutamide activity also seems to be cell-specific, where it down-regulates *mcl1* and *bcl2* and upregulates *bid* in MDA-MB-453 cells only. Our findings parallel an earlier report showing that BCL2 inhibition sensitizes TNBC to doxorubicin (Inao et al., 2018). In addition, breast cancer cells that overexpress AR become more resistant to tamoxifen and bicalutamide reverses this effect and sensitizes the cells to the drug (De Amicis et al., 2010). Variations in BCL-2 levels have already been shown to influence apoptotic susceptibility as a mechanism of evading apoptosis and have been associated with resistance to several cytotoxic drugs used in the treatment of TNBC, such as paclitaxel, doxorubicin and cyclophosphamide (Nedeljković & Damjanović, 2019). Similarly, Mehta et al. found that bicalutamide, an androgen antagonist, stimulated the expression of *bik*, *bok* and *enog* genes (Mehta et al., 2015). AR inhibition by enzalutamide has the potential to reverse the effect of androgen by acting as a tumor suppressor in AR-positive TNBC (Zhu et al., 2016). It is promising to note that the induction of apoptosis by enzalutamide and its amplification of the cytotoxic effect of doxorubicin in addition to its inhibition of the DHT-induced increase in the expression of *mcl1* in both cell lines suggesting its potential as a target in the treatment of TNBC (Alsawalha et al., 2022).

AR activation by DHT has proliferative effects on TNBC cells (Jiang et al., 2016; Pietri et al., 2016; Lyons & Traina, 2017; Shen et al., 2018). The findings presented in this study regarding the effect of DHT on the proliferation of MDA-MB-231 and MDA-MB-453 cells are compatible with previous studies by Ahram et al. (Ahram et al., 2021) and others as well, whereby it has an influence on the proliferation of MDA-MB-453 cells but not MDA-MB-231 cells (Chavez et al., 2010; Chottanapund et al., 2013; Ahram et al., 2018; Al-Othman et al., 2018). The effect of DHT on the growth of MDA-MB-453 cells may be due to the high expression of AR in these cells (Guedj et al., 2012; McNamara et al., 2013; Ahram et al., 2018). The role of AR in the development of breast cancer is complex, where it can perform its actions at the genomic level as a ligand-dependent transcription factor, regulating gene expression. Or, it can function in the non-genomic signaling pathways by suppressing G-protein receptor signaling in TNBC (Shen et al., 2018) or blocking apoptosis (Park et al., 2019). The underlying molecular processes for these modifications have been addressed, illustrating that androgen can activate several signaling pathways linked to DHT's anti-apoptotic activity (Santer et al., 2015; Nedeljković & Damjanović, 2019).

On the other hand, enzalutamide negatively affects the proliferation of both cell lines. This goes in line with the apoptotic activities of enzalutamide on breast cancer in general (Nadiminty et al., 2013; Gerratana et al., 2018) and, more specifically, in TNBC (Barton et al., 2015; D'Amato et al., 2016; Leone et al., 2020). In addition, previous studies have provided evidence that the treatment of breast cancer with first-generation non-steroidal anti-androgenic medications, such as flutamide and bicalutamide, reduces the development of several cancers (Yuan et al., 2019; Anestis et al., 2020). In a recent study carried out by Park and colleagues, treatment of MDA-MB-231 and MDA-MB-453 cells with 30 μM enzalutamide blocked AR nuclear localization (Park et al., 2019). Treating breast cancer cell lines with enzalutamide lowers the expression of several cell cycle proteins that promote cell cycle progression and increases the expression of the cell cycle inhibitor, P27 (Xia et al., 2019). It is interesting, though, this AR antagonist had more effect on MDA-MB-231 cells than on MDA-MB-453 cells when taking into consideration that the former cells express little AR compared with the high expression of AR in the LAR cell line (McGhan et al., 2014; Thike et al., 2014; Al-Othman et al., 2020). Interestingly, DHT induces dysregulation of the expression of a larger number of

microRNA molecules in MDA-MB-231 cells in comparison to MDA-MB-453 cells (Ahram et al., 2017; Al-Othman et al., 2017). Our findings revealed that miR-26b reduced cell proliferation in the LAR-positive TNBC MDA-MB-453 cells, thus being compatible with Tan et al., which found that estrogen suppressed the expression of miR-26a and miR-26b in ER+ breast cancer cells. At the same time, miR-26a/b deletion boosted the growth of ER+ breast cancer cells and thus proved the role of miR-26b in growth inhibition (Tan et al., 2014). In our research, we found that MDA-MB-453 cells that were transfected with miR-26b increased the chemoresponse of cells toward doxorubicin and thus concomitant with Wang et al. In their research on human colorectal cancers, they revealed that miR-26b increased the sensitivity of colorectal cancer cells to 5-FU *in vitro* and boosted its efficacy via inhibiting the expression of the P-gp (Wang et al., 2018).

Overexpression of anti-apoptotic genes *mcl1* and *bcl2* have been reported in various cancers (Pilling & Hwang, 2019). Moreover, the role of *mcl1* in drug resistance has been previously reported (Hutchinson et al., 2018). Our findings revealed that miR-26b decreased the expression of *mcl1* and *bcl2* in MDA-MB-453 cells only and thus suggested that miR-26b might serve as a tumour suppressor. By downregulation, *mcl1* and *bcl2* thus activate the intrinsic pathway of apoptosis and increase the activity of executive caspases; caspase 3/7.

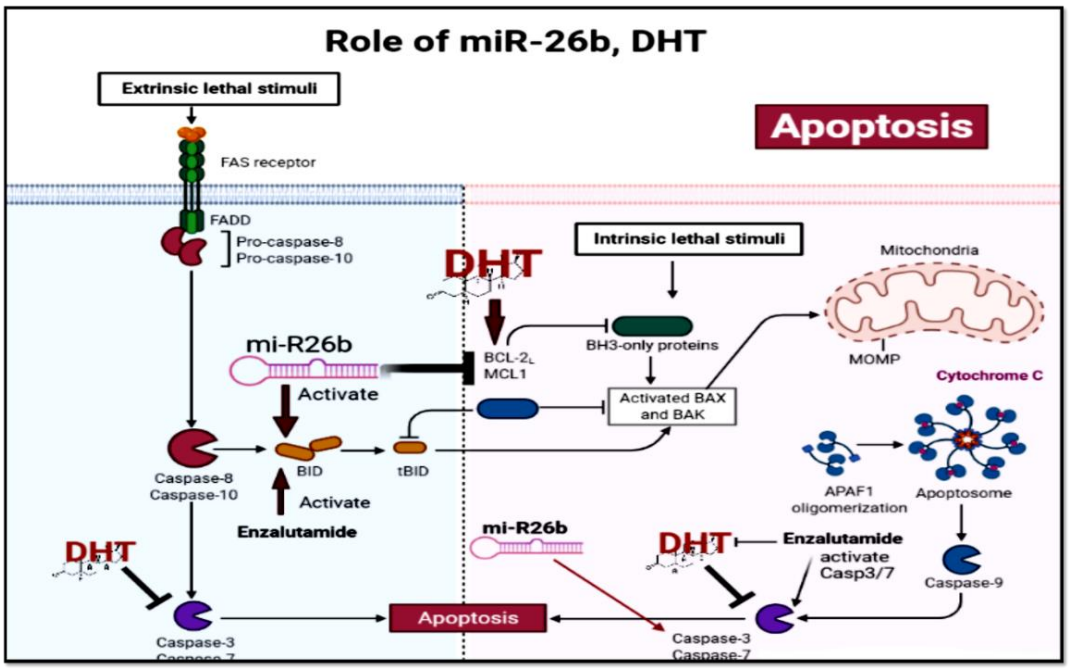
CHAPTER VI

Conclusions

Currently, the only therapeutic option for advanced TNBC is non-specific cytotoxic chemotherapy. The findings presented here offer a better understanding of the mechanism of androgen-induced chemoresistance and may facilitate the identification of alternative therapeutic interventions in TNBC that are positive for the downstream AR signaling pathway. AR-targeted therapies may allow patients with advanced or metastatic TNBC to be treated with less toxic endocrine drugs.

Figure 28

Summary of the effect of DHT ,Enzalutamide and miR-26b on apoptosis



DHT increases anti-apoptosis genes (mcl1 and bcl2), inhibits Bax/Bak that will inhibit MOMP from releasing cytochrome c, which causes escape from apoptosis and cell survival. On the other hand, enzalutamide and miR-26b increases cell sensitivity by sensitizing TNBC to apoptosis through the apoptosis gene bid that activate bax and bak that activate MOMP to release cytochrome c that leads to activation of the executive caspases; caspase 3/7. That will appear in the late stages of apoptosis in DNA degradation detected by the TUNEL assay. This figure was created using BioRender.com

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APPENDICES

Appendix A

Enzalutamide Overcomes Dihydrotestosterone Induced Chemo-Resistance In Triple-Negative Breast Cancer Cells via Apoptosis

Laila Alsawalha [1], Mamoun Ahram [2], Mohammad S Abdullah [2], Ozlem Dalmizrak [1]

- PMID: 35579133
- DOI: 10.2174/1871520622666220509123505

- **Abstract**

Background: Triple-negative breast cancer is challenging to treat due to its heterogeneity and lack of therapeutic targets. Hence, systemic chemotherapy is still the mainstay in TNBC treatment. Unfortunately, patients commonly develop chemo-resistance. Androgen signaling through its receptor is an essential player in breast cancer where it has been shown to confer chemo-resistance to TNBC cells Objective: To elucidate the mechanistic effects of enzalutamide in the chemoresponse of TNBC cells to doxorubicin through the apoptosis pathway.

Method: MDA-MB-231 and MDA-MB-453 cells were used as model systems of TNBC. Cell viability and apoptosis were investigated upon treatment of cells with doxorubicin in the presence of dihydrotestosterone (DHT) and/or enzalutamide. Caspase 3/7 activity and TUNEL assays were performed to assess the induction of apoptosis. The expression of apoptosis-regulatory genes were assayed by qPCR for the detection of expression changes.

Results: Enzalutamide decreased the viability of MDA-MB-231 and MDA-MB- 453 cells and reduced DHT-induced chemo-resistance of both cell lines. It also increased the chemo-sensitivity towards doxorubicin in MDA-MB-231 cells. Increasing DNA degradation and caspase 3/7 activity were concomitant with these outcomes. Moreover, enzalutamide downregulated the expression of the anti-apoptosis genes, mcl1 and bcl2, in MDA-MB-231 cells. Moreover, increase the pro-apoptotic gene bid. On the other hand, DHT upregulated the expression of the anti-apoptosis genes, mcl1 and bcl2, in both cell lines.

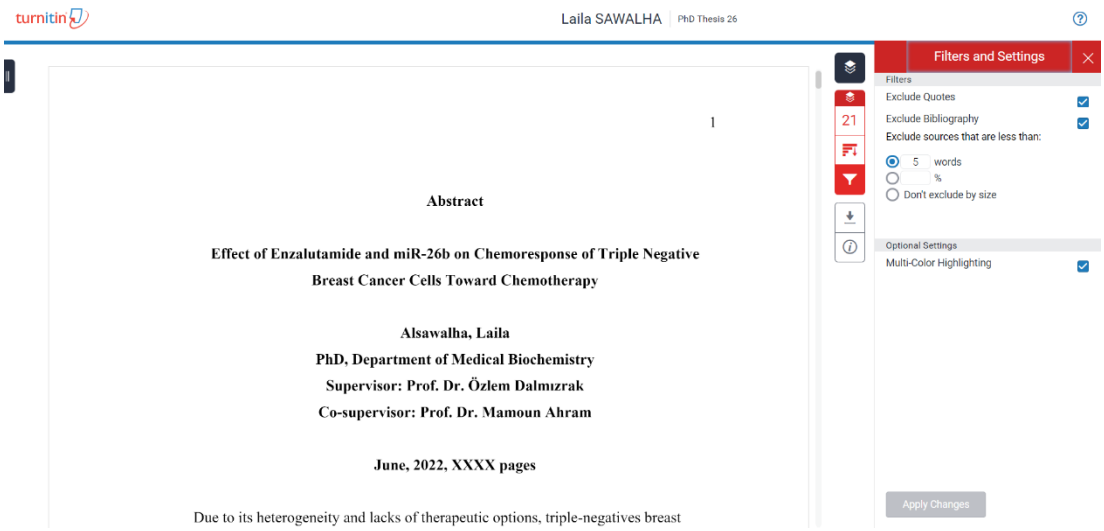
Conclusion: DHT increases the expression of the anti-apoptosis mcl1 and bcl2 in the TNBC cells, presumably leading to cell survival via the prevention of doxorubicin-induced apoptosis. On the other hand, enzalutamide may sensitize the cells to doxorubicin through downregulation of the bid/bcl2/mcl1 axis that normally

activates the executive caspases, caspase 3/7. The activities of the latter enzymes were apparent in DNA degradation at the late.

Keywords: DNA degradation; Triple-negative breast cancer (TNBC); androgen receptor; caspase 3/7; chemoresponse;; dihydrotestosterone(DHT); enzalutamide.

Appendix B

Turnitin Similarity Report



Appendix C

CV

CURRICULUM VITAE

Name	Laila	**Surname**	Sawalha
Place of Birth	Jordan	**Date of Birth**	9/7/1980
Nationality	Jordanian	**Tel**	00962775389344
E-mail	Laila.sawalha@gmail.com		

Educational Level

	Name of the Institution where she was graduated	**Graduation year**
Postgraduate/Specialization	Health Science Institute	2022
Masters	Yarmouk University	2005
Undergraduate	Yarmouk University	2002
High school	Ministry of Education/Scientific Stream	1998

Job Experience

	Duty	**Institution**	**Duration (Year - Year)**
Biochemistry Lecturer		Al Farabi Medical College	2010-2016
Teacher Assistant		Yarmouk University	2002-2005

Foreign Languages	**Reading comprehension**	**Speaking***	**Writing***
English	6	6.5	5.5

Foreign Language Examination Grade								
YDS	UDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
		6.0						

Computer Knowledge

Program	Use proficiency
very good	

*****Evaluate as very good, good, moderate, poor.

CONFERENCES AND SYMPOSIUM:

Presentations and Abstract: S-101

L. Sawalha et al., (2021, 27-30 October). Enzalutamide, An Androgen Receptor Antagonist, Overcomes Chemoresistance By Sensitizing Triple Negative Breast Cancer Cells To Apoptosis. [paper presentation].
TBS International Biochemistry Congress 2021,32nd National Biochemistry Congress; Gaziantep, Turkey

EMBO/EMBL Symposium: The Non-Coding Genome (6072)-(Fri, 2021,15 October); Heidelberg, Germany, mi-R26b and Androgen Receptor Inhibitor Overcome Chemoresistance By Sensitizing Triple Breast Cancer Cells To Apoptosis

TRAINING AND OTHER CERTIFICATES

Bioinformatics Fall School 2018-2019
Excellence In Laboratory Quality Management Workshop
Experimental Animal Models Course: From Gene To Function.
Preparing For ISO 15189 for Clinical Laboratories
Introduction to E-Learning
Practical Sessions and Skills Training
Exam Question Banking and Test Construction
Item Analysis, Result Analysis and Normalization Of Scores
Course Report Workshop
The Construction of MCQS
Blueprinting
Problem Based Learning (PBL)
The Facilitation of Small Group Sessions
Managing the Integrated Curriculum
Development Teaching Methods
Teaching Methods Assessment
Teaching Portfolio