To Evaluate the Role of Estrogen Receptor in Myeloid Leukemia

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Dedicated to

My Father for his immense support

And

My Mother for all her prayers and sacrifices.

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LIST OF ACRONYMS

A		
	APL	Acute promyelocytic leukemia
	ATRA	All trans retinoic acid
	ATO	Arsenic trioxide
B		
	BM	Bone marrow
	Bp	Base pairs
C		
	cDNA	Complementary DNA
	CML	Chronic myeloid leukemia
	CR	Complete remission
	CSCs	Cancer stem cells
D		
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	dNTPs	Deoxyribonucleotide triphosphates
	DTP	Drug-tolerant persisted
F		
	FISH	Fluorescence in situ hybridization
H		
	HSCT	Hematopoietic stem cell transplantation
I		
	IL	Interleukin
J		
	JAK	Janus Kinase
L		
	LICs	Leukemia initiating cells.

LSCs	Leukemic stem cells

M

M	Molar
mM	Millimolar
µl	Microliter
µM	Micromolar
MRD	Measurable residual disease
MTT	Tetrazolium salt
MPPs	Multipotent progenitors

N

NF water	Nuclease free water NUP214 Nucleoporin 214

O

OS	Overall survival

P

PCR	Polymerase chain reaction
PLZF	Promyelocytic leukemia zinc finger
PML/RARα	Promyelocytic leukemia/retinoic acid receptor

R

RA	Retinoic acid
RBCs	Red blood cells
RNase	Ribonuclease
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RTK	Receptor tyrosine kinases

T

t (15;17)	Translocation of chromosome 15 and 17
TAE	Tris acetate EDTA
TRM	Treatment-related mortality

U

UV	Ultraviolet

W

WBCs	White blood cells
Wnt	Wingless-related integration site

+	Positive
-	Negative
%	Percent
°C	Degree Celsius

ABSTRACT

The malignancy of white blood cells (WBCs) is called leukemia. Leukemia is divided into acute and chronic myeloid leukemia based on its rate of growth. Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML) that is principally defined by t (15; 17), a balanced translocation that results in the fusion of the PML/RAR fusion gene, account for 10-15% of newly diagnosed acute myeloid leukemia. Chronic Myeloid Leukemia occurs due to the Philadelphia chromosome which was first identified as a truncated version of chromosome 22 and later as a t (9;22) translocation. All-trans Retinoic Acid, Arsenic Trioxide, chemotherapy, and combinations are now used to treat APL. The emergence of resistance, differentiation syndrome, and ATO toxicity makes these treatments less effective. Tyrosine kinase inhibitors (TKIs) have marked a ground-breaking finding in the treatment of CML. However, it became resistant to imatinib, which now requires special attention. The role of Endocrine signaling, specifically that of estrogen receptor (ER) is very significant in progression of different cancers and has been linked to Wnt/Beta Catenin pathway which is indispensable for APL and CML. We therefore wanted to learn more about how endocrine signaling functions in APL (NB4) and CML (K562) mice. We demonstrated that NB4 and K562 are negative for ER and PR utilizing in silico, biochemical, and pharmacological techniques. Fulvestrant showed off target effects on our models Our results suggest that Fulvestrant interferes with proliferation of NB4 and K562 cells by downregulating Wnt target genes including *c-myc* (p-value < 0.001) and *axin 2* (p-value < 0.01) in both AML and CML. In conclusion our results suggest that Fulvestrant also targets other potential candidates which show its off-target activity and need to be further explored in different AML and CML models.

Keywords*: Leukemia, Wnt β-Catenin, Estrogen Receptor, Crosstalk, NB4 cell line, K562 cell line, Blood cancer*

1) INTROUCTION

1.1 Hematopoiesis

High cell production is achieved during hematopoiesis, a controlled process that produces mature blood cells through cell division and differentiation from hematopoietic stem cells with self-renewal capabilities. From the primary hematopoietic stem cell at the top of the hierarchy, cells are differentiated into myeloid and lymphoid lineages during the process of hematopoiesis (Kim, Stachura, & Traver, 2014). While a number of early and origin-specific factors are in command of the discrepancy of those hematopoietic stem cells through the generation of genes from their respective origins, this process involves a number of early and origin-specific growth factors and their receptors (Steffen, 2005).

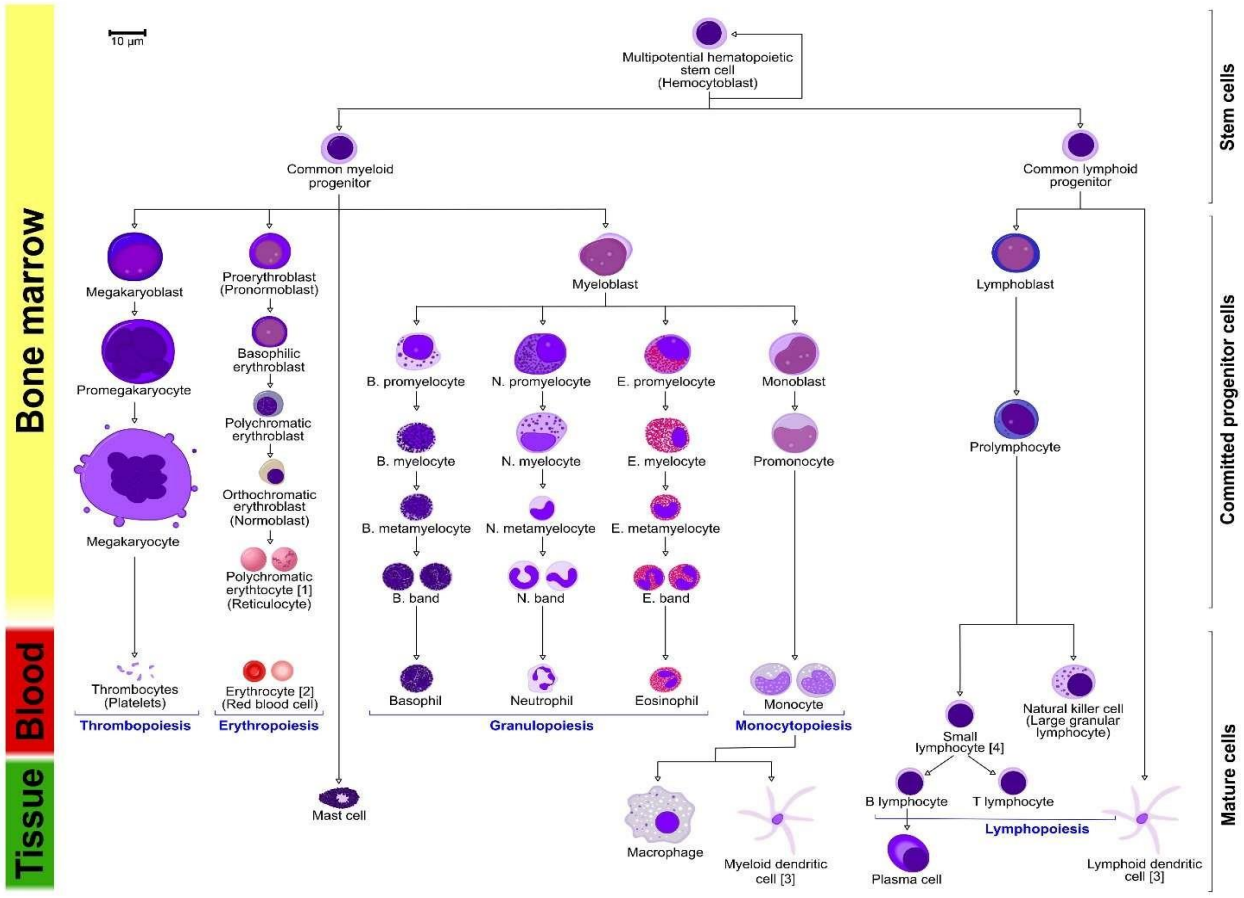

Figure 1. 1: The process of Hematopoiesis (Iskander, 2021)

Multipotent progenitor cells rapidly multiply and can still give rise to any type of hematopoietic cell, while losing their capacity to self-renew. (Iwasaki & Akashi, 2007). Red blood cells (RBC), granulocytes, platelets, macrophages/monocytes, and other distinct blood cells make up the myeloid lineage, whereas lymphoid cells are made up of different cells. These mature cells have a limited lifespan, they are continually being replaced. It gradually loses its capacity for self-renewal as committed blood cells continue to develop (M. Kondo et al, 2010). Human disorders, such as anemia and leukemia, are caused by errors in the hemopoiesis process (Kumar & Evans, 2015). Chromosomal changes caused by erroneous gene expression in hematogenic stem cells or multipotent originator cells lead to variety of hematopoietic cancers.

1.2 Leukemia

Anemia is the earliest sign of leukemia and is caused by the WBC cancer known as leukemia, which begins in the hematopoietic stem cells (Adamietz et al., 2009). Leukemia is a common blood cancer that typically manifests as liquid tumors with an abundance of undifferentiated erythroid, myeloid, or lymphoid cells (Hsieh., 2014). Normally only 5% of these undifferentiated malignant blast cells are present in bone marrow but the number increases up to 30 to 100% in leukemia (Adamietz et al., 2009.When these cancerous blasts travel to other areas of the body, the function of the liver, spleen, and the blood circulation is severely damaged. The two types of blasts are myeloblast and lymphoblast; myeloblasts develop into myeloid cells while lymphoblasts develop into lymphocytes. Like this, myeloid leukemia, which develops from myeloblasts, impairs blood cell production (Seipelt et al., 1998). However, leukemia that develops in the lymphoblast is known as lymphocytic leukemia and has an impact on lymphocyte production (Hematology, 2019).

1.3 Classification of Leukemia

Depending on the cell lineages involved as well as the structure and physiology of leukemic cells, leukemia can also be categorized into myeloid and lymphoid subtypes. CLL and CML are primarily cancers that affect older persons. (CLL is not reported in children while CML accounts for a few percent of all leukemias in children;). AML is divided into 8 groups (FAB 0-7) by the FAB approach on the basis of characteristics of the blast cells lineage. The classification and accurate diagnosis of leukemia are necessary for the effective and right selection of treatment

options. Leukemia must be diagnosed by examining the morphology of the disease's cells, according to the World Health Organization (WHO), the FAB and Committee for Standards in Hematology (Gisslinger et al., 2016).

1.3.1 Acute myeloid leukemia (AML)

AML is a diverse condition that affects hematopoietic progenitors and is caused by acquired genetic abnormalities that impair normal cell division, self-renewal, and differentiation processes (Ben-Batalla et al., 2013). Its hallmarks include aberrant proliferation and a diminished competence of HSCs to develop to mature cells. This type of acute leukemia is the most common. Due to the buildup of immature myeloid precursors (myeloblasts) as a result, the production of healthy RBCs, WBCs, and platelets are decreased. AML is indicated if the peripheral blood contains more than 20% myeloblasts (Harris et al., 1999).

APL, a type of AML, makes about 5–15% of all AML subtypes. Anemia, infections, and hemorrhages are among the signs and symptoms of APL, cells can be seen infiltrating organs (Anna Laurenzana, 2006). Balanced t(15;17)(q22;q12), which is caused by the fusion of the PML N-terminus with the RAR transcription factor C-terminus, is the most common chromosomal translocation in most instances of APL (Borrow et al., 1990). The nuclear receptor RAR's ligand (RA) is retinoic acid. RAR employs corepressors and histone deacetylases when its ligand RA is not present. In the presence of RA, RAR transforms influencing the expression of genes crucial for myeloid progenitor development (Lallemand-Breitenbach et al., 2011). All trans-RA (ATRA) and arsenic trioxide (ATO) have been found to be the most effective therapy options for APL, according to an extensive study (Ng et al., 2017).

Despite being a promising treatment for APL, ATRA induces differentiation in immature promyelocytes as well as in healthy stem cells, leading to differentiation syndrome, which is characterized by an abnormal rise in the WBC count brought on by the differentiation of cells that release pro-inflammatory cytokines (Degos et al., 2001). ATRA also lacks the ability to specifically target and eliminate leukemic initiating cells (LICs) (Zheng et al., 2007). Relapse is observed in patients who solely get ATRA as APL therapy after achieving CR. As a result, combined therapy (ATRA + chemotherapy) is used as an advanced treatment option for individuals with newly diagnosed APL (Asou, 2017). The primary problems in the treatment of

APL nowadays are arsenic poisoning (Degos et al., 2001), the mutation in PML creating resistance of PML/RAR to ATO therapy, and the inability of ATRA alone as a therapy for APL (Warrell as al., 1991).

1.3.2 Chronic myeloid leukemia (CML)

Juvenile myelomonocytic leukemia and Philadelphia chromosome positive (Ph+) myeloid leukemia are the two main types of chronic myeloid leukemia. Ph+ CML is linked to the BCR-ABL1 oncogene with constitutive TK activity in HSCs (Jabbour et al., 2007). This reciprocal translocation results in constitutive proliferation, activation of pro-survival signaling pathways, and repression of apoptosis, all of which contribute to genomic instability (Kuepper et al., 2019). Compared to childhood diseases, adults are more frequently affected by CML, which is distinguished by the growth of Ph+ HSC clones. Anemia-related fatigue, splenomegaly, high WBC levels, weight loss, and night sweats are all signs of CML. Nevertheless, many patients remain asymptomatic and are only identified through other diagnostic tests (Chereda and Melo, 2015). Imatinib, a kinase inhibitor has increased the patient's survival rate, however some patients develop de novo resistance to treatment, while others don't react when their disease worsens (Gorre et al., 2001). It may be possible to anticipate the emergence of imatinib resistance by observing the rate of active β-catenin in CML progenitors (Jamieson et al., 2004). For the patient population that requires third-line treatment and beyond, new treatment alternatives are required.

1.4 Endocrine Signaling

Specialized endocrine cells secrete signaling molecules (hormones), which are subsequently transported through the bloodstream to act on target cells in distant physiological regions. This process is known as endocrine signaling. Steroid hormones like progesterone and estrogen, which are produced by the ovary and aid in the development and maintenance of the female reproductive system as well as secondary sex characteristics, are a prime example (Cooper GM., 2000).

The principal female sex hormones, estrogens, are in charge of regulating both secondary sexual traits that emerge during puberty as well as the operations of the female reproductive system. The estrogen receptors (ERs), which are the receptors that estrogens bind to, are responsible for

regulating gene expression via activating signaling mechanisms and/or transcriptional processes. Non-genomic effects (effects not caused by direct DNA binding) or genomic effects may be responsible for these effects (Fuentes et al., 2019). Estrogen receptors control a wide range of biological and physiological processes. The discovery of ER and the development of a method to identify its expression using hormone binding assays in tumor samples allowed for the clinical research necessary to establish ER as a prognostic marker for response to hormone therapy. As a result, it is now common practice in clinical oncology to check the ER status of tumor samples (Williams C., et al 2013). The bulk of breast cancers have elevated levels of ER, and this protein's expression is indicative of hormone-dependent tumor growth. In comparison, tumor cells have lower ER levels. While ER is unquestionably connected to prognosis and response to endocrine therapy, there is no concrete evidence that ER expression is related to clinical characteristics in breast cancer. This might be because it is challenging to measure ER protein amounts precisely using the tools and methods that are currently available. While estrogen treatment of ER-positive breast cancer cells promotes proliferation, exogenously introduced ER has been shown in some studies to both inhibit ER-induced transcriptional activity and proliferation while also causing independent transcriptional and functional changes (Williams C & Lin CY 2013).

During the menstrual cycle and pregnancy, The development, proliferation, and differentiation of female reproductive tissues are significantly regulated by the progesterone receptor (PR). Breast cancer that is endocrine-dependent is facilitated by PR. In response to binding progesterone, a steroid hormone with which it shares a cognate receptor, PR controls networks of target gene expression (Sandra L et al., 2016). The activity of estrogen receptors (ER) in breast cancer is impacted by progesterone receptor (PR), an enhanced target gene of ER whose expression is dependent on estrogen. PR is a helpful prognostic biomarker for the disease, particularly in cases of hormone-positive breast cancer. In tumors with a good pre-treatment prognosis, PR expression is more frequently observed. One study found that postmenopausal women with higher levels of circulating progesterone had a 16% increased risk of breast cancer.PR is a direct estrogen-tempting gene that can collaborate with or compete with ER to influence following biological processes in the majority of target tissues(Li Z. et al.,2022). Breast cancer can be identified by an increase in the proportion of proliferating ER/PR-positive cells, which could indicate a shift from paracrine to autocrine regulation of steroid hormones. An increase in the proportion of

proliferating ER/PR-positive cells is linked to hyperplasia and the emergence of DCIS in mice models of hormone-dependent mammary tumors (Sandra L. et al. 2016).

1.5 Wnt Signaling Pathway

Throughout embryonic development, the Wnt signaling system regulates important processes. It is an ancient mechanism that has been preserved throughout evolution (Komiya Y & Habas R., 2008). Humans have a vast family of nineteen secreted glycoproteins called Wnts, which indicate a complicated regulation of signaling, function, and biological outcome (Komiya Y & Habas R., 2008). At the molecular and biochemical levels, these branches are currently being intensively dissected (Komiya Y. & Habas R., 2008). One of the key routes controlling development and stemness is wnt signaling, which has also been closely associated with cancer. (Zhan T et al., 2017). Although colorectal cancer has received the most attention for the role that Wnt signaling plays in carcinogenesis, aberrant Wnt signaling is found in many other cancer types as well. The Wnt secretory apparatus, Wnt co-receptors, parts of the β-catenin degradation complex, and nuclear co-factors are among the identified elements and procedures (Zhan T et al., 2017).

1.5.1 Wnt/β-Catenin Pathway

The cytoplasmic β-catenin destruction complex, which also consists of Axin, adenomatosis polyposis coli (APC) (Schwarz-Romond,.et al 2002) protein phosphatase 2A (PP2A) (Hsu,. Et al 1999), glycogen synthase kinase 3 (GSK3) (Kikuchi A,. 1999), and casein kinase 1 (CK1) (Kikuchi A,. 1999), breaks down cytoplasmic β-catenin in the absence of Wnt signaling (He X,. et al 2004). It is important to note that Wnt stimulation regulates Axin stability because one of its side effects is dephosphorylation of Axin and a decrease in Axin levels in the cytoplasm. (2008) (Komiya Y. & Habas R.)

a) **b)**

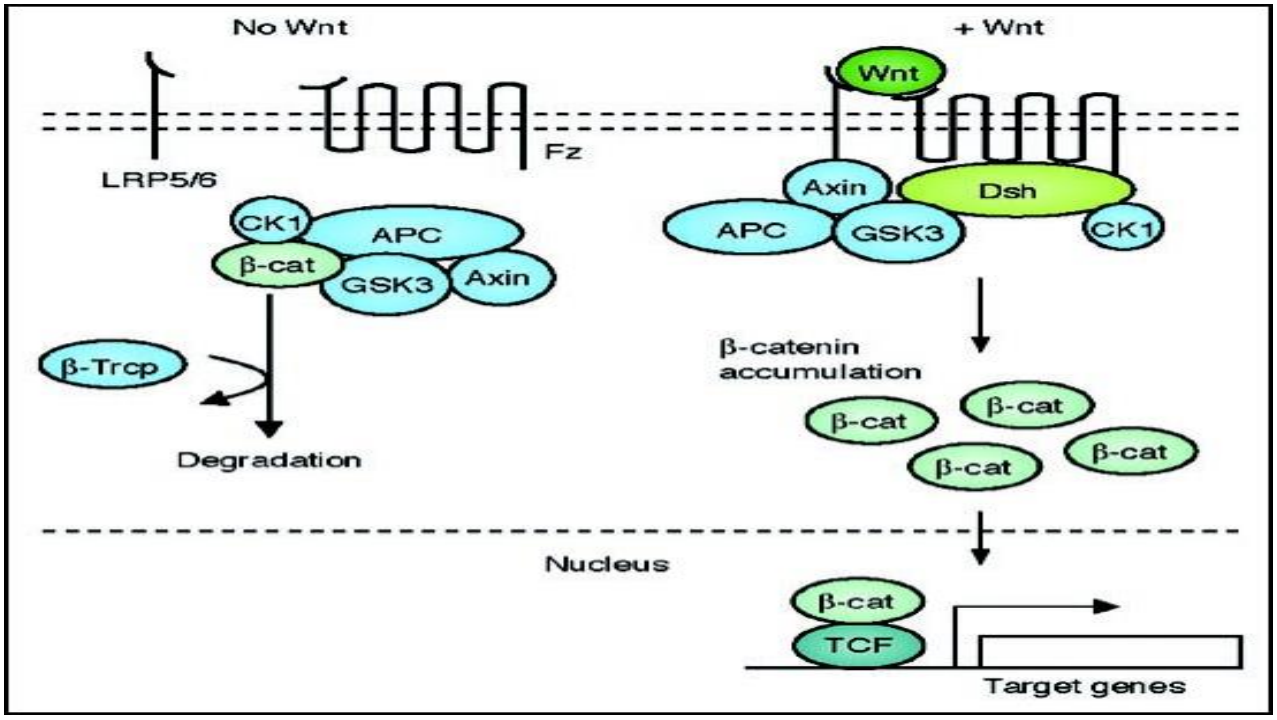

Figure 1.2: Wnt/β-Catenin Pathway a) In active pathway b) Activated pathway (Komiya Y & Habas R., 2008)

2) LITERATURE REVIEW

2.1 Acute Promyelocytic Leukemia

2.1.1 Introduction to APL

In 1957, Norwegian hematologist LK Hillestad first noted and defined APL. One of the numerous subtypes of AML, which makes up 10% to 15% of all AML cases, is APL (Tallman & Altman, 2008). In promyelocytes, APL is identified by maturation arrest and differentiation block. APL is caused by the creation of the oncogenic fusion protein PML-RAR, which is a consequence of the balanced reciprocal translocation of the RARA retinoic acid receptor gene on chromosome 17 and the PML gene on chromosome 15. (1991, Kakizuka et al.). Numerous cellular processes, such as tumor suppression, antiviral defenses, homeostasis, differentiation, angiogenesis, and DNA repair, are carried out by the PML protein. PML-NBs carry out these duties (Lallemand-Breitenbach, 2010). When it interacts to the retinoic X receptor, the ligand-dependent transcription factor RAR creates heterodimers that are transcriptionally active. Regulating gene expression is RARα's main purpose. Cell proliferation, survival, differentiation, and death are just a few of the physiological processes that genes with retinoic acid response elements (RARE) are engaged in. The disruption of PML-NBs and downregulation of RARα target gene transcription results in promyelocyte development (Grignani et al., 1998). Arsenic trioxide (ATO) and all-trans retinoic acid (ATRA) are crucial for the treatment of APL to achieve high cure rates. ATRA activation causes promyelocytes to develop into granulocytes (Breitman et al., 1980). Based on risk, there are three types of APL patients. Sometimes low and intermediate WBC counts are paired with WBC values of 10,000/ul. WBC counts more than 10,000/ul are indicative of high-risk patients (Sanz et al., 2000). Patients with moderate and low risk may benefit from receiving ATRA + ATO treatment. however cytotoxic chemotherapy is also used to treat high risk individuals because ATRA + ATO alone is insufficient (Osman et al., 2018). Patients with APL are more likely to experience recurrence; nonetheless, 10–20% of APL patients experience relapse regardless of risk classification (Vitaliano-Prunier et al., 2014).

2.1.2 Molecular Pathogenesis

In the molecular analyses of APL, the distinctive qualities of the PML-RARα fusion product have been shown to be crucial. The gene expression of hematopoietic progenitor cells, myeloid differentiation, and stem cell self-renewal are all disturbed by the PML-RARα fusion product. (Vitaliano-Prunier et al 2014). RARα and retinoid X receptors, nuclear hormone receptor proteins, normally form a heterodimer (Chambon, 2005). To regulate the expression of several genes, the RARα-RXR heterodimer interacts to retinoic acid response regions (RAREs, which are segments of DNA). These DNA sections regulate differentiation and self-renewal. If the retinoic acid (ligand) is lacking (SMRT), the heterodimer complex will engage corepressors such as nuclear receptor corepressors (NCoR) and silencing mediators for thyroid and retinoid hormone receptors. Additionally, histone deacetylases contribute to transcriptional regulation. Retinoic acid and RARa binding cause a conformational shift that releases these corepressors. Co-activators are then called upon to control chromatin remodeling and gene expression. (Kishimoto et al, 2006) The transcription of RARE is inhibited by PML-RARα fusion proteins, which interfere with the recruitment of coactivators (RAREs).

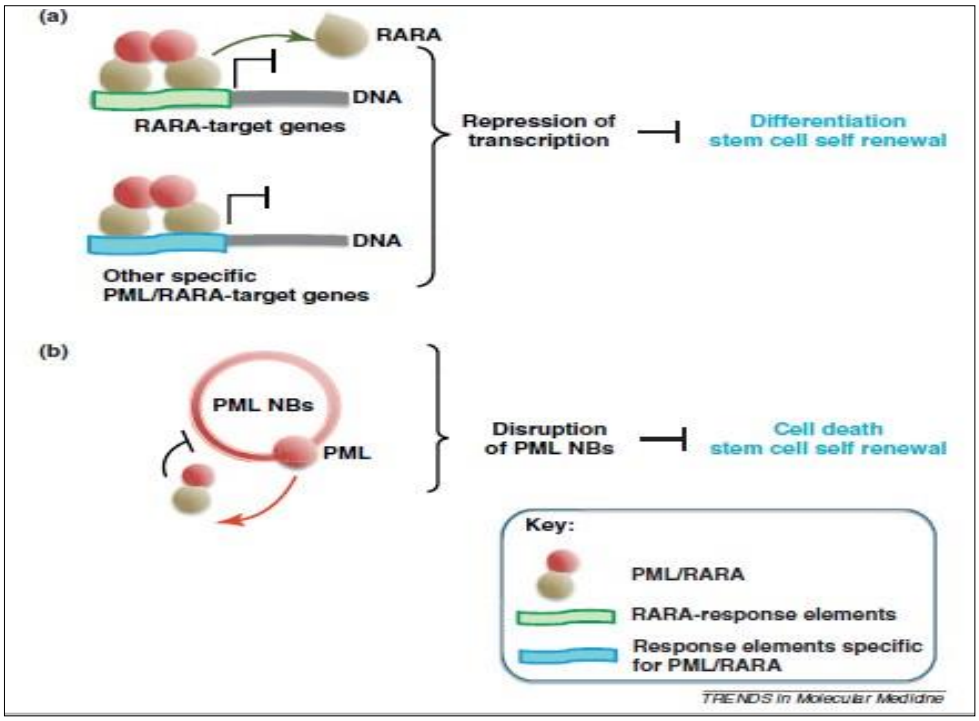

Figure 2.1: APL Pathogenesis (Lallemand-Breitenbach *et al.*, 2011)

2.1.3 Translocations in APL

New chromosomal translocations other than t(15;17) have been identified in one to two percent of APL patients. According to Baba, Pandith, Shah, and Baba (2019), there have been 12 discovered fusion variants of APL that are all connected to the RARA gene. The most prevalent APL variant, ZBTB16 (formerly PLZF)- RARA translocation, which is caused by the rearrangement t(11;17)(q23;q21), has been identified in more than 30 individuals (Z. Chen et al., 1993). While APL cells exhibit up-regulation of CD13 and CD117 as well as atypical expression of CD2 (T-cell linked antigen), which is associated with high leukocyte count and microgranular variant morphology, APL is different by the absence of various transmembrane glycoproteins like CD7, CD11a, CD11b, CD14, and CD18 expression, infrequent HLA-DR expression, and down regulation of CD34. Chemotherapy resistance and conventional ATRA are associated with CD56 expression. (Noguera et al. 2019).

2.1.4 PML-RARA Fusion Gene

The PML gene produces a number of mRNA transcripts of different lengths due to breakpoints in three exonic regions known as bcr. The long (L-) isoform of mRNA is produced when there is a break in the PML gene's intron 6 (bcr1), which subsequently results in the creation of PML6-RARA3 mRNA, which accounts for about 70% of APL cases. The short (S-) isoform of mRNA is produced when the PML gene's intron 3 (bcr3) breaks. The subsequent synthesis of PML3-RARA3 mRNA, which accounts for about 20% of APL cases, follows this break. The variable (V-) isoform mRNA forms as a result of a breakpoint in exon 6 (bcr2) and is then linked to RARA exon 3. 10% of the instances are this kind of APL. On the other hand, differences in the distribution of the three isoforms according to region or ethnicity have been described. (Liquori et al, 2020)

About 50% of pediatric and adult APL patients have additional chromosomal abnormalities, such as a 7q deletion and an 8-chromosome trisomy. Trisomy 8 is the accumulation of an extra chromosome and results in deregulation of the MYC gene in APL, which can upregulate the PML-RARA fusion gene and hasten the development of myeloid leukemic cells (Delgado, Albajar, Gomez-Casares, Batlle, & León, 2013)(Ronchini et al. (2017). According to research,

the prognosis of APL patients with extra chromosomal mutations remains unaffected, with the exception of those who have three or more of them (Labrador et al., 2019).

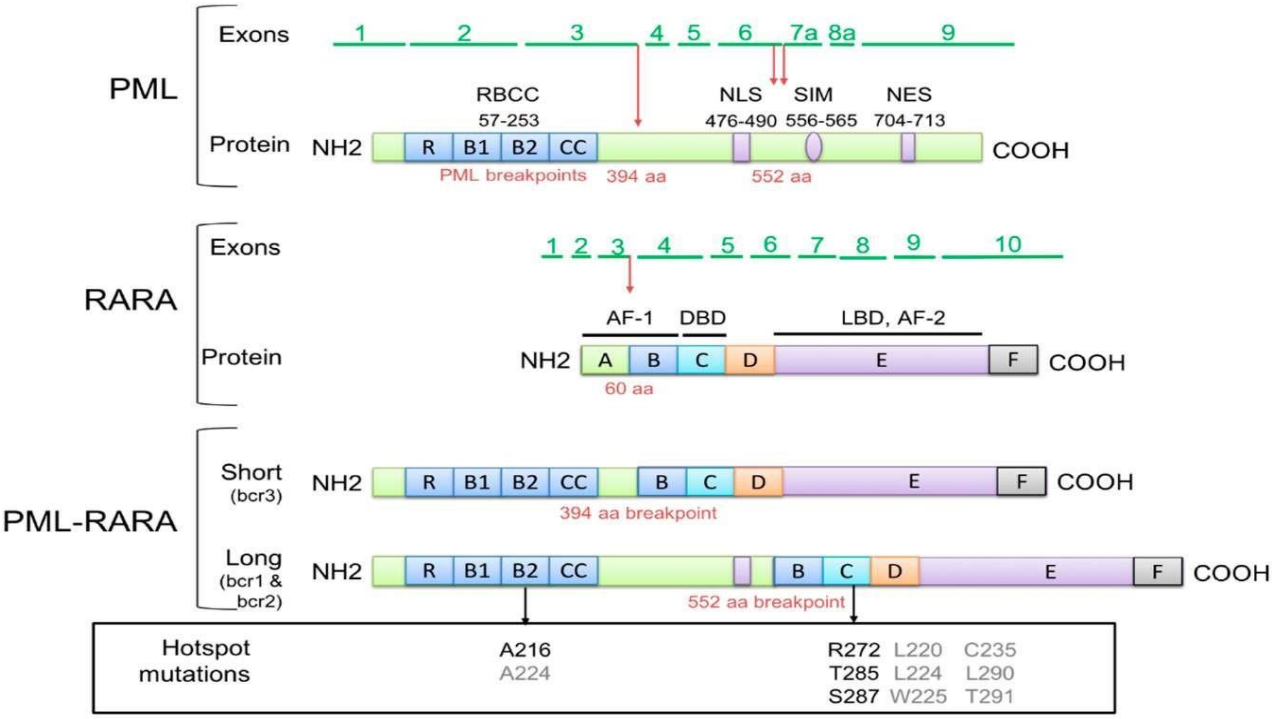

Figure.2.2: Structure of the APL primary event (Liquori et al., 2020)

Because of the disordering or disarray caused by the PML-RAR fusion in acute promyelocytic leukemia, which alters the structure of nuclear bodies, micro speckles form. (2010) Lallemand-Breitenbach. This condition is caused by the absence of an SUMO-binding motif in the PML component of the PML-RAR fusion gene. Due to PML-ability RAR's to multimerize with a variety of protein classes, this fusion gene can have both dominant-negative and gain-of-function carcinogenic effects (Pandolfi, 2001). On the one hand, PML-RAR prevents myeloid differentiation from continuing throughout the promyelocyte stage. This happens as a result of the transcription of genes necessary for myeloid development, like those necessary for granulocyte differentiation, being prevented. The sluggish accumulation of promyelocytes in the bone marrow of APL patients is brought on by PML-RAR, which, in contrast, encourages leukemic cell survival and proliferation (Kamashev, Vitoux, & De Thé, 2004).

2.1.5 Treatment Strategies

Acute promyelocytic leukemia, once thought to be the most lethal kind of acute leukemia in adults, is now one of the most curable types (Thomas, 2019). Regardless of how great or low their risk of getting the disease is, people with APL now have the option of chemo-free treatment. Without using chemotherapy, all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) are now regarded as the first-line and standard treatments for newly discovered cases of APL. Since the turn of the century, medical experts have been focusing on non-chemotherapeutic methods for treating newly discovered cases of APL (H.-H. Zhu, 2020).

2.1.6 Problems in existing Treatments

2.1.6.1 Early Death Rate

Early death is about passing away at any point during induction or within 30 days of diagnosis. (F Lo-Coco et al., 2013). Early death rate is a significant barrier to treating APL patients. According to the US SEER database, early death rates have decreased over time in the ATRA+ chemotherapy era. (Dinmohamed et al., 2016).If the ED rate is reduced further by the ATRA+ATO, it is unclear 5.5% ED rate was reported in an ATRA+ATO group trial with a n=758.(Murthy et al., 2020). Future research is necessary to determine whether the ED rates between the ATRA+ATO and ATRA+ Chemotherapy models differ.

2.1.6.2 ATRA+ATO Toxicity

Pain, GI toxicity, and liver damage are frequent side effects. Leukocytosis (WBS count more than 10 10'9/L) is the deadliest side effect of ATRA+ATO. In patients with APL, hyper leukocytosis may serve as a sign for ED, differentiation syndrome, and later recurrence. (Yoon et al., 2019).

2.1.6.3 Resistance

Treatment resistance is a significant obstacle for APL patients. Mutations in the retinoic acid receptor's ligand-binding domain cause ATRA resistance (Roussel & Lanotte, 2001). Due to these alterations, PML-RARA becomes unresponsive to retinoic acid, but it can still bind to RARE and block transcription, according to in vitro studies utilizing NB4 resistant cell lines. (Mozziconacci et al., 2002). Retinoic acid resistance has also been linked to PML/RARA degradation. (Fanelli et al.,1999). There have also been reports of in vitro and in vivo ATO resistance, which has been

linked to clustering mutations in the PML moiety's B2 domain, a direct binding site for arsenic. 2001 (Roussel & Lanotte).

2.2 Chronic Myeloid Leukemia

The BCR-ABL1 fusion gene linked to translocation t results in an increased generation of myeloid cells in chronic myelogenous leukemia (Kang et al., 2019). The (9;22) fusion oncoprotein mediates proliferation, apoptosis resistance, stimulation of the JAK/STAT, PI3/Akt pathways, and genomic instability through constitutive tyrosine kinase activity. (Caponetti and Bagg, 2019).

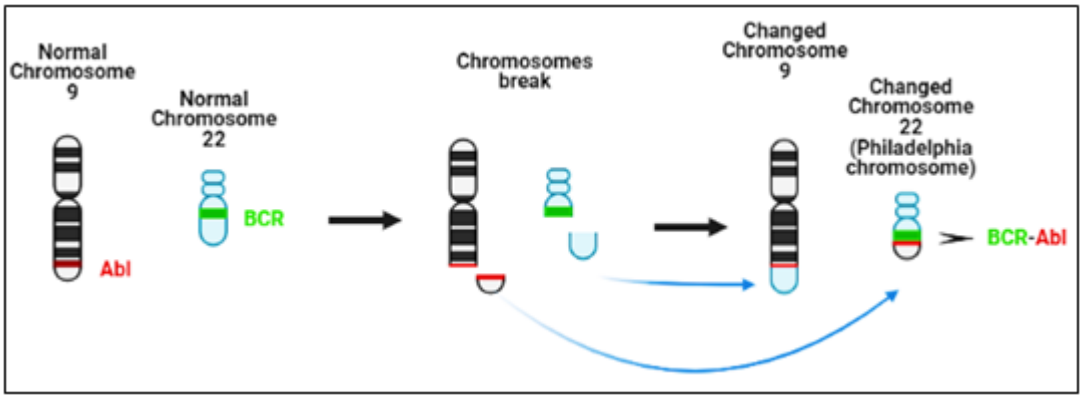

Figure 2.3: BCR-ABL fusion gene is formed on Philadelphia chromosome. Image created with BioRender.com

The t(9;22) translocation causes the BCR and ABL genes to be truncated. The ABL gene encodes a tyrosine kinase with tightly regulated activity, whereas the BCR gene encodes a phosphoprotein linked to serine/threonine kinase activity (Chandra et al., 2011). The BCR-ABL oncogene accelerates the development of leukemic progenitors while causing LSCs to colonize slowly (Michor et al., 2005). The basic basis of CML is unregulated signal transduction by the ABL kinase (Hehlmann et al., 2007).

The activation of signal pathways that result in greater proliferation, improved viability, altered migration, and altered homing is caused by the normal ABL Tyrosine Kinase (TK). These pathways are typically tightly controlled by several hematopoietic growth factors. The non-receptor tyrosine kinase ABL1 protein is present throughout hematological development, but when myeloid maturation occurs, its levels decrease. The neutrophil oxidative burst is caused by the normal BCR gene (Haider MZ., et al 2022). The synthesis of BCR/ABL1 results in

constitutive activation of tyrosine kinase. Cell differentiation is prevented, unchecked cell division occurs, and cell death results. Only a few of the processes connected to the pathophysiology of the BCR-ABL1 gene (Haider MZ., et al.2022).The BCR-ABL kinase must be active for CML to progress. Despite being present at the molecular level in every CML patient, the BCR-ABL fusion gene is not only present in CML (Ayatollahi H., et al 2018).

2.2.1 CML Treatment

Small kinase inhibitor Imatinib is quite effective for treating early-stage CML, while interferon is a different option for those who cannot tolerate Imatinib. Up to 87% of patients achieve full cytogenetic remission with imatinib treatment (400mg daily) (Hehlmann et al., 2007) (Weisberg et al., 2007). Imatinib inhibits phosphorylation of substrates and the activation of downstream signal transmission by occupying an ATP-binding site in the kinase domain of ABL. Myelosuppression, edema, bone discomfort, skin rashes, gastrointestinal toxicity, and hepatotoxicity are some of the side effects of Imatinib (P. Lanzkowsky, 2011).

2.2.2 Therapeutic failure in CML

Leukemia stem cells that continue to proliferate throughout treatment are not eliminated by imatinib therapy (Michor et al., 2005). TKI treatment is ineffective against CML stem cells because they do not need BCR-ABL1 kinase activity to live and operate as a reservoir for relapse (Soverini et al., 2018). Ponatinib is the TKI with the best efficacy against the T315I gatekeeper mutation. First and second generation TKIs are ineffective in individuals with this mutation or two or more mutations in the same molecule (Soverini et al., 2014). However, ponatinib's safety information showed a higher risk of unfavorable cardiovascular events (Saussele et al., 2020). At nanomolar doses, a modified form of ponatinib known as PF-114 has proven effective against mutant BCR-ABL1, including the T315I mutation (Mian et al., 2015). A significant barrier to attaining enduring CMR is the poor response in patients who advance to AP/BC (Zuo et al., 2021). This necessitates the urgent need to discover alternative CML therapeutic alternatives.

The aggressive nature of AML necessitates new therapy approaches and pharmacological targets. ER targeting in AML is strongly supported by epidemiological and preclinical investigations, despite the fact that the disease is not assumed to be sex-hormone-related. Studies have shown that ER activation reduces leukemogenesis and leukemia cell proliferation while increasing the

efficiency of conventional chemotherapeutic drugs (Forsythe A., et al 2010, Li Q., et al 1999, Valk PJ., et al 2004). According to a study, both the male and female patient groups with BCR/ABL-positive CML had significantly lower serum ER levels (ng/ml) than the control group (El-Kaream SAA., et al., 2022). Under pathological conditions, constitutively elevated Wnt activity in LSCs enforces the continuous generation of leukemic myeloid cells. Restoring the LSCs' dysregulated Wnt activity slows the development of illness. (Kang et al., 2019). The reason why the β-catenin pathway is activated in CML & AML is unclear (Jamieson et al., 2004).

2.3Hypothesis

It was hypothesized that AML and CML positive NB4 and K562 cell lines respectively, might express ER which is involved in leukemogenesis via Wnt/β-Catenin signaling and use of selective estrogen receptor down regulator could be helpful in controlling leukemogenesis in our models. This study's objective is to do ER expression profiling in leukemic models. Pharmacological targeting of our models and their role in proliferation of cells & to Study the mechanism of anti-proliferative effect in our models and to find the potential targets of selective estrogen receptor down regulator (Fulvestrant) against AML and CML models using *insilico* approaches.

3) MATERIALS AND METHODS

3.1 *in silico* study

3.1.1 Target Gene Selection

Gene expression profiles for the NB4 and K562 cell lines were retrieved using the databases National Center for Biotechnology Information (NCBI), Cancer Cell Line Encyclopaedia (CCLE), GeneCards, and literature study (Miramontes., 1992).

3.1.2 Therapeutic Target Library

To find sequence similarity in protein structure, all the chosen genes were sent to Uniprot, where FASTA sequences were extracted and then exposed to PSI-BLAST. The protein structures that shared 98% or more identity were chosen. (Van., 2002).

3.1.3 Protein 3D Crystallographic Structure

Target proteins' accession numbers were obtained from PSI-BLAST and then submitted to Protein Data Bank (PDB) for 3D protein structure collection. The PDB format of the protein 3D crystal structures was downloaded (Danchin *et al*., 1991).

3.1.4 Purification of Protein Structure

UCSF Chimera 1.14 was used to isolate PDB protein structures. To obtain a pure protein structure, extra or identical protein chains, water molecules, and all other non-standard amino acids were eliminated. (Danchin *et al*., 1991).

3.1.5 Binding Pockets Identification

DoGSiteScorer, an online automated pocket recognition and analysis tool that predicts the binding sites based on hydrophobic interactions, was used to identify the possible active drug binding pockets in target protein structures. The highest scoring binding pockets (close to 1) were chosen (Kuntz *et al*., 1982).

3.1.6 Preparation of Ligand

The 2D ligand structure was retrieved from the PubChem database in SDF format, and then translated using the programme Open Babel to mol2 format (Sousa *et al*., 2006).

3.1.7 Molecular Docking

The preferred interaction of a ligand with its protein receptor was predicted using molecular docking using the molecular modelling simulation software Autodock 4.2. By incorporating hydrogen atoms, calculating charges, and removing water molecules, the ligand and macromolecule were created. The Autogrid was configured using binding pocket scores that had previously been computed. Lamarckian Genetic Algorithm was chosen with default parameters to dock ligand with protein (Kitchen *et al*., 2004).

3.1.8 Analysis and Visualization of Binding Pose

The docking output file was converted using Cygwin commands into a PDB file format and then displayed using Discovery Studio Visualizer v4. After considering all binding postures and estimated binding energies, protein-ligand complexes with the lowest binding energy were chosen for additional research (Kitchen et al., 2004).

3.1.9 Protein Ligand Interaction Profiler

To define the interaction features at the atomic level, interactions among ligand-protein complexes with the lowest free binding energies for each target were visualized and thoroughly detected using PLIP (Protein Ligand Interaction Profiler).

3.2 In Vitro Study

3.2.1 Culturing of Cell Line

Suspension cell lines were used for all experiments:

1.NB4: APL study model with the t (15;17) PML-RARα gene fusion.

2.K562: A model for chronic myeloid leukemia with the Bcr-Abl b3-a2 fusion gene on the Ph chromosome.

The cells were cultured in 6-well tissue culture plates with pre-warmed RPMI. (Gibco by Life Technologies). For NB4 and K562, 10% Fetal Bovine Serum (Gibco by Life Technologies) was additionally added to the media. Additionally, 1% of Penstrep was used to prevent contamination due to microorganisms. The culture plates were kept in a humidified, water-jacketed incubator at 37°C and 5% CO2 to encourage the growth of the cell lines. The cells were always maintained at a density of less than 1.0 x 106/ml to avoid contact inhibition (Z. Chen et al., 1993).

3.2.2 Freezing & Thawing

Two solutions were created for the freezing or cryopreservation of cells: freezing solution I comprise RPMI 70% (v/v) and FBS 30% (v/v), whereas freezing solution II contains RPMI 80% (v/v) and DMSO 20% (v/v). Cells were collected in a 2 ml Eppendorf tube and centrifuged for 5 minutes at 1200–1400 rpm. After being washed with PBS, the cell pellet underwent another round of centrifugation. After discarding the supernatant, the cell pellet was resuspended in 2 ml of freezing solution I, and 1 ml of this was added to each cryovial. Following this, 1ml of solution II was dropped into each cryovial one at a time. The cryovials were then put in an isopropanol-filled cryo box and stored at -80°C. The vials were moved into a different box the following day and stored at -80 C. Cryopreserved cells were promptly defrosted in a 37°C incubator after being taken out of the liquid nitrogen freezer before being resuspended in growth medium. Cells were given a single PBS wash to eliminate the DMSO. After being resuspended in fresh growth medium and plated in culture plates, the pellet was then put into a CO2 incubator at 37 °C.

3.2.3 Trypan Blue Exclusion Essay

To ascertain the number of cells present per ml of the media, this test was conducted prior to the MTT assay. This was done to ensure a steady supply of cells that would be viable for the MTT experiment and yield reliable findings. The cell suspension was produced in a 1:1 dilution with 0.4 percent trypan blue staining solution (Gibco by Life Technologies). A hemocytometer's counting chambers were filled with 10µl of the dilution. The four chamber grids' four cells were counted in their whole. Cells that weren't alive were dyed blue. The following calculations were made to determine how many cells would be needed for the MTT assay**:**

$$\frac{\text{Number of cells in grid 1 + grid 2+ grid 3+ grid 4} \times \text{Dilution factor} \times 10^4 \text{ cells/ml}}{4}$$

3.2.4 Fulvestrant

The first estrogen receptor (ER) antagonist, Fulvestrant (also known as "Faslodex"), downregulates the ER without having any agonist-like effects. Fulvestrant is FDA approved drug against breast cancer. Fulvestrant, a 7-alkylsulphinyl analogue of 17-oestradiol, has a considerably different chemical makeup from the nonsteroidal structures of tamoxifen, raloxifene, and other SERMs. Fulvestrant, whose binding affinity is 89% that of estradiol, inhibits estradiol binding to the ER competitively (C K Osborne et al., 2004).

3.2.5 Drug Dilutions

To create a 20mM starting drug stock, the drug Fulvestrant was dissolved in DMSO. Different drug concentrations were produced in 10% FBS medium from stock solutions. The concentration increased geometrically and logarithmically at each subsequent stage because the dilution factor was constant. The 20mM stock solution was then serially diluted.

3.2.6 MTT Assay

Through the formation of Formazan from the tetrazolium salt, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide], this test was utilized to assess the viability and

proliferation of cells. Since mitochondrial dehydrogenase enzymes catalyze this reduction, it measures the cell's vitality (Riss TL, 2013).

In a 96-well plate, cells were seeded at a density of 1 x 104 cells/50 l. Next, 50µl/well of three different concentrations of the drugs fulvestrant (20 mM) (Med Bio Express), ATRA (3.3 mM), and Imatinib (1 mM) (Sigma Life Technologies) were added, each in triplicate. For roughly 48–72 hours, Fulvestrant, ATRA, and imatinib were allowed to work on the cells. After the required amount of time had passed, the cell viability was determined by adding 15µl of filter sterilized MTT (5 mg/ml in PBS) to the microtiter wells containing cells and concentrations of Fulvestrant, ATRA, and Imatinib. The plates were then once more kept in a CO2 incubator for about three to four hours to allow crystals to form. After 3–4 hours, 90µl of the media was carefully removed without disturbing the crystals. 100µl of DMSO were used to dissolve the crystals. After the crystals had completely dissolved, the absorbance at 550 nm was measured using a spectrophotometric plate reader.

3.2.7 Treatment of cells with Fulvestrant for the mechanism study

Cells (NB4and K562) were grown in liquid media (RPMI + 10% FBS + 1% L-Glutamate and 1% Penstrep) for 72–96 hours while also being exposed to various concentrations of fulvestrant (5µM, and 10µM). Following RNA extraction, cDNA was created for real-time PCR gene expression analyses (qPCR).

3.2.8 RNA Extraction for gene expression studies

About 0.5-1 million cells were placed in a 2 ml Eppendorf and centrifuged at 1500 rpm for 5 minutes. The particle was then obtained after the supernatant was removed. After adding 1ml of TRIZOL LS reagent (Life Technologies) to the cell pellet, the cells were homogenized by being pipetted up and down. The homogenized cells were then incubated on ice for around 5 minutes to ensure the separation of nucleoprotein complexes. Then 200 l of chloroform was added, and the mixture was vigorously shaken for 15 seconds. After that, the tube was permitted to stand on ice for approximately 10 minutes. The next step was to centrifuge the sample at 12,000 g for 20 min. at 2-4 C. After centrifugation, the mixture was divided into three phases: an upper aqueous phase carrying the necessary RNA, an interphase holding DNA, and a pink organic phase containing proteins. A second Eppendorf with 500µl of cooled isopropanol was added after the carefully

selected aqueous phase, and the tubes were agitated. Following a further 15 minutes of incubation on ice, a 20 minute centrifugation at 12,000 g at a temperature of 2-4 °C was performed. After being vortexed and washed with 1 ml of 75% cooled ethanol, the pellet was centrifuged at 7500 g for 5 minutes at 2-4 °C. The supernatant was then discarded. After the supernatant was removed, the RNA pellet was air dried in the hood at ambient temperature. In order to continue processing, the RNA pellet was then resuspended in 20 l of nuclease-free water and stored at -80°C.

3.2.9 RNA quality and quantity check

The quality of the bands was evaluated visually using a 2% agarose gel electrophoresis to assess the RNA's quality. The purity of the isolated RNA was verified using a 260/280 ratio check, and it was quantified using Nanodrop 2000 (ThermoScientific, USA).

3.2.10 Complementary DNA synthesis

For the reverse transcription of the template to cDNA, 1000 ng of RNA template was used. The following procedure was followed: A 0.2 ml microtube was initially filled with 1 l of oligo dT primers (10 M), then 2 l of dNTPs, 1µl of RNase inhibitor, 1µl of DTT, and 4 l of first strand synthesis buffer. Following the addition of 1µl of reverse transcriptase and 1µl of NF water as necessary to create a total volume of 20µl, 1000 ng of RNA template were added. The tubes were then put in a thermocycler, and the manufacturer's recommended conditions were followed.

3.2.11 cDNA quality check and primer optimization

Conventional PCR was used to optimize primers for the housekeeping gene GAPDH, the c-myc and the Axin2 gene. The quality of the cDNA was also examined. c-myc, AXL, and Axin2 primers were chosen after doing literature research (Y. Jin et al., 2017)

In 0.2 ml tubes, a reaction mixture (20µl) for PCR was made using 1000 ng of cDNA as a template. 1000ng of sample cDNA, 2.5µl (10X) of PCR buffer, 2µl of 25 mM MgCl2, 1.5µl (10 mM dNTPs), 1µl each of forward and reverse primers, 0.4µl of Taq DNA polymerase, and 11.6µl of nuclease-free water made up the reaction mixture. Thermocycling settings included 35 cycles of PCR amplification after 5 minutes of denaturation at 95 °C. Each cycle also included three steps: denaturing the template DNA for one minute at 95 degrees Celsius, annealing the primers for 45 seconds at 60 degrees Celsius, and then extending the cDNA strand from the annealed

primers for one minute and 45 seconds. Taq polymerase was allowed to synthesize unexpended strands for 10 minutes at 72 °C. After that, PCR products were kept at -20°C. Using a 2 % agarose gel and the Dolphin Gel Doc System, PCR results were examined for bands using gel electrophoresis.

3.2.12 Gel Electrophoresis

The validation of cDNA synthesis was performed by running the PCR result on a 2% agarose gel for analysis. 1g of agarose was dissolved in 50ml of 1x TAE buffer after 49ml of distilled water and 1ml of 50X TAE buffer were combined. The solution in the flask was cooked in the microwave for 30 to 40 seconds until it was clear. It was then allowed to cool for a minute before 4 ul of ethidium bromide was added. The casting tray was then filled with gel, which was then given time to set. Combs were then taken out, and wells were then loaded with 4ul of PCR product and 2ul of loading dye. Thermo Scientific's 1 kb DNA ladder was utilized as the marker. At 90 volts and 500 amps, gel was operated for 40 minutes.

3.2.13 Real-time PCR (qPCR) for gene expression analysis

Real-time PCR was used to examine primers for the target genes for specific product amplification at 10 mM. (Applied Biosystems 7300). 0.5 l of cDNA, 0.5ul of forward and reverse primers (10 mM each), and 2ul of SYBR Green Master Mix (5 X) (Solis BioDyne) were used to create the reaction mixture. The reaction volume was increased to 10 l by the addition of 6.5 l of water free of nuclease. There were three main steps in the reaction. The reaction mixture was first heated to 50 degrees Celsius for two minutes, then heated to 95 degrees Celsius for ten minutes, followed by 40 cycles of PCR. Three incubation phases, each lasting 15 seconds at 95 ℃, 45 seconds at 60 ℃, and 30 seconds at 72 ℃, were used to complete each cycle. The dissociation step was introduced for melt curve analysis. For the dissociation stage, three incubations were used: the first at 95 ℃ for 30 seconds, the second at 60 ℃ for 30 seconds, and the third at 95 ℃ for 30 seconds. The information was gathered at 72 ℃. SDS software for the ABI 7300 system was used for data analysis. As a housekeeping gene for normalization, GAPDH primer was utilized. To determine the relative quantification of gene expression, the 2-C method was used.

3.2.14 Statistical Analysis

Results for all experiments that were carried out in triplicate (MTT assay) or replication (Real-time PCR) are displayed as mean SEM. A p-value of less than 0.05 was regarded as a significant difference when using one-way and two-way ANOVA to analyze the data. The Graph pad Prism 5.01 program was used to create the graphic data.

3.2.15 DNA Fragmentation

Cells (NB4 and K562) treated with fulvestrant were collected in a 2 ml Eppendorf tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was then discarded, and the pellet was resuspended in 500 ul of lysis buffer. They were flipped over to mix the tubes, and then incubated for five to ten minutes at room temperature. The tubes were then centrifuged for 1 minute at 13000 rpm. The pellet was once more resuspended in 400 l of lysis solution, centrifuged at 13000 rpm for one minute, and incubated at 60 °C for five minutes after the supernatant was discarded. After the sample had cooled, 500µl of chloroform-isoamyl alcohol was added, and 10 minutes of 13000 rpm centrifugation followed. Select the top aqueous phase with care, then put it into a 1.5ml Eppendorf. Equal parts of cold isopropanol were added to facilitate DNA precipitation, and tubes were repeatedly turned upside down. The DNA pellet was washed in 200 ul l of 70 percent ethanol and centrifuged again for 7 minutes at 13000 rpm after the supernatant had been spun down for 10 minutes at that speed. The DNA pellet was dried in the air for roughly 30 minutes after the ethanol was entirely removed. The DNA pellet was dissolved in 40 ul l of nuclease-free water. Thermo scientific USA's Nanodrop 2000 was used to quantify the DNA that had been extracted. After running the 1.5 percent agarose gel with the DNA samples combined with loading dye, the gel was inspected using an ultraviolet gel documentation system (Rahbar Saadat et al., 2015).

4) RESULTS

4.1 In Silico Analysis

4.1.1 Ligands Structure

The structure of Fulvestrant was obtained from PubChem

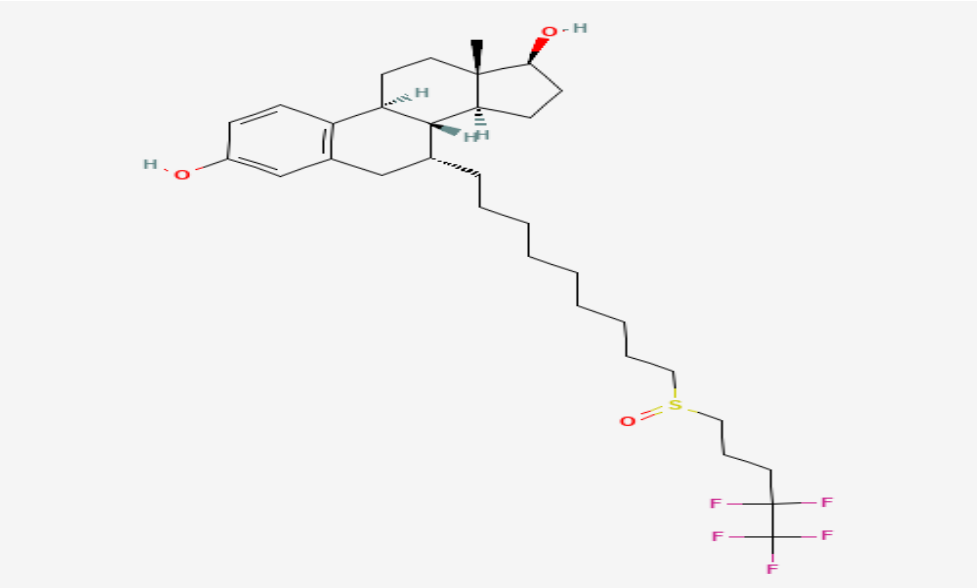

Figure 4.12: 2D structure of Ligand Fulvestrant

Figure 4.13: 3D structure of Ligand Fulvestrant

4.1.2 Target Proteins

As previously noted, the NCBI data bank was utilized to choose the target proteins for docking, and protein structures were obtained from PDB.

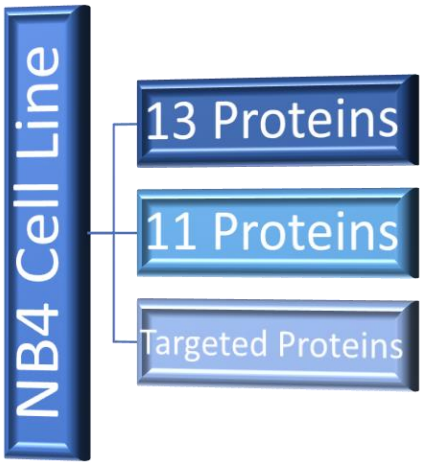

Figure 4.14: Hierarchical Model of Proteins in NB4 Dark blue box represents the total number of proteins expressed in NB4 cells taken from NCBI gene database, light blue box represents the shortlisted proteins on the basis of % identity and 3Dstructure available and then the protein targets with lowest ligand-protein binding energies**.**

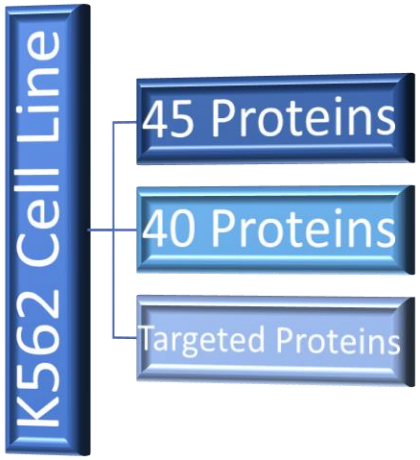

Figure 4.15: Hierarchical Model of Proteins IN K562 Dark blue box represents the total number of proteins expressed in K562 cells taken from NCBI gene database, light blue box represents the shortlisted proteins on the basis of % identity and 3Dstructure available and then the protein targets with lowest ligand-protein binding energies**.**

4.1.3 Molecular Docking Analysis

Computer-based drug design is the main objective of molecular docking; it primarily forecasts the best possible interaction between the ligand and the targeted proteins. Automated molecular docking makes structurally and energetically based predictions about molecular recognition. According to this theory, docking allows for accurate calculation of binding strength and easy prediction of ligand structure within the confines of a receptor's binding site (Waszkowycz, Clark, & Gancia, 2011).

The ligand Fulvestrant was docked with the target proteins of NB4 and K562 cell lines. Those targets having low binding energies were selected after molecular docking.

Table 4.1: Binding Energies of all possible Proteins targets of NB4

NB4 Cell Line			
Gene ID	**Description**	**PDB**	**Binding Energies (kcal/mol)**
RARA	Retinoic acid receptor alpha	3a9e	-13.94
CDK2	Cyclin-dependent kinase 2	5uq3	-10.76
PML	PML nuclear body scaffold	1bor	-5.88
CTNNB1	Crystal Structure of a beta-catenin	2gl7	-6.57

Table 4.2: Binding Energies of all possible Proteins targets of K562

K562 Cell Line			
Gene ID	**Description**	**PDB**	**Binding Energies (kcal/mol)**
MAPK3	Mitogen-activated protein kinase 3	4qtb	-12.25
CDK2	Cyclin-dependent kinase 2	5uq3	-10.76
BCR	Breakpoint cluster region protein	5n7e	-10.86
CTNNB1	Crystal Structure of a beta-catenin	2gl7	-6.57
ABL	Tyrosine-protein kinase ABL1	4xey	5.07

4.1.4 Protein-Ligand Interaction Profile

PLIP, the protein ligand identification profiler, primarily uses a quantum mechanics (QM)-based technique to identify non-covalent interactions between macromolecules and ligands, such as ionic interactions, hydrogen bonds, and van der Waals (Raha et al., 2007). Sometimes simple potential energy functions are employed in place of QM calculations to speed up the procedure.

4.1.5 Protein- ligand complex of Beta Catenin-Fulvestrant

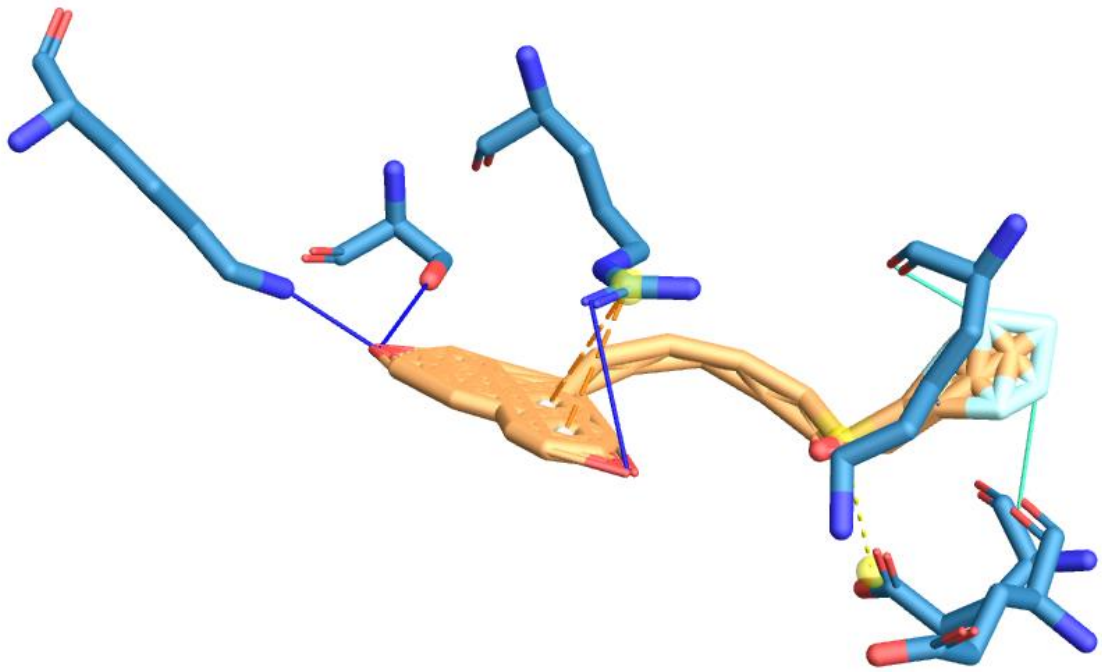

Figure 4.16: Protein ligand interaction profile image indicating the binding pose of Fulvestrant with B-catenin protein in NB4.

Table 4.3: Hydrogen Bonds between Fulvestrant and B-catenin ligand protein complex.

Hydrogen Bonds

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	435A	LYS	1.81	2.74	149.81			2196 [N3+]	3865 [O3]
2	469A	ARG	2.91	3.68	136.6			2448 [Ng+]	3866 [O3]
3	469A	ARG	3.49	3.68	103.2			3866 [O3]	2448 [Ng+]
4	473A	SER	3.07	3.46	124.4			3865 [O3]	2480 [O3]

Table 4.4: Hydrophobic interactions between Fulvestrant and B-Catenin ligand protein complex.

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	508A	LYS	3.29	3861	2761

Table 4.5: Halogen Bonds between Fulvestrant and B-catenin ligand protein complex.

Halogen Bonds

Index	Residue	AA	Distance	Donor Angle	Acceptor Angle	Donor Atom	Acceptor Atom
1	508A	LYS	2.53	151.32	105.23	3870 [F]	2759 [O2]
2	568A	GLU	2.77	136.16	107.63	3871 [F]	3138 [O2]

4.2 In vitro Analysis

4.2.1 Expression Analysis of ER & PR

Estrogen is involved in proliferation of cell via estrogen receptor through genomic and non-genomic pathways (Fuentes N., et al, 2019). Also, the crosstalk between ER and Wnt signaling has been reported to interfere with the cancer biology in mammal models (Gao Y., et al 2013). Keeping it in mind we analyzed the expression of Estrogen and Progesteron receptor in the APL & CML cell lines using MCF7 which is receptor positive Breast cancer cell line, as control. RT PCR results have shown no expression of these receptors.

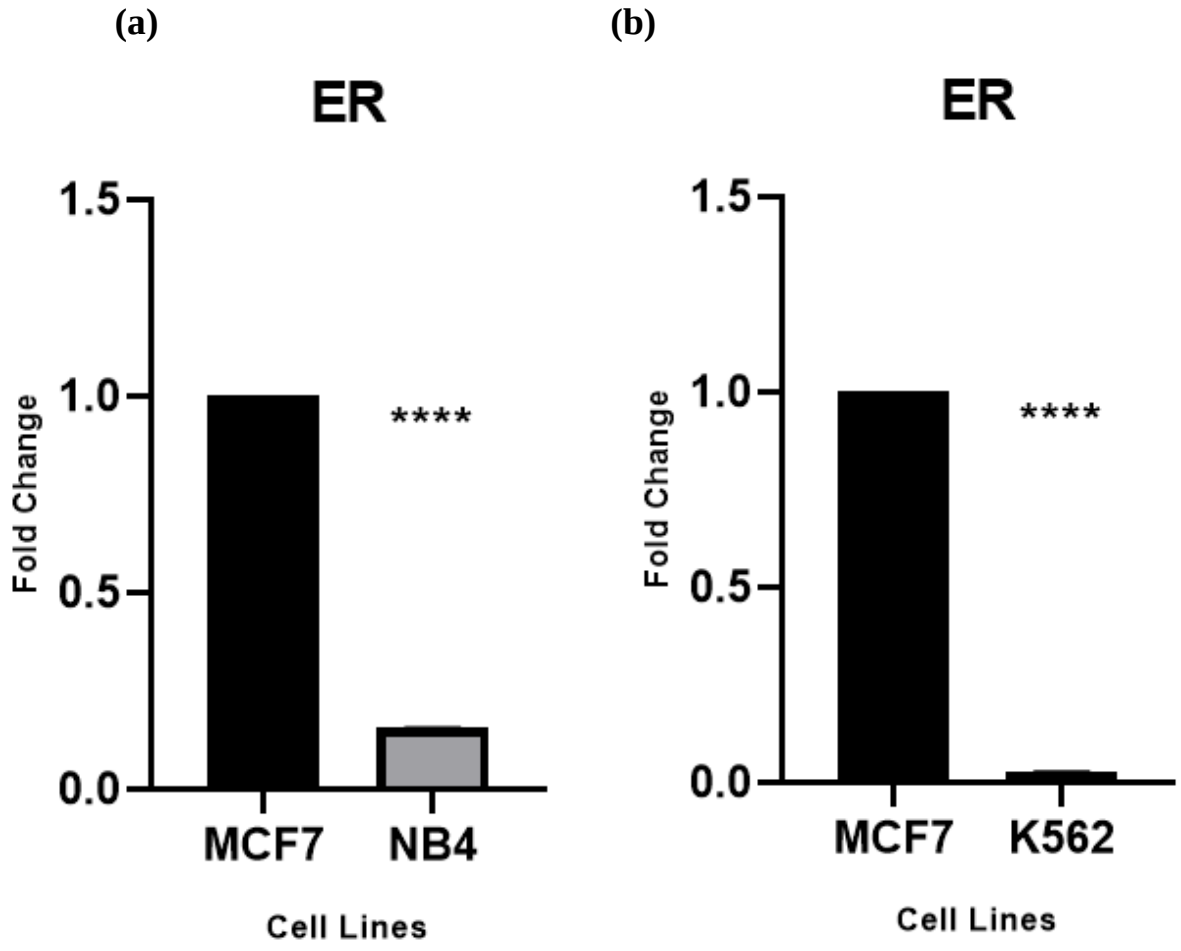

Figure 4.1: Expression of ER in (a) NB4 cells (b) K562 cells

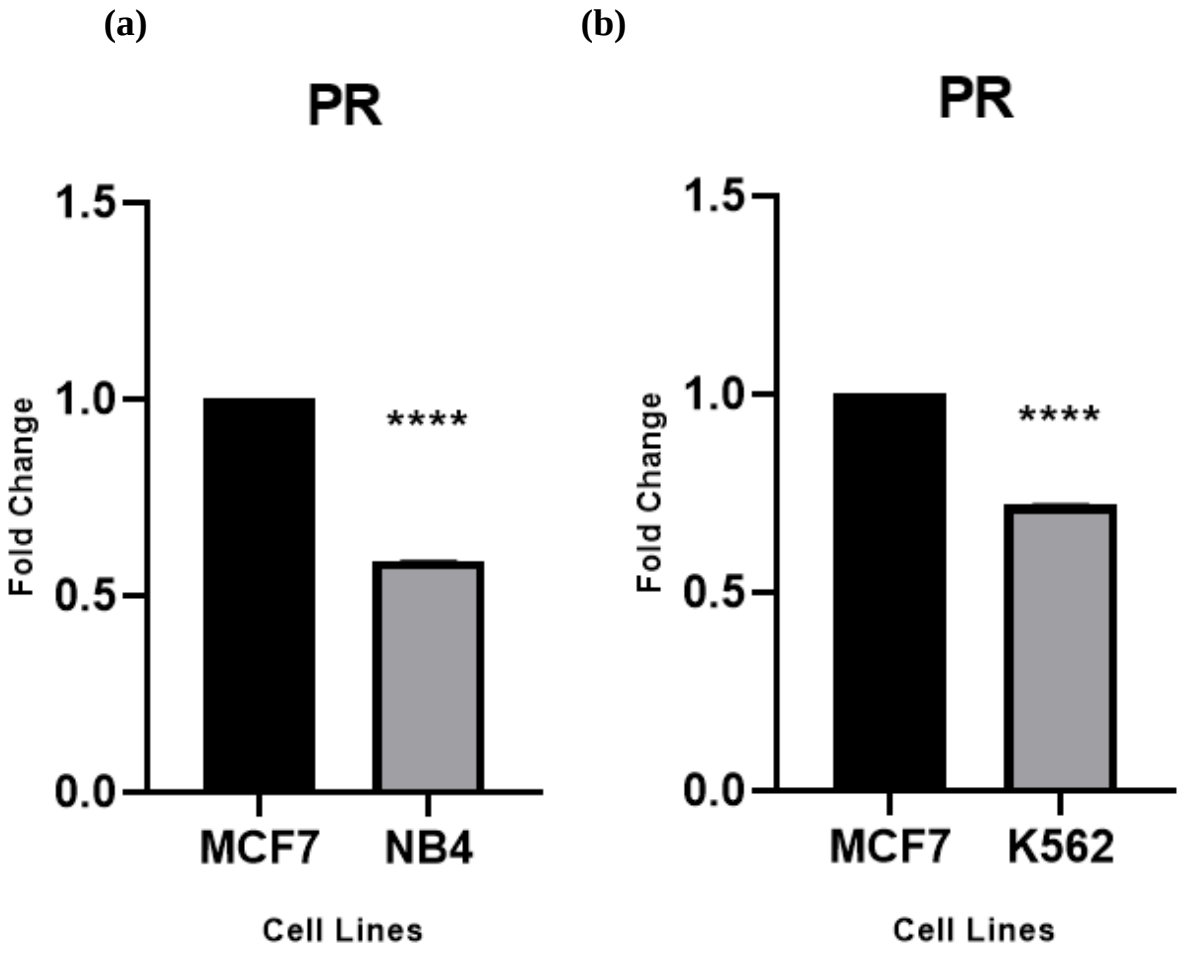

Figure 4.2: Expression of PR (a) NB4 cells (b) K562 cells

Analysis of real time PCR result for Expression of Estrogen Receptor show that Estrogen Receptor is not expressed in NB4 and K562 model (figure4.1 & 4.2). Although there is expression of Progesteron Receptor but as Fulvestrant is selective estrogen receptor down regulator specifically and it indirectly downregulates the effect of progesteron signaling thus its expression can't be considered.

4.2.2 Pharmacological Targeting of PML/RARα- positive APL cells with Fulvestrant

Fulvestrant is well-known for its potential against estrogen receptor thus showing anti-proliferative effect in breast cancer models (C K Osborne et. al., 2004). As shown by in silico results Fulvestrant targets different potential genes present in NB4 cells, thus the action Potential fulvestrant was tested against the NB4 cell line, different concentrations of fulvestrant were given to the cell and MTT assay was performed for 72 hours to check the proliferation.

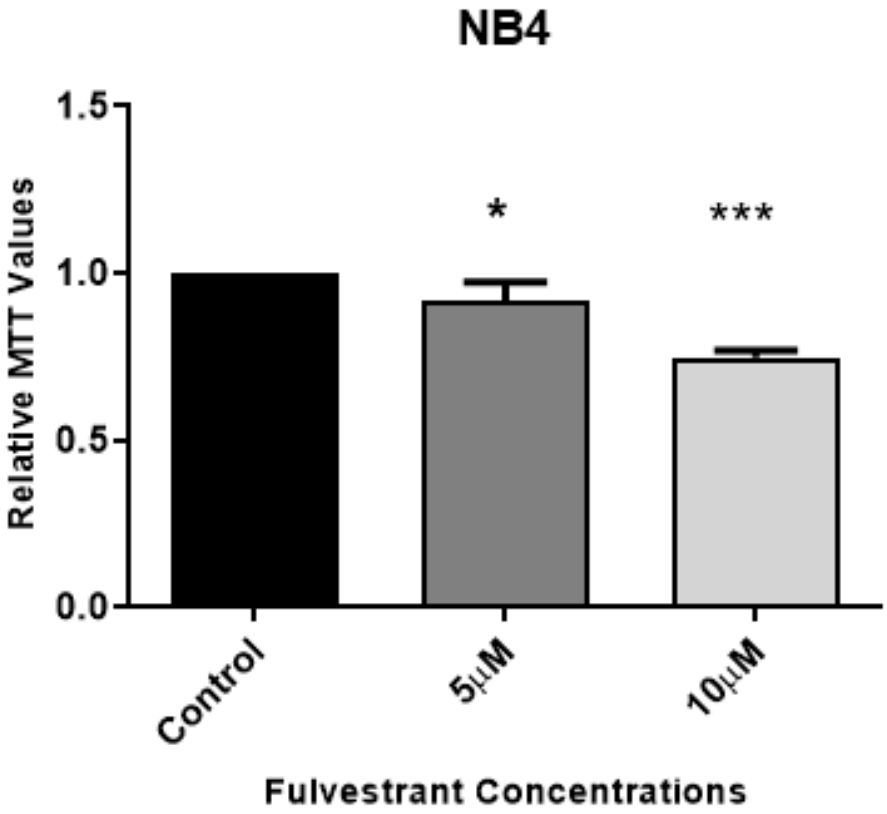

Figure 4.3: Effect of Fulvestrant on the proliferation potential of NB4 cells through MTT assay.

To ascertain the proliferation capability of NB4 cells in the presence of 0.01% DMSO and the prescribed concentration of Fulvestrant, cells were grown in liquid medium (RPMI + 10% FBS + 1% L-Glutamate and 1% Pencillin and Streptomycin). One-Way ANOVA was used to assess for statistical significance (p-values less than 0.001 are considered statistically highly significant). Bars display mean and SEM.

4.2.3 Pharmacological Targeting of BCR/ABL-positive K562 cells with Fulvestrant

As shown by in silico results Fulvestrant targets different potential genes present in NB4 cells, thus the action Potential fulvestrant was tested against the K562 cell line, different concentrations of fulvestrant were given to the cell and MTT assay was performed for 72 hours to check the proliferation.

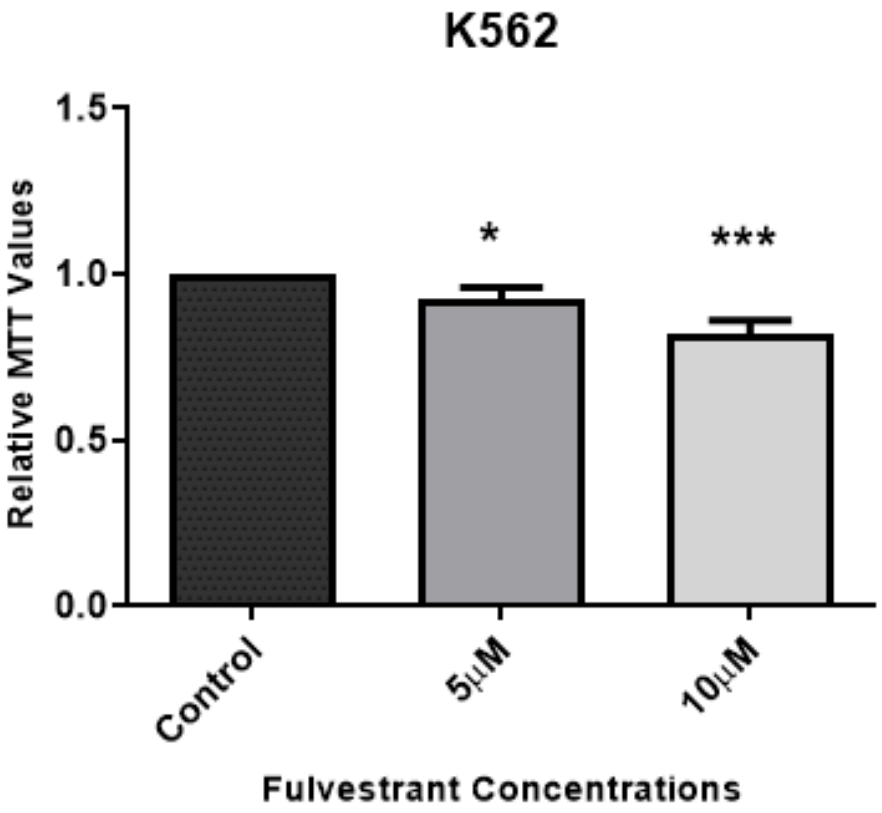

Figure 4.4: Effect of Fulvestrant on the proliferation potential of K562 cells through MTT assay.

To ascertain the proliferation potential of K562 cells in the presence of 0.01% DMSO and the prescribed concentration of Fulvestrant, cells were grown in liquid medium (RPMI + 10% FBS + 1% L-Glutamate and 1% Pencillin and Streptomycin). One-Way ANOVA was used to assess for statistical significance (p-values less than 0.001 are considered statistically highly significant). Bars display mean and SEM.

4.2.4 Comparison between the anti-proliferative effect of Fulvestrant and ATRA on NB4 cells

The standard of care for PML/RAR-positive APL has been ATRA since it was first made available as a treatment option for APL more than three decades ago, which has improved the clinical prognosis of this lethal condition (Ng & Chng, 2017). Nevertheless, a cure rate of more than 80% was attained when ATRA and chemotherapy were both used (Francesco Lo-Coco & Cicconi, 2014). However, it is well documented that ATRA causes differentiation syndrome in APL patients along with a sharp increase in leukocyte count. Therefore, it is seen that patients who take ATRA exclusively as APL therapy after achieving CR experience relapse (Asou, 2017). So, we compared the effect of Fulvestrant and ATRA on different concentration by apply MTT assay for 72 hours.

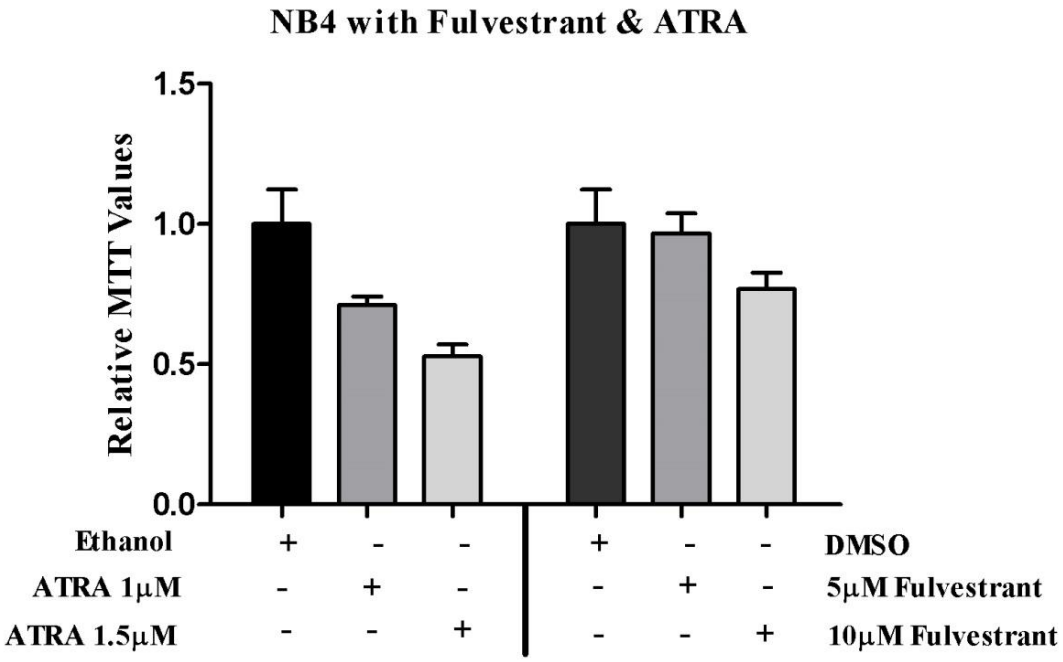

Figure 4.5: Comparison between Fulvestrant and ATRA.

We evaluated the effects of fulvestrant and ATRA on the ability of PML/RAR-positive NB4 cells to proliferate. To ascertain the proliferation capability of NB4 cells in the presence of 0.01% DMSO and specified concentration of Fulvestrant and ATRA, cells were grown in liquid medium (RPMI + 10% FBS + 1% L-Glutamate and 1% Pencillin and Streptomycin). After 72 hours, cell growth was evaluated using the MTT test.

4.2.5 Comparison between the anti-proliferative effect of Fulvestrant and Imatinib on K562 cells

Leukemia stem cells that continue to proliferate throughout treatment are not eliminated by imatinib therapy (Michor et al., 2005). TKI treatment is ineffective against CML stem cells because they do not need BCR-ABL1 kinase activity to live and operate as a reservoir for relapse (Soverini et al. 2018). So, we compared the effect of Fulvestrant and imatinib on different concentration by apply MTT assay for 72 hours.

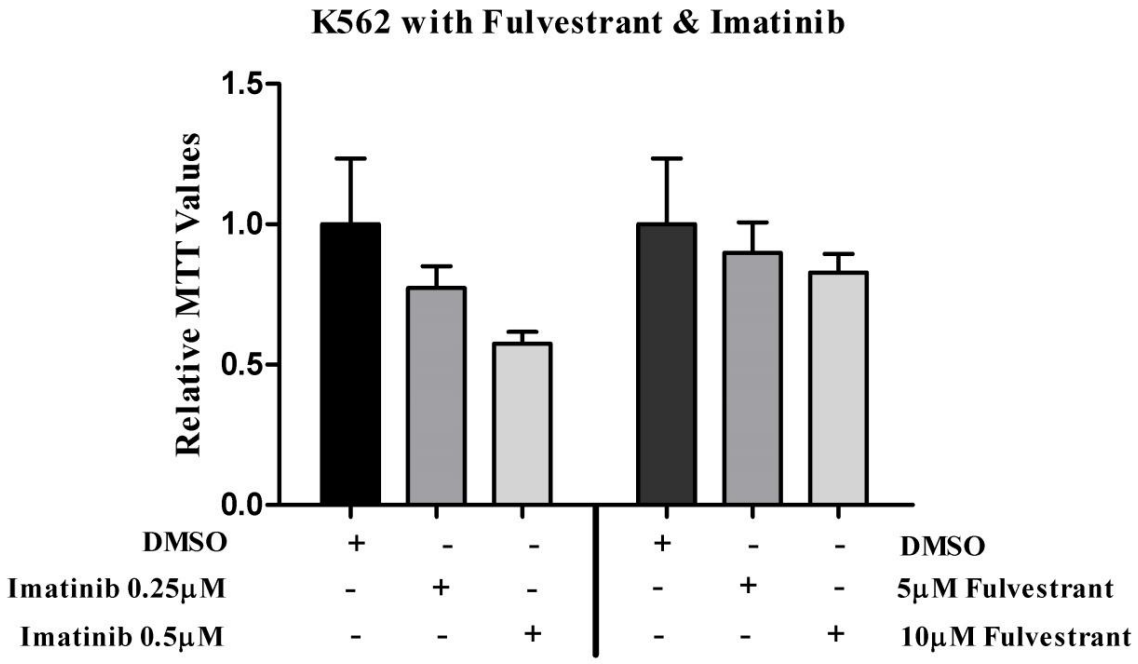

Figure 4.6: Comparison between Fulvestrant and Imatinib.

The effects of fulvestrant and imatinib on the capacity of BCR-ABL positive K562 cells to proliferate were contrasted. To assess the proliferative potential of K562 cells in the presence of 0.01% DMSO and specified concentrations of fulvestrant and imatinib, cells were grown in liquid medium (RPMI + 10% FBS + 1% L-Glutamate and 1% Pencillin and Streptomycin).

4.2.6 Combinatorial effect of Fulvestrant and ATRA on PML/RARα-positive NB4 Cells

To increase the overall effectiveness of the treatment, two medications are frequently combined. Although there are many cancer treatments available, their usefulness is constrained by their

toxicity to normal cells. In addition, over time, cancer cells can become resistant to a particular therapy that was once a successful treatment. Due to its promising outcomes, ATRA is known to be the standard of care for PML/RARα positive APL patients. However, as was mentioned above ATRA is known to cause differentiation syndrome in APL patients, which is accompanied with a sharp increase in leukocyte numbers. As a result, recurrence is observed in individuals who solely get ATRA as APL therapy after achieving CR. Therefore, a combination therapy is required in order to reduce the concentration of ATRA while still achieving good outcomes. With this in mind, we looked into the effects of combined treatment to see if there was any Combined consequence on the propagation of NB4 cells compared to single treatment as well as if combined treatment at different concentrations in μM could reduce the concentration of ATRA, in order to reduce the differentiation induction caused by administration of ATRA. After 72 hours, the proliferation was evaluated using the MTT test.

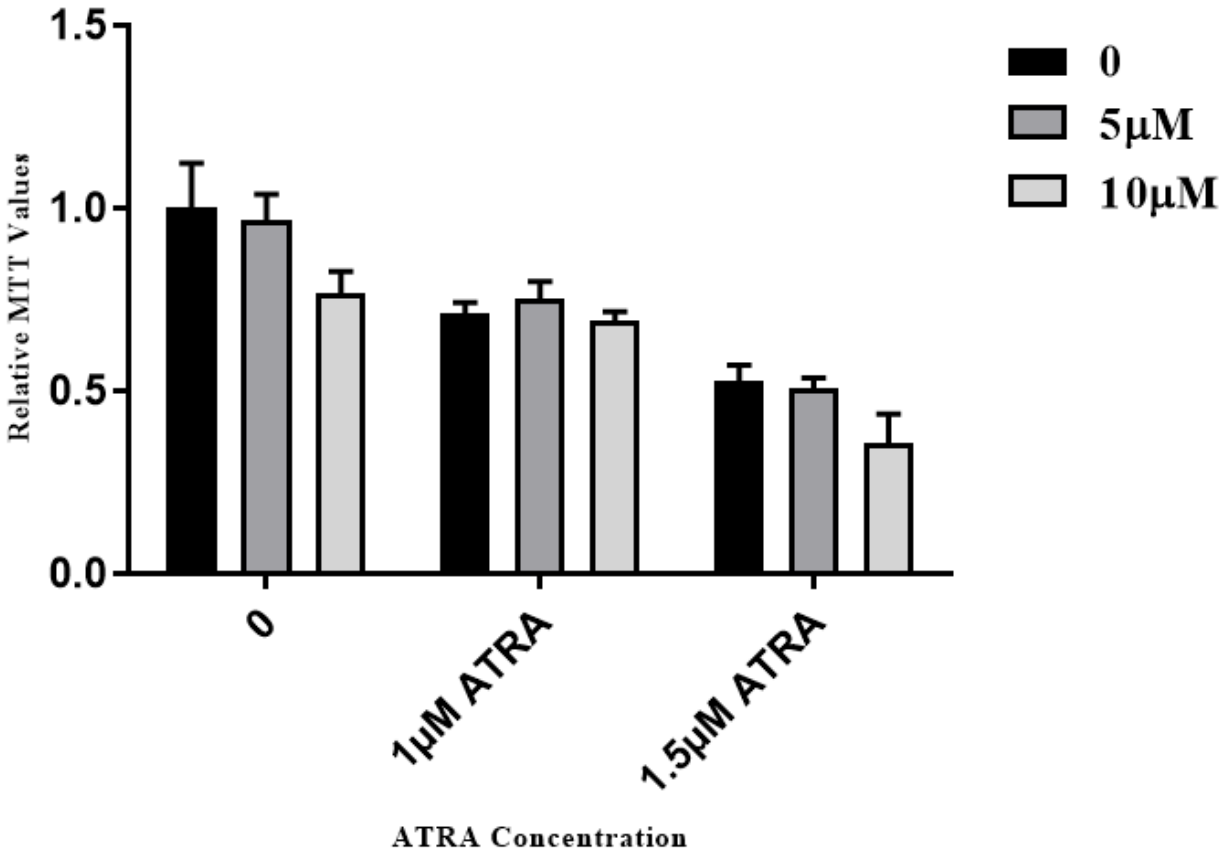

Figure 4.7: Effect of the combo therapy on the ability of NB4 cells that are PML/RAR-positive to proliferate. Combined Fulvestrant and ATRA Therapy

Our data shows a little amount of additive effect of combinatorial treatment against the proliferation potential of NB4 cells.

4.2.7 Combinatorial effect of Fulvestrant and Imatinib on BCR-ABL-positive K562 Cells

Similarly, to improve the level of treatment through additive or synergistic affect K562 cells were treated with combination of Fulvestrant and Imatinib at different concentrations and proliferation of cells using MTT assay was observed after 72 hours.

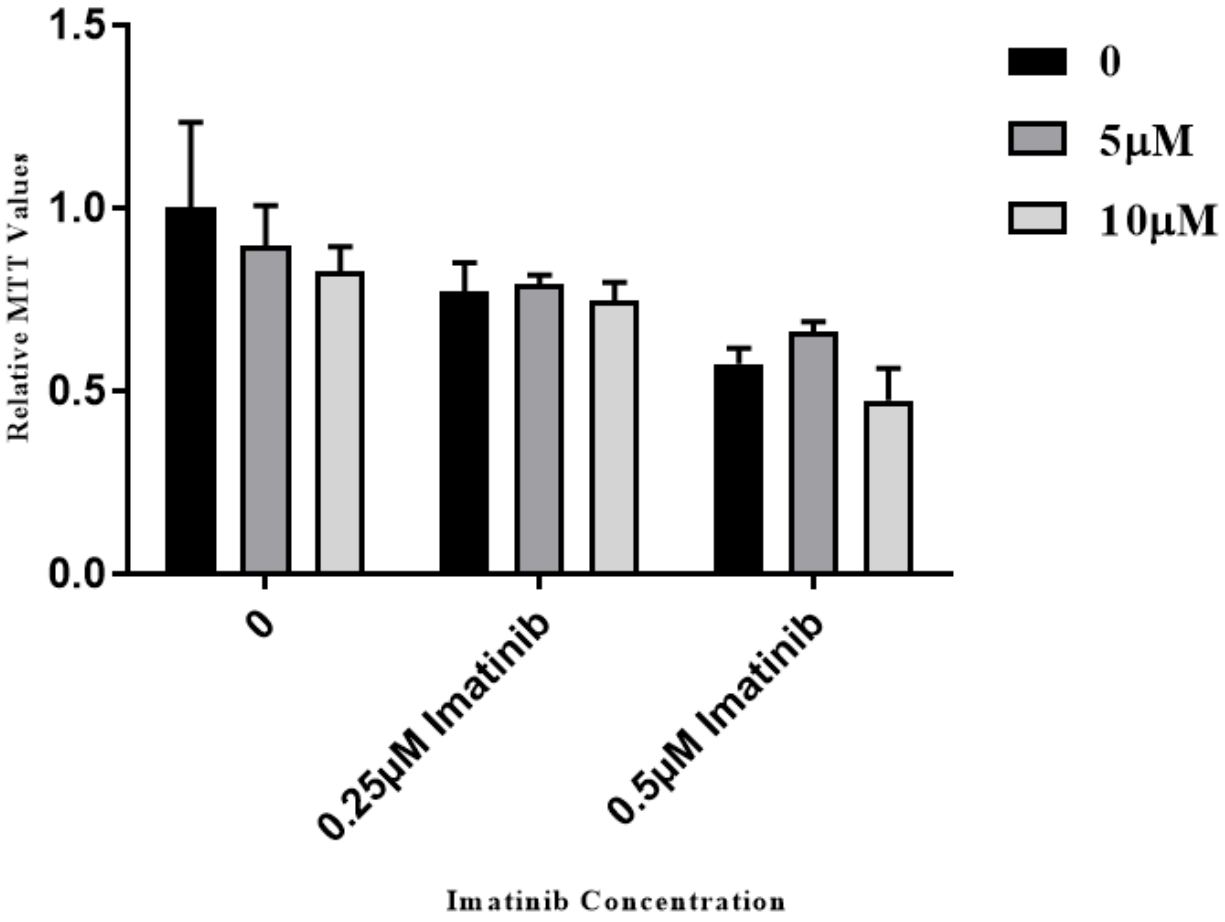

Figure 4.8: Effect of the combination treatment on the proliferation potential of BCR-ABL-positive K562 cells. Combine Treatment with Fulvestrant and Imatinib

Our data shows a little amount of additive effect of combinatorial treatment against the proliferation potential of K562 cells.

4.2.8 Effect on downstream signaling cascade

We also studied the impact of targeting the fusion gene and Estrogen receptor as described in our in-silico studies on downstream signaling pathways as the aforementioned results demonstrate a reduction in the proliferative potential of the APL & CML cell lines. Axin-2 was first assessed before moving on to the downstream signaling.

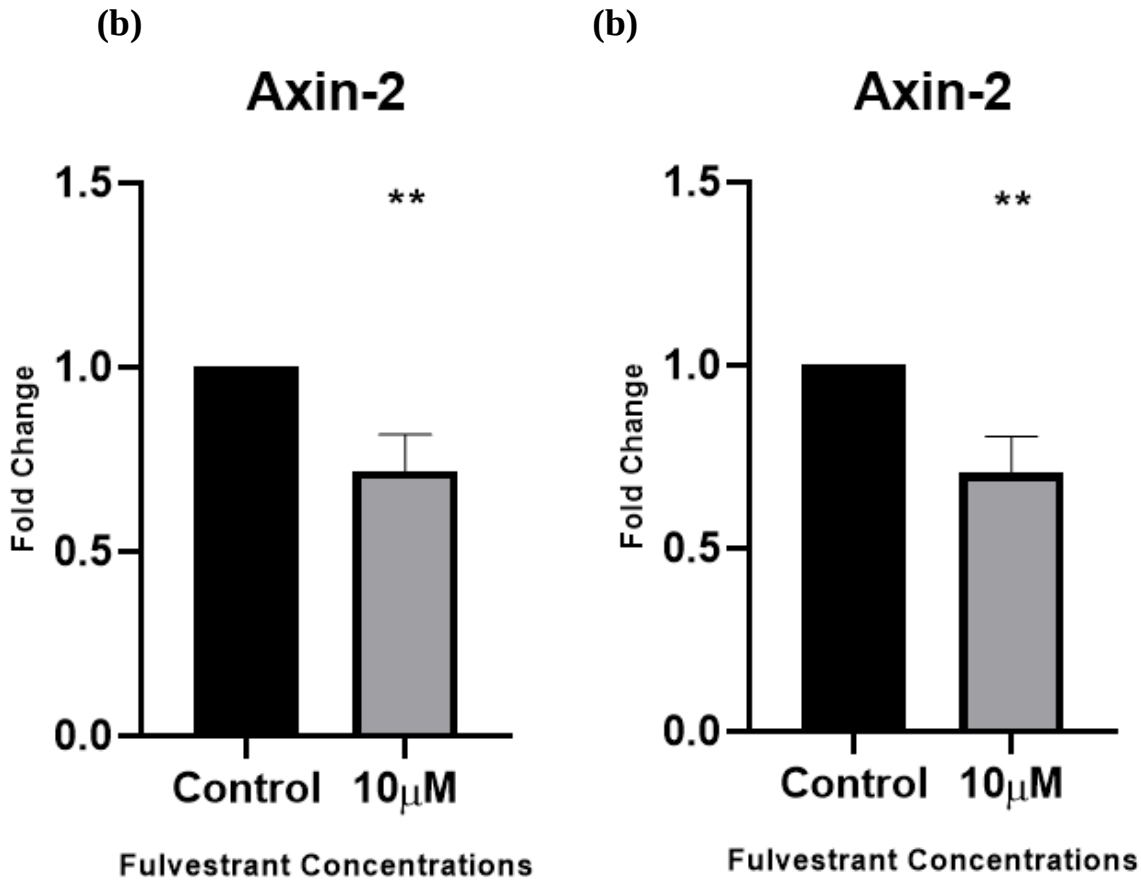

Figure 4.9: Expression of Axin-2 in (a) K562 cells (b) NB4 cells

The indicated concentration of Fulvestrant was added to liquid media (RPMI + 10% FBS + 1% L-Glutamate + 1% Penstrep) together with the indicated concentrations of Fulvestrant. Real-time PCR was used to analyze expression.

In NB4 and K562 cells there was observed an downregulation of expression of Axin-2 which indicates the involvement of Wnt/β- catenin target genes via Estrogen Receptor. Also we know that the stabilization of β-catenin by the fusion protein PML/RARα has a role in the activation or up-regulation of Wnt-target genes including c-Myc, Axin2, etc. (Müller-Tidow et al., 2004). Real-

time PCR was used to assess the expression of the c-Myc, β-catenin target gene after the cell had been exposed to a specific concentration of Fulvestrant for approximately 72 hours.

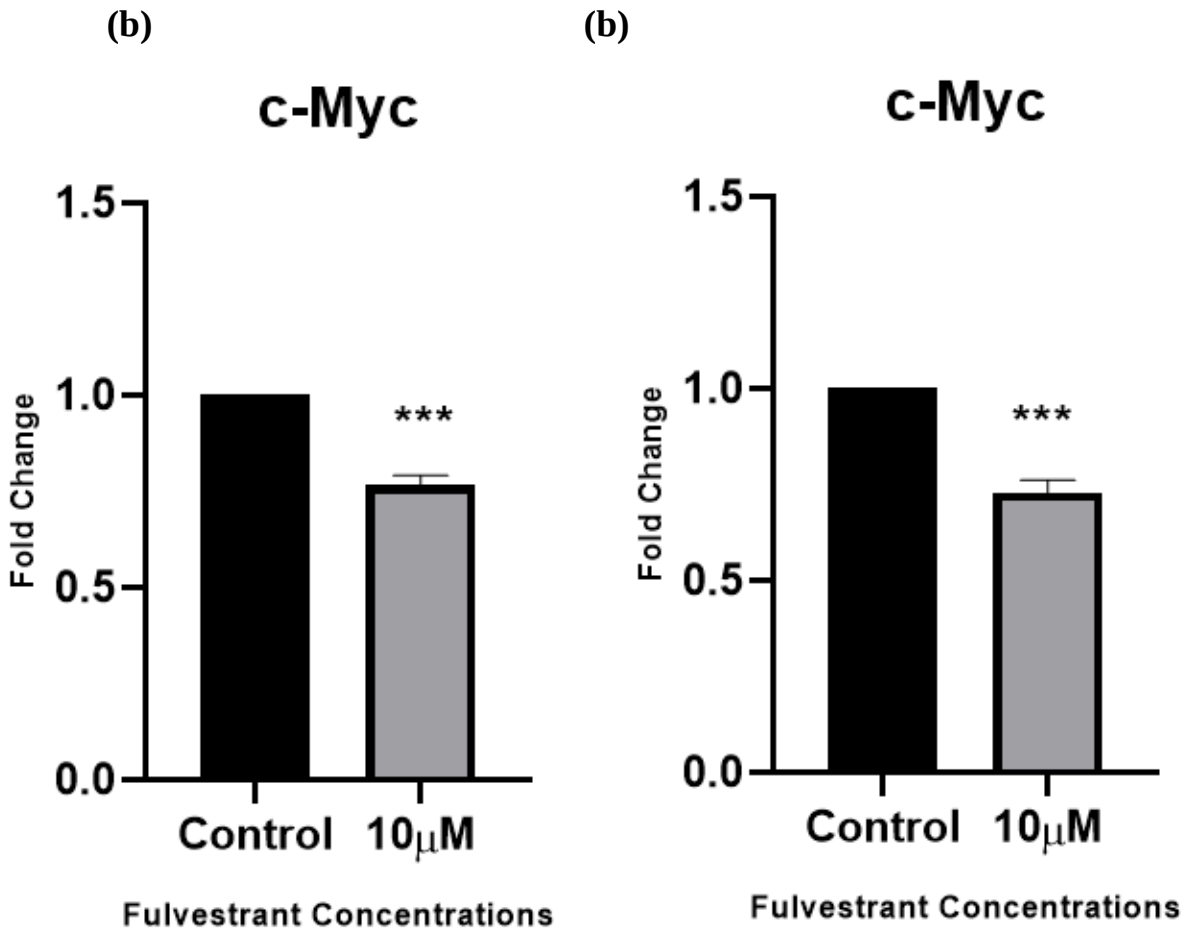

Figure 4.10: Expression of c-myc in (a) K562 cells (b) NB4 cells

The indicated concentration of Fulvestrant was added to liquid media (RPMI + 10% FBS + 1% L-Glutamate + 1% Penstrep) together with the indicated concentrations of Fulvestrant. Real-time PCR was used to analyse expression.

Down regulation of both c-myc and axin-2 genes indicates the involvement of Wnt/β catanin pathway via Estrogen receptor. As Fulvestrant blocks the Estrogen pathway thus Wnt target genes are down regulated, decreasing the proliferation potential of NB4 and K562 cells.

4.2.9 Apoptosis Check in PML/RARα-positive NB4 cells and BCR-ABL-positive K562 cells after treating with Fulvestrant

Fulvestrant inhibits the ability of PML/RAR-positive NB4 cells and BCR-ABL-positive K562 cells to proliferate, as shown in figure 4.10. Therefore, a DNA Fragmentation Assay was conducted to determine whether the cell death is caused by apoptosis or cell cycle arrest. Since one of the fundamental indicators of cellular death is DNA degradation and breakage (Saadat, Saeidi, Vahed, Barzegari, & Barar, 2015). After giving cells a 10µM dose of fulvestrant, cells were treated, and 48–72 hours later, DNA was retrieved. The extracted DNA was then electrophoresed on a 1.5% agarose gel. Figure 4.15 shows the degradation and fragmentation of DNA in NB4 and K562 cells after Fulvestrant treatment. Therefore, we can infer that Fulvestrant's ability to inhibit cell proliferation is caused by the induction of apoptosis.

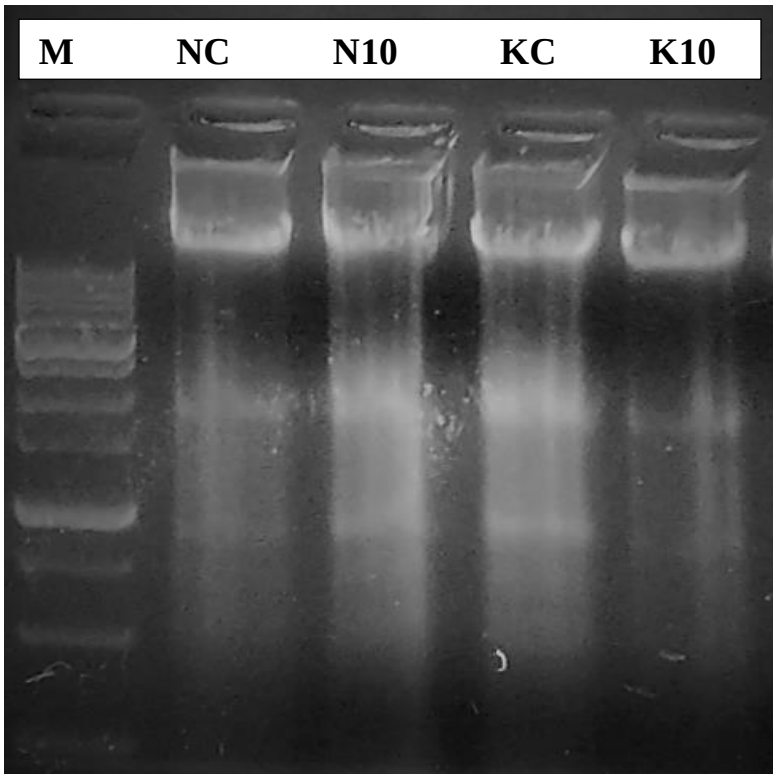

Figure 4.11: Gel image for DNA fragmentation assay for NB4 and K562 cells.

For 72 hours, cells were grown in liquid media (RPMI + 10% FBS + 1% L-Glutamate + 1% Penstrep) with 0.01% DMSO and 10 M Fulvestrant (M = 1 kb ladder, C = DMSO/ Ethanol Control). After that, DNA was extracted and run on a 1.5% agarose gel to be seen.

5) DISCUSSION

This study sought to evaluate the function of endocrine signaling, particularly that of the estrogen receptors, in myeloid leukemia. In RT PCR analysis there was no expression of these receptors. Secondly, we checked the pharmacological effect of Fulvestrant and we discovered that Fulvestrant could prevent the proliferation of BCR-ABL positive K562 and PML/RAR-positive NB4 cells. Thus, we looked for the off targets of Fulvestrant using in silico approaches and it was concluded that downregulation of β-catenin target genes may be responsible for Fulvestrant's anti-proliferative effects.

The aggressiveness of AML calls for novel therapeutic strategies and pharmacological targets. ER targeting in AML is strongly supported by epidemiological and preclinical investigations, despite the fact that the disease is not assumed to be sex-hormone-related. According to studies, ER activation boosts the effectiveness of traditional chemotherapeutic agents and inhibits leukemogenesis and leukemia cell proliferation (Forsythe A., et al 2010, Li Q., et al 1999, Valk PJ., et al 2004). According to a prior study, both the male and female patient groups with BCR/ABL-positive CML had significantly lower blood ER levels (ng/ml) than the control group (El-Kareem SAA., et al 2022). Keeping it in mind Expression of ER & PR was analyzed but there was no expression of these receptors in our models which is contrary to the previous findings.

Tamoxifen, a selective estrogen receptor down-regulator, either caused malignant cells to undergo apoptosis or normalized HSC levels. It's interesting to note that tamoxifen-induced AML cell death did not occur until after the reduction in mitochondrial respiration and spare reserve capacity, which are essential for AML cell survival, had taken place. Despite this, given tamoxifen has ER-dependent effects on HSCs, its pro-apoptotic actions are probably a result of a combination of ER signaling and metabolic targeting (Sriskanthadevan S., et al 2015, Skrtić M,. et al 2011). Similarly, Fulvestrant does not affect the proliferation of BCR/ABL-positive K562 cells but decreased its resistance towards different drugs according to a study (Zalcberg JR,. et al 1993). In this retrospect pharmacological effect of Fulvestrant was checked in our models and there was considerable decrease in cell proliferation suggesting that Fulvestrant effects our models but via its off targets not via ER pathway.

The cells undergo apoptosis and perish when c-Myc expression is deregulated. (Gandhi et al.,

2014). Since DNA fragmentation is a fundamental characteristic of apoptosis, it was discovered by the DNA fragmentation assay that the inhibition of cell proliferation of PML/RAR-positive NB4 cells is related to apoptosis (Saadat et al., 2015). Treatment with ATRA was evaluated in the DNA fragmentation assay, and it was discovered that ATRA is not linked to the production of apoptosis as previously stated in (Moosavi & Djavaheri-Mergny, 2019). In accordance with our research, pharmacological targeting with Fulvestrant may have decreased cell proliferation in our models, possibly inducing apoptosis.

To find the off targets of Fulvestrant in our models in silico analysis was performed. Our results have shown good binding affinity between fulvestrant and certain genes that are part of Wnt- β-catenin pathway. Although cdk2 & axin have shown very low binding energies with fulvestrant but as cdk2 is already downregulated in PML/RARα Positive NB4 cells (Meidan et al.,2017), also axin is negative down regulator of Wnt/β-Catenin Pathway (Komiya Y & Habas R., 2008). Thus, both of these genes couldn't be considered as potential targets of fulvestrant. On the other hand, Fulvestrant has a good binding affinity (-6.57) towards β-catenin, which is important in PML/RARα mediated APL and BCR-ABL mediated pathogenesis in CML, according to our molecular docking data. Therefore, we used Fulvestrant to pharmacologically target the NB4 and K562 cell lines, compared its outcomes to those of the currently used medication, imatinib, and looked at how the two treatments combined affected the inhibition of cell growth. According to the investigation's findings, Imatinib, the current standard of care for CML, and Fulvestrant both had a somewhat similar ability to inhibit K562 development. Additionally, studies have demonstrated that the combination therapy (Fulvestrant + Imatinib) had a marginally additive effect on reducing K562 cells' propensity to proliferate.

Similarly, in NB4 cell compared its outcomes to those of the standard of Drug, ATRA, and looked at how the two treatments combined affected the proliferation of the cell line. According to the investigation's findings, Fulvestrant can effectively and roughly as efficiently as the present treatment for APL, ATRA, interfere with the proliferation of NB4. Additionally, studies have demonstrated that the combination therapy (Fulvestrant + ATRA) had a marginally additive effect on reducing NB4 cell's proliferative potential.

The Wnt pathway's transcriptional target, the proto-oncogene c-Myc, is essential for the pathogenesis of leukemia. Nuclear β-catenin levels were higher in the progenitor cells of a

CML patient who was experiencing a blast crisis and was imatinib-resistant, and its activation in these cells increased their capacity for self-renewal (Jamieson et al., 2004). As a result of chromosome translocations, APL, a distinct subtype of AML, encodes an oncogenic fusion protein involving the RAR gene. Expression of PML-RARα hinders differentiation and increases survival in hematopoietic precursor cell lines (L.-Z. He et al., 1997; Puccetti & Ruthardt, 2004; Vitoux, Nasr, & de The, 2007). C-Myc is linked to human cancer because it promotes cell growth while simultaneously regulating apoptosis and differentiation. It is also linked to treatment resistance in AML (Lemaitre, Buckle, and Méchali (1996) and Uribesalgo, Benitah, and Di Croce (2012). According to Cardona-Echeverry and Prada-Arismendy (2020), if Wnt is not suppressed, -catenin may boost the expression of c-Myc, which is regarded to be a factor in treatment resistance. Cell division and other biological processes are promoted by the unspecific Wnt/-catenin target gene c-Myc (Dang, 2012). As a "master regulator," the c-Myc gene controls cellular metabolism and proliferation. Because it is triggered by a large number of oncogenic pathways, which in turn causes a large number of metabolic alterations that can result in malignant transformation. Studies have shown that mitogenic stimulation is necessary for the activation of Wnt-target genes such c-Myc and Axin2 (Coluccia et al., 2007; Miller, Thomas, Islam, Muench, & Sedoris, 2012; Müller-Tidow et al., 2004; K. Wang et al., 2010).According to Dang et al. (2006), C-MYC is a transcription factor that plays a significant role in encouraging the expression of genes whose products encourage cellular proliferation. It is evident from the MTT assay that Fulvestrant prevents the proliferation of NB4 and K562 cells. The PCR results showed that Fulvestrant treatment inhibited Wnt signaling, which in turn decreased the expression of c-Myc and Axin2 genes in PML/RARα positive NB4 APL and BCR-ABL-positive K562 CML cell lines.

CONCLUSION

Endocrine therapy is one of the remedies to a lot of diseases nowadays including the treatment of different types of cancer. Keeping it in mind expression of ER and PR was checked in PML-RARA positive NB4 cells and BCR-ABL positive K562 cells. Both of these receptors were absent in our models, but we checked the pharmacological effect of Fulvestrant on our models. Pharmacological targeting prompted us to look for off targets of Fulvestrant. In silico approaches have shown that Fulvestrant targets β-catenin which is part of Wnt/β-catenin pathway. Expression of its downstream genes was also downregulated which identifies that Fulvestrant have anti-proliferative affect against leukemia alone as well as in combination with standard drugs. Further validation of our result on resistant models is required.

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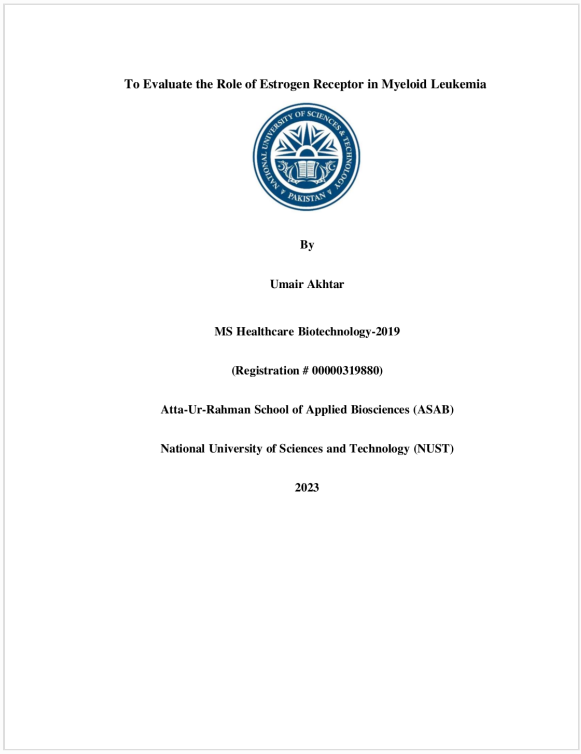

To Evaluate the Role of Estrogen Receptor in Myeloid Leukemia

By

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Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

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