To Evaluate the Anti-Proliferative Effect of Imidazole Derivatives in Acute Promyelocytic Leukemia

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Dedicated to

My Dada Abu for his immense support

And

My Amii for all of her prayers and sacrifices.

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Bazla Binte Nadeem

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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 α
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 commencement of leukemia is associated with continual proliferation of undifferentiated and immature white blood cells as cells do not undergo terminal differentiation due to maturation arrest. Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML) accounting 10-15% of newly diagnosed AML, and predominantly characterized by a balanced translocation; t (15; 17) leads to PML/RARα fusion gene. The PML/RARα fusion onco-protein induce leukemia by blocking differentiation at promyelocytic stage. Currently, APL is treated with All trans retinoic acid (ATRA), Arsenic trioxide (ATO), chemotherapy, and combinations, ATRA and ATO both targets fusion protein PML/RARα, ATRA induces differentiation while ATO induce apoptosis and promotes differentiation. But resistance development, differentiation syndrome and poisoning by ATO are still major issues need to be overcome. In this regard, imidazole derived compounds are of great interest among scientists for development of potential anticancer drugs as they have multiple biological activities including anticancer, antifungal, , antibacterial, anti-inflammatory, antineuropathic, and many more. The present research was designed to test anti-cancerous potential of four novel imidazole derived compounds L7, L4, R-35, and R-NIM04 both *in-silico* and *in-vitro* against PML-RARα positive APL. *In-silico* results of the study indicated RARα protein as potential drug target in APL. Furthermore, *in-vitro* results showed that treatment with L7, L4, R-35 & R-NIM04 at the different concentrations considerably reduced the proliferation of PML-RARα positive APL (p-*values <0.001)*. The expression analysis through Real-time PCR indicated that these imidazole derivatives interferes with the β-catenin dependent leukemiogenesis and downregulates AXL-RTK in APL cells with the expected downregulation of downstream *c-Myc*, related to the Wnt/β-catenin signaling. The past/present researches provide significant evidences of imidazole derivatives as a targeted anti-cancer therapy in future, but to open up new horizons in the field of targeted therapies more *in-vitro* and *in-vivo* research is needed.

1) INTRODUCTION

Acute Promyelocytic Leukemia

Acute promyelocytic leukaemia (APL) is a subtype of acute myeloid leukaemia distinguished as a block of differentiation during the promyelocytic stage. As a result, immature and undifferentiated white blood precursor cells start accumulating in the bone marrow. APL is an acute myeloid leukaemia (AML) (Jimenez, Chale, Abad, & Schally, 2020). APL occurs as 10-15% of all AML. (Tallman & Altman, 2008). The condition is known as AML-M3 in the classification system formulated by the French, Americans, and British (FAB). In 1957, APL was firstly reported by LK Hillestad (a Norwagian haematologist). J.Bernard in 1959, identified 20 APL patients and provide extensive description of APL by describing promyelocytes proliferation. (Francesco Lo-Coco & Cicconi, 2011). The most of APL intances are defined by a reciprocal translocation between chromosomes 17 and 15, t (17; 15) (q24; q21), which leads to the production of a PML-RARA hybrid protein (Kakizuka et al., 1991). The fusion protein PML-RARA give rise to a dominant negative mutation, as it prevents apoptosis and allows leukemic progenitors to proliferate while causing differentiation block.(Rogaia, Grignani, Nicoletti, & Pelicci, 1995). The fusion protein PML-RARα not only disrupts PML's homoeostasis function, but it also affects RARα target gene transcription. RAR α is a transcription factor involved in transcription of genes having pivotal roles in several physiological processes like cellular growth, survival, differentiation and cell death(Di Masi et al., 2015). PML recruits a peculiar molecular apparatus called as nuclear bodies. These PML-NBs performed different cellular functions including tumor suppression and homeostasis, response to viral infections, senescence, differentiation, angiogenesis and genomic stability maintenance(Hadjimichael et al., 2017). The fusion protein PML- RARα causes

disorganization of PML-nuclear bodies and downregulates the expression of RARα target genes resulted in myeloid progenitor's proliferation leading to maturation block at promyelocytic stage (Grignani et al., 1998) (K. Wang et al., 2010).

Depending on risk and WBC count, patients with APL are stratified into low, intermediate, and high-risk patients. The low as well as intermediate risk patients are defined by having 10,000/μL WBCs while patients with WBC count more than 10,000/ μL are under the definition of high-risk APL patients (Sanz et al., 2000). All Trans Retinoic Acid (ATRA) as well as Arsenic trioxide (ATO) have remarkable cure rates and are crucial in APL treatment. ATRA is a differentiation agent and targets PML-RARα complex, causes its degradation and mediates signals for promyelocytes differentiation into mature granulocytes (Breitman, Selonick, & Collins, 1980; de Thé & Chen, 2010). ATO mediates its treatment response by inducing apoptosis and promoting APL cells differentiation by targeting PML-RARα fusion gene. Patients who have recently been diagnosed with APL typically receive only ATRA and ATO, with no chemotherapy (H.-H. Zhu, 2020). Due to recent advancements in therapeutic strategies, the APL subtype of adult AML is now considered the most treatable form of the disease (Thomas, 2019). Despite the accessibility of standard and first line treatment problems like differentiation syndrome, treatment resistance, relapse and early death still exists. Differentiation Syndrome, also referred as the ATRA syndrome, is a potentially fatal treatment complication that typically manifests itself in the early stages of treatment. The differentiation syndrome is characterised by the following symptoms: fever, excess weight, hypotension, pleural effusion, peripheral edoema, and severe renal failure. (Yilmaz, Kantarjian, & Ravandi, 2021). Relapse and Early death are still major obstacles in curing high-risk APL patients(Stahl & Tallman, 2019)

The side effects, drug toxicity, and resistance profiles associated with currently available anticancer therapeutics necessitate the development of new anticancer or antiproleferative drugs. In this regard, scientists have been given considerable attention to synthesis of imadizole derrivatives and evaluation of their anti-cancer potential (Sharma, LaRosa, Antwi, Govindarajan, & Werbovetz, 2021).

Imidazoles are nitrogen containing heterocyclic rings and are present in several bioactive compounds and displays different pharmacological activities including anticancer (Ali, Lone, & Aboul-Enein, 2017), antiviral (Zhan et al., 2009), antibacterial (Rani, Sharma, & Singh, 2013), antiepileptic (Mishra & Ganguly, 2012) and antifungal activities (Rani, Sharma, Kumar Gupta, & Singh, 2013). Several available anti-cancer drugs contain imidazole as their structural component like dacarbazine, fludarabine phosphate, nilotinib, and ponatinib. Investigators have identified several biological targets through which imidazole and imidazole derrivatives exhibit anti-cancer properties. The targets proposed for imidazole include various kinases, microtubules/ tubulin, histone deacetylases, and the other expression regulating proteins (Sharma et al., 2021). Imidazole halts cell division by interacting with DNA through covalent or non-covalent interactions (X. Wang, Li, Gong, & Fu, 2002). Imidazole can easily bind to proteins and synthesis of some essential components of cell membrane can be inhibited by some imidazole drugs at higher concentrations (Ali et al., 2017). These interesting anticancer properties exhibited by imidazole inspired for the development of imidazole derrivatives with the aim of lowering the side effects and increasing efficacy of treatment.

1.1 Hypothesis

The present study was designed to test the anti-cancer potential of four novel imidazole derived compounds L7, L4, R35, and R-NIM04 against acute promyelocytic leukemia in

NB4 cell line. As differentiation syndrome, relapse, drug toxicity and resistance to current therapeutics is the main problem in APL treatment, the development of more effective targeted therapy is of great interest. Imadizoles have shown great potency as anticancer, antifungal, antineuropathic and many more. Owing to the broad spectrum of biological activities performed by imidazole/imidazole fused compounds; the imidazole derived L4, L7, R-35 and R-NIM04 were predicted to reduce the proliferation of APL cells by targeting key player; the fusion gene PML/RARα and downregulates the known downstream signaling pathways involved in APL leukemiogenesis.

1.2 Aims and Objectives

- To find out potential candidate targets of imidazole derrivatives in APL using *Insilco* approaches
- To evaluate anti-proliferative properties of imidazole derrivatives against APL using vet lab experiments and NB4 cell line.
- To analyze the effect of pharmacological targeting on downstream cancer signaling pathways.

2) LITERATURE REVIEW

2.1 Hematopoiesis

Hematopoiesis is a controlled process that leads to the production of mature blood cells and involves a high through put of cells through cell division and differentiation from hematopoietic cells having self-renewal property. Haematopoiesis occurs by differentiating cells into myeloid and lymphoid lineages starting from the main haematopoietic stem cell located at the apex of hierarchy (Kim, Stachura, & Traver, 2014) (Fig. 2.1). Several early and origin specific growth factors with their receptors are involved in this process while different early and origin specific transcription factors are responsible for the differentiation of those hematopoietic stem cells through the expression of origin specific genes. Any disturbance in this setup forms the basis for AML pathogenesis(Steffen, Müller-Tidow, Schwäble, Berdel, & Serve, 2005).

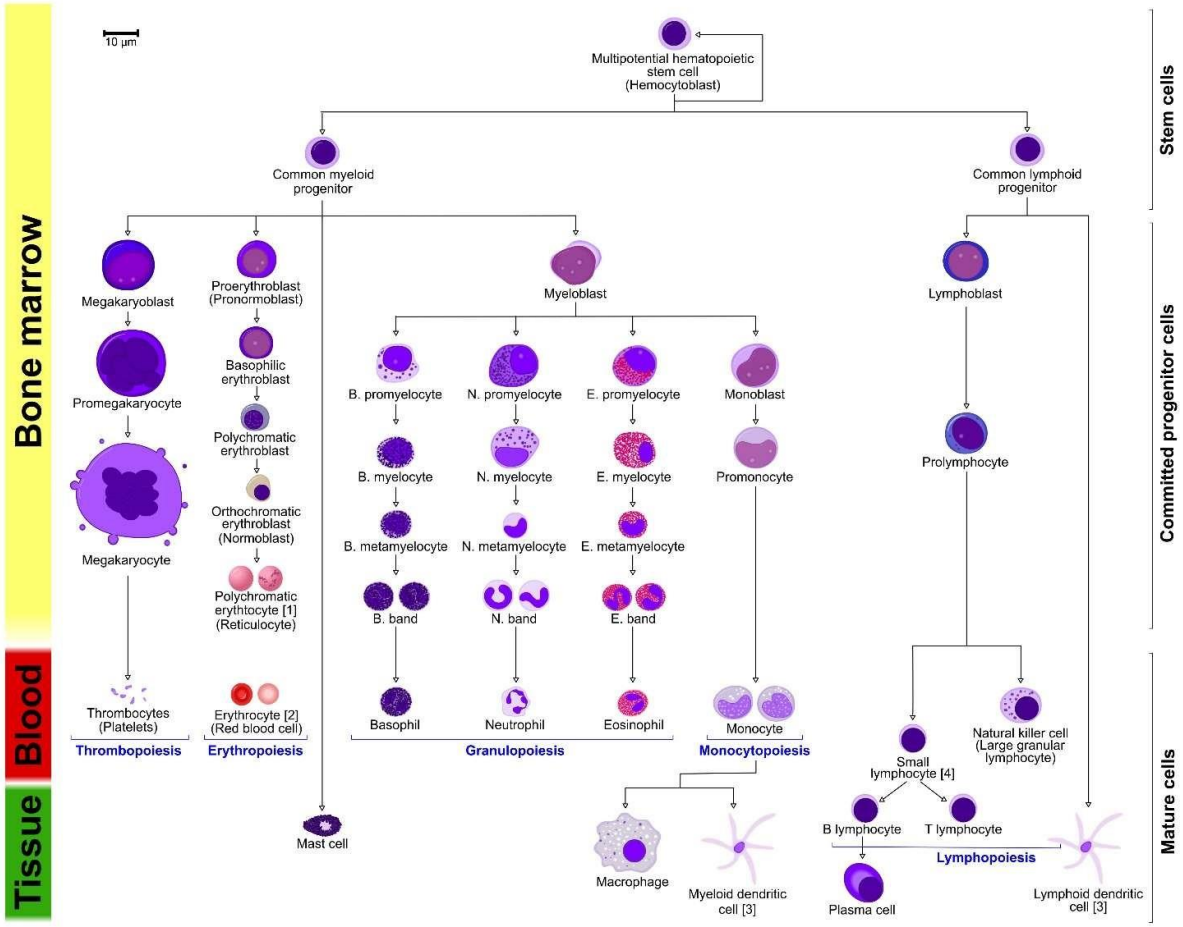

Figure 1 Figure 2. 1: The process of Hematopoiesis (Iskander, 2021)

Haematopoietic stem cell (HSCs) are pluripotent stem cells with self-renewal capability as well as can differentiate asymmetrically to form any type of blood cell. These cells are in responsible of initiating the multistep process of haematopoiesis. HSC differentiates into its immediate descendant, multipotent progenitor cells (MPP) which then differentiate into lineage committed myeloid and lymphoid progenitor cells that extensively proliferate and differentiate (Iwasaki & Akashi, 2007). Multipotent progenitor cells rapidly amplify and retain the ability to differentiate into any haematopoietic cell type whereas, loses the self-renewal capacity. Distinct blood cells forming myeloid lineage include red blood cells, granulocytes, platelets, and macrophages or monocytes while T cells, B cells, natural killer

cells and dendritic cells make up the lymphoid cells. These mature blood cells have finite life span and therefore, their numbers are constantly replenished. Regarding self-renewal ability, it gradually declines as committed blood cells keep on forming (M. Kondo, 2010).

Human diseases including anemia and leukemia occur due to defect in haematopoiesis process (Kumar & Evans, 2015). Abrupt gene expression in hematopoitic stem cells or multipotent progenitor cells leads to chromosomal alterations which bring about a variety of hematopoitic malignancies.

Of all the three major classes of Haematopoiesis cancers/ malignancies, Leukemiogenesis is the process by which leukaemia develops. Leukemias are the cancers that came from hematopoietic stem cells transformation of blood or bone marrow. In bone marrow, the transformed cells of cancerous progeny usually enter into the blood. Therefore, leukemias often arise as "liquid tumors" which are demonstrated by markedly increased lymphoid, myeloid, or rarely cells of erythroid lineage in bone marrow or in blood. Myelomas are tumors that arise in fully differentiated plasma cells and found either as dispersed clones or as solid masses in the bone marrow, tissue or blood. Dissimilar to normal plasma cells, which do not undergo division once they differentiate, the myeloma cells keep on proliferated in an unimpeded manner and continue to synthesize large number of Ig chains. HCs arise when a target cell assist different genetic changes to DNA repair genes, tumor suppressor genes or, oncogenes and have clonal nature. (TSGs)(Hsieh, 2014).

In1980s, researchers observed the chronic and acute myeloid leukemias as an abnormality of HSCs, in which the cells that begin these leukemias are innately stem cell-like as they can self-renew but cannot differentiate properly.

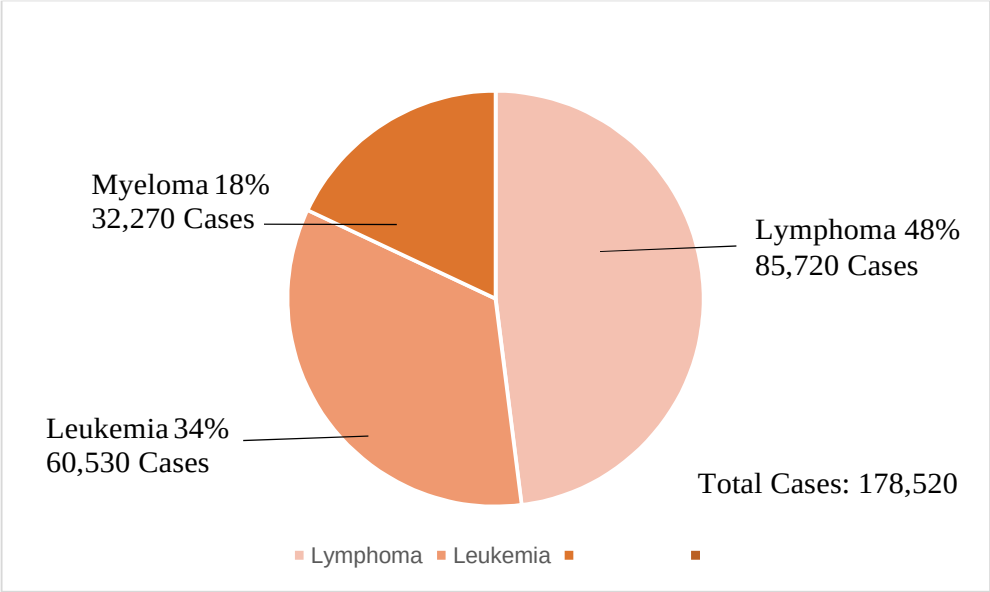

Fig.2.2 Estimated % new cases of leukemia Source: *Cancer Facts & Figures, 2020*. American Cancer Society; 2020.

2.2 Prevalence of Leukemia in Pakistan

Prevalence In Pakistan Owing to its fatality, leukaemia has become a global health concern with an increase in morbidity and mortality overtime. In Pakistan, leukaemia affects both genders with increased rate in males (5.2%). In 2018, leukaemia accounted for 4.1% of all cancer related cases and ranked 5th in cancer derived mortalities in Pakistan (WHO, 2019) while another study revealed AML as the most common leukaemia type in Pakistan followed by CML (Ahmad, Yusuf, & Burney, 2015).

2.3 ACUTE PROMYLEOCYTIC LEUKEMIA

2.3.1 Introduction to APL

In 1957, a Norwegian haematologist, LK Hillestad was the first one to report and describe Acute Promyelocytic Leukemia. Two years later, J.Bernard provides more extensive description of APL by describing proliferation of promeylocytes.(Francesco Lo-Coco & Cicconi, 2011). Acute myeloid leukaemia (AML) has several subtypes, one of which is APL,

which accounts for 10-15% of all AML case (Tallman & Altman, 2008). APL is recognized by maturation arrest and differentiation arrest in promyelocytes. APL is caused by PML-RARα fusion protein expression; a consequence of PML-RARα fusion gene which is a fusion product of reciprocal and balanced translocation between PML gene located on chr;15 and RARA retinoic acid receptor α gene present on chr;17. (Kakizuka et al., 1991). PML protein normally performs many cellular functions including tumor suppression, antiviral functions, homeostasis, differentiation, angiogenesis and also involved in DNA repair. These functions are exerted by PML-NBs(Lallemand-Breitenbach, 2010). The ligand-dependent transcription factor RAR forms transcriptionally active heterodimers when it binds with the retinoic X receptor. The primary function of RAR is to regulate gene expression. Genes having retinoic acid response element (RARE) are involved in different physiological activities of cells including cells growth, survival, cellular differentiation and death. Kinase signaling pathways are also activated by retinoid receptors(Di Masi et al., 2015). PML-RARα fusion protein downregulate the RARα target genes transcription resulting in proliferation of promyelocytes and disturbs PML-NBs(Grignani et al., 1998). ATRA (All-trans retinoic acid) as well as ATO (arsenic trioxide) are crucial for APL treatment having remarkable cure rates. ARTA activity is responsible for promyelocytes maturation into granulocytes (Breitman et al., 1980). Depending on risk APL patients are classified into three groups. Intermediate and low are sometimes grouped together and have WBC count as less than 10,000/ul. Patients who are at high risk have WBC count more than 10,000/ul (Sanz et al., 2000). Patients at intermediate as well as low risk can be treated with ATRA + ATO but in case of high risk patients, cytotoxic chemotherapy is also involved as ATRA + ATO alone are not sufficient(Osman et al., 2018). Relapse is more likely in APL patients who are considered to be at high risk; however, 10–20 percent of APL relapse aside from risk status (Vitaliano-Prunier et al., 2014).

2.3.2 Molecular Pathogenesis:

The characteristic features of PML-RARα fusion product has been proven of vital importance in the molecular studies of APL. PML-RARA fusion product derange the hematopoietic progenitor cell gene expression, myeloid differentiation and stem cell self-renewal. (Vitaliano-Prunier et al., 2014). In a normal situation, RARa will form a heterodimer with the retinoid X receptors, which are nuclear hormone receptor proteins (Chambon, 2005). The heterodimer RARa-RXR binds with retinoic-acid response elements (RARE) to control the expression of various genes (RAREs, which are segments of DNA). These DNA regions regulate differentiation and self-renewal. If the retinoic acid (ligand) is not present, the heterodimer complex will bind with corepressors like nuclear receptor corepressors (NCoR) as well as silencing mediators for thyroid and retinoid hormone receptors (SMRT) (Kishimoto et al., 2006) Histone deacetylases are also involved in the transcriptional repression process. These corepressors are released due to conformational change triggered by retinoic acid and RARa binding, subsequently co-activators are recruited which regulates the chromatin remodelling and gene expression. (Kishimoto et al., 2006). PML-RARa fusion proteins interferes recruitment of coactivators and inhibit retinoic acid response elements transcription (RAREs).

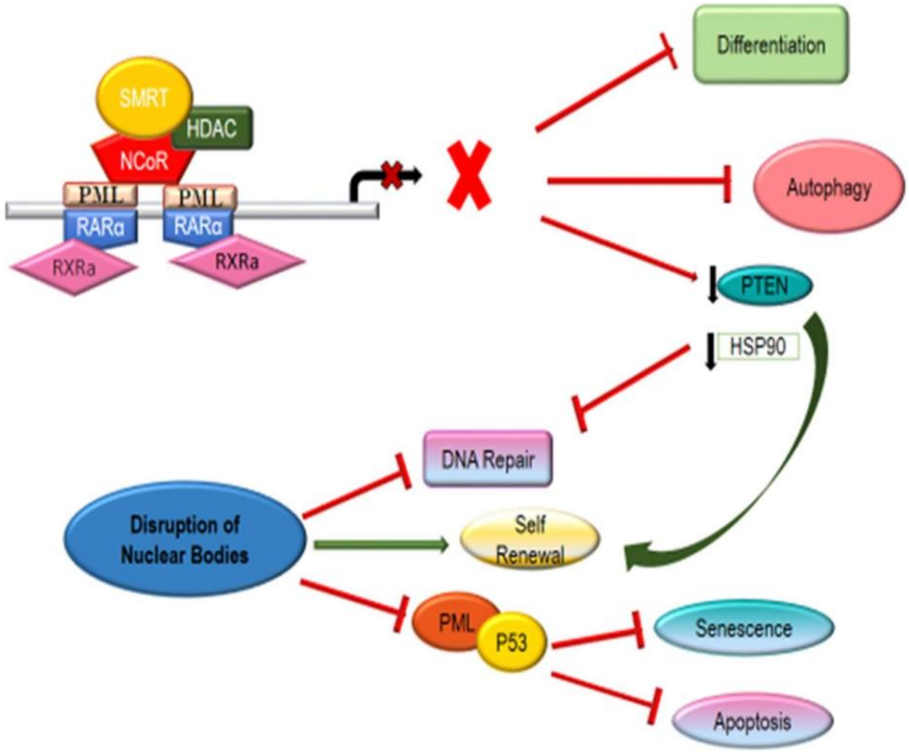

Figure 2.3 Molecular mechanisms involved in APL

The PML-RARA hybrid protein significantly hinders the synthesis of PML nuclear bodies (Vitaliano-Prunier et al., 2014). PML nuclear bodies have the shape of spheres and are hooked to the matrix of nucleus. They are responsible for regulation of a wide variety of nuclear functions, such as epigenetic silencing, translation, replication, and p53 signalling and senescence, probably through the control of proteolysis and sumoylation (de Thé, Pandolfi, & Chen, 2017; Vale′ rie Lallemand & de Thé, 2010).

The key regulator of these domains is PML gene which recruits a number of other proteins such as DAXX which is a key modulator of apoptosis and translation repressor. The PML protein go through a few post-translational adjustments, like phosphorylation or sumoylation which is important for recruitment of functionally associated proteins(Vale′ rie Lallemand & de Thé, 2010). The presence of a PML-RARA-RXR complex is linked to regulate APL

pathogenesis (J. Zhu et al., 2007). The RXRA sumoylation is a critical step in the transformation that occurs in APL cells.

2.3.3 Translocations in APL

New cytogenetic translocations other than t(15;17) have been identified in one to two percent of APL cases on average.

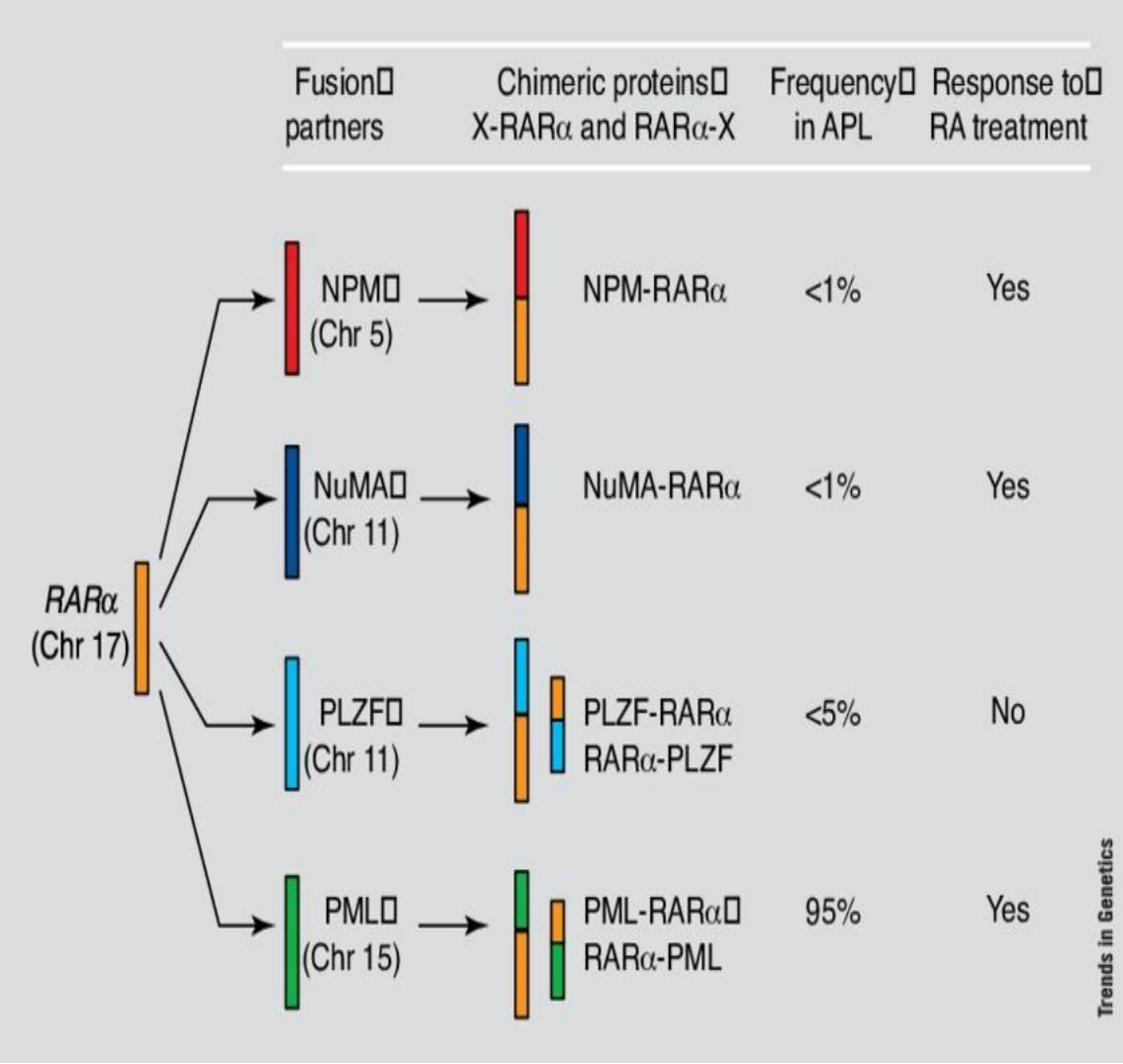

Figure 2.4 APL translocations involving RARα (Lin, Egan, & Evans, 1999)

Uptill now, 12 fusion variations of APL are reported, all associated with RARA gene (Baba, Pandith, Shah, & Baba, 2019). The most frequent APL variant ZBTB16 (previously PLZF)-

RARA translocation, acquire from the rearrangement t(11;17)(q23;q21) (Z. Chen et al., 1993), has been identified in more than 30 patients. Several other identified translocations leads to the fusion of RARα with following genes;

Table 2.1. Additional genes involved in APL

Sr.No	Gene	Reference
1.	NPMI	(Corey et al., 1994)
2.	NUMAI	(Wells, Catzavelos, & Kamel-Reid, 1997)
3.	STAT-5B	(Arnould et al., 1999)
4.	PRKAR-1A	(Catalano et al., 2007)
5.	FIP1L1	(T. Kondo et al., 2008)
6.	BCO R	(Yamamoto et al., 2010)
s7.	NABP1 (once OBFC-2A)	(Won et al., 2013)
8.	TBL1XR1 (once TBLR1)	(Y. Chen et al., 2014)
9.	GTF2I	(Li et al., 2015)
10.	IRF2BP2	(Yin et al., 2015)
11.	FNDC3B	(Cheng et al., 2017)

APL can be identified by absence of various transmembrane glycoproteins such as CD7, CD11a, CD11b, CD14, and CD18 expression, infrequent HLA-DR expression and downregulation of CD34 while APL cells show upregulation of CD13 and CD117 and atypical expression of CD2 (T-cell linked antigen) involved in high leukocyte count and microgranular variant morphology. CD56 expression is linked with standard ATRA and chemotherapy resistance. (Noguera et al., 2019).

2.3.4 Functions of PML:

The gene PML, which is present on chromosome 15q24 and contains nine exons, produces a variety of spliced transcripts. The C-terminal exon portions of the PML gene's isoforms differ due to splicing. The N-terminus section of the PML gene, which is encoded by exons 1-3 of the PML gene and consists of a RING B Box Coiled_coil/tripartite motif domain is the same for all isoforms of PML gene. These exons are in-charge of encoding the PML gene. These isotypes can be found in the cell's cytoplasm as well as its nucleus (Jensen, Shiels, & Freemont, 2001). PML's two primary functions are genomic instability and tumour suppression, and these activities are carried out through interactions, either temporary or constitutive, with over 170 proteins (Lallemand-Breitenbach, 2010). PML isoform-specific domains primarily mediate these connections, which govern several processes such as stem cell self-renewal, transcription, and HSC epigenetic alterations (G. Wang, Tian, Hu, Xiao, & Chen, 2019) as well as p53 apoptosis (either p53 dependent or p53-independent) (Matt & Hofmann, 2018). The following are some of the most important PML functions:

- PML protein basically act as a tumour suppressor which is required for formation and organization of macromolecular nuclear-complexes known as promyelocytic leukaemia nuclear bodies. These nuclear bodies are to blame for the disease's emergence. It is found in the nuclear bodies of promyelocytic leukaemia in healthy individuals (PML_NBs).

- PML_NBs are distinct nuclear bodies that are located in the nuclei of most mammalian cells and range in size from 0.2 to 1.0 micrometres. These nuclear foci may be detected in most mammalian cells. The number of bodies that they contain per nucleus ranges from one to thirty on average, but this number can change based on the type of cell, the cell cycle phase and the stage of differentiation.

- PML_NBs are charismatic structures that go through considerable change in the amount, dimensions, and placement during the course of the cell cycle and as cellular stress response factors such as senescence induction and DNA damage (Bernardi & Pandolfi, 2007).

- PML-NBs have been found in close proximity to other organelles of nucleus like Cajal bodies, and they have been linked to genomic regions involved in transcriptional processes. Furthermore, it has been demonstrated that a specific relationship exists with specific chromosomal locations.

- A large number of proteins can be discovered in PML-NBs, both temporarily and permanently. Consequently, PML_NBs are associated with the control of various cellular processes. These processes involve apoptosis and senescence induction, proliferation suppression, genomic stability maintenance, and antiviral responses.

- According to recent evidence, PML-NBs are composed of a variety of different structures, and different PML-NBs can govern different processes depending on the protein composition of their nuclei, their location in the nucleus, and their mobility. This is due to the fact that different PML-NBs are located in different parts of the nucleus and have varying mobility (Bernardi & Pandolfi, 2007).

2.3.5 Functions of RARA:

The RARA gene, which can be found on chromosome 17q21, contains ten exons and encodes RARA1 and RARA2. Because they use different promoters and exons, these isotypes have distinct Activation Function 1 domains (AF-1) at their N-termini (Zelent, Guidez, Melnick, Waxman, & Licht, 2001). The RARA protein belongs to the superfamily of nuclear receptors and shares up to 90% of its sequence with the RARB and RARG proteins. These are both nuclear receptor families. When the ligand is present, the retinoid X receptor (RXR) and the

retinoic acid-receptor alpha (RARA) form a heterodimer and then attached with retinoic-acid responsive elements (RARE) at the promoter of target genes. These RAREs have tandem sites 5'-AGGTCA-3' called as DR1-DR5, which regulate the transcription of a variety of transcripts (Collins, 2002).

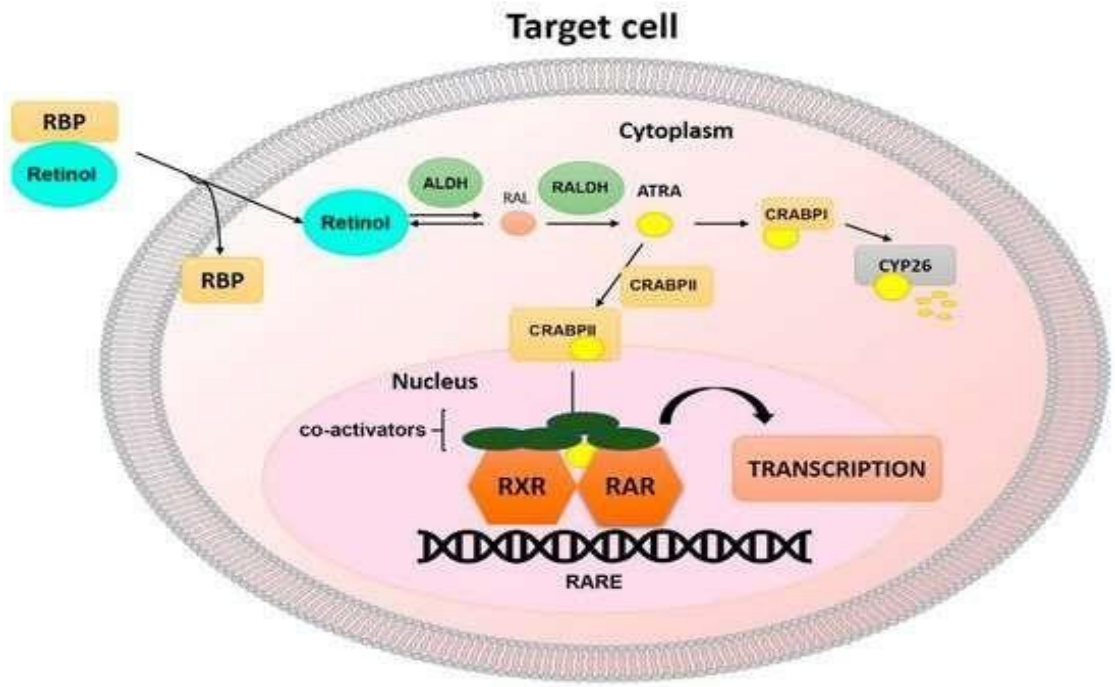

Figure 2.5 Mechanism of Retinoic Acid Receptor signaling (Conserva, Anelli, Zagaria, Specchia, & Albano, 2019)

When no ligand is present, RARA and RXR interact with a multi-protein complex that includes corepressors. This interaction causes chromatin condensation, histone deacetylation, and transcriptional inhibition through nuclear receptor corepressors (N-CoR) and thyroid and retinoic hormone receptor silencing mediators (SMRT).RARA causes myeloid haematopoietic cell differentiation and functions as a nuclear transcription factor. RARA, along with HDAC3, HDAC5, and HDAC7, is responsible for the regulation of microRNA-10a suppression, which enhances an inflammatory response (Liquori et al., 2020).

2.3.6 The fusion Gene; PML-RARA:

Because the gene PML contains breakpoints in three exonic regions known as bcr, several mRNA transcripts of varying lengths are produced. A break in the PML gene's intron 6 (bcr1) causes the formation of the long (L-) isoform mRNA, which then leads to the production of PML6-RARA3 mRNA, which is responsible for approximately 70% of APL cases. A DNA break in the PML intron-3 (bcr-3) yields in the production of the short (S-) isoform mRNA. This break then results in the production of PML3-RARA3 mRNA, which is responsible for approximately 20% of APL cases. A breakpoint within exon 6 (bcr2) causes the variable (V-) isoform mRNA to form, which is then joined to RARA exon 3. This type of APL accounts for 10% of all incidents. On the other hand, there have been descriptions of how the occurrence of the three isoforms can vary depending on the region or the ethnicity (Liquori et al., 2020).

Additional chromosomal abnormalities, such as a 7q deletion and a trisomy at chromosome 8, are found in roughly half of paediatric and adult APL patients. The acquisition of an additional chromosome, which occurs in trisomy 8, causes dysregulation of the MYC gene (Delgado, Albajar, Gomez-Casares, Batlle, & León, 2013) in APL that can upregulate the PML-RARA fused gene to accelerate the development of myeloid leukemic cells.(Ronchini et al., 2017). With the exception of three or more extra chromosomal changes, research has shown that the presence of extra chromosomal alterations does not affect the prognosis of APL patients (Labrador et al., 2019)

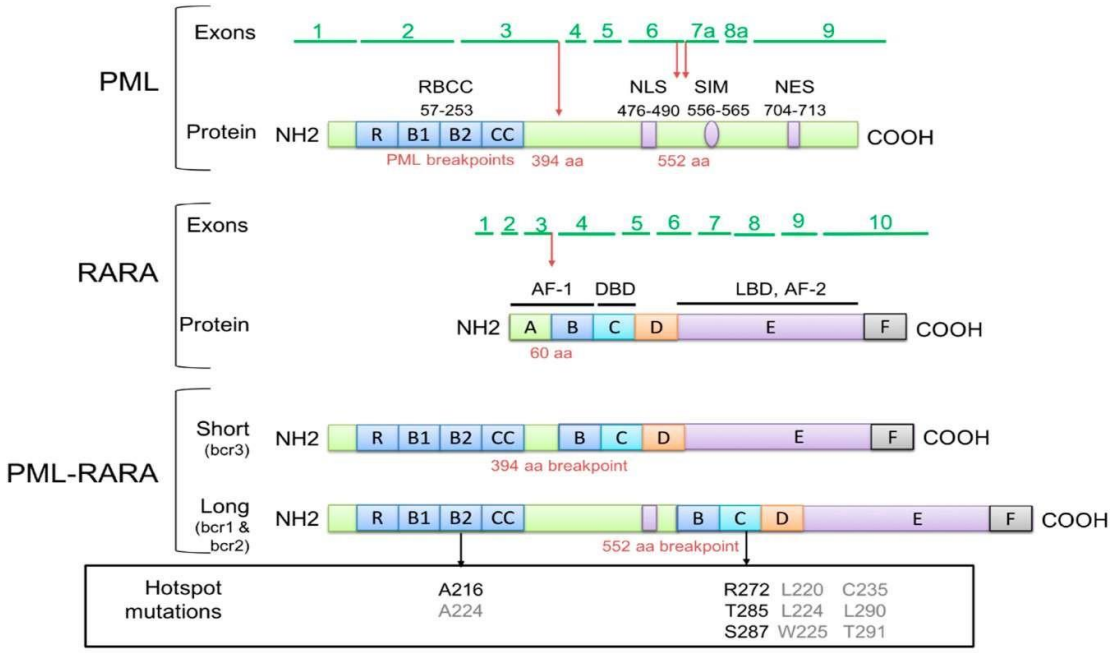

Fig.2.6 Structure of the APL primary event

(Breakpoint sites (shown as red), the hotspot-mutations in proteins PML and RARA, as well as their related fusion protein PML-RARA (in the box below; black are shown mostly mutated regions, and rarer changes are represented in grey). The RING-finger_(R), the B box (B1 and B2), the coiled coil domain_ (CC), the nuclear-localization signal (NLS), the SUMO Interacting Motif (SIM), the nuclear export-signal are all found in PML (NES). RARA N terminus domain (A and B) contains activation function-domain _1 (AF_1) as well as the DNA binding domain_(C), the hormone binding domain_(E), other regulatory domains_(D , F) (Liquori et al., 2020)

2.3.7 Leukemiogenesis of PML/RARα:

Because the PML-RARα fusion in APL alters the structure of PML NBs, microspeckles form as a result of the disordering or disarray caused by this. (Lallemand-Breitenbach, 2010). Because the PML portion of the fusion gene PML-RARα lacks an SUMO- binding motif, this phenomenon occurs. Because PML-RARα has a proclivity to multimerize with a wide range of protein classes, this fusion gene is capable of producing oncogenic effects via both gain of function and dominant-negative effects (Pandolfi, 2001). On the one hand, PML-RAR causes an interruption in myeloid differentiation during the promyelocyte stage. This happens because

it stops the transcription of genes that are necessary for myeloid development, such as genes that are crucial for the granulocytic differentiation. On other side, PML-RARα encourages the cell proliferation and survival of leukemic cells, as a result gradual build-up of promyelocytes inside bone marrow of APL patients (Kamashev, Vitoux, & De Thé, 2004).

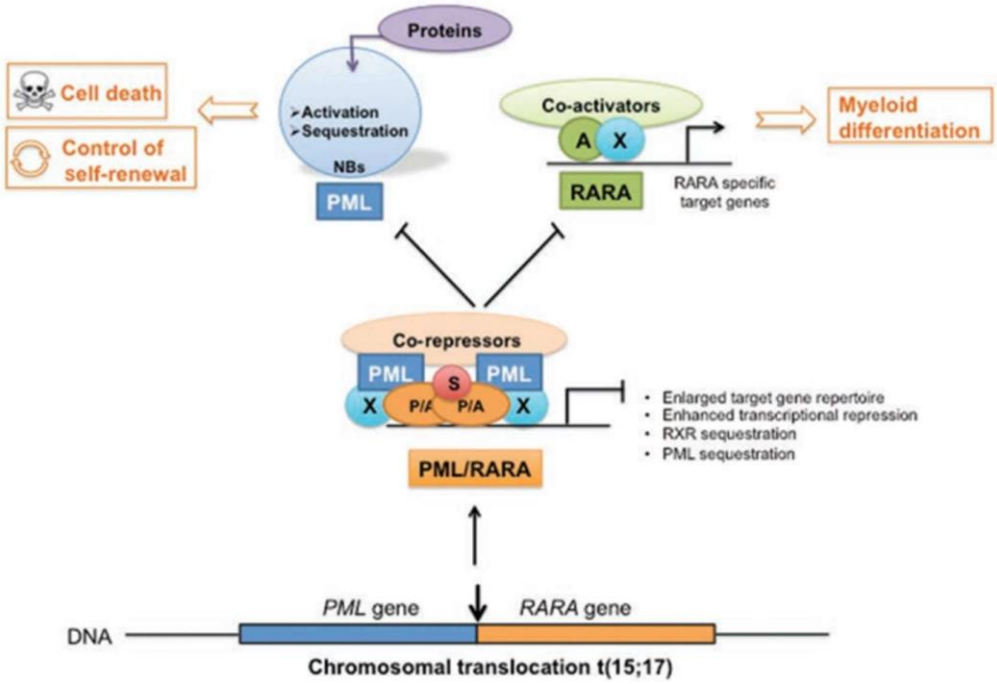

Fig.2.7 The Leukemiogenesis of fusion product PMLRARA

In addition to acting as repressor of transcription, the PML-RARA fusion can cause disruptions in PML nuclear bodies. In addition to binding RXR (X) and PML, PML/RARA (P/A) is sumoylated (S). Repression of targeted genes is achieved by PML/RARA via hiring co-repressors. This prevents RARA (A) targets from taking effect, which is relevant to myeloid differentiation. This also prevents the construction of PML NBs, which are the domains which attract a large number of partner proteins to increase the posttranslational modifications of those proteins, which in turn allows those proteins to be activated or sequestered. Defects in the control of apoptosis or in the capacity of stem cells to self-renew have been linked to defective nuclear bodies.(J. Zhu, Nasr, Ablain, & Lallemand-Breittenbach, 2015).

Recent research paints a complicated picture of the process by which APL pathogenesis occurs. PML-RAR tetramers can form binding interactions with a wide variety of DNA locations that are not normally recognised by the RAR-RXR receptor. The PML-RAR protein can recognise

a non-canonical DNA location that may be involved in widespread transcriptional dysregulation (Martens et al., 2010). Furthermore, the PML-RAR fusion product contains significant beneficial domains that are involved in leukemiogenesis and can be affected by retinoic corrosive and arsenic trioxide (de Thé, Chomienne, Lanotte, Degos, & Dejean, 1990). The RAR gene includes the RXR binding-domain, the hormone-binding domain and DNA binding-domain whereas the PML gene includes both coiled-coil and RING finger sites (Poddighe & Weghuis, 2016).

2.3.8 Treatment Strategies

It was formerly thought that acute promyelocytic leukaemia was the most deadly type of acute leukaemia; however, recent advancements in treatment have rendered this subtype of acute myeloid leukaemia relatively treatable in adults (Thomas, 2019). Patients with APL, whether at low or high risk of the disease, can now benefit from the chemo-free treatment concept. The combination of all Trans Retinoic Acid (ATRA), and arsenic trioxide (ATO) is considered currently to be the first-line and standard-treatment for newly diagnosed cases of APL. This treatment does not involve the use of chemotherapy. Since the turn of the century, scientists in the medical field have been focusing their attention on developing treatments for newly diagnosed cases of APL that do not need the use of chemotherapy drugs (H.-H. Zhu, 2020).

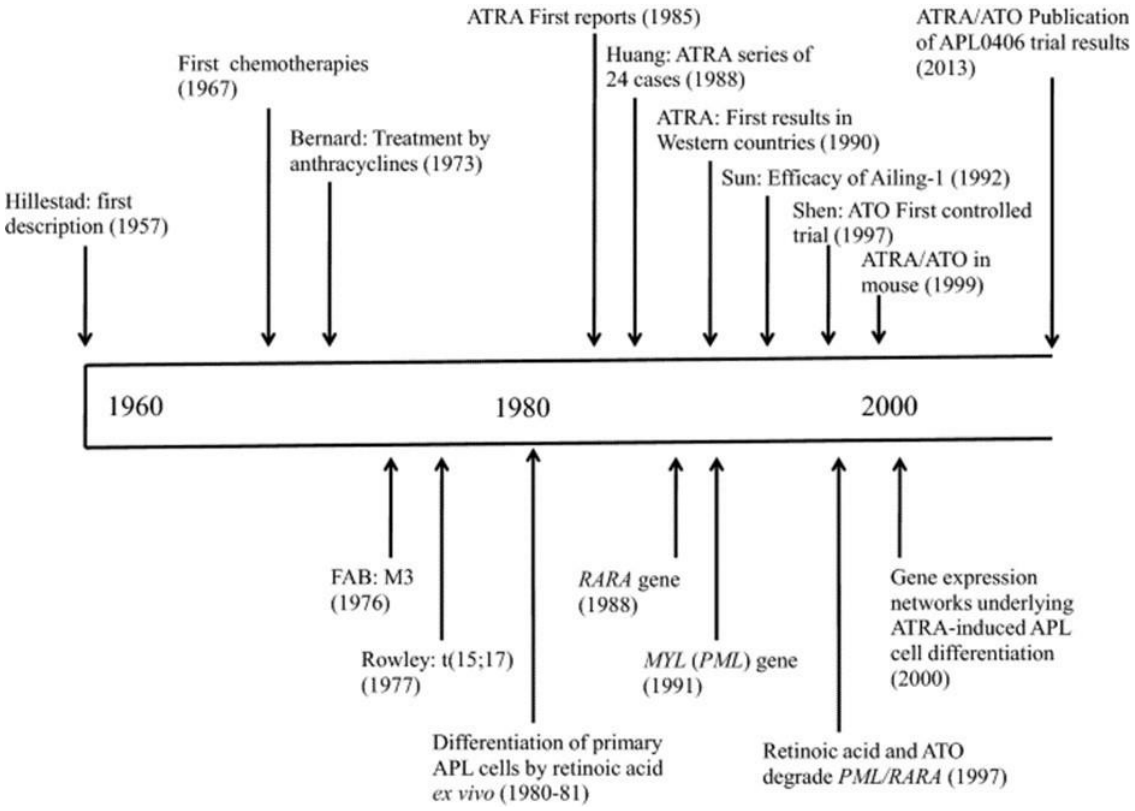

Fig.2.8 Events in the APL Treatment History (Thomas, 2019)

2.3.8.1 All -Trans Retinoic Acid Receptor (ATRA)

The FDA approved ATRA as the first treatment for APL. It works by targeting the PML-RARa complex and causing its degradation through a proteasome-ubiquitin or caspase-dependent pathway. This activates the RAR/Retinoid X receptor, which is the receptor that mediates signals for granulocyte differentiation (de Thé & Chen, 2010). Because of the changes in PML-conformation, RARa's transcriptional co-activators are recruited to the mutant, resulting in the activation of differentiation genes and, as a result, promyelocyte maturation (Liu et al., 2000).

A Chinese journal reported in 1987 that giving ATRA alone to four newly diagnosed and two chemotherapy-refractory APL patients resulted in complete remission for all of them (Huang et al., 1987). A year later, the Shanghai Institute of Haematology reported remarkable success

in 24 APL patients. While 23 of these patients achieved CR with ARTA alone, one patient required low-dose cytosine arabinoside in addition to ATRA (Z.-Y. Wang & Chen, 2008).

2.3.8.2 Treatment with ATRA alone

After initial report of Shanghai group (Huang et al., 1987) , many other institutions of world including the Hospital Saint-Louis in Paris (Degos et al., 1990) and Memorial Sloan Kettering Cancer Centre in the New York (Warrell Jr et al., 1991) carried the confirmatory studies regarding efficacy of ATRA alone. These studies describe the high rates of CR in APL patients as well as rapid enhancement in coagulopathy. Another remarkable attribute was that in BM promyelocytes completed the process differentiation. Despite of continuous ATRA treatment, relapse within few months was observed in the patients who achieved CR.

2.3.8.3 ATRA+ Chemotherapy

In the early 1990's, several non-randomized studies (Fenaux et al., 1992; Kanamaru et al., 1995; G. Sun et al., 1994) and a randomized research (Fenaux et al., 1993) were conducted in which significant better results were observed in relapse and complete remission in APL patients given with the ATRA and then chemo-therapy was done rather than those treated alone with ATRA or chemotherapy. Later, European APL group conducted a randomized study (Fenaux et al., 1999) shown the superiority of ATRA use combining with chemotherapy simultaneously to the chemotherapy after ATRA. This was later confirmed during the trials.(Mandelli et al., 1997; Sanz et al., 2004; Sanz et al., 1999). Based on these results, the European LeukemiaNet established the standard care for newly diagnosed patients of APL with the induction of ATRA plus Chemotherapy after which 2-3 chemotherapy courses for the consolidation therapy.(Sanz et al., 2009)

2.3.8.4 Arsenic Trioxide (1974-2002)

Initial studies reveals that ATO mediates its response by disappearing PML-RARa fusion protein. ATO induced complete remission rate is high (87%) in treatment of APL. Action mechanism of ATO involves either promoting APL cells differentiation at low levels or apoptosis induction at high levels. ATO- induced APL cells differentiation involves PML-RARa degradation, which allows promyelocytes to vanquish maturation block. ATO also induces apoptosis via different mechanisms which are apparently independent of fusion protein and involves cytochrome C release from mitochondria into cytosol, thus activating caspases activation. (Miller Jr, 2002)

In the early 1970's, the ATO as potential anti-cancer drug was identified in China by a group of Harbin Medical University. In 1992 it was reported that out of 32, 21 patients of APL treated with Ailing-1 (an anticancer solution with 1% ATO) achieved CR after 10 years with survival rate of 30%.(H. Sun, Ma, Hu, & Zhang, 1992). These findings were afterwards proven to be true by Shanghai group(G.-Q. Chen et al., 1997). In a multicentre trial conducted in USA, ATO was administered in relapsed patients after treatment with ATRA + Chemotherapy(Soignet et al., 2001). 85% patients achieved CR with ATO alone. These studies established the ATO as effective therapy for relapsed APL patients as approved by FDA in 2000. Between 1974 and 1985, one ATO injection was administered in 32 recently diagnosed APL patients and long-_term follow-up findings were studied. (Ohno, Asou, & Ohnishi, 2003). The results showed 50% 5-year overall survival rate, 50% complete remission and 19% partial remission. The result was later confirmed in a study in which 124 patients from same group were treated with pure ATO. (P. Zhang et al., 2000). Lu et al. first reported excellent 100% CR rate and 76.6% disease-free survival rate of 3 years in 19 patients under study by using oral As4S4 (tetra-arsenic tetra sulphide)(Lu et al., 2002). The quality of patient's life was affected by total arsenic

course which was more than 3 years in both studies. This led to the development of ATO+ATRA chemo-free model.

2.3.8.5 ATO + ATRA (2002- now)

Estey et al. was first who investigated ATO +ATRA chemo-free model during induction and post-remission treatment(Estey et al., 2006). 82 patients were included in this study and their post-induction treatment involved four courses of ATO+ATRA. The results showed 92% complete remission, 85% 3-year overall survival, and 9% early death rate. Confirmation of this result was later done on the same group by a long-term follow up which provided basics for the APL0406 study.

APL0406, a randomized trial was conducted by Lo-Coco for low-risk APL patients (newly diagnosed) using ATRA+idarubicin vs. ATRA+ATO.(F Lo-Coco et al., 2013; Platzbecker et al., 2017). The ATRA+ATO group demonstrate 100% CR rate and 99% overall survival of 2 years with a follow-up of 34.4 median. Another trial NCRI AML17 that was irrespective of patient's risk status showed 94% CR and 92% 5-year OS.(Burnett et al., 2015).

The result of this research supported ATRA plus ATO being standard treatment for APL patients either at high or low-risk. APL is characterized by epigenetic and repression of RAR target genes transcription. *TGM2* and *RARβ* both are involved in differentiation and are heavily methylated during leukemogenesis. Huynh et al. recently suggested that ATRA+ATO combinational treatment leads to expression of TGM2 and RARβ and then terminal differentiation of NB4 promyelocytes.(Huynh et al., 2019)

2.3.8.6 Oral ATO+ATRA

A group from Hong Kong, administered oral ATO first time and a series of clinical trials were done by them.(Foa et al., 2011; Gill et al., 2019; Gill et al., 2018). Grill et al. studied follow up of 15 years in 73 patients of relapsed APL and reported 79.5% 5yr OS and 67.3% 10yr OS

rate.(Gill et al., 2018). ORH-2014 is an oral arsenic has completed phase 1 indicating that 15mg is safe. (Ravandi et al., 2020). In future phase 2 and 3 trials10mg dose is recommended. ALLG phase 1 study is also evaluating an oral ATO developed in Australia.

2.3.9 Problems in existing Treatments

2.3.9.1 Early Death Rate

Death at any time during induction or within 30 days after diagnosis is termed as early death.(F Lo-Coco et al., 2013). Early death rate is major hurdle in curing APL patients. In the ATRA+chemotherapy era have improved early death rates over time, as according to US SEER database.(Dinmohamed et al., 2016). It is uncertain whether the ATRA+ATO further reduce ED rate. Zhu et al. reported 5.5% ED rate in ATRA+ATO group study where n= 758.(Murthy et al., 2020). Future investigation is required whether ED rates between ATRA+ATO and ATRA+ Chemotherapy model are different.

2.3.9.2 ATRA+ATO Toxicity

Headache, GI toxicity, and liver damage are common. The fatal adverse effect with ATRA+ATO is leucocytosis (WBS count over 10 × 10’9 /L). Hyperleukocytosis can be a predictive marker for ED and subsequent relapse, differentiation syndrome in APL patients.(Yoon et al., 2019).

2.3.9.3 Resistance

Resistance to treatment is major hurdle in treating APL patients. ATRA resistance is caused by active mutations in the ligand-binding domain of the retinoic acid receptor.(Roussel & Lanotte, 2001). Because of these mutations, PML-RARA has lost its sensitivity to retinoic acid, but it is still able to bind to RARE and suppress transcription. This was discovered through in vitro research conducted using a resistant NB4 cell line.(Mozziconacci et al., 2002) PML and RARA degradation has also been reported in the process of mediating RA resistance. (Fanelli et al.,

1999). In vitro and in vivo, ATO resistance has also been seen and associated with clustered mutations in the PML moiety's B2 domain., which is a direct arsenic binding site (Roussel & Lanotte, 2001)

Table 2.2 Treatment strategies for APL and their shortcomings

s/n	Treatment	Shortcomings	Reference
1.	ATRA alone	Relapse within 5 months despite continuous ATRA treatment	(Sanz & Barragán, 2021)
2.	ATRA + Chemotherapy	Differentiation Syndrome	(Yilmaz et al., 2021)
3.	ATO alone	Relapse, ATO related toxicities	(Ghavamzadeh et al., 2011)
4.	ATRA+ ATO	Early Death rate, Overloaded fluid even in the absence of Differentiation Syndrome	(Yilmaz, Naqvi, & Ravandi, 2019)
5.	Tamibarotene	Disease free survival is still short	(Sanford et al., 2015)

2.4 Imidazole and their derivatives

Because of their unique characteristics, such as increasing polarity & their active ability to form hydrogen bonds and consistency and stability, nitrogen-containing heterocyclic imidazole structures can interfere with various biological molecules. A wide range of biological activities

have been reported for imidazole and fused imidazole-containing compounds (Sharma et al., 2021).

The imidazole is a five membered aromatic ring i.e. heterocycle that occurs in both naturally and synthetic molecules. Because of the distinctive structural characteristic of the imidazole ring, which has the advantageous feature to be rich in electrons, Imidazole compounds have the ability to rapidly engage with a wide number of enzymes or receptors in living organisms by engaging in a wide variety of non covalent bonding. This allows imidazole derivatives to have a wide range of bioactivities. Multiple imidazole-based compounds, in particular, have been extensively utilised as clinical medications to cure a wide range of disorders with high therapeutic effectiveness, demonstrating the compounds' immense development potential (Ling Zhang, Peng, Damu, Geng, & Zhou, 2014). Many clinical medications based on imidazole have been playing an important part in curing various diseases, and novel imidazole variants with therapeutic potential are now a days the subject of development and intensive research all around the world. An amphoteric hetero-cycle with high polarity is characterised by its imidazole ring, which is an aromatic ring of five carbons having two atoms of nitrogen (Molina, Tárraga, & Otón, 2012).

2.4.1 Characteristics of Imadizole compounds and their use in Medicine

Because of the unique structural properties of the imidazole ring, its derivatives are able to quickly attach with a wide range of receptors and enzymes in living organisms. This binding can take place through hydrogen bonds, ion–dipole, coordination, van der Waals forces, cation– , π– stacking, hydrophobic effects, and so on; as a result, these derivatives display a wide range of biological properties. In point of fact, the imidazole ring is widely distributed throughout naturally occurring products as well as a wide variety of bioactive chemicals produced by human metabolism (Aleksandrova, Kravchenko, & Kochergin, 2011). The fact

that nature chooses to include this specific type of imidazole ring in a large number of biomolecules, like histamine, haemoglobin, DNA, and vitamin B12 to carry out a wide range of biological activities suggests that imidazole ring is essential to the physiological function of significant bio-processes. Imidazole-based medicinal chemistry has recently received a lot of attention due to the unique physiological properties and unusually important roles that imidazole molecules play in key processes (Boiani & González, 2005). Because its two nitrogen atoms can easily form hydrogen bonds, When it comes to aromatic compounds, the inclusion of an imidazole ring may be favourable for improving water solubility to a certain level. The imidazole ring, as a potential attractive binding site, may binds with a wide range of an-ion and cat-ion ions, and biological compounds found in living bodies. The imidazole ring has thus been frequently assimilate into fluorescent skeletons in order to generate synthetic compounds (fluorescent) for use as diagnostic tools that monitor the metabolic process of biologically significant molecules and ions in biological systems as pathologic probes in order to comprehend biological phenomena. All of the examples above demonstrate the enormous potential of imidazole containing compounds in medicine, and an increasing number of researchers are focusing their efforts on developing imidazole-based compounds that are both practical and prolific in a variety of fields.

Notably, various imidazole containing complexes as therapeutic drugs like anticancer (dacarbazine, azathioprine, Tipifarnib, and zoledronicacid), antifungal (miconazole, ketoconazole, oxiconazole, and clotrimazole), antiparasitic (secnidazole, ornidazole, benznidazole, and metronidazole), antihistaminic (imetit, thioperamide, immepip, and cimetidine), antineuropathic (dexmedetomidine, fipamezole, and nafimidone), and also antihypertensive (olmesartan ,losartan, and eprosartan) drugs with high therapeutic efficacy have been extensively utilized to treat various diseases, demonstrating the wide development value of these medications. (Ling Zhang et al., 2014). This has had the effect of substantially

supporting a significant amount of attempts to concentrate on imidazole containing clinical drugs, expanding research and advances are developing and progressively busy subject that has almost expanding over the range in the field of medicine.

2.4.2 Imadizole being anticancer compounds

Cancer is among most serious threats to health of humans today, and as a result, it has received an unusual amount of focus from people all over the world. The discovery of efficient anticancer therapies has been the focus of a significant amount of research, which has included the coordinated application of various surgical procedures, radiation treatment, and chemotherapy. Over the course of the past sixty years, a great number of advancements and important breakthroughs have been produced. Despite the large number of anticancer medications, including natural compounds, that are currently being tested in clinical settings (e.g., camptothecins, taxols) (Cragg, Grothaus, & Newman, 2009) and synthetic substances like alkylating agents (e.g., chlorambucil, mechlorethaminoxide), (Kapuriya et al., 2011; Tang, Zhang, Zhang, Geng, & Zhou, 2012) porphyrin drugs (e.g., visudyn, photofrin,), (Yu, Zhou, & Li, 2007) azole agents (e.g., fadrozole, letrozole), (Chang et al., 2011) and inorganic metal complexes (e.g., cisplatin, carboplatin), (Pattabiraman & Bode, 2011) the majority of the medical need is not being adequately satisfied as a direct result of the numerous limitations of existing treatments, which include inadequate curative effects, limited selectivity, high cytotoxicity, & multiple resistances. As a result, considerable effort has been expended in the development of new medicines that are not cross resistant and are more specific to tumors. These therapuetics should be able to reduce cancer cell migration in a targeted manner while also making tumour cells more sensitive to lethal drugs. A significant amount of research indicates that imidazole derivatives have a significant amount of potential as prospective anticancer medicines. In general, imidazoles have the potential to obstruct DNA synthesis by using weak contacts such as- stacking, hydrogen bonds, and coordination bonds, resulting in

the cessation of cell development and division. Imidazoles, on the other hand, attaches with proteins much more readily than the other heterocycles. Furthermore, at high doses, several imidazole medications can directly reduce the formation of critical components of cell membrane without interfering with sterol esters and sterols (Shalini, Sharma, & Kumar, 2010). Till now , various imidazole comounds are being used as anticancer agents like (1) dacarbazine, (2) zoledronic acid, (3) azathioprine, (4) tipifarnib, and (5) nilotinib have had a great deal of use in the medical context (Fig. 1). They have been proved to play crucial roles in the control and cure of a variety of malignancies.

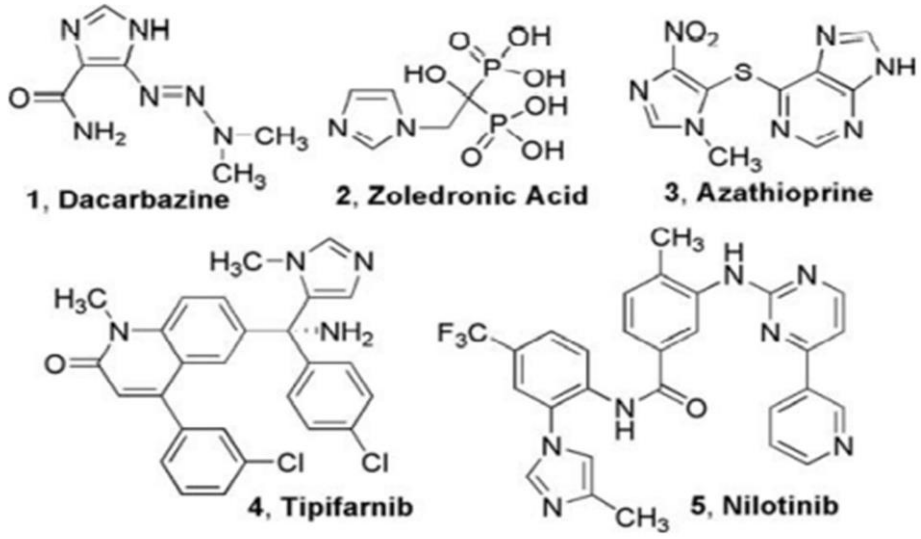

Figure 2.9 Some imidazole containing anti-cancer drugs.

3) MATERIALS AND METHODS

3.1 In silico study

3.1.1 Target Genes Selection

The databases including National Center for Biotechnology Information (NCBI), Cancer Cell Line Encyclopaedia (CCLE), GeneCards and literature review were used sfor retrieving gene expression profile for NB4, K562, U937 and FKH1 cell lines.

3.1.2 Therapeutic Target Library

All the selected genes were subjected to Uniprot and FASTA sequences were retrieved which were further subjected to Position-Specific Iterative Basic Local Alignment Tool (PSI-BLAST) to obtain sequence similarity in protein structure. The protein structures with 98% or above percent identity were selected.

3.1.3 Protein 3D Crystallographic Structure

Accession numbers of target proteins were obtain from PSI-BLAST and further subjected to Protein Data Bank (PDB) for acquisition of 3D structure of protein. The protein 3D crystallographic structures were downloaded in PDB format.

3.1.4 Purification of Protein Structure

PDB protein structures were purified by using UCSF Chimera 1.14. Extra or identical chains of proteins were deleted, water molecules and all other non-standard amino acids were removed to get purified structure of protein.

3.1.5 Binding Pockets Identification

The potential active drug binding pockets in target protein structures were obtained by the use of DoGSiteScorer, an online automated pocket detection and analysis tool which gives

prediction of the binding sites based on hydrophobic interactions. Binding pockets with highest binding (near to 1) score were selected.

3.1.6 Preparation of Ligand

PubChem database was used for retrieving 2D structure of ligand in SDF format which was then converted to mol2 format by using a software Open Babel.

3.1.7 Molecular Docking

Autodock 4.2 (a molecular modelling simulation software) was used for performing molecular docking to predict the favoured binding of ligand with its protein receptor. Ligand and macromolecule was prepared by addition of hydrogen atoms, computing charges, and removal of water molecules. Previously calculated binding pocket scores were used to set the Autogrid. For docking of ligand with protein, Lamarckian Genetic Algorithm were selected with default parameters.

3.1.8 Analysis and Visualization of Binding Pose

Cygwin commands were used for converting docking output file into PDB file format, and further visualized by using Discovery Studio Visualizer v4. All the binding poses with calculated binding energies were assessed and for further analysis Protein-Ligand complexes having lowest binding energy were selected.

3.1.9 Protein-Ligand Interaction Profiler

PLIP (Protein Ligand Interaction Profiler) was used for visualization and comprehensive detection of interactions among ligand-protein complexes having lowest free binding energies for each target to characterize the interaction details on atomic level.

3.1.10 Ligands used in study

Table: 3.1 Imidazole derived inhibitors used in study

Sample code	***Molecular formula***	***Molecular weight***	***Solvent***
L-4	$C_9H_8N_2S$	176.24g/mol	Ethanol
L-7	$C_7H_6N_4S_2O$	226.00g/mol	Ethanol
R-35	$C_{27}H_{18}Br_2N_2$	530.25g/mol	DMSO
R-NIM04	$C_{32}H_{24}N_2O$	452.55g/mol	DMSO

3.2 In *Vitro* Study

NB4 Cell Line

Used as a model for PML-RARα positive Acute Promyelocytic Leukemia

3.2.1 Culturing of Cell Line

Cells were growm in a 6-well culture plate using RPMI 1640 media (Gibco Life Technologies) provided with 10% Fetal Bovine Serum (Gibco Life Technologies) and 1% of Penstrip (Biowest) to avoid any contamination with microorganisms. The culture plates were placed in water-jacketed humidified-incubator at 5% CO2 and 37℃ for optimum growth of cells. To prevent any contact inhibition 1×10^6 /ml cells were maintained in culture.

3.2.2 Freezing

For cryopreservation of cells freezing solution I (70% RPMI 1640, 30% FBS) and freezing solution II (80% RPMI 1640, 20% DMSO) were prepared.

Cells were taken in a centrifuge tube, centrifugation was done at 1400 rpm for 5 minutes. Pellet was washed with PBS. Supernatant was removed and pellet obtained was re-suspended in 2ml freezing solution I. After pipetting up & down, 1ml of resuspension was added into each cryovial. Then 1ml freezing solution II was added dropwise in each cryovial. The cryovials were then placed at -80℃ in Mr. Frosty that contained Isopropanol which lowers the temperature 1℃ per minute. Next days, cryovials were shifted in cryobox and stored at -80℃.

3.2.4 Thawing

Cryovials were taken out from -80℃ liquid nitrogen freezer and placed at 37℃ in incubator for rapid thawing. The cryovial was then properly sprayed with ethanol and moved inside biosafety cabinet hood. The cell suspension of cryovial was then transferred into a falcon and 4-5ml pre-warmed fresh 10% media was added. Centrifuged the falcon to get cell pellet followed by PBS washing to remove DMSO content. Pellet was then resuspended in 10% culture media. Cells were placed in a six-well culture plate then placed at 37℃, 5%CO2 in incubator.

3.2.4 Trypan-Blue Exclusion Assay

For counting the number of viable cells, the trypan blue exclusion assay was performed each time before plating MTT assay, in order to plate/ seed the constant number of cells per well to get accurate results. Cells from all wells of 6-well culture plate were taken out in a centrifuge tube, centrifuged it to obtain pellet, resuspended in 1ml 10% culture media. Resuspended cells were vortexed and 10ul of them was dropped on a paraffin strip, which was then diluted with 10ul of trypan blue dye. Homogenized it with pipetting up and down. Finally 10ul of dilution was loaded on haemacytometer. Non-viable cells appear blue as they uptake the dye due to compromised plasma membrane while the viable cells exclude the dye, thus appear white. All

the viable cells were carefully counted in four grids of chamber and following formula was used to get the exact cell count per ml:

$$\frac{\textit{Number of cells in grid1 + grid2 + grid3 + grid4} \times \textit{Dilution Factor} \times 10^4 \textit{ cells/ml}}{4}$$

3.2.5 Drug Dilutions

The drug compound L4 and L7 were dissolved in ethanol solvent while R-35 and R-NIM04 were dissolved in DMSO to obtain 10mM initial drug stock. From stock solution different concentrations of drug were prepared in 10% FBS media. The dilution factor remained the same at each subsequent step; hence, the increase in concentration followed a logarithmic pattern that was geometric in nature. The 10mM stock solution was serially diluted according to following method:

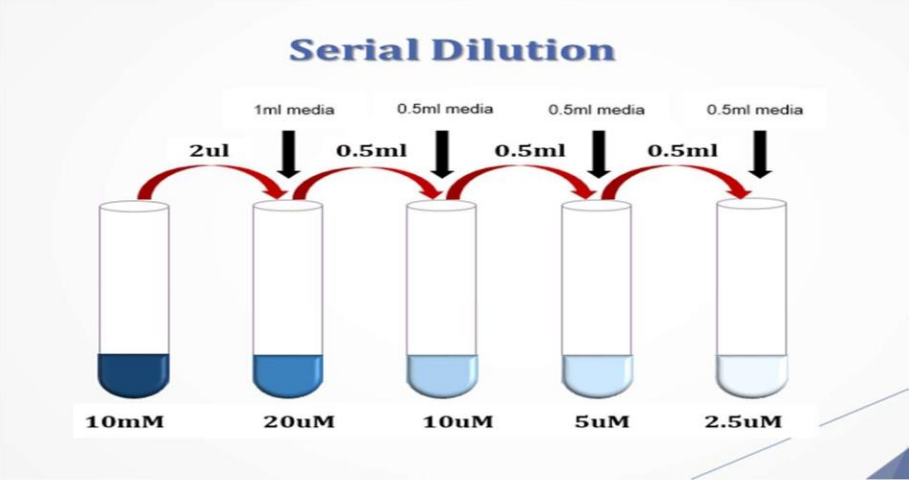

Fig 3.1: Serial Dilution of 10mM drug in 10% FBS media for NB4 cells

3.2.6 MTT Assay

1×10^4 cells / 50ul were plated in a 96 well plate. 50ul of different concentration (uM) of inhibitors L4, L7, R-35 and R-NIM04 (10, 5, 2.5, 1.25 and 0.625) were added in triplicate wells. In the –ve control triplicate, 50ul of cells were treated with 50ul of 0.01% ethanol. 100ul

culture media (used for making drug dilutions) was also plated in a triplicate. This was done to minus the effect of drug solvent and media if any. The 96 well plate was then placed at 37℃ and 5% of CO2 for 72 hours (doubling time of cell line, NB4) allowing the inhibitors to produce their effect. After completion of doubling time, 15ul of MTT dye (5mg/ml of phosphate buffer saline) was then added into all wells of 96 well plate containing cells and drug concentrations. This was done to assess the cells viability after drug treatment at different concentrations. After MTT dye addition, 96-well plate was moved back again into incubator for 3-4 hours allowing crystal formation. This is a colorimetric assay and based on cells metabolic activity, the viable cells convert the tetrazolium dye into purple-colored formazan insoluble crystals. After 3 hours, plate was taken out from incubator and 100ul media from wells was removed without disturbing the crystals. Then 100ul of DMSO was added into wells to dissolve formazan crystals. When crystals were fully dissolved, the absorbance at 550nm was measured using a spectrophotometric micro-plate reader.

3.2.7 Mechanistic Studies

3.2.8 Treatment of cells with L4, L7, R-35 and R-NIM04

In a 6-well plate, NB4 cells were cultured at a number of 1 million cells/ml. and treated with 1.25uM concentration of inhibitor and 0.01% ethanol for 72 hours. After treatment, RNA extraction from treated cells was done followed by cDNA synthesis for gene expression studies.

3.2.9 RNA Extraction

About 1-2 million treated cells / 2ml were taken in 2ml ependroff and for 5 minutes centrifugation was done at 1500rpm. After discarding of supernatant, 500ul chilled PBS was added and then centrifuged at 1500rpm for another 5 minutes. Supernatant was removed and the obtained pellet was suspended in 1ml TRIZOL (life technologies). Homogenization of cells

by up and down pipetting followed by ice incubation, for 5 minutes. Then 200ul chloroform was added with 15 seconds of vigorous shaking, followed by ice incubation for next 10 minutes. Then tube was again centrifuged at 12000^{x}g and 4℃ for 20 minutes. Mixture was separated into three layers, upper aqueous layer of RNA, interphase contains DNA and appeared as thin white layer and lower organic pink layer of proteins. Upper aqueous layer of RNA was carefully picked (approximately 350ul) and transferred into another tube, 500ul chilled isopropanol was added to it and shaking was done followed by ice incubation for 15 minutes. Centrifuged the tube at 12000^{x}g and 4℃ for 20 minutes. After discarding supernatant, obtained pellet was washed with 75% chilled ethanol, this time centrifuged at 7500^{x}g and 4℃ for next five minutes. Ethanol was removed carefully; pellet was air-dried inside hood at room temperature for 20 minutes. 20ul NF water was then added into tube to resuspend the pellet and placed at -80℃ until further cDNA synthesis.

3.2.10 RNA Quality and Quantity Check

The quantity of RNA was measured using Nanodrop 2000 (Thermoscientific, USA), quality was checked by 260/280 ratio for its purity confirmation.

3.2.11 Complementary DNA (cDNA) Synthesis

cDNA was synthesized by using 1000ng of RNA as template for reverse transcription. 1uL oligo dT20 (10uM) was taken in a 0.2mL micro tube. 1ul DTT(100mM), 1ul dNTPs (2.5mM), 2ul 10x Reaction Buffer, and 0.5ul RNase Inhibitor 40U/ul were added in it. 2ul template RNA was added followed by NF water addition to make 20ul total volume. Micro tubes were then placed in thermocycler and reaction profile was set according to kit protocol (wizbio solutions).

3.2.12 Confirmation of cDNA Synthesis

20ul reaction mixture was prepared for PCR containing 2ul of 1000ng cDNA, 2ul of 25mM $Mgcl_2$, 2.5ul 10X reaction buffer, 1.5ul 10mM dNTPs, 0.5ul Taq DNA polymerase, 1ul of

forward primer, 1ul of reverse primer (GAPDH) and 9.5ul NF water in a microtube. PCR profile was set as, in stage 1, 5 minutes of initial denaturation was done at 95℃ followed by stage 2 of 35 PCR cycle, each consisting of denaturation for 1 minute at 95℃, annealing for 45 seconds at 60℃, and extension of cDNA strand for 45 seconds at 72℃. Final extension was given for 10 minutes at 72℃ in stage 3. PCR product was then placed at -20℃ till further processing.

3.2.13 Gel Electrophoresis

PCR product was analyzed by running on 2% agarose gel for confirmation of cDNA synthesis. 1ml of 50X TAE buffer was added in 49ml distilled water to get 50ml 1x TAE buffer and 1g of agarose was dissolved in it. Microwaved the flask for 30-40 seconds until solution become clear, rested it to cool for a minute and 4ul ethidium bromide was added in it. Gel was then poured in casting tray and allowed it to solidify. After it, combs were removed and wells were loaded with 4ul PCR product mixed with 2ul loading dye. 1kb DNA ladder (Thermo Scientific) was used as marker. Gel was run for 40 minutes at 90 volts and 500 amperes.

3.2.14 Real-Time PCR for Gene Expression Analysis

Primers for the gene of interest were analyzed by specific amplification of product at 10mM by Real-time PCR (Applied Biosystems 7300). Whole process was performed on ice. Each PCR strip was prepared for each primer. The reaction mixture was prepared by adding 0.5µl of cDNA, 0.5µl of each forward & reverse primers (10mM) and 2µl of SYBR-Green Master Mix (5X) (Solis BioDyne). Reaction volume was made up to 10µl by adding 6.5µl NF water. The reaction was completed in three major steps. Following a two-minute incubation at 50 degrees Celsius, the reaction mixture was subjected to a ten-minute incubation at 95 degrees Celsius, followed by 40 cycles of PCR. There are three rounds of incubation in each round of PCR: 1) 1 minute at 95 degrees Celsius 2) 45 seconds at 60° Celsius, and 3) 30 seconds at 72°

Fahrenheit. A dissociation stage was added to the melt curve to help with further analysis. Three different incubations were performed during the dissociation stage: first initial stage at 95 °C for 30 seconds, second stage at 60 °C for 30 seconds, and third stage at 95 °C for 30 seconds. The data was collected at a temperature of 72 degrees. The data analysis was carried out using the ABI 7300 system SDS software. B-Actin primer was used as a housekeeping gene for normalisation purposes. The $2^{-\Delta\Delta C}$ method was used to perform relative gene expression measurements.

Gene/Primer	**Sequence**	**GC%**	**Annealing Temperature**
□-actin-FW	CATGTACGTTGCTATCCAGGC	52.38%	59.13°C
□-actin-RV	CTCCTTAATGTCACGCACGAT	47.62%	58.46°C
AXL-FW	GTCGGACCACTGAAGCTACC	60.0%	60.1°C
AXL-RV	CATCGTCTTCACAGCCACCT	55.0%	60°C
EYA3-FW	GGGCAAGAGGAAAGCTGA	55.6%	59.9°C
EYA3-RV	GCCACTGAAACCATCTGTTG	50%	60.4°C
c-myc-FW	CAGCGACTCTGAGGAGGAAC	60.0%	59.8°C
c-myc-RV	TCGGTTGTTGCTGATCTGTC	50.0%	58.2°C

3.2.15 DNA Fragmentation Assay for Apoptosis Analysis

In order to determine if the compound being investigated causes apoptosis or an arrest in the cell cycle, 1 million cells/ml were first plated, then treated with 2.5uM of the medication, and then incubated at 37 degrees Celsius for three days. After that, the genomic DNA of the cells that had been treated was extracted. In order to obtain pellet, treated cells were taken in a 2 ml Eppendorf tube and then centrifugation was done at a speed of 1500 rpm for 5 minutes. After that, 200 ul of PBS was then added, for 5 minutes the mixture was centrifuged at a speed of 2000 rpm. After removing the supernatant and adding 1 millilitre of solution A, the mixture was vortexed and inverted 4-6 times before incubation for ten min. at the room temperature. After that, the tube was centrifuged for one minute at a speed of 13,000 revolutions per minute.

After discarding the supernatant, an additional 500 ul of Sol A was added to the mixture. Then performed one minute of centrifugation at 13000 rpm. After removing the supernatant, 500 ul of Sol B was added, then 20 ul of 20 percent SDS, 5 ul of Proteinase K (Thermo ScientificTM), and the mixture was given a vortex. It was incubated at 37 degrees Celsius for a full 24 hours. After incubation, 500 ul of phenol (Sol C) and 500 ul of chloroform isoamyl alcohol (Sol D) were added to it, and then it was centrifuged at a speed of 13000 rpm for ten minutes. After carefully transferring the aqueous phase into a fresh tube, 500 ul of Sol D was added to it. Centrifugation of the mixture was done at a speed of 13000 rpm for ten minutes. Once more, the aqueous phase was transferred into a second tube, and then sodium acetate in a volume of 50–55 ul and isopropanol in a volume of 500 ul were added to it. Inverted, then mixed several times while being vortexed. Centrifuged at 13000rpm for 10 minute. After getting rid of the supernatant, we were left with the pellet. And the pellet obtained was air-dried in the air for one hour after being rinsed with ethanol at a concentration of 70%. After that, 10-20 ul of TE 1X Buffer was then added, the DNA was then placed at -20 degrees Celsius.

Solution (A)	**Solution (B)**	**Solution (C)**	**Solution (D)**
0.32M sucrose Tris (10mM)of pH 7.5 $Mgcl_2$ (5mM) Triton (X-100) 1% (v/v)	Tris (10mM) of pH 7.5 $NaCl_2$ (400mM) EDTA (2mM) (pH 8.0)	phenol	Chloroform 24 volume + Isoamyl alcohol 1 volume

3.2.15.1 DNA Quantification

The extracted genomic DNA was quantified using Nanodrop 2000 (Thermoscientific USA)

3.2.15.2 Apoptosis Analysis

For DNA fragmentation analysis, extracted DNA samples were mixed with loading dye in 2:1 and run on 1.5% agarose gel. 1kb DNA ladder (Thermo Scientific) was utilized as a marker. The gel was run for 45 minutes at 90 volts and 5000 amperes and visualized through UV illuminator.

3.2.16 Statistical Analysis

All the experiments (MTT assays) were carried out in triplicates, All Real-Time PCR reactions were performed in duplicates and results are presented as ± SEM. The data was analysed on the basis of statistical tests i.e. one-way ANOVA, and student's t-test. The P-value < 0.05 was taken as significant one. The software GraphPad Prism 5.01 was used to generate graphical data.

4) RESULTS

4.1 In silico Analysis

4.1.1 Ligands Structure

The structure of L4, L7, R-35 & R-NIM04 obtained from PubChem were as follow:

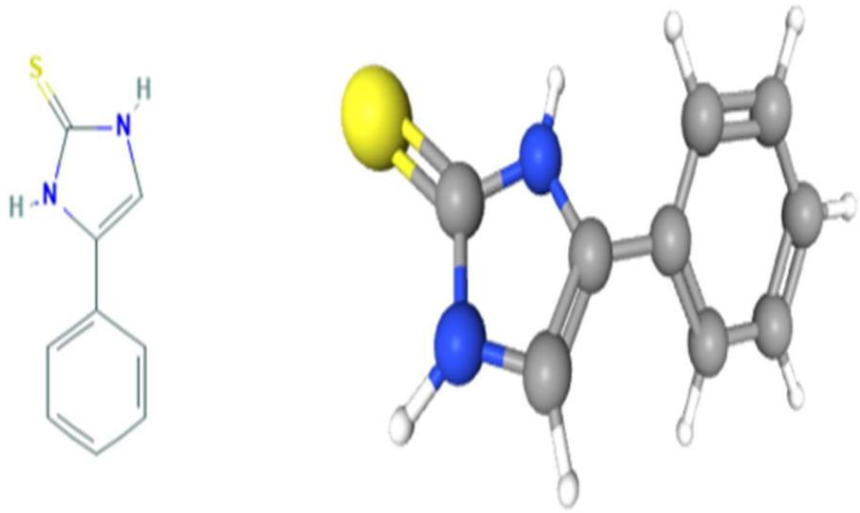

Figure 4.1: Structure (2D &3D) of Ligand L4

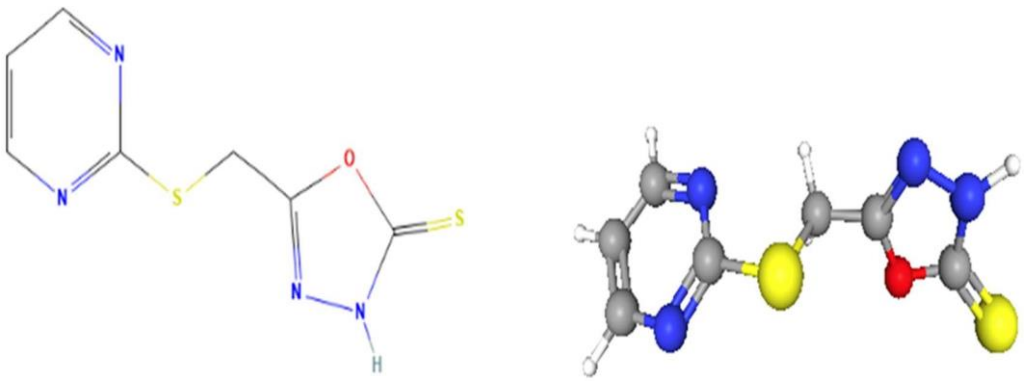

Figure 4.2: Structure (2D &3D) of Ligand L7

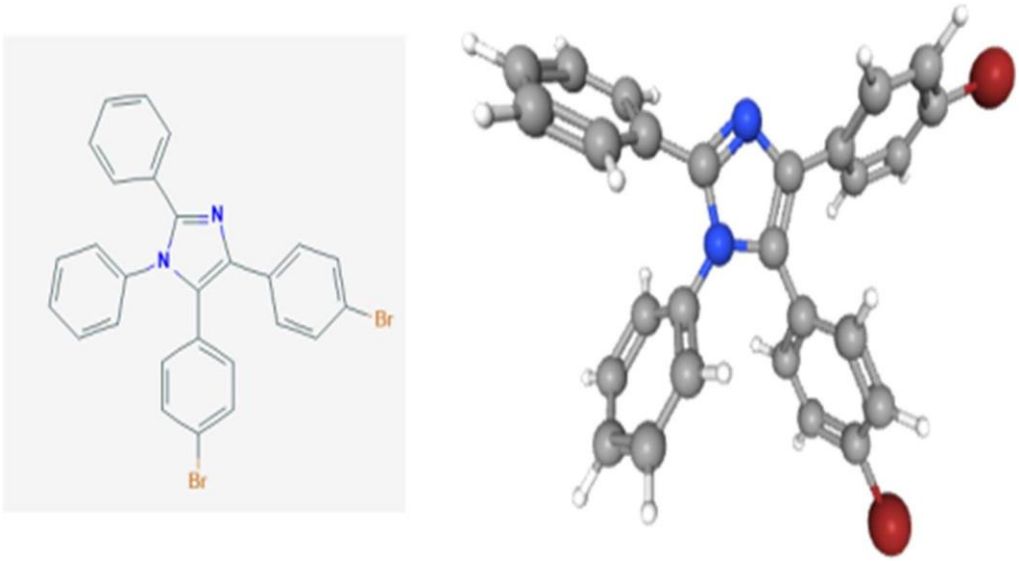

Figure 4.3: Structure (2D &3D) of Ligand R-35

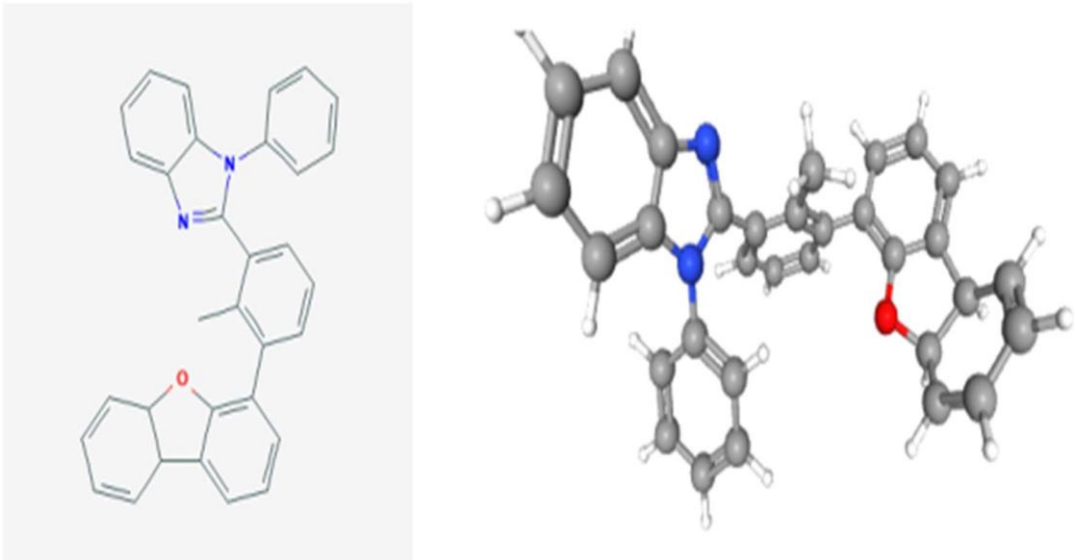

Figure 4.4: Structure (2D &3D) of Ligand R-NIM04

4.1.2 Target Proteins

For the selection of target proteins for docking as mentioned earlier, NCBI data bank was used and protein structures were obtained from PDB.

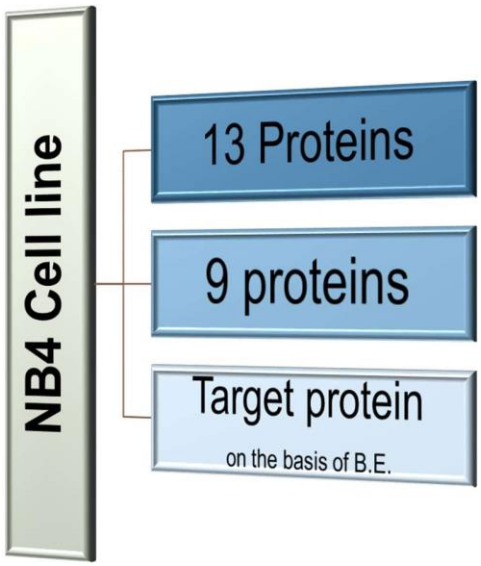

Figure 4.5: Hierarchical Model of Proteins

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**.**

4.1.3 Molecular Docking Analysis

The major goal of molecular docking is computer-based drug-design; it predominantly predicts the best possible interaction between the ligand (that can be a drug in future) with targeted proteins. The automated molecular docking predicts molecular recognition, both structurally and energy based. It is thought that through docking the structure of ligand can be predicted

easily within the boundary of a receptor binding site along with the correct estimation of binding strength (Waszkowycz, Clark, & Gancia, 2011).

The ligands L7, L4, R-35 and R-NIM04 were docked with the targeted proteins of NB4 cell line. The possible drug targets after molecular docking were selected on the basis of low binding energies (Table 4.1).

Table 4.1: The Binding Energies of all possible Proteins targets

Ligands	**Shortlisted Proteins of NB4 (With lowest binding energy)**			**Binding Energies (kcal/mol)**
	Gene ID	**Description**	**PDB**	
L7	**RARA**	Retinoic acid receptor alpha	3kmr	-6.55
	PML	PML nuclear body scaffold	1bor	-5.1
	TGM2	Transglutaminase 2	4pyg	-5.74
L4	**RARA**	Retinoic acid receptor alpha	3kmr	-6.19
	PML	PML nuclear body scaffold	1bor	-5.81
	TGFB1	Transforming growth factor beta 1	6p7j	-6.19
	TGM2	Transglutaminase 2	4pyg	-6.35

R-35	**RARA**	Retinoic acid receptor alpha	3kmr	-11.85
	PML	PML nuclear body scaffold	1bor	-8.15
	TGFB1	Transforming Growth Factor Beta 1	6p7j	-12.76
	TGM2	Trans-glutaminase 2	4pyg	-10.56
	VAV	vav guanine-nucleotide exchange factor	6nf1	-8.41
R-NIM04	**RARA**	Retinoic acid receptor alpha	3kmr	-12.18
	PML	PML nuclear body scaffold	1bor	-7.06
	TGFB1	Transforming growth factor beta 1	6p7j	-12.78

4.1.4 Protein-Ligand Interaction Profile

PLIP, the protein ligand identification profiler basically identifies the non-covalent interactions between macromolecules and ligands like ionic interactions, hydrogen bonds & van der Waals by quantum mechanics (QM) based approach (Raha et al., 2007). Sometime to fasten up the process, instead of QM calculations simple potential energy functions are used.

4.1.4.1 Protein- ligand complex of RARα-L7

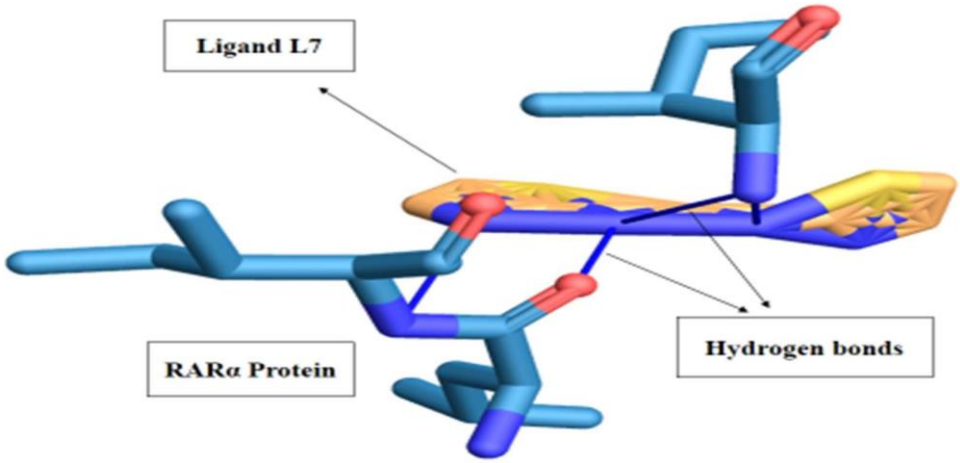

Figure 4.6: Protein ligand interaction profile image indicating the binding pose of L7 with RARα protein in NB4.

Table 4.2: Hydrogen Bonding between L7 and RARα complex.

Index	Residue	Amino acid	Distance (H-A)	Distance (D-A)	Donor-Angle	Donor Protein	Side chains	Donor atom	Acceptor atom
1	269A	LEU	2.12	2.73	167.75	No	No	3704 [Npl]	688 [O2]
2	270A	ILE	3.37	3.76	104.52	Yes	No	693 [N3]	3703 [Nar]
3	273A	ILE	3.32	3.89	118.85	Yes	No	720 [Nam]	3704 [Npl]
4	273A	ILE	2.56	3.56	168.61	Yes	No	2571 [N3]	3705 [N2

Existence of four hydrogen bonds between L7 and RARα complex indicate good binding of ligand with protein.

4.1.4.2 Protein- ligand complex of RARα-L4

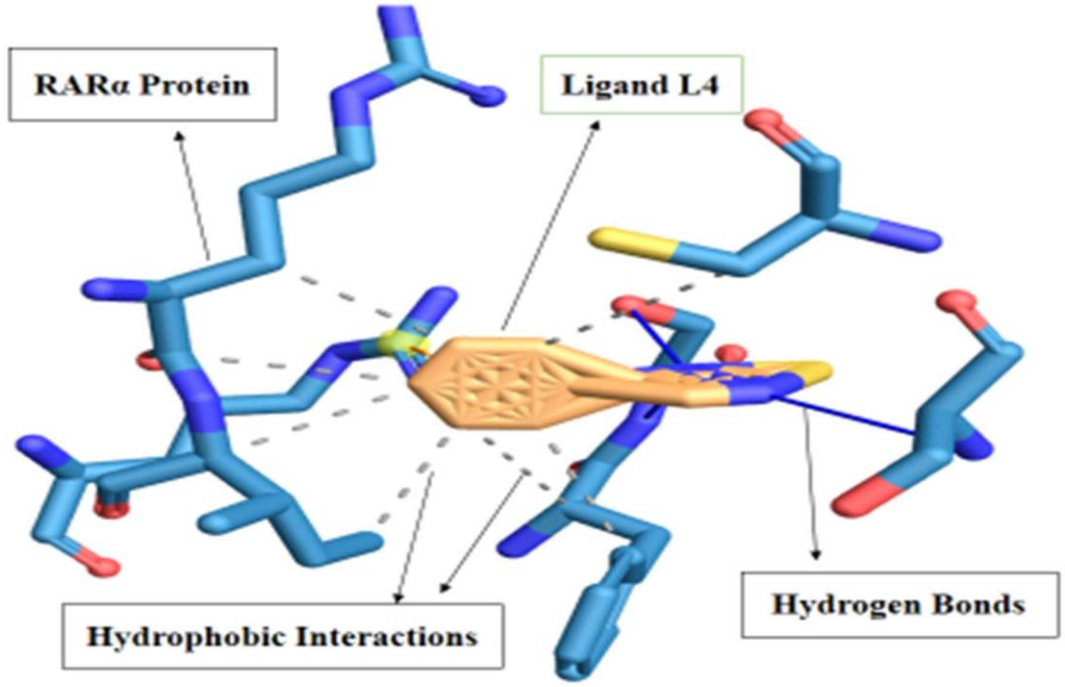

Figure 4.7: Protein ligand interaction profile image indicating the binding pose of L4 with RARα protein in NB4.

Table 4.3: The hydrophobic interactions & hydrogen bonding between L4 and RARα complex.

π-Cation Interactions

Index	Residue	AA	Distance	Offset	Protein charged?	Ligand Group	Ligand Atoms
1	276A	ARG	4.36	1.63	yes	Aromatic	3708, 3709, 3710, 3711, 3712, 3713

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	235A	CYS	3.94	3709	432
2	272A	ARG	3.43	3710	713
3	272A	ARG	3.51	3711	711
4	273A	ILE	3.4	3711	721
5	273A	ILE	3.16	3712	727
6	286A	PHE	3.43	3713	833
7	286A	PHE	3.82	3712	2678

Hydrogen Bonding

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	232A	SER	3.48	4.01	114.43	yes	no	2257 [N3]	3704 [Nam]
2	232A	SER	3.41	4.01	164.77	no	no	3704 [Nam]	2257 [N3]
3	287A	SER	2.81	3.15	100.22	yes	no	837 [N3]	3703 [Nam]
4	287A	SER	2.21	3.15	153.95	yes	no	2688 [N3]	3703 [Nam]
5	287A	SER	2.11	2.76	123.38	yes	yes	2693 [O3]	3703 [Nam]
6	287A	SER	2.18	2.76	155.63	no	yes	3703 [Nam]	842 [O3]

4.1.4.3 Protein- ligand complex of RARα-R-35

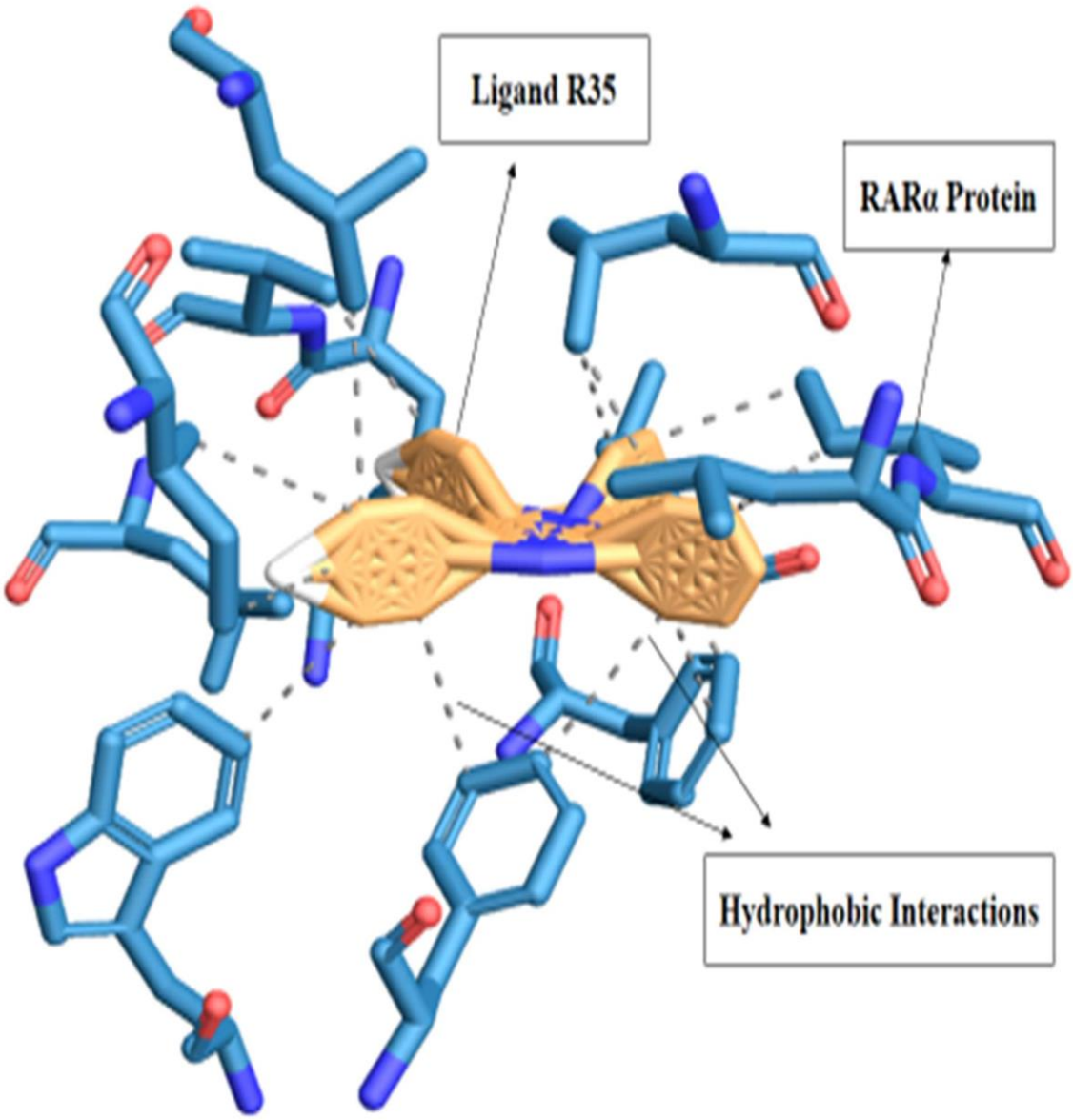

Figure 4.8: Protein ligand interaction profile image indicating the binding pose of R-35 ligand with RARα protein in NB4.

Table 4.4: Hydrophobic interactions between R-35 and RARα ligand protein complex.

Hydrophobic interactions					
Index	Residues	Amino acids	Distances	Ligand - Atom	Protein – Atom
1	225A	TRP	3.77	3730	353
2	228A	PHE	3.65	3731	381
3	228A	PHE	3.75	3725	382
4	266A	LEU	3.22	3719	668
5	266A	LEU	3.27	3721	668
6	269A	LEU	2.78	3722	689
7	270A	ILE	3.19	3718	700
8	270A	ILE	3.12	3722	698
9	302A	PHE	2.75	3716	957
10	302A	PHE	3.58	3725	958
11	305A	LEU	3	3717	976
12	394A	ARG	3.91	3710	1683
13	395A	VAL	3.31	3709	1695
14	398A	LEU	3.41	3708	1717
15	410A	ILE	3.85	3728	1806
16	410A	ILE	3.84	3729	1807
17	414A	LEU	3.65	3728	1840

Presence of only hydrophobic interactions between R-35 and RARα protein complex indicate relatively less strong binding relative to hydrogen bonding.

4.1.4.4 Protein- ligand complex of RARα-R-NIM04

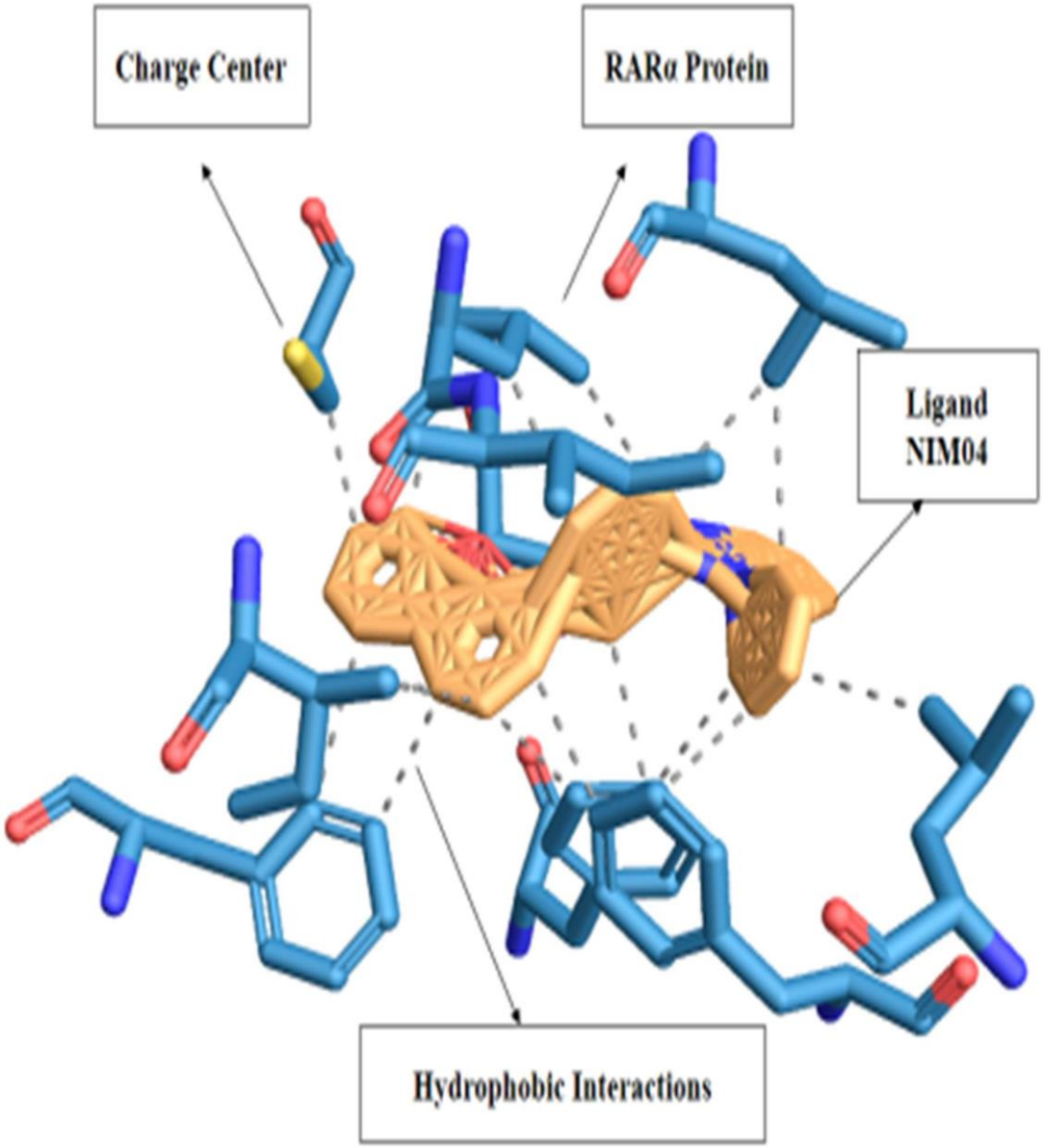

Figure 4.9: Protein ligand interaction profile image indicating the binding pose of R-NIM04 ligand with RARα protein in NB4.

Table 4.5: The Hydrophobic interactions and the hydrogen bonding existing between R-NIM04 and RARα complex.

Hydrophobic Interactions					
Index	Residues	Amino acids	Distances	Ligand - Atom	Protein – Atom
1	228A	PHE	3.09	3736	382
2	228A	PHE	2.86	3720	382
3	228A	PHE	3.5	3716	380
4	232A	SER	3.88	3711	2258
5	235A	CYS	2.92	3715	432
6	266A	LEU	3.3	3732	668
7	266A	LEU	2.91	3721	668
8	269A	LEU	3.3	3721	691
9	269A	LEU	2.94	3723	689
10	269A	LEU	3.39	3711	692
11	270A	ILE	3.43	3722	698
12	273A	ILE	3.17	3716	726
13	273A	ILE	3.23	3712	724
14	286A	PHE	3.32	3710	833
15	286A	PHE	3.25	3712	835
16	302A	PHE	3.19	3735	957
17	302A	PHE	2.97	3713	958
18	305A	LEU	3.46	3734	976

Hydrogen Bonds									
Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	232A	SER	2.75	3.62	149.52	**yes**	**yes**	411 [O3]	3704 [Nar]

4.2 *In vitro* Analysis

4.2.1 Imidazole derivate L7, L4, R35, R-NIM04 reduces the proliferation of PML/RARα-positive NB4 cell line

Cells were treated with different concentrations of L7, L4, R-35 and R-NIM04 and MTT assay was performed after 72-hours to assess the proliferation (Fig 4.10)

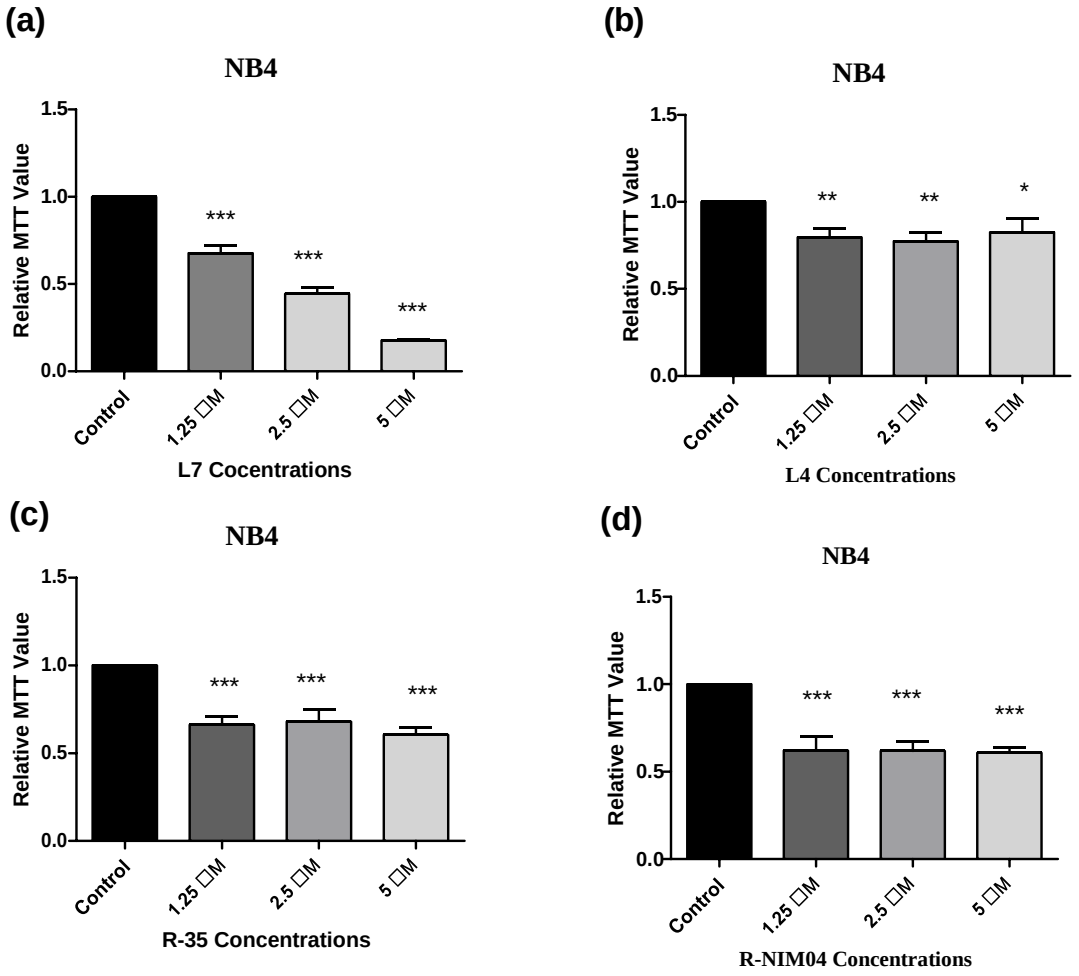

Figure 4.10: Effect of imidazole derivatives on the proliferation potential of NB4 cells through MTT assay.

(a)L7 (b) L4 (c) R-35 (d) R-NIM04 Cells were grown in a liquid medium containing RPMI given with 10% FBS, 1% L-glutamate, and 1% pen-strip to test their proliferative capacity in the presence of 0.01 percent DMSO and given concentrations of L7, L4, R-35, and R-NIM04. To test for statistical significance, one-way ANOVA was used (p-values of 0.001 or lower are considered to be statistically highly significant). The bars represent the Bars show mean ± SEM.

The imidazole derivatives L7, L4, R-35 & R-NIM04 interferes with the proliferation of PML-RARα-positive NB4 cells.

4.2.2 Comparison between the anti-proliferative effect of L7, L4, R35, R-NIM04 and ATRA on NB4 cells

ATRA, as previously stated, is the current treatment of choice for PML/RAR-positive APL. This is because, more than three decades ago, the introduction of ATRA as a potential therapeutic option for APL resulted in an improvement in the clinical outcome of this fatal disease (Ng & Chng, 2017). Given the fact that ATRA was used in conjunction with chemotherapy, a cure rate of more than 80% was achieved (Francesco Lo-Coco & Cicconi, 2014). But ATRA causes differentiation syndrome in patients with APL with a swift rise in leukocytes number. So the patients who receive ATRA only as APL treatment after attaining CR, are seen to undergo relapse (Asou, 2017). We have shown (Figure 4.10) that imidazole derivatives L7, L4, R-35, and R-NIM04 are able to reduce the NB4 cells proliferation. So, we compared the inhibitory effects of ATRA and these inhibitors on the cell proliferation potential of NB4 at different concentrations in μM and proliferation was checked through MTT assay after 72 hours. L7 significantly reduces the proliferation ability of NB4 in a dose dependent manner when compared to ATRA.

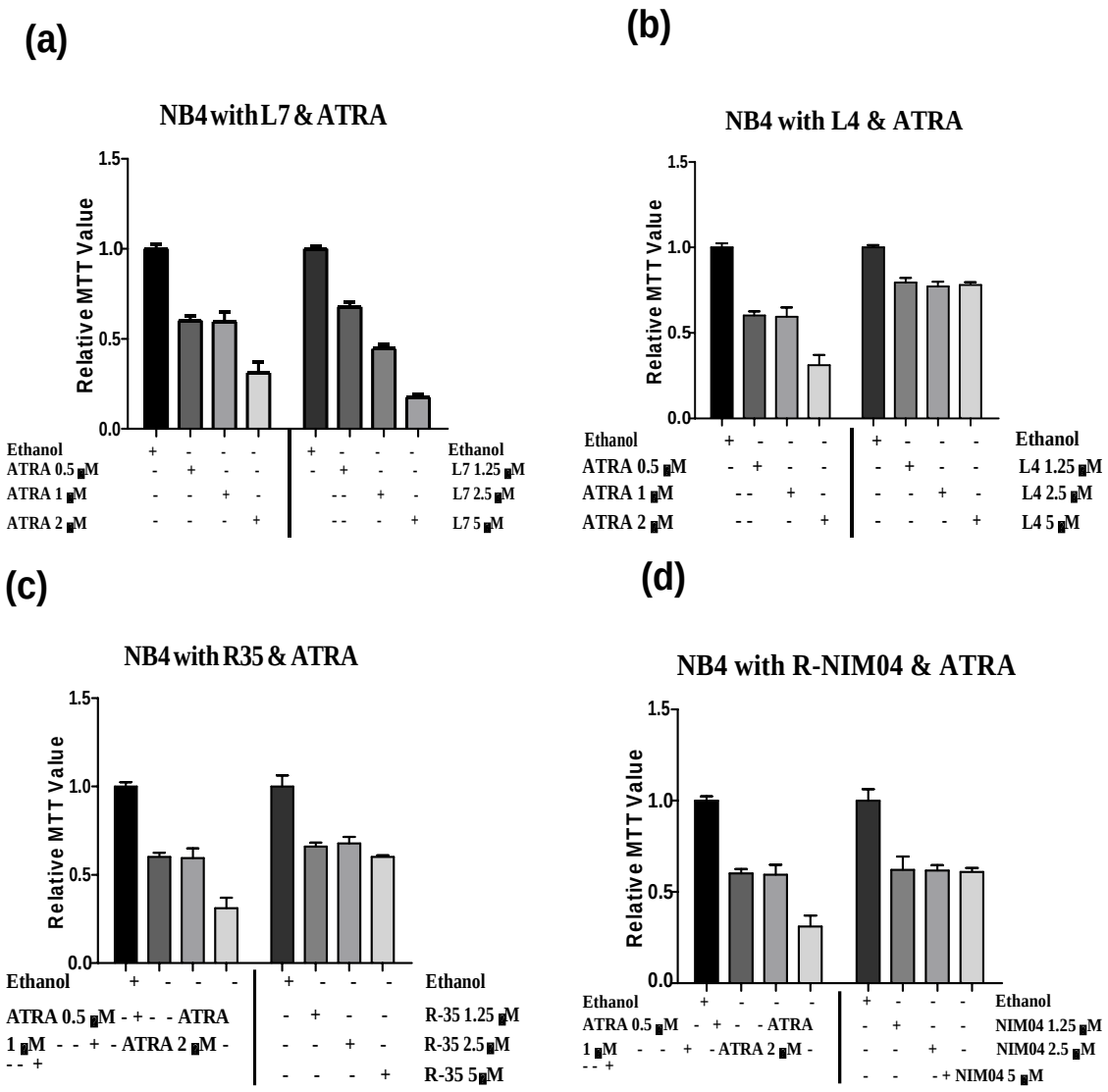

Figure 4.11: Comparison between L7, L4, R35, R-NIM04 and ATRA.

Cells were grown in a liquid medium containing RPMI given with 10% FBS, 1% L-glutamate, and 1% pen-strip to test their proliferative capacity in the presence of 0.01 percent DMSO and given concentrations of L7, L4, R-35, and R-NIM04. To test for statistical significance, one-way ANOVA was used (p-values of 0.001 or lower are considered to be statistically highly significant). The bars represent the Bars show mean ± SEM.

Imidazole derivatives L7, L4, R35 and R-NIM04 work at much higher concentrations than ATRA but the down-regulation can be seen (Fig 4.8).

4.2.3 Effect on downstream signaling cascade

As the above results indicates the decrease in the proliferation potential of APL cell line, so further we investigated the effect of targeting the fusion gene as mentioned in our *In-silico* results on downstream signaling pathways. Leading towards the downstream signaling, AXL Receptor Kinase expression was first evaluated.

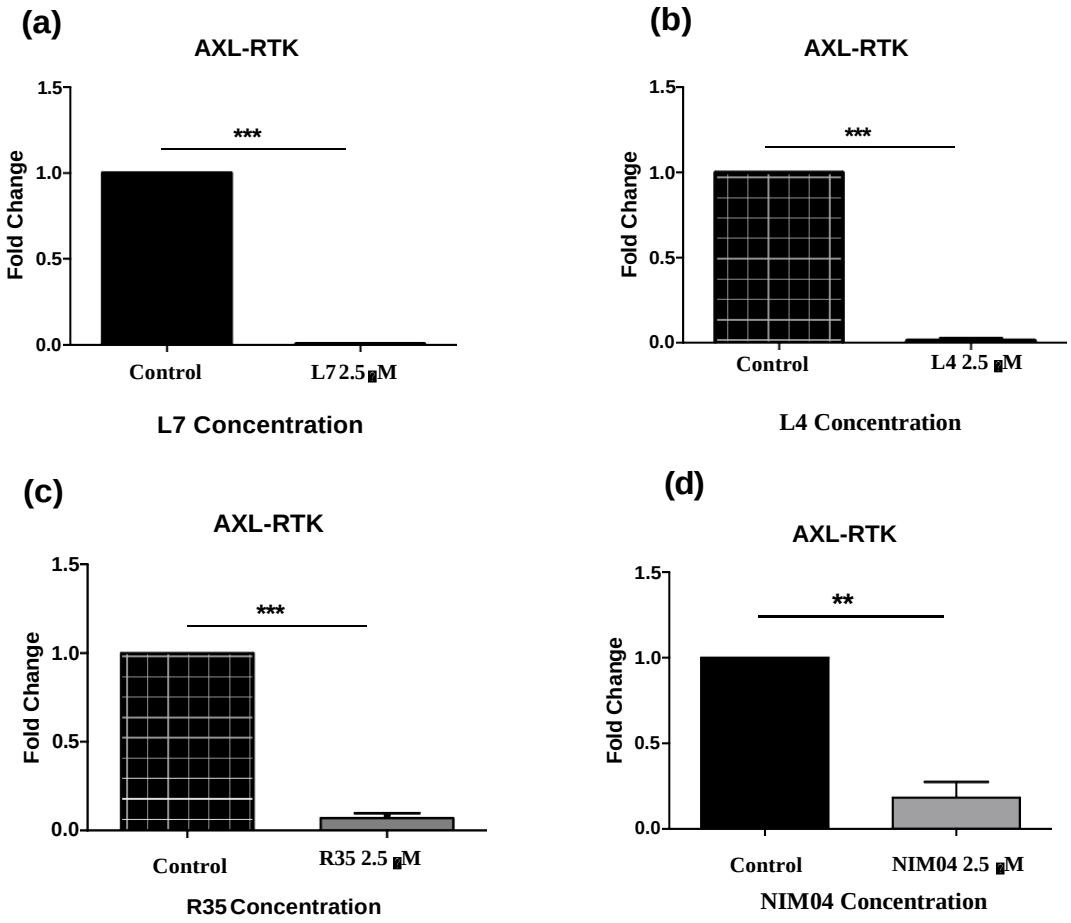

Figure 4.12: Expression of AXL-RTK in NB4 cells after treating with (a) L7 (b) L4 (c) R35 and (d) R-NIM04.

Cells were grown in liquid media (RPMI +10% FBS+ 1% L-Glutamate and 1% Pen-strep) and in presence of DMSO (0.01%) and given concentrations of L7, L4, R35 and R-NIM04. Expression analysis was done through real time PCR.

Expression of AXL receptor tyrosine kinase was downregulated in PML-RARα-positive NB4 cells (Figure 4.12). As described before AXL-RTK is a crucial component of leukemogenesis.

The fusion-protein PML/RARα is concerned in the stabilization of β catenin, hence involved in the activation/up-regulation of Wnt-target genes like c-Myc, Axin2 etc. (Müller-Tidow et al., 2004). So, β-catenin target gene c-Myc expression was analyzed using Real-time PCR, after treating the cell for about 72 hours with given concentrations of L7, L4, R35 and R-NIM04.

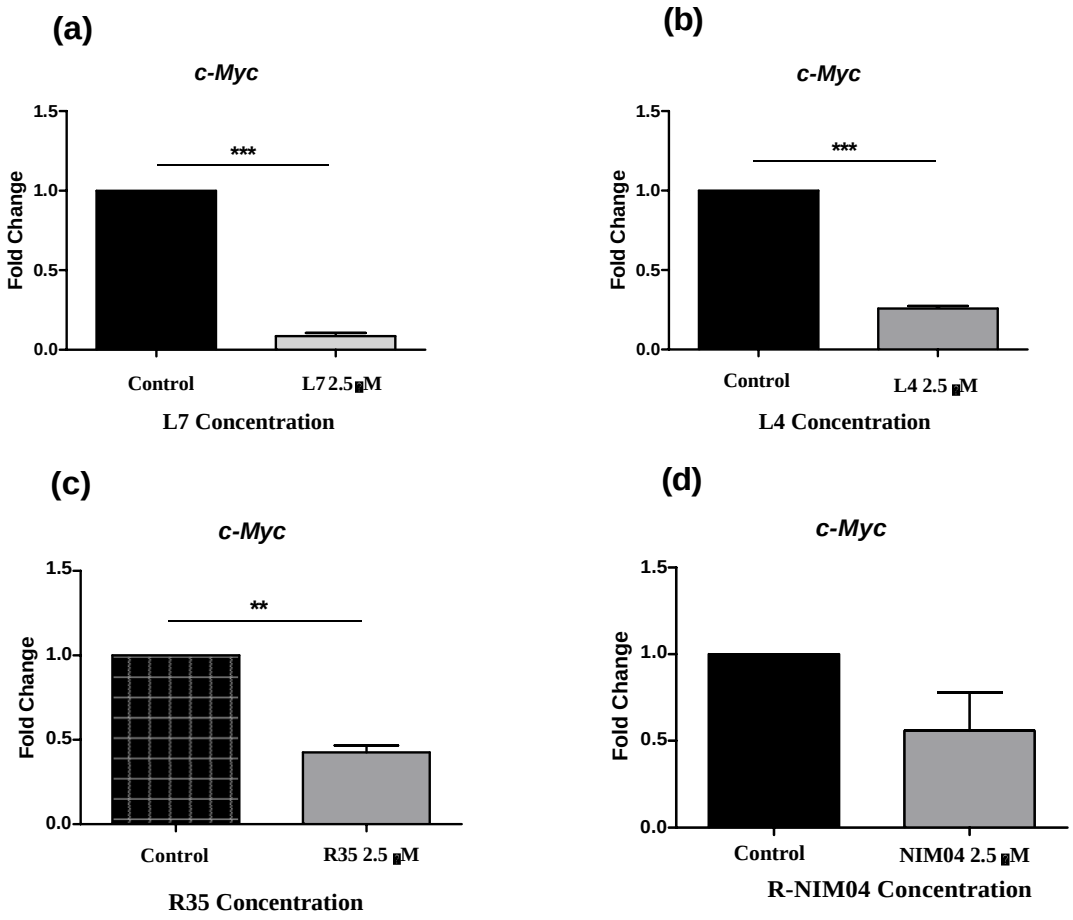

Figure 4.13: Expression of *c-Myc* in PML/RARα-positive NB4 cells after treatment with (a) L7 (b) L4 (c) R35 and (d) R-NIM04.

Cells were grown in liquid media (RPMI +10% FBS+1% L-Glutamate and 1% Pen-strep) and in the presence of DMSO (0.01%) and given concentrations of L4, L7, R-35 & R-NIM04. Expression analysis was done through real time PCR.

In Figure.4.13 we can see that effect of β catenin target genes in PML/RARα-positive NB4 cells, since L7, L4, R35, and R-NIM04 were able to reduce the proliferation of NB4 cells; the function of *c-Myc* in cell proliferation, a significant downregulation of *c-Myc* was seen in

treated vs control cells. This was due to the fact that treated cells experienced a significant increase in *c-Myc* levels. As the PML/RARα leukemogenesis is dependent on canonical Wnt β -catenin signalling, the fact that downregulation in AXL expression reduces the NB4 cells proliferation can indicate that AXL-RTK plays a role in Wnt/ β -catenin signalling in APL. This can be shown by the fact that the downregulation of AXL expression reduces the proliferation of NB4 cells. This Wnt/ β -catenin route is the primary regulator of the CSCs' ability to maintain their own self-renewal (Fatima et al., 2021).

The Eya genes code for a family of multi-functional proteins which operate as co-activators of transcription and haloacid dehalogenase family of Tyr phosphatases. These proteins are encoded by the Eya genes. Eya3 is a PP2A regulator of, which is a significant cellular Ser-Thr phosphatase. It also plays a role in maintaining the stability of *c-Myc*, which is an essential oncogene. As a result of Eya3's partnership with PP2A, *c-Myc* stability and tumour growth are induced (Lingdi Zhang et al., 2018), expression of Eya3 was also checked in NB4 cells after treatment with L7, L4, R-35, and R-NIM04. Except R-NIM04, all other three compounds showed significant decrease in Eya3 expression.

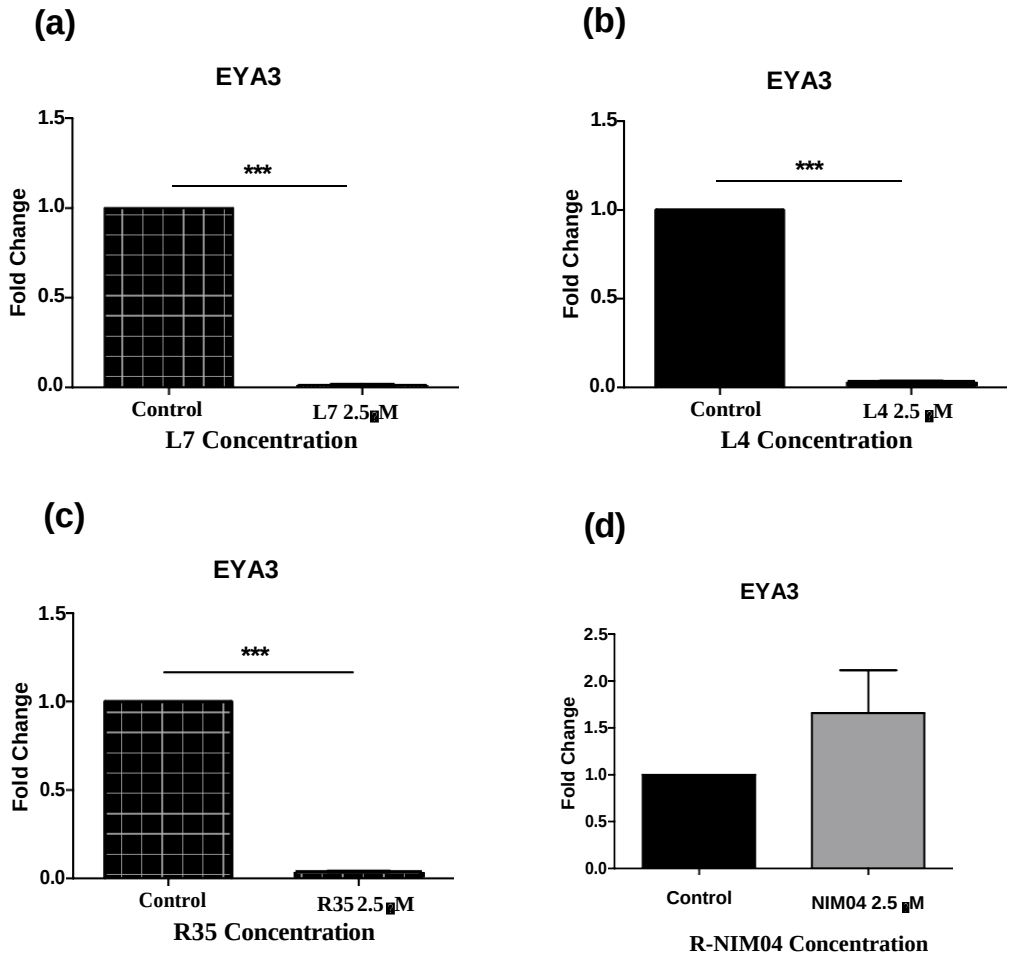

Figure 4.14: Expression of EYA3 in PML/RARα-positive NB4 cells after treated with (a) L7 (b) L4 (c) R35 and (d) R-NIM04.

Cells were grown in liquid media (RPMI +10% FBS+1% L-Glutamate and 1% Pen-strep) and in the presence of DMSO (0.01%) and given concentrations of L4, L7, R-35 & R-NIM04. Expression analysis was done through real time PCR.

4.2.4 Apoptosis Check in NB4 cells after treating with L4, L7, R35 and R-NIM04

As seen in figure 4.10 imidazole derrivatives L7, L4, R-35 and R-NIM04 interferes with the proliferation of PML-RARα-positive NB4 cells, so in order to investigate whether the cell death is due to apoptosis or cell cycle arrest; DNA Fragmentation Assay was performed. Since DNA degradation and fragmentation are basic indicators of cellular apoptosis (Saadat, Saeidi, Vahed, Barzegari, & Barar, 2015). Cells were treated with 1.25 µM concentration of L7, L4, R-35 and R-NIM04 and DNA was extracted after 48-72 hours. Extracted DNA was then run on 1.5% agarose gel through electrophoresis.

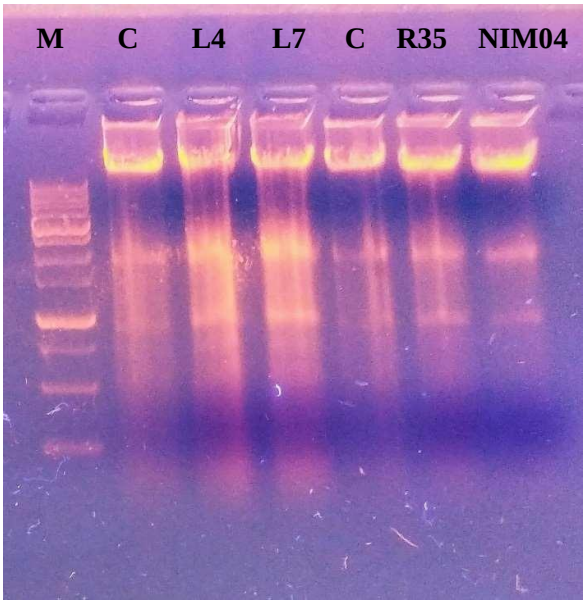

Figure 4.15: Gel image for DNA fragmentation assay for NB4 cells.

Cells were grown in liquid media (RPMI +10/20% FBS+1% L-Glutamate and 1% Pen-strep) and in the presence of DMSO (0.01%) and 2.5 µM concentrations of L7, L4, R35 & R-NIM04 (M= 1kb ladder, C= DMSO/ Ethanol Control) for almost 72 hours. DNA extraction was done then and run on 1.5% agarose gel for visualization.

In figure 4.15, degradation and fragmentation of DNA can be seen in NB4 cells treated with L7, L4, R-35 & R-NIM04. Hence we can say that reduction in cell proliferation ability of NB4 by these imidazole derived inhibitors is due to the induction of apoptosis.

4.2.5 Combinatorial effect of L7, L4, R35, R-NIM04 and ATRA on PML/RARα-positive NB4 Cells

Combining two drugs is a very common strategy to enhance the overall treatment efficacy. Although there are many cancer drugs but effectiveness of cancer drugs is limited by their toxicity to normal cells and also with the passage of time cancer cells also develop resistance to certain drug that was an effective treatment before. So, in context to this issue it is reasonable to use several drugs in combination for treatment.

ATRA plus chemotherapy is considered as standard treatment for PML/RARα positive APL patients due to its promising results but as discussed above ATRA causes differentiation syndrome in patients with APL and associated with a swift rise in leukocytes number. So the patients who receive ATRA only as APL treatment after attaining CR, are seen to undergo relapse. So there is a need of combination therapy in which the concentration of ATRA can be minimized and yet effective results can be obtained. So keeping in view that L7, L4, R35 and R-NIM04 significantly reduces the proliferation of NB4 cells, we investigated the effect of combined treatment to see if there is any additive effect of these inhibitors with ATRA on the proliferation of NB4 cells in comparison to the single treatment and also if combined treatment at different concentration in μM can reduce the concentration of ATRA, so as to reduce the differentiation induction due to administration of ATRA with or without chemotherapy. Proliferation was assessed by MTT assay after 72 hours.

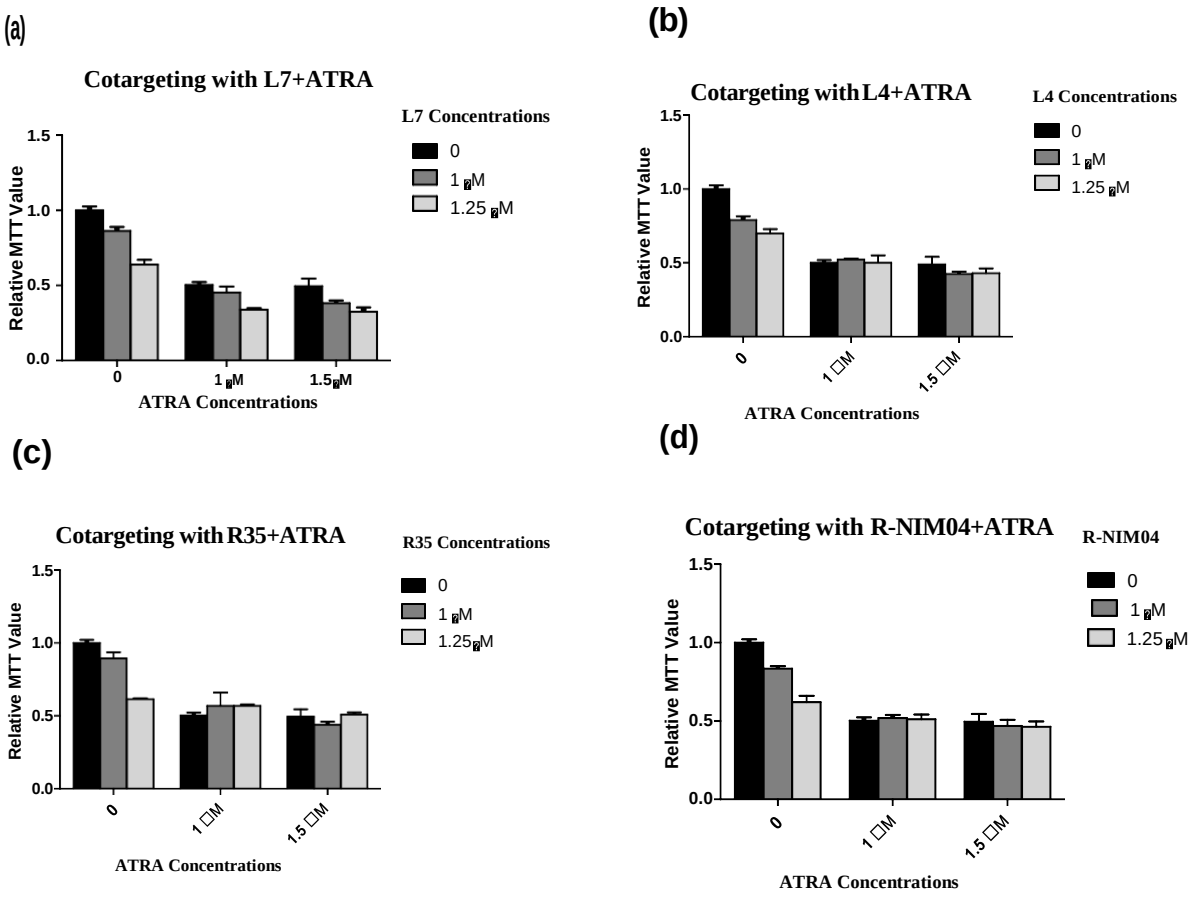

Figure 4.16: Effect of the combination treatment on the proliferation of NB4 cells.

(a) Combine treatment with L7& ATRA. (b) Combine treatment with L4 & ATRA. (c) Combine treatment with R35 & ATRA. (d) Combine treatment with R-NIM04 & ATRA. Cells were grown in liquid medium (RPMI + 20% FBS+1% L-Glutamate and 1% Pen-strep) to assess the proliferation of NB4 cells, in the presence of DMSO (0.01%) and given concentrations of L7,L4, R35, R-NIM04 and ATRA. And the proliferation was analyzed by MTT assay after treatment of 72 hours**.**

Taken together our data showed (Figure 4.16 (a)) that combined treatment (L7+ATRA) had a little additive effect on the cell proliferation of NB4 cells. While treatment with other combinations of ATRA+L4, ATRA+R35 and ATRA+R-NIM04 showed no additive effect on proliferation ability of NB4 cells. (Figure 4.16 (b), (c), and (d).)

4.2.6 Effect of Combinatorial treatment with L7+ ATRA on downstream signaling cascade

As the above results indicate that L7+ATRA has some additive effect on proliferation of PML/RARα-positive NB4 cells when given together. Next, we investigated the expression of AXL-RTK, *c-Myc* & EYA3 after treating the cells with 1 µM concentration of L7 plus 1 µM concentration of ATRA. The results showed the significant down regulation of AXL-RTK, *c-Myc* and EYA3.

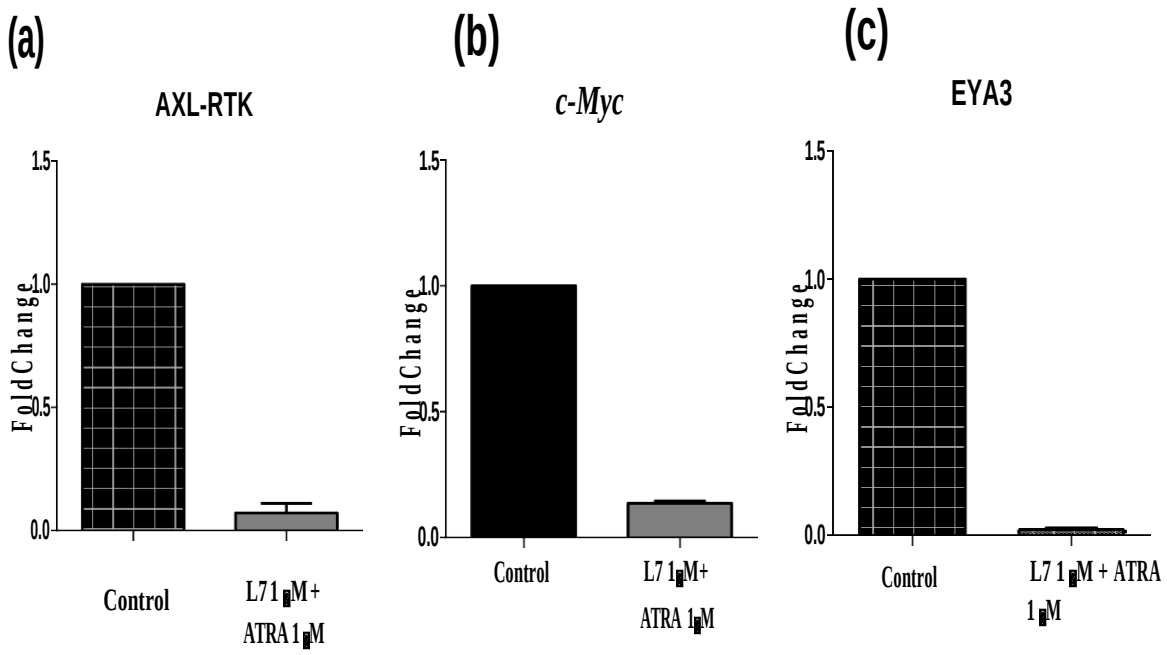

Figure 4.17: Expression of AXL (a), *c-Myc* (b) and EYA3 (c) in NB4 cells after combine treatment with L7 1 µM + ATRA 1 µM.

Cells were grown in liquid media (RPMI +10% FBS+1% L-Glutamate and 1% Pen-strep), in the presence of DMSO (0.01%) and given concentrations of L7+ATRA. Real time PCR was used to check the expression level.

4.2.7 Apoptosis Check in NB4 cells after co-targeting with L7 + ATRA

Cells were given with 1 µM concentration of L7 and 1 µM concentration of ATRA. DNA was extracted after 48-72 hours of incubation. Extracted DNA was then run on 1.5% agarose gel through electrophoresis.

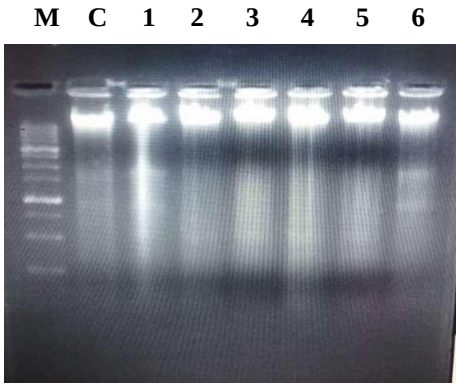

Figure 4.18: Gel image for DNA fragmentation assay for NB4 cells co-targeted with L7+ATRA.

Cells were grown in liquid media (RPMI +10/20% FBS+1% L-Glutamate and 1% Pen-strep), in the presence of Ethanol (0.01%) and given concentrations of L7 and ATRA (M= 1kb ladder, Ethanol Control, 1= L7 1 µM, 2= ATRA 1 µM, 3 = L7 1 µM + ATRA 1 µM, 4 = ATRA 1.5 µM, 5 = L7 1 µM + ATRA 1.5 µM, 6= L7 1.5 µM) for almost 72 hours. The DNA was then extracted and run on 1.5% agarose gel for visualization.

5) DISCUSSION

The current study aims to test the anti-proliferative effect of four novel compounds L7, L4, R35, & R-NIM04 *invitro* and *in-silico* against APL. These compounds L4, L7, R35, and R-NIM04 are imidazole derrivatives, upon testing *in vitro* reduces the proliferation of PML-RARα-positive APL cells. In context to *in-silico* study, the structure of these compounds were docked with all the possible protein targets from APL cells, finalizing the potentially best targets based on lowest ligand-protein binding energy.

Because of their unique properties, such as higher polarity and their ability to take part in hydrogen bonding as well as coordination chemistry, imidazole structures can bind with various biomolecules. The biological activities of compounds containing imidazole or fused imidazole are said to be diverse. Chemical compounds containing imidazole or fused imidazole have been shown to have selectivity for a variety of targets and significant activity in cancerous cell lines when compared to normal cell lines. In some instances, promising novel anticancer activities can be produced by making only minor changes to a specific scaffold or by including imidazole via scaffold hopping (Sharma et al., 2021).

Due to chromosome translocations, APL, a distinct subtype of AML, involves the expression of an oncogenic fusion protein which involves the PML and RARA gene. In hematopoietic precursor cell lines, expression of PML-RARA prevents differentiation and promotes survival (L.-Z. He et al., 1997; Puccetti & Ruthardt, 2004; Vitoux, Nasr, & de The, 2007). We found that L7, L4, R35, and R-NIM04 were able to reduce the proliferation of APL cells although L7 show anti-proliferation more effectively as compared to the other compounds L4, R-35, and R-NIM04 for APL. Also it was found that the combination treatment (L7 + ATRA) produces an additive-effect on proliferation potential of APL cells. Due to the differentiation induction by

ATRA and also differentiation syndrome (Asou, 2017), combinatorial therapy (L7+ ATRA) might be a better targeted therapy than the current therapy ATRA.

Our results showed L7, L4, R35, & R-NIM04 were interfering with the proliferation of APL cells. So next we investigated the effect of targeting the fusion genes on the downstream signaling in PML/RARα NB4 cells. Decrease in the expression of AXL was reported after treating APL cells with L7, L4, R-35, and R-NIM04, AXL the receptor tyrosine kinase, plays a vital role in oncogenesis and transformation of CML(O'bryan et al., 1991). It mediates intracellular signaling through PI3K/Akt and Erk pathways resulting in enhanced cell survival and proliferation (Graham, DeRyckere, Davies, & Earp, 2014; Schmidt, Ben-Batalla, Schultze, & Loges, 2012). AXL- receptor tyrosine kinase is involved in leukemiogenesis of PML/RARα positive APL and can be a possible therapeutic target (Fatima et al., 2021).

Up-regulation of the transcriptional activator c-Myc has been linked to human cancer because it stimulates cell proliferation while also regulating apoptosis and differentiation. This is also linked to AML treatment resistance (Lemaitre, Buckle, & Méchali, 1996; Uribesalgo, Benitah, & Di Croce, 2012). *c-Myc* is a gene that has been linked to treatment resistance. β -catenin has the potential to increase Wnt expression if it is not regulated (Cardona-Echeverry & Prada-Arismendy, 2020). c-Myc is a non-specific Wnt/β-catenin target gene that facilitates cell proliferation as well as other cellular processes (Dang, 2012). As a "master regulator," the c-Myc gene is responsible for regulating both cellular metabolism and proliferation. Because it is turned on by a wide variety of oncogenic pathways and consequently sets off a great deal of the metabolic rewiring that can result in the development of cancer, this gene has been called the "cancer gene." Expression and function of c-Myc are dependent on the presence of mitogenic stimulation under normal conditions (Coluccia et al., 2007; Miller, Thomas, Islam, Muench, & Sedoris, 2012; Müller-Tidow et al., 2004; K. Wang et al., 2010), hence involved in

the activation of Wnt-target genes like c-Myc and Axin2 etc. *c-MYC* is a well-characterized Wnt/b-catenin target gene (T.-C. He et al., 1998; Yochum, Cleland, & Goodman, 2008). The MYC transcription factor is largely responsible for activating the expression of genes whose products are responsible for driving cellular proliferation (Dang et al., 2006).

The canonical Wnt/ β -catenin signalling cascade is a pro-survival signalling pathway that has intense interferences with other signalling pathways like signal transducer and activator of transcription (STAT), the mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt signalling pathways. More recently, Wang et al (Dunne et al., 2014) showed that AXL inhibition decreased the amount of nuclear -catenin in MCF7 breast cancer cells, but AXL activation by Gas6 increased β -catenin levels and stimulated its nuclear translocation. Overall, the findings suggest that AXL is involved in cancer progression and beta-catenin stabilisation. The AXL-RTK regulate canonical Wnt signalling by stabilising - β catenin, and activating a variety of signalling cascades, including PI3K, AKT, mTOR, Ras, Raf, MAPK, ERK, and others (Corno et al., 2016) likewise the downregulation in the expression of AXL-RTK can affect β-catenin stabilization and downregulation of its target genes as indicated in our results. Since L7, L4, R35, and R-NIM04 inhibited the proliferation of APL cells, so c-myc being a important cellular proliferation regulator (Miller et al., 2012) was seen to be downregulated.

Using MTT assay, it is clear that L7, L4, R35, and R-NIM04 inhibit proliferation of APL cells. The PCR results demonstrated that treating NB4 with L7, L4, R35 and R-NINM04 downregulate AXL-RTK which as a result reduces the expression level of beta-catenin dependent genes, c-Myc in PML-RARA-positive APL cells.

It was found through the DNA fragmentation assay that the inhibition of the cell proliferation of PML-RARα-positive NB4 cells is related to the apoptosis since fragmentation of DNA is a

basic indicator of late-stage apoptosis (Saadat et al., 2015). In DNA fragmentation assay, treatment with ATRA was also assessed and it was seen that ATRA is not associated induction of apoptosis as described previously in (Moosavi & Djavaheri-Mergny, 2019).

Our *in- silico* results showed that, RARα (retinoic acid receptor alpha) was the best possible potential protein target of APL cells. The RARA protein belongs to nuclear receptor superfamily, act as a nuclear transcription factor (Liquori et al., 2020). PML-RARα causes an arrest in differentiation of promyelocytes and gives a survival as well as a proliferative benefit to leukemic cells. Direct targeting the RARα protein can lead to inhibition of PML-RARα-positive APL leukemogenesis, downregulation of AXL-RTK as our results indicated with the suppression of activation of aberrant Wnt/β-catenin signaling pathway and hence cancer stem cells renewal (Fatima et al., 2021). As Wnt beta catenin pathway is the main pathway in regulation of the self _renewal of normal as well as CSCs (Clevers, 2006), therefore, additional research on the inhibitory role of these compounds and involvement of AXL RTK in PML/RAR-positive cancer stem cells could be helpful in eliminating CSCs and, as a result, preventing a recurrence of cancer. Collectively, cancer progression is all about cross-talk between different cell to cell signal cascades that leads to survival and maintenance of cancer cells. Therefore, designing a therapy that will target the main drivers of leukemia is needed. So, by validating the above-mentioned findings and more *in-vitro* and *in-vivo* research, these imidazole derived compounds L7, L4, R-35, and R-NIM04 can be considered as a targeted therapy for leukemia in future.

6) CONCLUSION

As a result of the chemical, structural, and biological properties of imidazole, there is a significant level of interest in researchers for development of imidazole-based therapies as risk-free alternatives to chemotherapy for the treatment of cancer. It can be concluded from the findings of this study that imidazole derived L7, L4, R-35, and R-NIM04 have a strong anti-proliferative effect on PML/RARα-positive APL. Additive effects on the proliferation of APL cells can be achieved through combination treatment of L7 with ATRA for APL. Since our *In-silico* results indicated RARα to be the main target of our imidazole derived compound and this target protein is the main key player of PML/RARα-positive APL leukemiogenesis, furthermore the downregulation in the AXL-RTK expression was seen that stabilize/regulate β-catenin leads to downregulated β-catenin target genes expression and β-catenin being the main player in regulation of the CSCs self-renewal, so this study may pave way in eradicating CSCs, eventually avoiding the cancer relapse. However, for investigating effectiveness and efficacy of L7, L4, R-35, and R-NIM04 in APL further research is needed.

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