# Molecular Targeting of AXL-Receptor Tyrosine Kinase in the subtypes of Acute Myeloid Leukemia (AML)



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**Molecular Targeting of AXL-Receptor Tyrosine Kinase in the subtypes of Acute Myeloid Leukemia (AML)**

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*Dedicated to*

*My beloved parents*

# ACKNOWLEDGEMENTS

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# LIST OF ACRONYMS

## A

ABL              Abelson murine leukemia virus homology gene

ALL              Acute lymphatic leukemia

AML             Acute myeloid leukemia

AML1/ETO      Acute Myeloid Leukemia 1/ Eight Twenty One

APL              Acute promyelocytic leukemia

ATRA           All Trans retinoic acid

ATO              Arsenic trioxide

## B

BCR             B cell receptor

BCR/ABL        Breakpoint cluster region/Abelson murine leukemia virus

bp                Base pairs

## C

CBFβMYH11     Core binding factor β myosine heavy chain 11

CD               Cluster of differentiation

cDNA           Complementary DNA

CFU             Colony forming unit

CLL              Chronic lymphatic leukemia

CLP             Common lymphoid progenitor

CML            Chronic myeloid leukemia

CR               Complete remission

CSC             Cancer stem cells

## D

DMSO         Dimethyl sulfoxide

DNA            Deoxyribonucleic acid

| | |
|---|---|
| DNMTs | DNA methyl transferases |
| dNTPs | Deoxynucleoside triphosphate |
| DTT | Dithiothreitol (threo-1,4-dimercapto-2,3-butandiol) |

**E**

| | |
|---|---|
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal regulated kinase |

**F**

| | |
|---|---|
| FAB | French American British |
| Fak | Focal adhesion kinase |
| FBS | Fetal Bovine serum |
| FLT3 | Fms like tyrosine kinase 3 (CD 135) |
| FLT3ITD | Fms like tyrosine kinase 3/internal tandem duplications |
| FOX | Forkhead Box |

**G**

| | |
|---|---|
| G-CSF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte macrophage- colony stimulating factor |
| Grb2 | Growth factor receptor-bound protein 2 |
| GSK3β | Glycogen synthesis kinase 3 beta |

**H**

| | |
|---|---|
| HDAC | Histone deacetylase |
| HSCs | Hematopoietic stem cells |
| HSCT | Hematopoietic stem cell transplantation |
| HSPCs | Hematopoietic stem and progenitor cells |
| Hif-1α | hypoxia-inducible factor 1 alpha |

**I**

IL            Interleukin

**K**

KDa         Kilodalton(s)

**L**

LEF1        Lymphoid enhancer factor 1

LIC          Leukemia initiating cell

LSC         Leukemic stem cell

LT           Long term

LT-HSC    Long-term hematopoietic stem cells

**M**

M            Molar

MAPK       Mitogen activated protein kinase

M-CSF     Macrphage-colony stimulating factor

mM         Millimolar

mTOR       Mammallian target of rapamycin

µl           Microliter

µM          Micromolar

MTT        Tetrazolium salt

**N**

NF-κB      Nuclear factor kappa-light-chain-enhancer of activated B cells

NUP214   Nucleoporin 214

**O**

OS          Overall survival

**P**

| | |
|---|---|
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PI3K | Phosphatidylinositol-3-kinase |
| PKC | Phospho kinase C |
| PLZF | Promyelocytic leukemia zinc finger |
| PLZF/RARα | Promyelocytic leukemia zinc finger/Retinoic acid receptor α |
| PML | Promyelocytic leukemia protein |
| PML/RARα | Promyelocytic leukemia/retinoic acid receptor α |

**R**

| | |
|---|---|
| RBC | Red blood cells |
| RA | Retinoic acid |
| RAR | Retinoic acid receptor |
| RAS | Rat sarcoma |
| Rnase | Ribonuclease |
| rpm | Revolution per minute |
| RPMI | Roswell Park Memorial Institute |
| RT | Reverse transcriptase |
| RTK | Receptor tyrosine kinases |
| RUNX1 | Runt related transcription factor |

**S**

| | |
|---|---|
| STAT | Signal transducers and activators of transcription |
| ST-HSC | Short-term hematopoietic stem cells |
| SCT | Stem cell transplant |

**T**

| | |
|---|---|
| t(6;9) | Translocation of chromosome 6 and 9 |
| t(15;17) | Translocation of chromosome 15 and 17 |
| TAE | Tris acetate EDTA |
| TEF | T cell enhancer factor |

**U**

UV          Ultraviolet


**W**

WBC          White blood cell count


+          Positive

-          Negative

%          Percent

°C          Degree Celsius

# ABSTRACT

Leukemia refers to the cancer of white blood cells (WBCs) in which aberrant proliferation of immature blasts is seen due to the differentiation blockage, hence effecting the production of normal WBCs. Acute myeloid leukemia (AML), the most common form of acute leukemia, is a malignant disorder that is identified due to the aberrant growth and differentiation of hematopoietic stem cells (HSCs), leading to the accumulation of immature myeloid precursors (myeloblasts) in the bone marrow and peripheral blood. AML is a heterogeneous disease consisting of various combinations of genetic aberrations, two of them are t(15;17) and t(6;9) that results in the formation of chimeric genes encoding leukemia associated fusion proteins (LAFP); PML/RARα associated with AML subtype acute promyelocytic leukemia (APL) that has good prognosis and DEK/CAN associated with subtype high risk AML that has poor prognosis. PML/RARα is involved in the stabilization and up regulation of β-catenin through activation of Wnt pathway and hence involved in the leukemogenesis of APL. The current therapy for PML/RARα-positive AML is ATRA and Arsenic trioxide. Both ATRA and arsenic trioxide target PML/RARα fusion protein. ATRA induces differentiation, resulting in differentiation syndrome while arsenic trioxide induces apoptosis and promote differentiation. But due to resistance development, differentiation induction and poisoning by ATO, it is important to explore such therapies and targets that are less toxic and can overcome the resistance to the current therapies. Currently no therapy other than standard chemotherapy is available for DEK/CAN positive AML. So there is a need for effective targeted therapy for DEK/CAN-positive AML. AXL receptor tyrosine kinase belongs to TAM family of receptors that are known for their active role in developing resistance to therapy. R428 is the first specific AXL-RTK inhibitor to enter the clinical trials. So the current study aims to explore

the therapeutic potential of AXL-RTK by targeting with R428. ATRA resistant PML/RARα-positive NB4 cell line was developed to study the role of AXL-RTK in resistance development. Upregulation of AXL-RTK was found in R-NB4. We showed that pharmacological targeting of AXL receptor strongly interfered with leukemogenic potential of PML/RARα- and DEK/CAN-positive AML ($p<0.05$). Furthermore resistance to ATRA was also overcome by targeting AXL receptor with R428 in APL ($p <0.05$). The anti-proliferative effects of targeting AXL-RTK were related to down regulation of target genes including *c-myc* ($p < 0.001$), *Axin2* ($p < 0.001$), and *Hif-1 alpha* ($p < 0.001$ and $< 0.01$). Hence AXL-RTK might be a suitable therapeutic target for PML/RARα-positive and DEK/CAN-positive AML. Further studies of this receptor and its inhibitor on LSCs and clinical samples may pave the way in proposing an effective therapy for AML without differentiation induction in PML/RARα-positive APL and especially for DEK/CAN-positive high risk AML that currently lacks an effective therapy.

*CHAPTER 1*

## INTRODUCTION

The word leukemia refers to the cancer of white blood cells that begins in the hematopoietic stem cells (blood forming cells) in the bone marrow (Adamietz *et al.*, 2009). Under the normal circumstances, mature blood cells i.e. RBCs, WBCs and platelets are formed due to differentiation and maturation of blast cells. In case of leukemia, there is accumulation of malignant blast cells in the bone marrow due to differentiation blockage as a result of some mutation. This affects the production of normal blood cells leading to anemia (Adamietz *et al.*, 2009), the first symptom of leukemia. Such blast cells are also referred to as leukemic blasts or cells (Kodappully Sivaraman Siveen, 2017; Siveen *et al.*, 2017). The normal amount of blasts in the bone marrow is about <5% whereas this amount is increased to about 30% -100% during leukemia (Adamietz *et al.*, 2009). When these malignant blasts migrate to other sites in the body like circulating blood, spleen, liver etc., they affect their function as well. Myeloblast and lymphoblast are the two forms of a blast i.e. myeloblasts mature into myeloid cells whereas lymphoblast mature into lymphocytes. Similarly, leukemia that originates from myeloblasts is known as myeloid leukemia and deteriorates the production of red blood cells, white blood cells and platelets (Seipelt *et al.*, 1998). On the other hand, leukemia that begins in the lymphoblast is called as lymphocytic leukemia and affect the production of lymphocytes (Hematology, 2019).

Being a growing disease in Pakistan, leukemia affects both the genders with increased rate in males while it affects people of all age-groups equally. Pakistan ranks 7th in the world in incidence of leukemia (Khan S, 2017). As per a report by WHO, 7,139 new leukemia cases

were reported in Pakistan in 2018 with highest percentage of males of all age groups being affected (WHO, 2019).

Leukemia is divided into chronic and acute based on the progression and maturation level of abnormal cells (Seipelt *et al.*, 1998). When mature white blood cells accumulate and cancer progression is slow, then the leukemia is referred to as chronic leukemia. Chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML) are the two major types of chronic leukemia. In acute leukemia, there is accumulation of the immature blast cells in the bone marrow that migrate to the circulating blood (Adamietz *et al.*, 2009) Infiltration of these blast cells is also seen in other bodily tissues, ultimately leading to the failure of bone marrow. Acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL) are two major types of acute leukemia (Seipelt *et al.*, 1998)

Acute myeloid leukemia (AML) refers to a heterogeneous disease condition that involves hematopoietic progenitors identified due to gained genetic aberrations leading to disturbance in the normal processes of division, self-renewal and differentiation (Ben-Batalla *et al.*, 2013). It is the most common form of acute leukemia and is a malignant disorder that is identified due to the aberrant growth and reduced ability for differentiation of hematopoietic stem cells (HSCs) into mature cells. This leads to the accumulation of immature myeloid precursors (myeloblasts) in the bone marrow and peripheral blood, reducing the production of normal RBCs, WBCs and platelets. If >20% myeloblasts are circulating in the peripheral blood, then it indicates AML (Harris *et al.*, 1999). This reduction in the normal mature blood cells formation and increase in malignant blasts formation exerts different effects on the body such as anemia, bleeding disorders, bone marrow failure, increased susceptibility to the infections and ultimately death (Daniel V.T. Catenacci, 2005). Significant research has been carried out with the aim of

developing improved therapies that can produce some effective outcomes in AML. The standard therapy for most subtypes of newly diagnosed AML is the 7 + 3 regimen that is the combination of cytarabine with an anthracycline (daunorubicin or idarubicin). High-dose cytarabine (HiDAC) consolidation is recommended to low risk patients, whereas allogeneic stem-cell transplant (SCT) to in first remission to the high risk ones (Kadia *et al.*, 2016).

Acute promyelocytic leukemia (APL) is a subtype of AML that constitute 5–15% of all AML subtypes. It is identified by the particular growth of immature myeloid precursors whose differentiation is blocked at the promyelocytic stage. Signs and symptoms include anemia, infections and hemorrhages, whereas leukemic cells are also seen invading the organs in some cases of APL (Anna Laurenzana, 2006). Balanced chromosomal translocation t(15;17)(q22;q12) is the most abundant one in majority of the APL cases and it is caused due to the fusion of the promyelocytic leukemia protein (PML) N-terminus to the retinoic acid receptors (RARα) transcription factor C-terminus (Borrow *et al.*, 1990). RARα is a nuclear receptor whose ligand is retinoic acid (RA). RARα acts as a transcription repressor of the target genes in the absence of its ligand RA through the recruitment of corepressors and histone deacetylases. In the presence of RA, there is conversion of RARα from transcriptional repressor to transcriptional activator, regulating the expression of the genes that play major role in the differentiation of myeloid precursors (Lallemand-Breitenbach *et al.*, 2011) . Considerable research has been conducted for the therapeutic approaches of APL, the most suitable ones being the all trans RA (ATRA) and arsenic trioxide (ATO) (Ng *et al.*, 2017). Both these agents when given in combination with chemotherapy and improved transfusion therapy, have shown promising outcomes due to which this deadly disease is now transformed into a treatable one (Z.-Y. Wang *et al.*, 2008).

Despite being a promising agent against APL, ATRA induce differentiation in immature promyelocytes as well as in the normal stem cells, causing differentiation syndrome, in which there is an aberrant increase in the WBCs count due to the release of pro-inflammatory cytokines by the differentiating cells (Degos *et al.*, 2001). Also, ATRA alone is unable to target and eradicate the leukemic initiating cells (LICs) and their self-renewal ability (Zheng *et al.*, 2007). Therefore, ATRA in combination with cAMP was shown to produce an escalated effect on LICs which depicts the importance of targeting these LICs for an effective APL treatment (Nasr *et al.*, 2008). Patients who receive ATRA only as APL treatment after attaining CR, are seen to undergo relapse. Due to this, combination therapy (ATRA + Chemotherapy) is employed for the newly diagnosed APL patients as the advanced therapy option (Asou, 2017). Hence inability of ATRA alone as a therapy for APL (Warrell *et al.*, 1991), mutation in the B2 domain of PML causing resistance of PML/RARα to ATO therapy (H.-H. Zhu *et al.*, 2014) and arsenic poisoning (Degos *et al.*, 2001) are the main issues faced today in the treatment of APL.

High risk AML is a rare subtype of AML that comprises 1-5 percent of overall AML with t(6;9)(p23;q34) chromosomal translocation that leads to the formation of DEK-CAN (NUP214) fusion protein whereas 90% of patients with this translocation are positive for internal tandem duplications of the tyrosine kinase FLT3 (FLT3-ITD). Due to poor understanding of the biology of this subtype and poor prognosis, high risk AML forms a distinct subtype of AML (Sandén *et al.*, 2013). It was shown through molecular analysis that this t(6;9)(p23;q34) chromosomal translocation consists of the *dek* gene at 6p23 and *can* gene at 9q34 (Soekarman *et al.*, 1992). As a result of disorganization of these genes, Dek/can fusion gene is formed due to the recombination of the two genes, occupying position on the imitative 6p chromosome. One particular intron on each chromosome contains the breakpoints on chromosomes 6 and 9,

transcribing a chimeric mRNA and translating a novel protein of 165-kd that plays an important role in the leukemogenesis (Ko *et al.*, 2006).

DEK consists of three binding sites where it binds the DNA. Despite many studies on this protein, its biology and connection between its structure and function is not well understood (Sandén *et al.*, 2013). *DEK* gene that encodes a nuclear protein of 375 amino acid, localizes to the nucleus and is associated with chromatin. It may also have a function in RNA metabolism as per a study, some of it associates with RNA of unknown kind (Privette Vinnedge *et al.*, 2013). The *CAN* gene translates into a 214 kDa nuclear pore complex protein. DEK promoter controls the fusion protein (DEK/CAN) expression and this fusion protein is linked to the poor prognosis of AML, forming a high risk set of AML patients (Shearer *et al.*, 2005). But, very limited information has been explored about the leukemogenesis of DEK/CAN. As reported, the fusion protein fails at blocking the differentiation, rather it increases the protein synthesis in myeloid progenitors and cells (Ageberg *et al.*, 2008). There is no particular treatment currently for the high risk AML due to the limited information on the leukemogenic ability of this fusion protein and response to chemotherapy is poor in patients with DEK/CAN (Ishiyama *et al.*, 2011). Although many studies have shown early allogeneic hematopoietic stem cell transplantation (HSCT) to have better results in first CR with special mention to the patients with FLT3-ITD (Ishiyama *et al.*, 2011).

AXL is a transmembrane receptor that belongs to the TAM family of distinctive receptor tyrosine kinases which includes Tyro3, AXL and Mertk. Beginning of Axl expression is seen in late embryogenesis (O'Bryan *et al.*, 1991). Activation of Axl can occur by varying pathways, of which the most usual is the activation through binding of ligand Gas6 to the Axl receptor in the form of a dimer (Myers *et al.*, 2016). Receptor activation is followed by the activation of

different signaling cascades including PI3K, MAPK and PKC and as per many studies JAK/STAT too. Axl activation, whether in the presence of ligand or its absence, is known to activate these signaling cascades in different cancers like prostrate, lung, ovarian, head and neck etc. (Scaltriti *et al.*, 2016). Activation of these signaling networks leads to the activation of transcription factors that are involved in controlling cellular functions like survival and proliferation e.g. transcription of anti-apoptotic genes, *survivin, cyclin D1, FAK* etc. by NF-kB happens as a result of IkBα-NF-kB complex disruption directed by AKT leading to nuclear translocation of NF-kB (Scaltriti *et al.*, 2016). Bone marrow derived stem cells (BMDSCs) are educated by AML cells in order to release Gas6 that ultimately aids in proliferation of leukemic cells and developing therapeutic resistance due to the activation of Axl-RTK (Ben-Batalla *et al.*, 2013). AXL expression is also seen to be significantly high in drug resistance AML patients and that too after chemotherapy (Hong *et al.*, 2008).

Variety of AXL-RTK inhibitors have been reported in literature but the main problem is that these inhibitors are not specific for Axl (Myers *et al.*, 2016). R428, also called as BGB324, is known to be the strongest, most specific and well-studied Axl inhibitor. Its mechanism of action involves blockage of Axl auto-phosphorylation on Tyr821 that is the docking site of its C-terminal, at nanomolar concentrations. It is the first inhibitor of Axl to enter the clinical trials in 2014, on the basis of inhibition of cancer cells metastases, both *in vitro* and *in vivo* (F. Chen *et al.*, 2018).

Of the many signaling cascades that regulate the process of hematopoiesis, canonical Wnt pathway is also the one that is required for the maintenance of the HSCs (Lento *et al.*, 2013). Hematopoiesis is promoted when there is activation of β-catenin due to the inhibition of GSK3β. Disruption in this physiological process contributes to the hematological cancers due to

disturbance in the target gene functions like *c-myc, Axin2* etc. (Chung *et al.*, 2002). This is the major pathway in controlling the self-renewal process of normal as well as leukemic stem cells. Therefore inhibition of this pathway might ultimately inhibit the self-renewal ability of LSCs that are the main players in the relapse of cancer.

Several treatment options for AML are available including chemotherapy, and some are undergoing clinical trials. But, the main challenge faced in the treatment of AML now-a-days is to develop an efficient targeted therapy that can overcome the challenge of therapeutic resistance as well as to avoid relapse by directly target the CSCs.

Toxicity of the current cancer therapeutics as well as their side effects, both long and short term, are restricting the cancer treatment options in aged patients. To overcome this, the concept and practice of targeted therapies, with special mention of tyrosine kinase inhibitors (TKIs), has not only lowered the issue of toxicity due to chemotherapy but has also increased the survival of many leukemia patients. However, the issue of therapy resistance is a major one and is still a challenge. Currently, some targeted therapies are available for AML patients with poor prognosis and overall survival of five year in about 26.6% vs 59.7% patients (Huey *et al.*, 2016). Keeping in view the scenario of issues at hand, new targets as well as targeted therapies for AML needs to be explored so as to increase the treatment options for AML patients. Such therapies might be able to reduce the dosage as well as toxicity of the current therapies with the achievement of promising and effective results and reduced side effects. Moreover, administration of such targeted therapies in combination with the standard therapies may have the ability to lower the resistance developed from single targeting.

**1.1 HYPOTHESIS**

The main problem faced currently in the treatment of APL and high risk AML is the availability of an effective therapy that can destroy leukemic cells as well as overcome the adaptive resistance to the current therapeutics. Deregulation of Wnt signaling pathway is the basis for abnormal self-renewal of CSCs including LSCs in APL (Mikesch *et al.*, 2007). PML/RARα, the main culprit in the APL, is involved in the leukemogenesis of APL through upregulation of β-catenin that is a key effector in the Wnt pathway (Müller-Tidow *et al.*, 2004). It has been shown previously that targeting the Wnt signaling pathway can eradicate leukemic cells (Ashihara *et al.*, 2015). This forms the basis for the maintenance therapy using compounds and related signaling cascades against APL. DEK/CAN fusion gene is known to activate signaling pathways like Akt/PI3K, RAS/RAF and JAK/STAT and is involved in the leukemogenesis in AML. AXL, a unique receptor tyrosine kinase, is known to activate signaling pathways like Akt/PI3K, RAS/RAF and JAK/STAT and in the regulation of β-catenin in CML (Y. Jin *et al.*, 2017). But whether AXL-RTK signaling is involved in the leukemogenesis of both PML/RARα-positive and DEK/CAN-positive AML is still to be explored. Hence by determining the effects of the functional and pharmacological targeting of this AXL receptor and its signaling, a new therapeutic target against APL and high risk AML can be deduced and the therapeutic potential of AXL receptor can be determined.

**1.2 AIMS AND OBJECTIVES**

1. To study the expression pattern of AXL receptor in PML/RARα and DEK/CAN-positive AML

2. Pharmacological targeting, single for AXL and in combination for AXL receptor and its effects on the biology of PML/RARα, DEK/CAN and BCR/ABL induced leukemogenesis

3. To develop ATRA resistant NB4 cells and determination of the expression of AXL-RTK in ATRA resistant NB4 cells

4. To determine the effect of R428 on resistant NB4 cells

5. To study the effect of pharmacological targeting on the downstream signaling of AXL receptor in PML/RARα and DEK/CAN-positive AML

# LITERATURE REVIEW

## 2.1 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) refers to a disease condition that involves hematopoietic progenitors identified due to gained genetic aberrations leading to disturbance in the normal processes of division, self-renewal and differentiation (Ben-Batalla *et al.*, 2013). AML is the most common malignant, leukemic disorder characterized by an irregular growth and differentiation of hematopoietic stem cells (HSCs), which leads to an accumulation of abnormal myeloid precursors (myeloblasts) in the bone marrow and surrounding blood. If >20% myeloblasts are circulating in the peripheral blood, then it signifies AML (Khwaja *et al.*, 2016).

AML is marked due to males being effected commonly on western side with average age of about 70 years at the time of diagnosis (Juliusson *et al.*, 2009).

As per American Cancer Society's estimation, approximately 60,300 new cases of all types of leukemias was reported in 2018 in US whereas about 24,370 deaths from leukemia of all types are expected. As for AML specifically, approximately 19,520 new patients of acute myeloid leukemia mainly adults were expected to be reported in 2018 whereas about 10,670 deaths due to AML are expected. Despite the fact that AML is known to be the most frequent leukemia type in adults, slightly higher in males than in females, it is an uncommon cancer; constituting only about 1% of all cancers (Society, 2018). A study estimated AML incidence for next 10 years (2017-2027) for 45 countries that represents about 90% of the 2017 world population. The number of AML cases reported in Africa, Latin America, lower income Asia

Pacific countries, high-income Asia Pacific countries, Europe, and North America was found out to be 0.4, 1, 1, 2, 4 and 5 cases per 100,000/year (Hughes, 2017). The prevalence of AML in Africa is 1, in Latin America 4, in lower-income Asia Pacific countries it is 4, high-income Asia Pacific countries show prevalence of 7, Europe 10, and North America has the highest prevalence with 11 cases; per 100,000. Of all these regions, Africa is susceptible to an increase in prevalent cases of AML over the next ten year i.e. 33% by 2027. The number of patients affected by AML is expected to increase globally. On the other hand any improvements in the research for survival of AML patients, can save up to 81 thousand cases by 2027, worldwide (Hughes, 2017). Acute leukemias are a growing disease in Pakistan. In northern Pakistan, leukemia comes second as a common malignancy in males and is at third in cancers that are most common in females (Raziq, 2014).

The clinical signs and symptoms of AML are not specific instead they are directly linked to the leukemic take-over of the bone marrow, causing reduction in the number of mature blood cells (cytopenia) (Lowenberg *et al.*, 1999). The non-specific symptoms include fatigue, less or no appetite, being out of breath after some physical activity mostly due to anemia, frequent infections due to shortage of infections fighting neutrophils (neutropenia) and being more prone to injuries and ultimately bleeding due to lack of platelets (thrombocytopenia) (Khwaja *et al.*, 2016). Reduction in the number of RBCs, neutrophils and platelets is common among the AML patients, making them anemic.

Diagnosis of AML depends upon the morphological recognition of immature leukemic cells in Wright-Giemsa stained blood and bone marrow samples. Nuclei of such cells are observed to be round to asymmetrical, have a clear nucleoli and small amount of cytoplasm that usually can be seen consisting of azurophilic granules and different number of Auer bodies that are

basically rod structures consisting of azurophilic granules in lysosomes. For a definite diagnosis of AML, >30 percent of leukemic blasts in a bone marrow sample must be present before starting therapy but other specific diagnostic techniques must also be employed for a confirm diagnosis (Lowenberg *et al.*, 1999). Besides, modern techniques like immunophenotyping, cytogenetic and molecular characterization of myeloblasts are also employed to differentiate AML from other leukemias and to further identify subtypes of AML.

Origin diagnosis for acute leukemia can be done using flowcytometric immunophenotyping by checking for the expression of origin specific cluster differentiation (CD) markers. CD45, the common leucocyte antigen, is used to confine the population of immature blood cells (blasts) population and on this confined population, analysis of the expression of the other origin specific markers is carried out (Gajendra, 2016). Expression of CD45 varies in various sort of blast cells like B lymphoblasts are CD45 negative to slight positive, myeloid blasts are slightly CD45 positive but due to granular nature they show a higher side scatter. A tear drop pattern formed by abnormal promyelocytes can be observed in Acute Promyelocytic Leukemia (APL) whereas immature T-cells show diminished to same CD45 expression as that of normal lymphocyte population. For flowcytometric immunophenotyping of acute leukemia, some of the common antibodies used include stem cell/hematopoietic precursors (CD34, HLA-DR, and terminal deoxynucleotidyl transferase/TdT), myeloid markers (cMPO, CD13, CD15, CD117, CD33, monocytic markers (CD14, CD11b, CD64, CD11c, and lysozyme), erythroid (CD71, CD235a) and megakaryocytic (CD61, CD36, CD41) (Gajendra, 2016).

Expression of CD7, CD9, CD11b, CD13, CD14, CD33, CD34, CD56 and terminal deoxynucleotidyl transferase (TdT) are linked with poor prognosis in AML. CD34 and HLA-DR co-expression can aid in predicting the failure of achieving complete remission as per a study. A helpful prognosis can be achieved due to the presence of such blasts that express pan-myeloid markers i.e. myeloperoxidase (MPO), CD13, CD33, CDw65 and CD117 (Gajendra, 2016). In about 10-30% of patients, T-cell antigen, CD7, has been reported whereas B-cell antigen, CD19, is expressed atypically in about 3% of the patients (Khwaja *et al.*, 2016).

Among the risk factors associated with AML, no particular factors can be highlighted that can lead towards AML in majority of the patients. However, environmental factors like getting exposed to DNA-damaging agents like benzene, tobacco smoke and ionizing radiations such as therapeutic radiotherapy and chemotherapy (known as therapy related AML) can lead to a possible increase in the risk of developing AML (Shimizu *et al.*, 1990). People whose first degree relatives are patients of major blood cancers, they are at a high risk, about 5-7 folds, of getting that particular cancer (Khwaja *et al.*, 2016). However, no study has yet reported any strong proof of AML or MDS related to familial aggregation apart from the related persons of young AML patients, as reported by a study that consisted of a limited study group, who have a high risk of developing AML or MDS, concluding that in such patients germline genes may have a strong part (Goldin *et al.*, 2012). AML development risk is also linked with some hereditary disorders like Down syndrome, Fanconi anemia, Bloom syndrome (Seif, 2011).

**2.1.1 Classification of AML**

The earlier French-American-British (FAB) classification system and the more recent World Health Organization (WHO) classification system are the two systems used for the classification of AML.

As per FAB classification, AML is divided into 8 subtypes on the basis of the cell type from where leukemia originated and the degree of maturation of cells. This was done by the French, American and British leukemia experts back in the 1970-80s. This classification is as follows:

**Table 2. 1: FAB classification of acute myeloid leukemia (Kumar, 2011)**

| Acute Myeloid Leukemia | Morphological classification | % of all AML cases |
|---|---|---|
| Mo | Undifferentiated acute myeloblastic leukemia | 5 |
| M1 | Acute myeloblastic leukemia with minimal maturation | 15 |
| M2 | Acute myeloblastic leukemia with maturation | 25 |
| M3 | Acute promyelocytic leukemia | 10 |
| M4 | Acute promyelocytic leukemia | 20 |
| M4 eos | Acute promyelocytic leukemia with eosinophilia | 5 |
| M5 | Acute monocytic leukemia | 10 |
| M6 | Acute erythroid leukemia | 5 |
| M7 | Acute megakaryoblastic leukemia | 5 |

The prognostic factors that affects the patient outlook forms the basis for the WHO classification of AML. It was done in 2001 and was updated later in 2008.

**Table 2.2: WHO classification of acute myeloid leukemia (Vardiman, 2010)**

| WHO classification of acute myeloid leukemia | Subtype | |
|---|---|---|
| **AML with recurring cytogenic abnormalities** | With t(8;21)(q22;q22), (AML1/ETO) | Independent of Numbers of Blasts in PB or BM <br> PB: Peripheral Blood <br> BM: Bone Marrow |
| | With inv (16)(p13.1;q22) or t(16;16) (p13.1;q22), (CBFβ/MYH11) | |
| | Acute promyelocytic leukemia: with t(15;17)(q22;q12), (PML/RARα) and variants | |
| | AML with t(9;11)(q22;q23), MLLT3/MLL or other 11q23 (MLL) abnormalities | $\geq$ 20% Blast cells in PB or BM <br> PB: Peripheral Blood <br> BM: Bone Marrow |
| | AML with t(6;9)(p23;q34), DEK/NUP214 | |
| | AML with inv (3)(q21;q26.2), RPN1/EVI1 | |
| | AML (megakaryoblastic) with t(1;22)(p13;q13), RBM15/MKL1 | |
| | Temporary entities: AML with mutated NPM1 and AML with mutated CEBPα | |
| **AML with myelodysplasia related changes** | | |
| **Therapy related myeloid neoplasm** | | |
| **Myeloid sarcoma** | | |
| **Myeloid proliferation By Down Syndrome** | | |
| | M0 AML with minimal maturation | |

| AML, not Otherwise classified | M1 AML without maturation | |
|---|---|---|
| | M2 AML with maturation | |
| | M4 Acute myelomonocytic leukemia | |
| | M5 Acute monocytic leukemia | |
| | M6 Acute erythroid leukemia | |
| | M7Acute megakaryoblastic leukemia | |
| | Acute Basophilic leukemia | |
| | Acute panmyelose and Myelofibrose | |

## 2.1.2 Normal Hematopoiesis

Hematopoiesis is a strictly 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 (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 *et al.*, 2005).
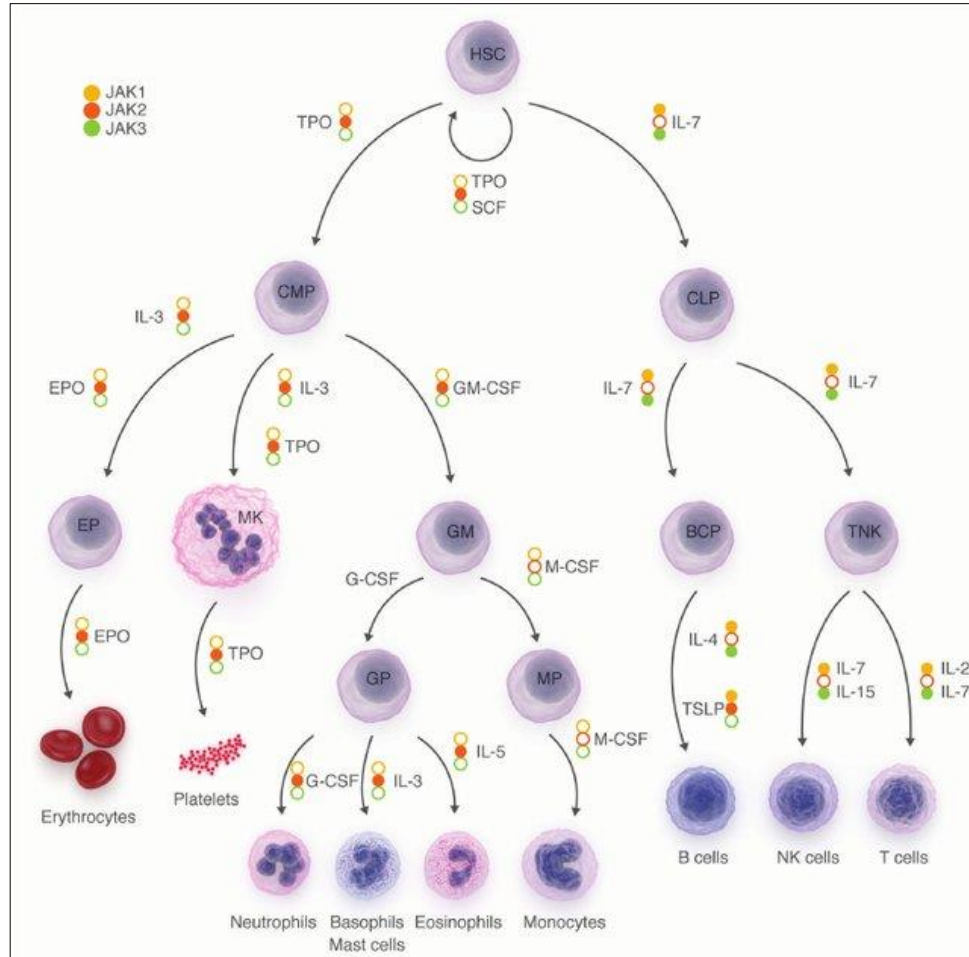
**Figure 2. 1: The Hematopoiesis (Springuel *et al.*, 2015)**

### 2.1.3 Growth factors and receptors in hematopoiesis

Two classes of receptors that control the hematopoiesis are: Receptor Tyrosine Kinase (RTK) with intrinsic tyrosine kinase activity and Cytokine receptors that lacks intrinsic tyrosine kinase activity.

Most RTKs expression can be found in human CD34+ hematopoietic stem and progenitor cells. Majority of the RTKs studied in HSPCs are members of class-III family, having an extracellular domain in the form of five Ig-like domains, for binding of ligand that also controls the substrate specificity, affinity and kinase function and an intracellular domain cleaved by a regulatory domain. Members of class-III family includes platelet-derived growth

factors (PDGFs) receptors A & B (PDGFRA & B), macrophage colony stimulating factor (M-CSF) receptor FMS, KIT receptor for Kit ligand SCF and FLT3 receptor for FL (Toffalini *et al.*, 2010). Signal transduction through these receptors begins when ligand binds to its specific receptor and activates intracellular receptor domain through phosphorylation (Lemmon *et al.*, 2010).
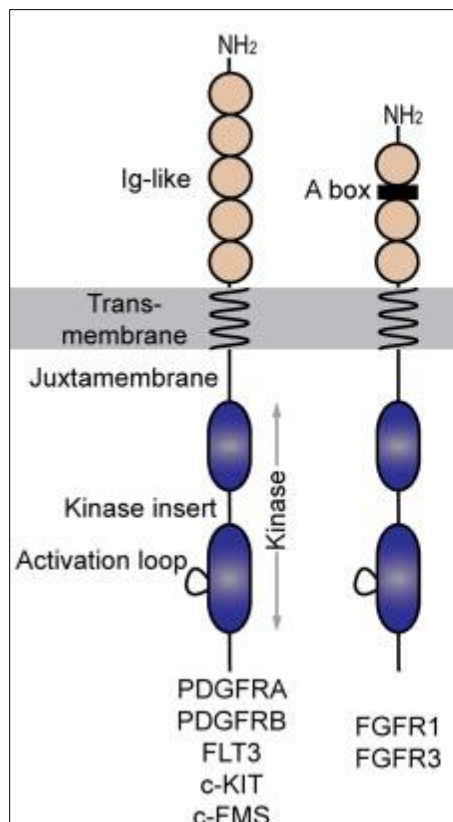


**Figure 2. 2: RTKs class-III family in hematopoiesis (Toffalini *et al.*, 2010)**

Type 1 cytokine receptors, also known as hematopoietin receptor family, are the class of receptors that lacks intrinsic kinase activity and have heterodimers of ligand binding alpha subunit and beta subunit for signal transduction (Steffen *et al.*, 2005) As a signal transduction subunit, beta-subunit relay the extracellular signal received due to ligand binding as an intracellular signal to the other signaling molecules by interacting with kinases so as to change the status from inactive to active of other players in the signaling pathway through

phosphorylation (Steffen *et al.*, 2005). Ligands for cytokine receptors include IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-1, IL-12, IL-13, IL-15, GM-CSF, G-CSF and Epo (Olofsson, 1991). JAK/STAT pathway is the most common downstream signaling pathway of this class of cytokine receptors (Touw *et al.*, 2000).
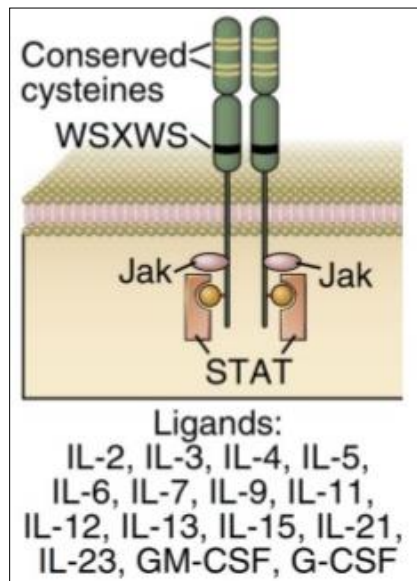


**Figure 2. 3: Type 1 cytokine receptor & its ligands (Lichtman, 1991)**

Activation of both RTKs and cytokine receptors further activates several downstream signaling networks that crosstalk and leads to the transcription of several target genes responsible for the proliferation and survival of cells (Fig.2.4). Any disruption in any of these pathways forms the basis for AML pathology.
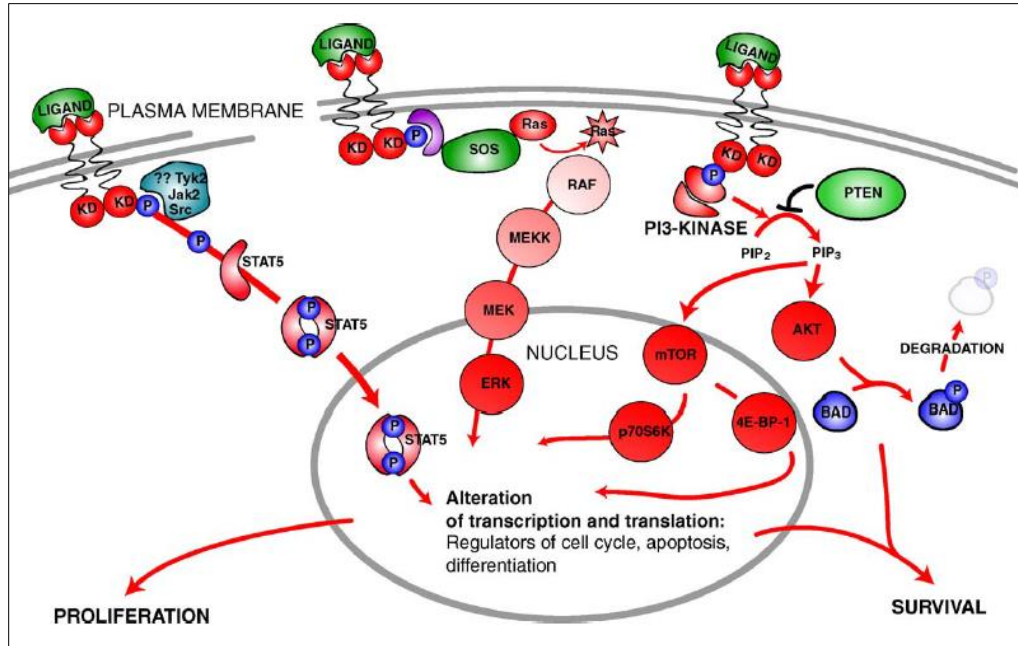
**Figure 2. 4: Signaling networks related to normal hematopoiesis (Steffen *et al.*, 2005)**

## 2.1.4 Transcription Factors in hematopoiesis

Just like for any other cellular process such as growth, proliferation, and survival, the differentiation of cells also requires transcription factors so that the signal received by upstream molecules is transcribed in the nucleus through gene expression. Such transcription factors are also required by the hematopoietic stem cells for their devotion of differentiation into diverse progenitors like myeloid and lymphoid that ultimately give rise to mature blood cells (Shivdasani *et al.*, 1996).

Two classes of myeloid transcription factors have been identified based on lineage specificity which include AML1 of class I and PU.1, c/EBPα and GATA-1 of class II (Fig.2.5) (Steffen *et al.*, 2005).

AML1 (now known as RUNX1) belongs to the CBF family and is an important transcription factor involved in the gene expression for the differentiation and maturation of mostly

monocytes (Tenen *et al.*, 1997). But AML1 is also considered non-specific transcription factor as it plays role in the development of cells of almost every hematopoietic stage and is the first factor responsible for hematopoietic stem cells formation (Licht, 2001; Steffen *et al.*, 2005). Therefore, any abnormality of this transcription factor can affect the whole process of hematopoiesis (Steffen *et al.*, 2005). This was concluded on the basis of several AML1 knock-out experiments in mice in which fetal death was observed due to loss of AML1 gene leading to serious CNS bleeding and hemorrhage (Licht, 2001). AML1 is the main gene that is observed to be disrupted in many cases of AML and translocation t(8;21) (q21;q22) is the most common translocation known in AML that leads to the production of fusion protein AML1-ETO (Licht, 2001).
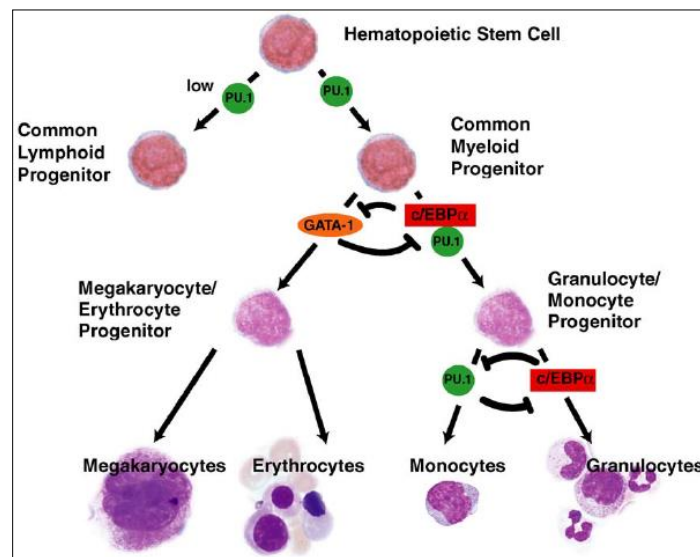


**Figure 2. 5: Role of transcription factors in different hematopoietic stages (Steffen *et al.*, 2005)**

PU.1 is another important transcription factor in hematopoiesis which is a product of SPI1 gene. This transcription factor is responsible for the differentiation of myeloid progenitors mostly through regulating expression of genes involved in the differentiation of these progenitors like the CSF1R gene encoding macrophage colony-stimulating factor receptor

(M-CSF receptor) and the gene for IL-7 receptor. However it is also observed to be involved in the differentiation of B-lymphocytes only if it is expressed in early HSCs (Takei *et al.*, 2019). In HSCs that are devoted to differentiate into myeloid progenitors, PU.1 plays role of a lineage specific transcription factor, promoting monocytes development while inhibiting the activity of c/EBPα that leads to blockage of granulocyte development (Fig.2.5) (Steffen *et al.*, 2005). Any abnormality or disruption in the function of this transcription factor can lead to blockage in the differentiation of myeloid precursors, forming the basis for AML pathogenesis. Its abnormality can also be the reason for increased self-renewal ability of erythroid progenitors and their differentiation blockage (Tenen *et al.*, 1997). Several PU.1 knockout studies have revealed stem cell failure in mice, depicting the importance of this transcription factor in hematopoiesis (Takei *et al.*, 2019).

c/EBPα is transcription factor that belongs to leucine zipper family and mainly plays its role in the differentiation of adipocytes by expressing in adipose tissues. But its expression has also been observed in hematopoietic organs like bone marrow, spleen and liver, where it is a player in the process of hematopoiesis with the role of promoting differentiation and maturation of granulocytes through the expression of G-CSF receptor and inhibiting erythroid and lymphoid cells maturation. It competes with PU.1 transcription factor by facilitating granulocyte maturation and inhibiting monocyte differentiation (Fig.2.5) (Takei *et al.*, 2019). This is justified by the study in c/EBPα knockout mice where lack of granulocytes was evident (Lourenço *et al.*, 2017) but other hematopoietic lineages i.e. myeloid was not disturbed instead myeloblasts buildup maybe seen in such mice (D.-E. Zhang *et al.*, 1997). Studies on c/EBPα have also revealed it to be a tumor suppressor since it prevents the cell proliferation (Tenen *et al.*, 1997).

The transcription factor responsible for the development of erythroid progenitors as well as megakaryocytes is the GATA-1 (Steffen *et al.*, 2005). It binds to the DNA consensus sequence (A/T)GATA(A/G) (Ferreira *et al.*, 2005). The significance of this transcription factor in the normal hematopoiesis is evident from different studies in chimeric mice in which GATA-1 was absent, due to which erythroid progenitors were unable to mature and were arrested at proerythroblast stage following apoptosis (Fujiwara *et al.*, 1996). Embryos of such mice were anemic and died at day 10. Similarly due to its role in the maturation of megakaryocytes, loss of GATA-1 can lead to incomplete maturation and functionality of megakaryocytes with an increased proliferation. As a result, there is low number of circulating platelets in blood (Ferreira *et al.*, 2005). During normal hematopoiesis, a balance is maintained between transcription factors which leads to correct differentiation of specific progenitors at the right time. During the early stage of myeloid progenitors, there is a balance between the expression of PU.1 and GATA-1 until the decision of which lineage to follow. If myeloid progenitors decide to commit for the erythroid lineage, then there is upregulation of GATA-1 that blocks the activity of PU.1, hence conducting the progenitors towards the lineage of megakaryocyte and erythroid (Fig.2.5) (Steffen *et al.*, 2005).
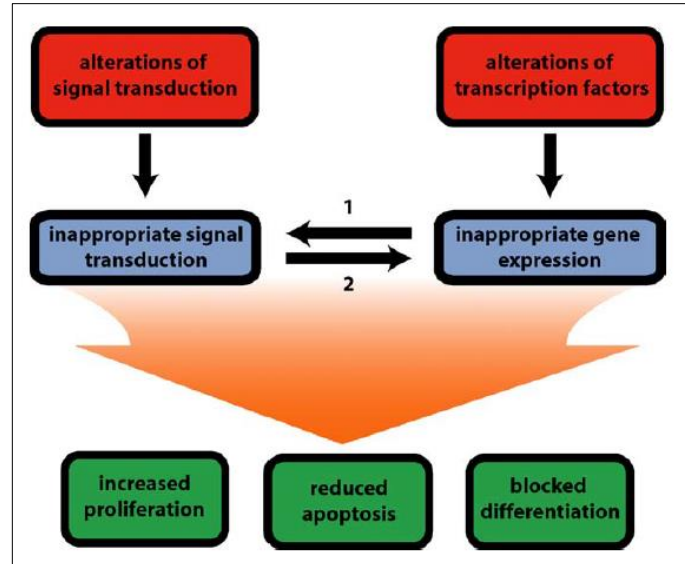
**Figure 2. 6: The interplay of different molecular alterations in the development (Steffen *et al.*, 2005)**

### 2.1.5 Molecular Pathogenesis of AML

Cell proliferation, survival, differentiation, DNA damage-repair, self-renewal ability and cell cycle control are the essential aspects of a cell's transformation and the AML pathogenesis involves alterations in almost all these aspects at the molecular level (Licht *et al.*, 2005).

Leukemogenesis leading to AML can result from two groups of molecular changings i.e. changes in the control of transcription in hematopoietic stem cells resulting in the changes in signal transduction molecules required for receptors of growth factors and mutations leading to the activation of signal transducing molecules inducing changes in many transcription factors functions and expression, that are important for normal differentiation of myeloid progenitors (Steffen *et al.*, 2005).

### 2.1.6 Abnormalities in signal transduction

Various signaling cascades are responsible for carrying out different cellular activities like proliferation, survival, differentiation, programmed cell death and any anomaly in these cascades at any point can lead to disruption of these normal cellular activities, ultimately

forming the basis for cancer. Similarly, in AML various components of these signalling pathways are seen to be mutated i.e. either upregulated due to gain-of-function mutations or downregulated due to loss-of-function mutations (Scholl *et al.*, 2008).

Of all the elements of a signaling network, most frequent activating alterations are observed in receptor tyrosine kinase (RTK) like Flt3 and c-Kit and these two RTKs serve an important role in the process of normal hematopoiesis (Steffen *et al.*, 2005). Mutations are also observed in other RTKs as well and some signaling molecules like K-Ras and N-Ras but they have little association with AML (Steffen *et al.*, 2005) JAK2 kinase and STAT. Abnormalities in any of these elements contribute to the uncontrolled proliferation of leukemic cells (Licht *et al.*, 2005).
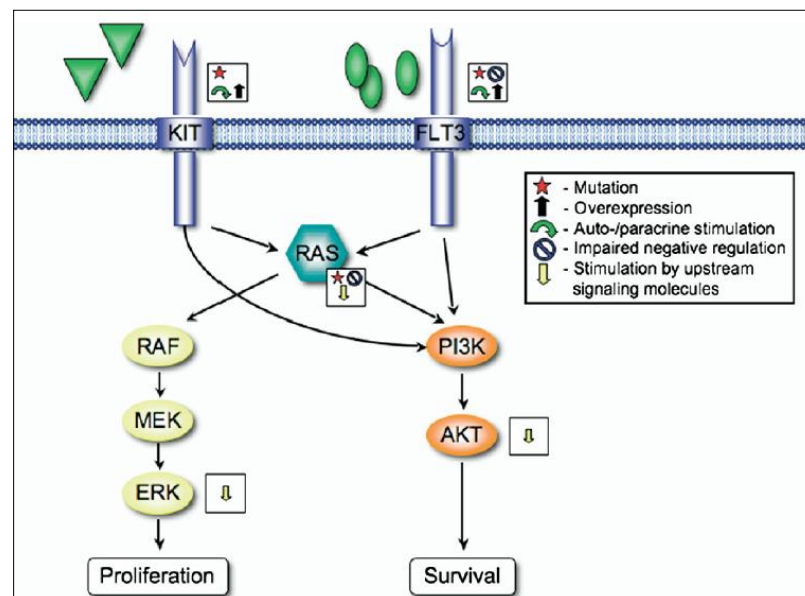


**Figure 2. 7: Abnormalities in signal transduction related to AML (Claudia Scholl, 2008)**

### 2.1.7 Abnormalities in transcription factors

Transcription factors holds great importance in normal hematopoiesis due to their role in directing the HSCs towards lineage specificity but as previously explained that this is possible

only when there is an equilibrium between the right transcription factors, leading to expression of right genes according to the specific lineage. Any disturbance in this equilibrium forms the basis for AML mainly by blocking differentiation.

Disruption of transcription factors can be a result of chromosomal translocations leading to the formation of fusion proteins (Licht *et al.*, 2005). Such fusion proteins, also referred to as oncofusion proteins, forms due to joining of a truncated transcription factor whose DNA binding domain has a function of transcriptional activation, with an irrelevant protein that functions as a transcriptional repressor. Hence the transcriptional repressor is positioned in such a way that it controls the transcription of transcriptional activator target genes that it inhibits their expression and ultimately affects their normal function (Steffen *et al.*, 2005). Out of the previously discussed balanced translocations and their products, only four are most common i.e. AML1-ETO, PML-RARα, CBFβ-MHY11 and fusions involving MLL.

### 2.1.8 Treatment Options

As the understanding of the biology of AML is achieved day by day, several novel therapies and strategies are being discovered to treat AML, whether it is through targeting the mutations at the molecular level or disrupted signaling cascades that lead to uncontrolled proliferation of immature blasts and help them escape apoptosis (Kavanagh *et al.*, 2017). The major aim in the treatment of AML is to avoid relapse by inducing remission, which means that there are <5% of leukemic cells/blasts in the blood or bone marrow followed by the improvements in the blood cells count. two phases of AML treatments are: Induction and Post induction (consolidation and maintenance) therapy (Lowenberg *et al.*, 1999).
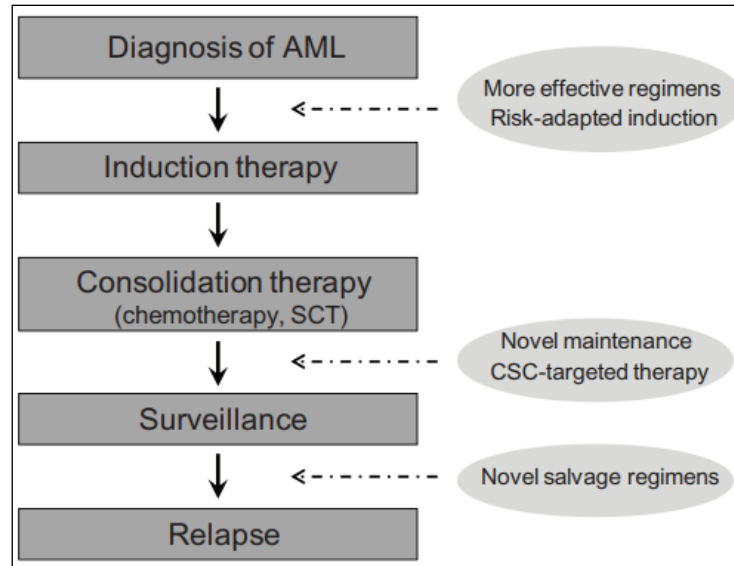
**Figure 2. 8: Abnormalities in signal transduction related to AML (Lin *et al.*, 2012)**

## 2.1.9 Induction Therapy

During induction therapy, immature blasts (leukemic cells) are eliminated and their number is brought to normal in the bone marrow as well as in the surrounding blood. During this, high doses of chemotherapeutic drugs, typically daunorubicin for 3 days and cytarabine for 7 days as per the US standard care for patients, are administered in the patient that destroy and eliminate leukemic cells (Lin *et al.*, 2012). Side effects of this therapy are killing of normal bone marrow cells as well as toxicity of other body cells like skin and GI tract. Achieving complete remission (CR) after administering induction therapy is the goal which means all kinds of tests indicate that the patient is free from leukemia.

Apart from standard induction therapy, researchers have employed strategies to develop intensive chemotherapy in order to improve response to induction therapy and to achieve complete remission at an increase rate. According to a phase III study run by the Eastern Cooperative Oncology Group, higher doses of daunorubicin were given to freshly diagnosed AML patients of ages 17-60 years (Fernandez *et al.*, 2009). An increase in CR rate (71 versus

57%, P, 0.001) and longer median survival (24 versus 16 months, P = 0.003) was seen in the patients that received elevated doses of daunorubicin, although toxicities related to different organs were also observed (Lin *et al.*, 2012).

But as per recent studies and cases, there is a need to discontinue such intensive induction therapy. Although CR rate following induction therapy in patients under 60 years of age is as high as about 70%, but it must be kept in mind that the rate of treatment related mortality (TRM) is also high with 13%, whereas the five year overall survival (OS) is low, around 30% (Mandelli *et al.*, 2009).

The main issue with the intensive induction therapy is more than its inadequate effectiveness. It is basically more detrimental than being effective. Why? Because this therapy only eliminate leukemic cells, not the preleukemic HSCs from the non-leukemic progenitors that later forms the basis for relapse after chemotherapy (Shlush *et al.*, 2014).
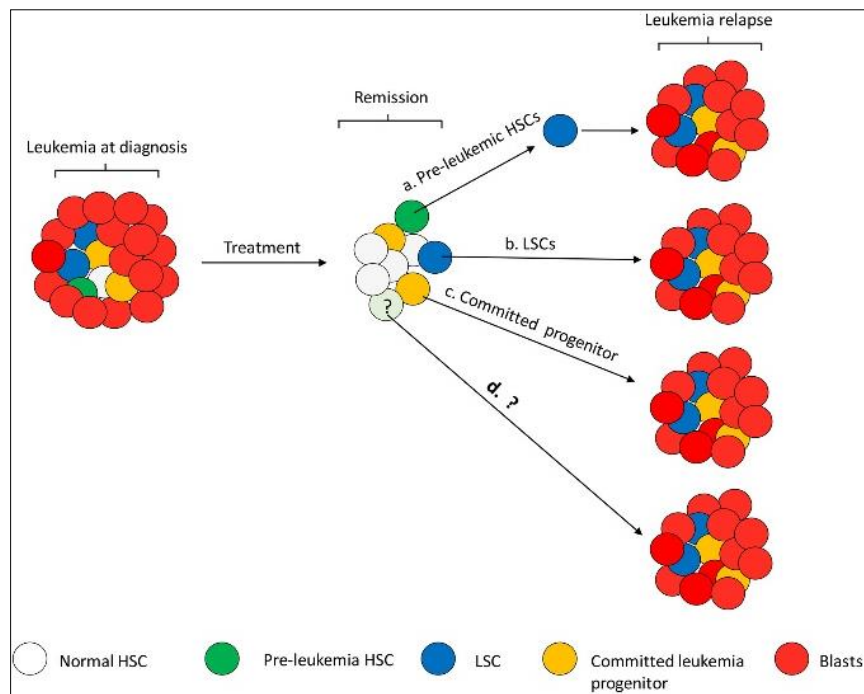


**Figure 2. 9: Relapse mechanism as understood in Leukemia (L. Jin *et al.*, 2018)**

It was found through gene expression studies of AML patients before and after cytarabine treatment that signaling cascades leading to resistance against chemotherapy were activated in such patients in response to induction chemotherapy which is also a main cause for patients going into relapse. Also LSCs heterogeneity was also seen in patients with unsuccessful induction therapy (Ho *et al.*, 2016). In conclusion, the intensive induction therapy developed to combat the AML might be aiding in making it more unfortunate and adverse. Therefore, some other strategies need to be developed for effective AML treatment.
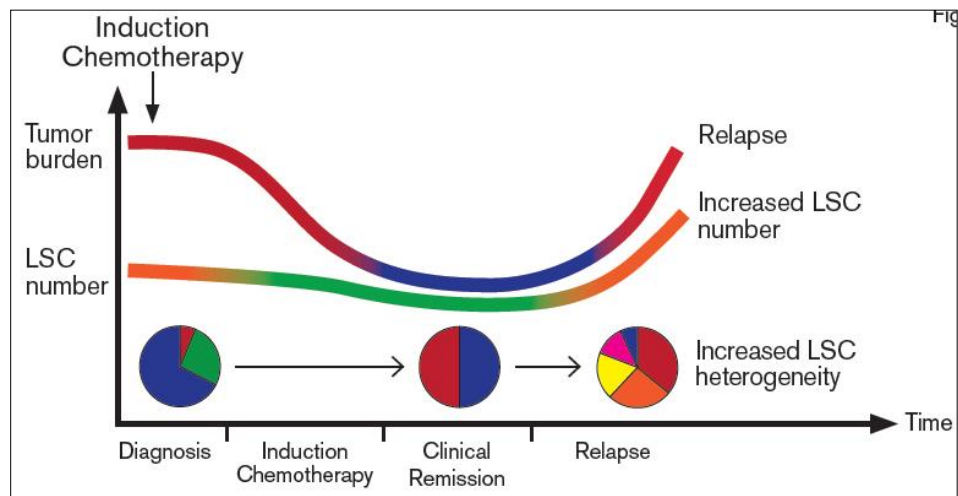


**Figure 2. 10: Effect of Intensive Induction Therapy on AML following relapse (Daniel A. Pollyea, 2017)**

### 2.1.10 Post Induction Therapy

After induction of remission, it is necessary to proceed with another intensive therapy in order to prevent relapse. Post induction therapy involves elevated dose consolidation chemotherapy and allogenic or autologous hematopoietic cells transplantation (Döhner *et al.*, 2017). In many cases, low dose maintenance therapy is also followed with the purpose of avoiding relapse (Rowe, 2009).

**2.1.10.1 High Dose Consolidation Chemotherapy**

Consolidation therapy refers to the therapy given to the patient in order to ensure complete eradication of any leukemic cells that may have been left in the body once leukemic cells have been eliminated through induction chemotherapy. It is necessary in order to complete the treatment because as per many studies, the intensive post induction therapy increases the probability of cure in patients, usually 55 to 60 years of adults, by extending the time period of remission (Rowe, 2009).

High doses of cytarabine alone and combination of different chemotherapeutics are given in consolidation therapy, both showing same results. For cytarabine the standard regime extensively followed is to administer almost 4 cycles of high-dose cytarabine (2000-3000 mg/m$^2$, 6 doses per cycle, regularly) (Döhner *et al.*, 2017). This is supported by a trial conducted by CALGB for collating three different cytarabine doses in a study that consisted of adults of up to 60 years that were given maintenance therapy afterwards. It was concluded with the results that by administering a high dose of cytarabine at 3000 mg/m$^2$ for 6 doses, a considerable refinement in the overall survival for the patients of age <60 years can be achieved. But it does not tell about the number of cycles to be given (Mayer *et al.*, 1994).

**2.1.10.2 Allogeneic Hematopoietic Stem Cell/ Bone marrow Transplantation**

In allogeneic HSC/bone marrow transplantation, stem cells from bone marrow of one individual are taken and are transplanted into leukemic patient in order to reintroduce healthy stem cells in the patient's body so that production of normal blood cells is reinitiated. Stem cells are usually taken from an HLA-compatible person in order to avoid rejection by the body. This treatment option is currently known to be the most effective and is followed for many years now. It is observed to cure leukemia in about 60% of the patients and the relapse

percentage in the patients who receive transplantation during first remission is <20% (Lowenberg *et al.*, 1999).

Despite it being a successful treatment option, not many patients opt for HSCT either due to age, organ toxicities due to previous therapies, comorbidities, unsuccessful remission and a quick relapse. A patient is usually suggested to go for allogeneic HSCT when the expected rate of relapse is >35% to 40% but without the procedure. More the expected relapse risk, more is the risk of non-relapse mortality (NRM) (Döhner *et al.*, 2017).

### 2.1.10.3 Autologous HSC/Bone marrow transplantation

During autologous HSCT, stem cells from the peripheral blood or patient's bone marrow are obtained before administration of chemotherapy by processing the blood/bone marrow in order to concentrate the stem cells. These stem cells can be cryopreserved for a long time. Then chemotherapy is given to the patient in order to destroy the leukemic cells. The stem cells obtained are then reinfused into the patient's body after the chemotherapy in order to protect the healthy stem cells from any toxic side effects of chemotherapeutic drugs.
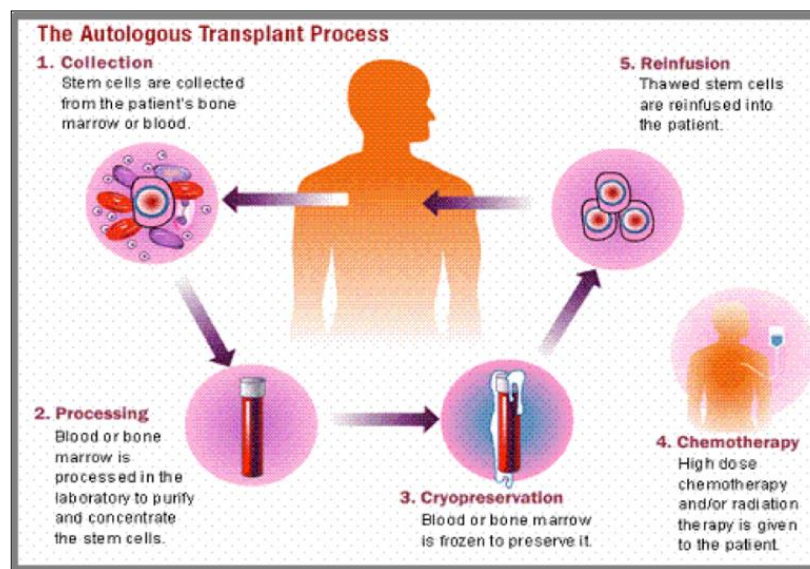


**Figure 2. 11: Autologous HSCT procedure**

More solid treatment option can be made through autologous HSCT following one cycle of intensive chemotherapy as indicated by a study which concluded that a preferable relapse free survival (RFS) and overall survival (OS) rate can be achieved through autologous HSCT (Vellenga *et al.*, 2011).

### 2.1.10.4 Maintenance Therapy

This phase of treatment is given to patients who are in remission after consolidation phase in order to make consolidation therapy completely successful and to avoid relapse. Low doses of chemotherapeutic drugs are given during this phase for months and in some cases for years. But currently this therapeutic phase is not involved in standard AML therapy since no significant evidence of its efficacy have been proven for AML (Döhner *et al.*, 2017). Its effectiveness is, however, somehow proven in acute promyelocytic leukemia (APL) cases (Embury *et al.*, 1977).

## 2.2 High Risk AML

This subtype of AML is a rare one and comprises 1% to 5% of overall AML with t(6;9)(p23;q34) chromosomal translocation that leads to the formation of DEK-CAN (NUP214) fusion protein (Shearer *et al.*, 2005) whereas 90% of patients with this translocation are positive for internal tandem duplications of the tyrosine kinase FLT3 (FLT3-ITD) (Slovak *et al.*, 2006). Due to poor understanding of the biology of this subtype and poor prognosis, high risk AML lacks an effective treatment (Sandén *et al.*, 2013). People of young age i.e.23 years, of both sexes equally are usually affected by this AML subtype. Median survival of such patients is low i.e. <1 year from the time of diagnosis (Chi *et al.*, 2008).

Immunophenotyping of t(6;9) positive AML blast cells revealed that they are positive for CD9, CD13, CD33, and HLA-DR; usually positive for CD45 and CD38; and may be positive for CD15, CD34, and terminal deoxynucleotidyl transferase (Oyarzo *et al.*, 2004).

**2.2.1 DEK-CAN (NUP214) Fusion Protein**

DEK is a distinctive non-histone, non-enzymatic protein of 43 kDa (375 amino acids) (Waldmann *et al.*, 2004). As showed by many studies, both structural and *in vitro,* DEK consists of three binding sites where it binds the DNA. Despite many studies on this protein, its biology and connection between its structure and function is not well understood. DEK localizes to the nucleus and having function in RNA metabolism as per a study, some of it associates with RNA whose kind is yet to be explored (Privette Vinnedge *et al.*, 2013). Most of the DEK protein have function in chromatin organization and through this finding it was further studied that DEK has function in different nuclear activities like replication of DNA, regulation of transcription, splicing of mRNA, DNA damage response etc. DEK on binding to its binding site on DNA is known to recruit proteins that play part in the processes of chromatin organization and DNA damage repair (Kappes *et al.*, 2001).

Nucleoporin 214 (NUP214), previously called as CAN, is a nucleoporin with FG‑repeats. This protein is found at the cytoplasmic side of the nuclear pore complex (Fornerod *et al.*, 1997). It functions in the cell cycle and is essential for the movement of the materials between the cytoplasm and the nucleus (Yang, 2014). This protein was first discovered due to chromosomal translocation between DEK or SET genes that was then found to be related to leukemia (Köhler *et al.*, 2010).

Rowley and Potter first discovered the chromosomal translocation t(6;9)(p23;q34) in AML (Rowley *et al.*, 1976). Chimeric fusion protein product DEK-CAN is formed as a result of translocation of *dek* gene at 6p23 and the *can* gene at 9q34 (Soekarman *et al.*, 1992). One particular intron on both the chromosomes contains the break points from where translocation takes place (C. Oancea *et al.*, 2010). As a result of this translocation, the normal function of these genes is hampered forming a dek-can fusion gene located on the chromosome 6p. This fusion gene encodes a chimeric mRNA that is translated into a unique 165-kd protein that plays a major role in leukemogenesis (Oyarzo *et al.*, 2004). The potential role of DEK/CAN in leukemogenesis has been demonstrated in a study by C Oancea et al in which it was shown that DEK/CAN possess leukemogenic potential, deploying its outcome on a very little subpopulation of Sca1$^+$/lin$^-$ cells (C. Oancea *et al.*, 2010).

Garcon *et al* examined 79 bone marrow and peripheral blood samples taken from 12 patients with DEK-NUP214 gene using quantitative PCR (qPCR) method. Ten out of these 12 patients had AML and two were patients of myelodysplastic syndrome. Five out of 12 patients were negative for DEK-NUP214 gene and went through allogeneic hematopoietic stem cell transplantation, four of these patients exhibited consistent molecular negativity with a follow-up time of 18.5 months (Garçon *et al.*, 2005). On the other hand, the other seven patients yielded to the disease after a median time of 12 months from diagnosis, because of not achieving DEK-NUP214 negativity. Quantitative PCR was deemed a useful method in this research for analyzing the DEK-NUP214 fusion transcript. As a result of this study it was observed that since four patients, positive for DEK-NUP214 gene survived earlier to transplantation hence allo-HSCT can potentially overcome the poor survival rate and prognosis of DEK-NUP214 fusion gene (Garçon *et al.*, 2005).

The Akt/mTOR pathway is constitutively active in DEK/CAN leukemic cells and is efficiently targeted by mTOR inhibitors. A study by Sanden *et al* showed how DEK-CAN fusion protein is involved in upregulation of mammalian target of rapamycin complex 1 (mTORC1) resulting in increased cell proliferation. However this increased cell proliferation by the chimeric DEK-CAN fusion protein is shown to be inhibited by the mTORC1 inhibitor, hence adding to the treatment options for the DEK-CAN fusion protein derived high risk AML patients (Sandén *et al.*, 2013).

## 2.3  Acute Promyelocytic Leukemia (APL)

Acute promyelocytic leukemia (APL) is a rare subtype of AML, constitute 5–15% of all AML subtypes. It is identified by the particular growth of immature myeloid precursors hindered from further growth at the promyelocytic stage (Anna Laurenzana, 2006). Signs and symptoms are anemia, infections and hemorrhages, whereas leukemic cells are also seen invading the organs in some APL cases (Anna Laurenzana, 2006). APL prognosis can be done with some aid of immunophenotyping. Over-expression of CD56 is observed in about 10% of APL cases and can be linked to increased number of WBCs. CD56 expression in different studies has been associated to an increased risk of relapse and inferior-relapse free survival (RFS), event free survival (EFS) and overall survival (OS). In about 24% of APL cases, CD2 expression was observed which in turn was linked to positive CD34 and increased number of WBCs (Ng *et al.*, 2017). Bright cytoplasmic myeloperoxidase can be seen in early malignant promyelocytes. Other markers include CD13 and CD33 while HLA-DR and CD11b are absent. Slight presence or absence of CD15, CD117 and CD34 can also be seen in malignant promyelocytes. CD9 expression can only be seen in APL out of all AML subtypes (Koshy, 2019).

**2.3.1 Etiology**

The gene actively present and always involved in APL is the RAR-alpha gene that codes for a nuclear hormone receptor that is also a transcription factor. It is based on the long arm of chromosome 17. Retinoic acid is the ligand of RAR-α and binding of RA to RAR-α aids in the expression of different genes. Majority of the APL cases i.e. about 95% involves the chromosomal translocation t(15;17) (q22;q21). Following the said translocation, the promyelocytic leukemia (*PML*) gene fuses with *RAR-α* gene and forms two fusion genes i.e. PML-RARα in case of head to tail fusion and RARα-PML in case of reciprocal fusion. In both cases, protein that is translated acts as an abnormal retinoid acid receptor.

Other chromosomal translocations associated with APL are t(5;17)(q35;q21), t(11;17)(q23;q21), t(11;17)(q13;q21), and t(17;17)(q11;q21) resulting in the fusion of RAR-alpha to the Nucleophosmin (NPM), Promyelocytic Leukemia Zinc Finger (PLZF), Nuclear Mitotic Apparatus (NuMA), and STAT5b genes, respectively, forming fusion proteins (Koshy, 2019).

**2.3.2 Role of PML/RARα in the pathogenesis of APL**

Retinoic acid (RA) have affinity for specific nuclear receptors and has a role in gene regulation after binding to these specific nuclear receptors. These nuclear receptors are three retinoic acid receptor RARα, β and γ and three retinoid X receptor RXRα, β and γ (Lo-Coco *et al.*, 2014). RXRs are required for the heterodimerization of RARs, thus initiating different signaling networks. Binding of RAR/RXR heterodimer to RA response element (RARE), that are unique DNA sequences, in the promoter regions of their target genes and through hyper acetylation and chromatin remodeling results in the transcriptional regulation of those genes (Anna Laurenzana, 2006; Lo-Coco *et al.*, 2014).

RAR/RXR heterodimer remain bound to RAREs on DNA and, also to co-repressors SMRT and NcoR, in the absence of ligand RA. As a result of this, through deacetylation of histones, there is transcriptional silencing of target genes due to increased condensation of chromatin. When the ligand RA binds to the binding site of RARα, there is conformational change in the ligand binding domain that allows the binding of co-activators TIF2 and SRC-1 that brings about the acetylation of histones and activates the transcription of target genes due to decondensation of chromatin, co-repressors are released (Fig.2.12) (Marlétaz *et al.*, 2006).
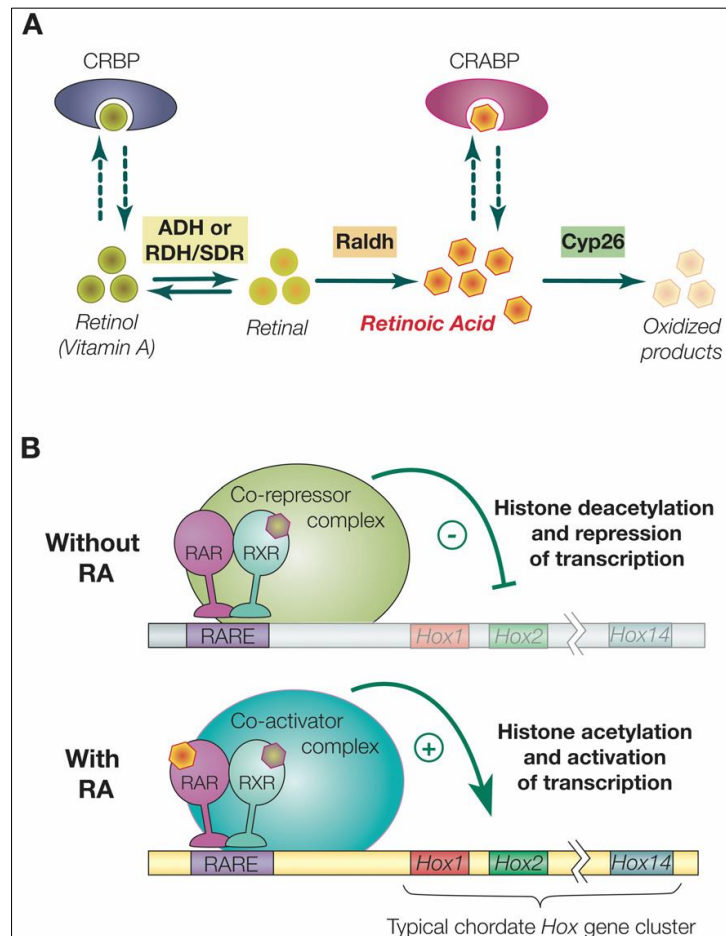


**Figure 2. 12: Retinoic Acid (RA) Signaling (Marlétaz *et al.*, 2006)**

Various membrane less structures are present in the nucleus, known as nuclear bodies (NBs) and PML-NBs are one of them. These NBs are involved in different pathways related to the

nurturing of genome like DNA damage response and repair, p53 induced apoptosis, telomere homeostasis etc. When DNA damage occurs, PML-NBs combine and increase in number by dividing through a fission process. Double-strand breaks (DSBs) in DNA are also repaired by these NBs through homologous recombination. PML protein is ubiquitous and was first founded in APL as a result pf its translocation and fusion to RARα nuclear receptor (Chang *et al.*, 2018).

It has been shown through different studies that PML/RARα fusion protein/receptor disturbs the activity of normal RARα, RXR and PML that are expressed within the same cell by normal alleles. PML/RARα forms heterodimer with RXR through the E domain of RARα part, leading to the sequestration of the RARα key partner and playing a role in the differentiation blockage of granulocytes as well as RARα-RXR pathway. PML/RARα, at normal levels of RA, associate with the corepressor complex in the nucleus (NCoR/ SMRT/HDAC), and is recruited to suppress the transcription of RAR target genes by binding strongly with PML/RARα as compared to RARα. For activation of the transcription of RARα target genes, corepressors need to be replaced with coactivators. For this replacement, much higher levels of RA are needed by PML/RARα than normal. But at pharmacological dose of RA, immature APL differentiate into granulocytes as result of PML/RARα conversion from transcriptional corepressor to coactivator of RAR target genes (Anna Laurenzana, 2006).
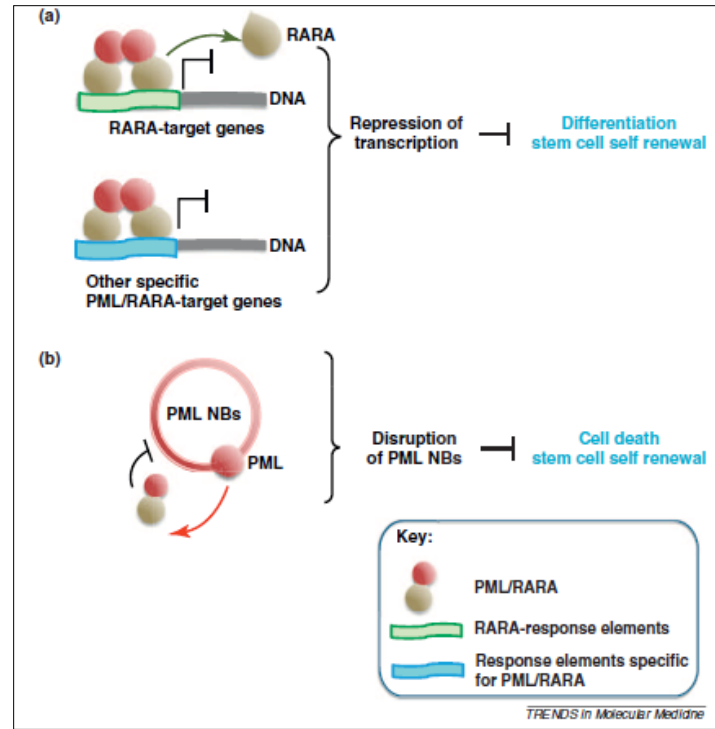
**Figure 2. 13: APL Pathogenesis (Lallemand-Breitenbach *et al.*, 2011)**

PML/RARα is an oncoprotein with gain of function as shown by different studies where it is known to bind to different DNA response elements not controlled by RARα. Hence not only there is myeloid differentiation blockage by PML/RARα but it also suppresses the DNA repair genes, whereas it leads to the activation of genes of the Wnt/Catenin and Jagged/Notch pathways, assisting multiplication and leukemic cells growth (Licht, 2006).

In order for its oncogenic potential to be fully explored, PML/RARα needs to be cleaved as shown in a study by Ley *et al* according to which neutrophil elastase cleaves PML/RARα at many positions. It was also shown that APL was developed at a much slower rate when mice that lack this protein, were crossed with mice in which neutrophil elastase was present (Lane *et al.*, 2003).

**2.3.3 Treatment Options**

Retinoic acid and arsenic trioxide are the two agents that both target PML/RARα, making APL a distinctive cancer biology model (Nasr *et al.*, 2008). Current therapy for APL is the all-trans retinoic acid (ATRA), as the inception of ATRA as a treatment option for APL for more than three decades ago now has improved the clinical outcome of this lethal disease (Ng *et al.*, 2017). ATRA along with chemotherapy is administered and a cure rate of more than 80% has been achieved (Lo-Coco *et al.*, 2014).

For extended clinical responses, it is required that RA must be in high concentrations in plasma. RA not only converts PML/RARα into a transcriptional activator from transcriptional repressor at molecular level but also promotes its breakdown which is dependent on PML/RARα cleavage by proteases. Part of RARα bound to RA also aids in the recruitment of proteases for degradation of PML/RARα (Nervi *et al.*, 1998). As reported by a study, RA is also known to block the further growth of leukemic-initiating cells (LICs) *ex vivo* while *in vivo* it plays role in removal of those LICs in PML/RARα positive APL mouse model (Nasr *et al.*, 2008).

ATRA is known to cause differentiation induction due to which a high CR rate is achieved (Asou, 2017). Several studies have demonstrated that ATRA cause no cross-resistance against chemotherapeutic drugs as observed in newly diagnosed APL patients as well as relapsed. But ATRA is also known to cause differentiation syndrome in APL patients with a swift rise in leukocytes number. Patients who receive only ATRA as APL treatment after attaining CR, are seen to undergo relapse. Due to this, combination therapy (ATRA + Chemotherapy) is employed for newly diagnosed APL patients as the advanced therapy option (Asou, 2017).

Arsenic of the arsenic trioxide ($As_2O_3$) targets and disrupts the PML/RARα fusion protein through proteasomal degradation which is important in attaining cure of APL (Lallemand-Breitenbach *et al.*, 2011) Activation of different kinases that target PML/RARα is done by arsenic which causes degradation of RARα by SUMOylation, following ubiquitination of PML part (J. Zhu *et al.*, 2005).
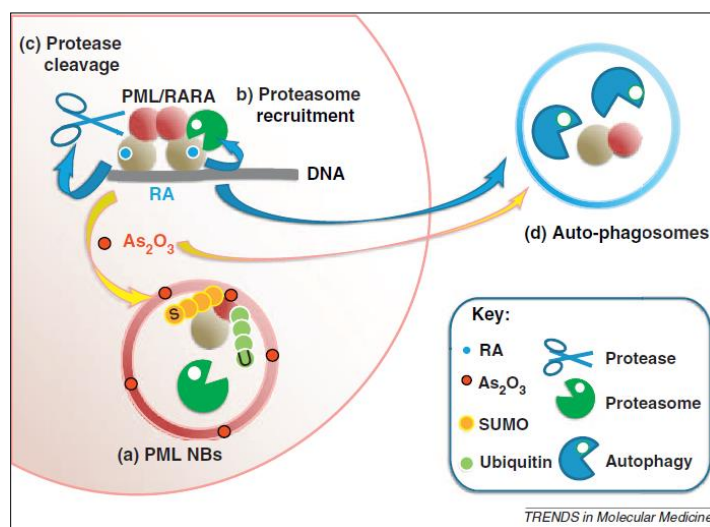


**Figure 2. 14: Four distinct pathways enforce PML/RARA degradation by As2O3 (Lallemand-Breitenbach *et al.*, 2011)**

It is shown by Ganesan *et al* that how significant micro-environment-mediated drug resistance (EMDR) to ATO is developed in APL. The method to control this EDMR was also explained, to combine ATO with a proteasome inhibitor like bortezomib. By doing so, a combined effect of the two compounds was observed *in vitro,* in Arsenic Trioxide-sensitive and Arsenic Trioxide-resistant APL cell lines. The mechanism behind this combined effect of the two compounds involved the NFκB pathway downregulation whereby increasing the unfolded protein response (UPR) and ROS production in malignant leukemic cells. Also, the combination these two compounds was more effective in clearing PML/RARα, despite bortezomib being involved in proteasome inhibition. Further studies showed that a p62-

dependent autophagy pathway aided PML/RARα. Also, induction of autophagy was supported by proteasome inhibition plus ATO (Ganesan *et al.*, 2016).
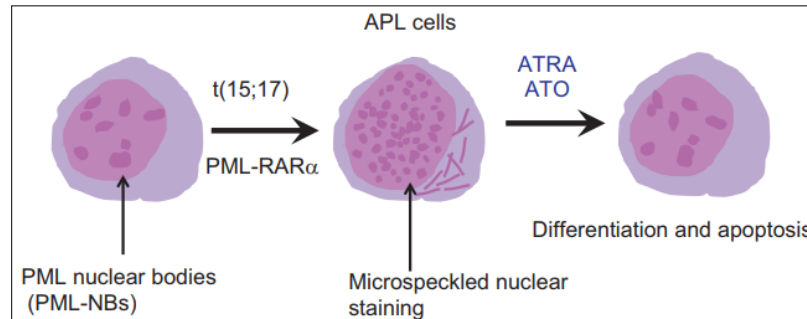


**Figure 2. 15: PML nuclear bodies and the effects of ATRA and arsenic (Asou, 2017)**

High efficacy with low hematologic toxicity has been shown in trial studies of treatment with arsenic trioxide with or without ATRA. It was shown by Lo. Coco *et al* that combination of ATRA and ATO is far more effective for the treatment of patients that have low-to-median risk APL than the combination of ATRA and chemotherapy. CR was attained in all the 77 patients that were administered ATRA plus ATO and were 100% evaluated (Lo-Coco *et al.*, 2014).

Apart from I.V administration, researches have now developed a form of ATO that can be given to patients orally as it was shown to have a good oral absorption and bioavailability of >90%, just equal to an I.V ATO dosage. This oral form of ATO was first used for the treatment of relapsed APL case, with high efficiency and toxic side effects just like I.V form of ATO (Coombs *et al.*, 2015).

An anti-CD33 monoclonal antibody, Gemtuzumab ozogamicin (GO) is known to have notable action when adjoined with toxin calicheamicin. It is due to the fact that there is high expression of CD33 target antigen on leukemic cells (Coombs *et al.*, 2015). There is uptake

of the immunoconjugate and hydrolytic delivery of calicheamicin due to binding of GO with CD33 causing permanent damage to the DNA leading to cell death. Efficacy of GO can be explained through different mode of actions in APL i.e. due to increased CD33 expression in maximum cases of APL, calicheamicin being a member of anthracycline family that is a highly effective chemotherapeutic agent against APL, lack of gp170 in APL blasts, a multidrug resistance glycoprotein that is a main cause of resistance in all AML cases except APL (Ferrara, 2010).

Tamibarotene, a synthetic retinoid, when compared to ATRA has been shown to have ten folds more potent activity by inducing differentiation of HL-60 and NB-4 cells. After administering daily, its plasma level does not drop, showing reliable pharmacokinetics (Coombs *et al.*, 2015). Shinagawa *et al* carried out a phase 3 study with the purpose of comparing Tamibarotene with ATRA, for newly diagnosed APL patients, as a maintenance therapy. CR was seen in 319 (93%) patients out of the 344 selected patients. 269 patients out of the 319, after completing three courses of consolidation therapy, underwent maintenance randomization (Shinagawa *et al.*, 2014).

## 2.4 AXL Receptor Tyrosine Kinase

AXL is a receptor tyrosine kinase that was discovered in cancerous cells about many years ago and this name AXL was originated from a Greek word *anexelekto* meaning uncontrolled. This receptor belongs to the TAM family of distinctive receptor tyrosine kinases that includes Tyro3, AXL and Mertk. Beginning of Axl expression is seen in late embryogenesis (O'Bryan *et al.*, 1991).

Having molecular weight between 100kDa and 140kDa, Axl is a transmembrane receptor that consists of an N-terminal extracellular domain containing two Ig and two fibronectin type III motifs and a C-terminal intracellular tyrosine kinase domain (Fig.2.16) (Korshunov, 2012).
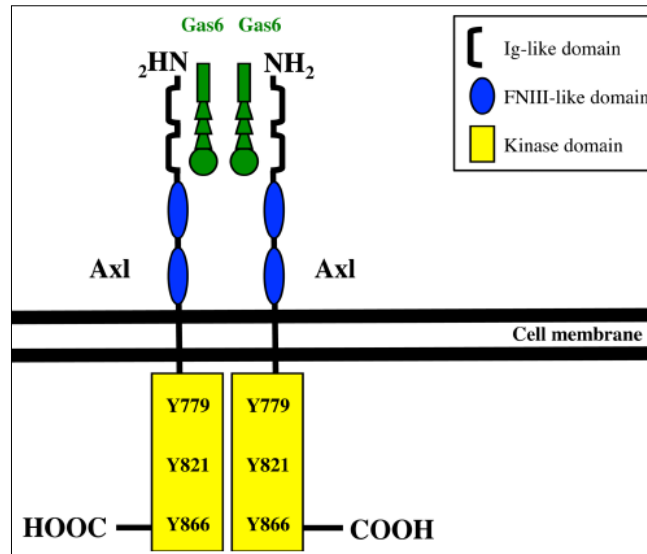


**Figure 2. 16: AXL RTK Structure (Korshunov, 2012)**

Activation of Axl can occur by different methods among which the most usual is the activation through binding of ligand Gas6 to the Axl receptor in the form of a dimer. In case of Axl overexpression or stress conditions, activation can occur even in the absence of Gas6 as observed in vascular smooth muscle cells (Myers *et al.*, 2016).

Receptor activation is followed by the activation of different signaling cascades including PI3K, MAPK and PKC and as per many studies JAK/STAT too. Axl activation, whether in the presence of ligand or absence, is known to activate these signaling cascades in different cancers like prostrate, lung, ovarian, head and neck etc. (Scaltriti *et al.*, 2016). Activation of these signaling networks leads to the activation of transcription factors that are involved in controlling cellular functions like survival and proliferation e.g. transcription of anti-apoptotic genes *, c-myc, survivin, cyclin D1, FAK* etc. by NF-kB happens as a result of IkBα-NF-kB

complex disruption directed by AKT leading to nuclear translocation of NF-kB (Scaltriti *et al.*, 2016).

GRB2 phosphorylation is followed by Axl activation, leading to the activation of RAS-RAF-MEK-ERK pathway, contributing to the proliferation of cancerous cells. Phosphorylation of PI3K by Axl activation results in increased levels of phosphatidylinositol trisphosphate leading to the recruitment and activation of AKT, which is involved in the activation of several pro-survival proteins like IKK, MDM2, or mTOR. AKT inhibits pro-apoptotic players like the BCL-2 family member BAD. Cancer cells migration and invasiveness is promoted due to the activation of FAK and phosphorylation of SRC due to direct Axl activation. Axl cross talk with other RTKs like VEGFR, EGFR, and MET may explain the role of AXL in drug resistance mechanisms as observed in few cancers. Axl role in inflammatory events is due to the upregulation of SOCS proteins and STAT-1 as a result of Axl phosphorylation (Myers *et al.*, 2016).
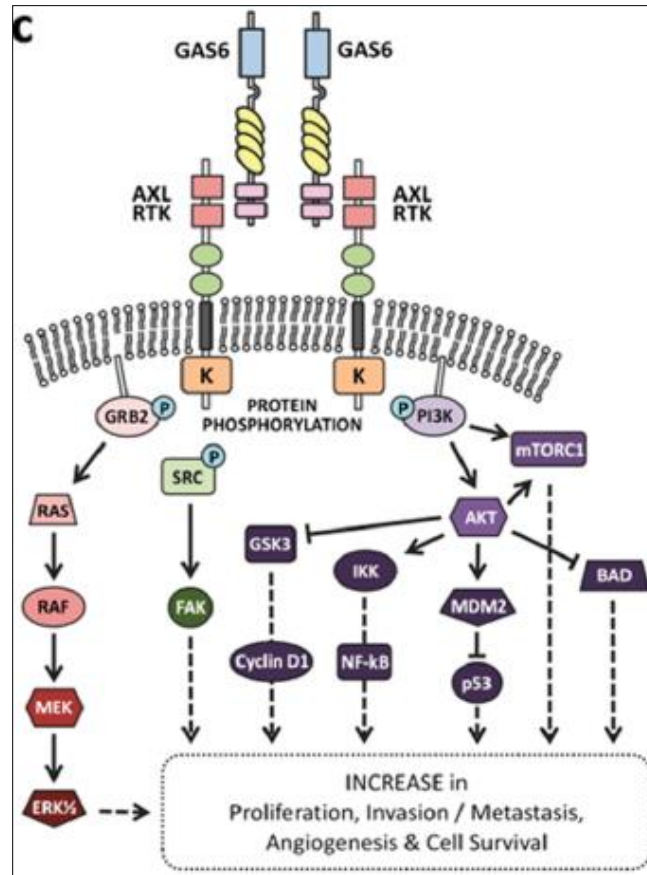
**Figure 2. 17: Downstream AXL signaling pathways due to GAS6-mediated activation (Myers *et al.*, 2016)**

Negative regulation of Axl leading to its activation occurs as a result of binding of soluble form of Axl receptor with the ligand Gas6, serving as a decoy receptor, ultimately lowering the ligand availability to bind to the transmembrane Axl receptor an,d hence blocking its activation. This soluble form of Axl receptor can be used as a biomarker for tracking the high Axl expression and beginning of drug resistance, as observed in hepatocellular carcinoma (Scaltriti *et al.*, 2016).

Importance of Axl activation was explained *in vivo* for FLT3 activation in a study and how there is growth suppression of FLT3-positive AML in humans by inhibiting Axl activation (Park *et al.*, 2013). As demonstrated in a study by Ben-Batalla *et al*, bone marrow derived stem cells (BMDSCs) are educated by AML cells in order to release Gas6 that ultimately aids

in proliferation of leukemic cells and developing therapeutic resistance due to activation of Axl-RTK (Ben-Batalla *et al.*, 2013). Hong *et al* showed that in drug resistance AML patients, expression of AXL was significantly high and that too after chemotherapy (Hong *et al.*, 2008).

Self-renewal of cancer stem cells is a major problem in cancer as these stem cells are the main agents for the relapse. Wnt/β-catenin signaling causes self-renewal of CSCs. Jin *et al* demonstrated that stabilization of β-catenin is AKT-dependent through ligation of Axl/Gas6 in CML CD34+ cells as Axl overexpression ultimately leads to increased self-renewal ability of CML LSCs, both *in vitro* and *in vivo* (Y. Jin *et al.*, 2017). Study by Wang et al concluded that aggressiveness in breast cancer is caused by Gas6/Axl with the aid of Akt/GSK-3β/β-catenin signaling (C. Wang *et al.*, 2016).

### 2.4.1 AXL Inhibitor-R428

Axl-RTK is known to be a promising target for cancer therapeutics due to its importance and role in cancer development, as previously described. For targeting Axl, small molecule inhibitors are now under research and a hot topic among researchers as a targeted therapy for several cancers (F. Chen *et al.*, 2018).

Variety of Axl-RTK inhibitors have been reported in literature but the main problem is that these inhibitors were not specific for Axl, rather they were meant for other RTKs. But due to similarities between the kinase domain of Axl and other RTKs like MET or MER, they also seem to target Axl (Myers *et al.*, 2016).

R428, also known as BGB324, is known to be the strongest, most specific and well-studied Axl inhibitor. Its mechanism of action involves blockage of Axl auto-phosphorylation on Tyr821 that is the docking site of its C-terminal, at nanomolar concentrations. It is the first

inhibitor of Axl to enter the clinical trials in 2014, on the basis of inhibition of cancer cells

metastases, both *in vitro* and *in vivo* (F. Chen *et al.*, 2018).
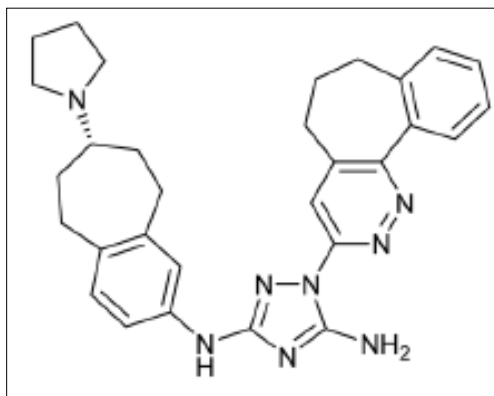


**Figure 2. 18: Structure of BGB324/R428 Axl Inhibitor (Myers *et al.*, 2016)**

A study by Hollande et al demonstrated how R428 works by blocking phenomenon that

depend on Axl like AKT phosphorylation, proinflammatory cytokines production, breast

cancer cells invasion etc. It was shown that when intracardiac mouse xenograft models of

breast cancer metastasis were given R428 orally, EMT transcriptional regulator SNAIL was

reduced in a dose-dependent manner with increased survival of the models. Also, there was

increase in the reduction rate of liver micro metastases in a murine model when combination

of cisplatin and R428 was administered (Myers *et al.*, 2016).

It was also demonstrated that when AML cell lines were treated with cytarabine and R428 or

a ligand sink that consisted of the soluble form of extracellular Axl domains, an increased rate

of apoptosis and dead cells were observed when compared to the cell lines that were treated

with cytarabine or R428 alone. Also, tumor growth was seen to be reduced in AML xenograft

model when treated with doxorubicin and R248 in combination. Though no significant effect

was to be seen on single treatment. This Axl targeted therapy can be beneficial for a large

population of AML patients as inhibition of Axl through R428 is successful despite the mutational level of FLT3 (Huey *et al.*, 2016).

## 2.5 Wnt/β-catenin Signalling

Wnt/β-catenin signalling is crucial for the self-renewal property of normal stem cells but this pathway is dysregulated in CSCs, hence leading to their abnormal self-renewal. It is classified as canonical pathway that is β-catenin dependent and non-canonical pathway that is β-catenin independent.

### 2.5.1 Activation of Wnt/β-catenin pathway

Activation of canonical Wnt/β-catenin pathway occurs when a cell secretes ligand Wnt and it binds to the Frizzled receptor along with the co-receptors, the low-density lipoprotein-related protein (LRP) 5 and LRP 6, on a nearby cell. In the absence of ligand Wnt, the scaffolding proteins Axin and adenomatous polyposis coli (APC), the kinase proteins glycogen synthase kinase-3β (GSK-3β) and casein kinase 1α (Ck1α) together forms a destruction complex that phosphorylates β-catenin leading to its ubiquitination through proteosomal degradation, hence maintaining lower levels of β-catenin in the cytoplasm (Matsui, 2016). On binding of Wnt to its receptor, intracellular domain of Lrp is phosphorylated followed by the attachment of GSK3-β and Axin to the Lrp along with the recruitment of scaffold protein Disheveled (Dvl). As result of this attachment, the destruction complex is dismantled leaving β-catenin free to move into the nucleus and activates transcription of various target genes by binding to the transcription factors, lymphoid enhancer factor (LEF)/T-cell factor (TCF) (Matsui, 2016). Morgan et al showed in myeloid leukemia that LEF-1 is a mediator of β-catenin nuclear localization (Morgan *et al.*, 2019).
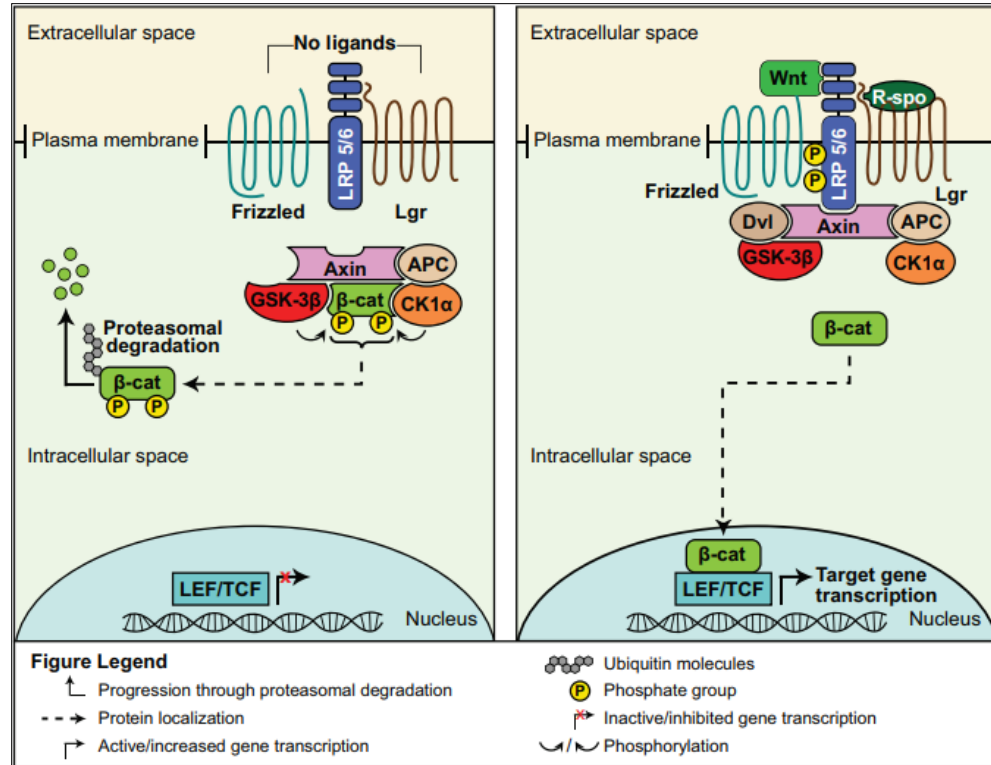
**Figure 2. 19: Canonical Wnt Signalling Pathway (Matsui, 2016)**

Fang et al demonstrated that β-catenin can have increased transcriptional activity through phosphorylation by AKT, as a result of which there is increased invasion of cancer cells and this shows how β-catenin depends on AKT for a role in cancer invasion and progression (Fang *et al.*, 2007).

Wnt pathway not only aids in the cellular proliferation and tissue development, but is also responsible for managing cellular fate/differentiation regulation (Clevers, 2006).

Various Wnt target genes have been discovered over the years in living systems, of which the most studied with respect to cancer are the *c-myc and cyclinD1* (Clevers, 2006).

## 2.5.2 CSCs and Wnt/β-catenin

The self-renewal property in both CSCs and non-CSCs is maintained by the Wnt pathway and activation of this pathway and beginning of the cancer are linked to each other. CSCs increase

in number as a result of the activation of not only Wnt /β-catenin but also Notch pathway, by activating protein-4 (AP-4), for the regulation of CSCs homeostasis as reported in colon cancer (Jaeckel *et al.*, 2018). For the stemness of skin cancer cells, maintenance of CSCs phenotype is important and is maintained by the Wnt/β-catenin signaling (Malanchi *et al.*, 2008). A positive regulator of Wnt/β-catenin pathway, Rap1 interacting factor 1 (RIF1), when over expressed, is responsible for the induction of cell cycle development that causes lung cancer cells to manifest CSC-like characteristics (Mei *et al.*, 2018). Upregulation of β-catenin cause induction of CSCs related attributes in the lung cancer cells through the action of Forkhead box C1 (FOXC1). CSCs have the ability to aid in the metastasis of cancer and that ability is due to the contribution of β-catenin. Also, switching of Wnt signaling to hedgehog also contributes in the cancer metastasis through CSCs progression (Cao *et al.*, 2018). Moreover, Wnt signaling activation through hepatocyte growth factor (HGF) released by the cancer-associated fibroblasts (CAFs) dedifferentiates cancer cells into CSCs (Najafi *et al.*, 2019). Wnt signaling was seen to be activated due to increased level of β-catenin in the cytoplasm, in the GMPs of CML patients and the blast cells of the patients that were resistant to therapy. Moreover, due to the abnormal Axin expression, β-catenin expression was inhibited that ultimately reduced the replating ability of leukemic cells *in vitro,* concluding the growth and self-renewal dependence of CML CSCs on Wnt/β-catenin signaling (Clevers, 2006).

<div align="right">

*CHAPTER 3*

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## MATERIALS AND METHODS

### 3.1 Culturing of Cell lines

All experimentation was performed on suspension cell lines:

- **NB4:** Study model for APL that carries t(15;17) PML-RARα fusion gene

- **FKH-1:** Study model for high risk AML that carries t(6;9)(p23;q34) leading to DEK-NUP214 (DEK-CAN) fusion gene

- **U937:** AML model. Used as a negative control for AXL expression

- **K562:** Model for chronic myeloid leukemia that carries Ph chromosome with Bcr-Abl b3-a2 fusion gene. Used as a positive control for AXL expression

The cells were cultured in 6-wells tissue culture plates in pre-warmed RPMI (Gibco by Life Technologies). The media was also supplemented with 10% Fetal Bovine Serum (Gibco by Life Technologies) for NB4, U937 and K562 whereas with 20% fetal bovine serum for FKH-1. Also, 1% of Penstrep (Gibco by Life Technologies) was added to cell culture medium to prevent contamination of micro-organisms.

The culture plates were kept in a humidified water-jacketed incubator at 37˚C temperature and 5% $CO_2$ to allow for cell lines growth. The cells were maintained at a density of less than $1.0 \times 10^6$ /ml at all times to avoid contact inhibition.

### 5.2 Freezing and Thawing

#### **Freezing**

For freezing/cryopreservation of cells, two solutions were made i.e. freezing solution I that contains RPMI 70% (v/v) and FBS 30% (v/v) whereas freezing solution II contains RPMI

80% (v/v) and DMSO 20% (v/v). Cells were taken in a 2ml Eppendorf and centrifuged at 1200-1400 rpm for 5 minutes. The cell pellet was then washed with PBS and centrifuged again. After discarding supernatant, the cells pellet was resuspended in 2ml of freezing solution I and 1ml of this was transferred into each cryovial. After this, 1ml of solution II was added drop wise in each cryovial and the cryovials were placed in a cryobox that contained isopropanol and stored at -80˚C. Next day the vials were shifted into another box and was placed at -80˚C.

## Thawing

Cryopreserved cells were taken out from the liquid nitrogen freezer and were rapidly thawed in incubator at 37˚C and were resuspended in culture medium. Cells were washed one time with PBS to remove DMSO and pellet was resuspended in fresh culture medium and plated in were culture plates and placed in $CO_2$ incubator at 37˚C.

## 3.3 Trypan Blue Exclusion Assay

This assay was performed before MTT assay to determine the number of cells present in the medium per ml of the medium. This was done to seed the constant amount of cells viable for MTT assay, to obtain accurate results.

A dilution of 1:1 of the cell suspension was prepared with 0.4% trypan blue staining solution (Gibco by Life Technologies). The counting chambers of a hemocytometer were loaded with 10μl of the dilution. All cells in the four grids of the chamber were counted. Non-viable cells were stained blue. Following calculations were done to calculate the number of cells to be used for MTT assay:

*Number of cells in grid 1 + grid 2+ grid 3+ grid 4 × Dilution factor x $10^4$ cells/ml*

*4*

## 3.4 MTT Assay

This assay was used to evaluate the cell viability and proliferation by the conversion of tetrazolium salt, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] to Formazan. This reduction is catalyzed by mitochondrial dehydrogenase enzymes and is therefore an indicator of cell viability (Riss TL, 2013).

Cells were seeded at a density of 1.0 x $10^4$ cells/50 µl into 96 well plate followed by the addition of 50µl/well of different concentrations of R428 (10mM) (Med Bio Express), ATRA (3.3mM) and Imatinib (1mM) (Sigma Life Technologies) in µM in triplicates. R428, ATRA and Imatinib were allowed to produce their effect on the cells for about 48-72 hours. After completion of the required time duration, cell viability was assessed by the addition of 15µl of filter sterilized MTT (5 mg/ml in PBS) to the microtiter wells containing cells and R428, ATRA and Imatinib concentrations and plates were again kept in $CO_2$ incubator for about 3-4 hours to allow crystals formation. 50µl of media was then removed carefully without disturbing the crystals after 3-4 hours. 150µl of DMSO was added to dissolve the crystals. After complete dissolution of crystals, the absorbance at 550 nm was measured with a spectrophotometric plate reader.

## 3.5 Treatment of cells with R428 for mechanism studies

Cells (NB4, FKH-1, K562 and U937) were cultured in liquid medium (RPMI +10/20% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and different concentrations of R428 (0.62µM, 1.25µM and 2.5µM) for 72-96 hours. RNA was then extracted and cDNA was then synthesized for gene expression studies through real time PCR (qPCR).

**3.6 Gene Expression Profile Analysis**

**3.6.1 RNA Extraction for Gene Expression Studies**

About 0.5-1 million cells per 2ml were taken in a 2ml Eppendorf and were centrifuged at 1500 rpm for 5 minutes. Supernatant was then removed and pellet was obtained. 1ml TRIZOL LS reagent (Life Technologies) was added in the pellet and homogenization of cells was done by pipetting the cells up and down. The homogenized cells were then incubated on ice for about 5 minutes to assure the separation of nucleoprotein complexes. 200µl of chloroform was then added and vigorous shaking was done for 15 seconds. The tube was then allowed to stand on ice for about 10 minutes. Centrifugation was then done at $12,000 \times$ g for 20 minutes at 2-4˚C. The mixture was separated into three phases as a result of centrifugation: a pink organic phase containing proteins, an interphase containing DNA and an upper aqueous phase containing the required RNA. Aqueous phase was picked carefully and transferred into another Eppendorf in which 500µl of chilled isopropanol was added and tubes were shaken. Another incubation on ice was done for 15 minutes followed by centrifugation at $12,000 \times$ g for 20 min at 2-4˚C. Supernatant was discarded and the pellet was washed with 1ml of 75% chilled ethanol followed by vortex and centrifuge at $7500 \times$ g for 5 min at 2-4˚C. Supernatant was then removed and RNA pellet was air dried at room temperature in the hood. 20µl of nuclease free water was then added to resuspend the RNA pellet and was stored at -80 ℃ until further processing.

**3.6.2 RNA quality and quantity check**

The quality of RNA was checked by 2% agarose gel electrophoresis by visually examining the quality of bands. The extracted RNA was quantified using Nanodrop 2000 (Thermoscientific, USA) and purity was confirmed through 260/280 ratio check.

**3.6.3 Complementary DNA synthesis**

1000 ng of RNA template was used for the reverse transcription of the template to cDNA. The procedure was carried out as follows: 1μl oligo dT primers (10μM) were added to a 0.2ml micro tube followed by the addition of 2μl dNTPs, 1μl RNase inhibitor, 1μl DTT and 4μl first strand synthesis buffer. 1000 ng of RNA template and 1μl of reverse transcriptase were then added followed by the addition of NF water as per required to make total volume of 20μl. The tubes were then placed in a thermocycler and the conditions were followed as per kit manufacturer's instructions (Solis BioDyne).

**3.6.4 cDNA quality check and primer optimization by conventional PCR**

cDNA quality was checked and primer optimization for *GAPDH* (housekeeping gene), *AXL*, *c-myc, Axin2 and Hif-1α* was done by conventional PCR. Primers for *AXL, c-myc* and *Axin2* were selected through literature review (Y. Jin *et al.*, 2017) whereas primer for *GAPDH* was kindly provided by Dr. Aneela Javed and that for *Hif-1α* by Dr. Maria Shabbir. Primer parameters were checked using Primer Blast.

**Table 3.1: Primers properties for the amplification of genes of interest**

| Primer | Sequence | GC% | Annealing Temperature |
|---|---|---|---|
| GAPDH-Forward | CCTGCACCACCACTGCTTA | 57.8% | 59.9˚C |
| GAPDH-Reverse | CATGAGTCCTTCCACGATACCA | 50.0% | 59.5˚C |
| AXL-Forward | GTCGGACCACTGAAGCTACC | 60.0% | 60.1˚C |
| AXL-Reverse | CATCGTCTTCACAGCCACCT | 55.0% | 60˚C |
| c-myc-Forward | CAGCGACTCTGAGGAGGAAC | 60.0% | 59.8˚C |
| c-myc-Reverse | TCGGTTGTTGCTGATCTGTC | 50.0% | 58.2˚C |

| Axin2-Forward | TCAAGTGCAAACTTTCGCCAACC | 47.8% | 62.8 ˚C |
|---|---|---|---|
| Axin2-Reverse | TAGCCAGAACCTATGTGATAAGG | 43.4% | 57 ˚C |
| Hif-1α-Forward | CAGATCTCGGCGAAGTAAAG | 50.0% | 60.4˚C |
| Hif-1α-Reverse | TCACAGAGGCCTTATCAAGATG | 45.5% | 60.8˚C |

1000 ng of cDNA was used as a template and reaction mixture (20μl) for PCR was prepared in 0.2ml tubes. Reaction mixture contained 1000ng of sample cDNA, 2.5μl (10X) of PCR buffer, 2μl of 25mM MgCl2, 1.5μl of 10 mM dNTPs, 1μl of each forward and reverse primers, 0.4μl of Taq DNA polymerase and 11.6μl of nuclease free water. Thermocycling conditions were; denaturation at 95˚C for 5 minutes followed by 35 cycles of PCR amplification. Each cycle further consisted of 3 steps: 1 minute at 95˚C for denaturation of template DNA, 45 seconds at 55˚C for annealing of primers to their targets on template and 1 minute and 45 seconds for extension of the cDNA strand from annealed primers. Unextended strands were allowed for synthesis by Taq polymerase for 10 minutes at 72˚C. PCR products were then stored at -20˚C. PCR products were analyzed using 2% agarose gel through gel electrophoresis and bands were visualized through Dolphin Gel Doc System.

### 3.6.5 Real time PCR (qPCR) for gene expression analysis

Primers for the genes of interest were analyzed for specific amplification of product at 10mM by real time PCR (Applied Biosystems 7300). The reaction mixture was prepared by adding 0.5μl of cDNA, 0.5μl of each forward and reverse primers (10mM) and 2μl of SYBR Green Master Mix (5X) (Solis BioDyne). Reaction volume was made up to 10μl by adding 6.5μl nuclease free water. The reaction was completed in three major steps. First the reaction mixture was incubated for 2 minutes at 50˚C, second incubation was at 95˚C for 10 minutes

followed by 40 cycles of PCR. Each cycle was completed in three incubation steps, one for 15 seconds at 95˚C and other for 45 seconds at 60˚C and third for 30 sec at 72˚. For melt curve analysis, dissociation stage was added. Three incubations were used for dissociation stage; first at 95˚C for 30 seconds, second incubation at 60˚C for 30 seconds and last incubation for 30 seconds at 95˚C. The data was collected at 72˚C. Data analysis was done by ABI 7300 system SDS software. GAPDH primer was used as a housekeeping gene for normalization. For relative quantification, $2^{-\Delta\Delta C}$ method was used for relative quantification of gene expression.

### 3.6.6 Statistical Analysis

All experiments were performed in triplicates (MTT assay) and replicates (Real-time PCR) and results are presented as mean ± SEM. The data was analyzed using one-way and two-way ANOVA and *p*-value less than 0.05 was taken as an indicator for significant difference. The graphical data was generated using the Graphpad Prism 5.01 software.

### 3.7 DNA fragmentation assay for apoptosis analysis

Cells (NB4, FKH-1, K562 and U937) treated with R428 were taken in a 2ml Eppendorf and were centrifuged for 5 minutes at 2000 rpm. The supernatant was then discarded and pellet was resuspended in 500µl lysis buffer. Tubes were mixed by inversion and were incubated at room temperature for 5-10 minutes. The tubes were then centrifuged for 1 minute at 13000 rpm. The supernatant was discarded and the pellet was again resuspended in 400µl of lysis buffer, followed by centrifugation at 13000 rpm for 1 minute, then incubation at 60˚C for 5 minutes. Then after cooling of the sample, 500µl of chloroform-isoamyl alcohol was added followed by centrifugation at 13000 rpm for 10 minutes. The upper aqueous phase was carefully picked and transferred into 1.5ml Eppendorf. Equal volume of chilled isopropanol

was added and tubes were inverted several times to facilitate DNA precipitation. Centrifugation was then done for 10 minutes at 13000 rpm followed by discarding of supernatant and washing of DNA pellet with 200μl of 70% ethanol and again centrifugation at 13000 rpm for 7 minutes. Ethanol was discarded completely and DNA pellet was air dried for about 30 minutes. 40μl of nuclease free water was used to dissolve the DNA pellet. Quantification of the extracted DNA was done on Nanodrop 2000 (Thermoscientific USA). The DNA samples mixed with loading dye were then run on 1.5% agarose gel and the gel was examined on ultraviolet gel documentation system (Rahbar Saadat *et al.*, 2015).

# RESULTS

## 4.1 Culturing of Cell lines

All the four cell lines i.e. NB4, FKH-1, K562 and U937 were successfully revived, cultured

and maintained at the conditions described previously with maximum viability.
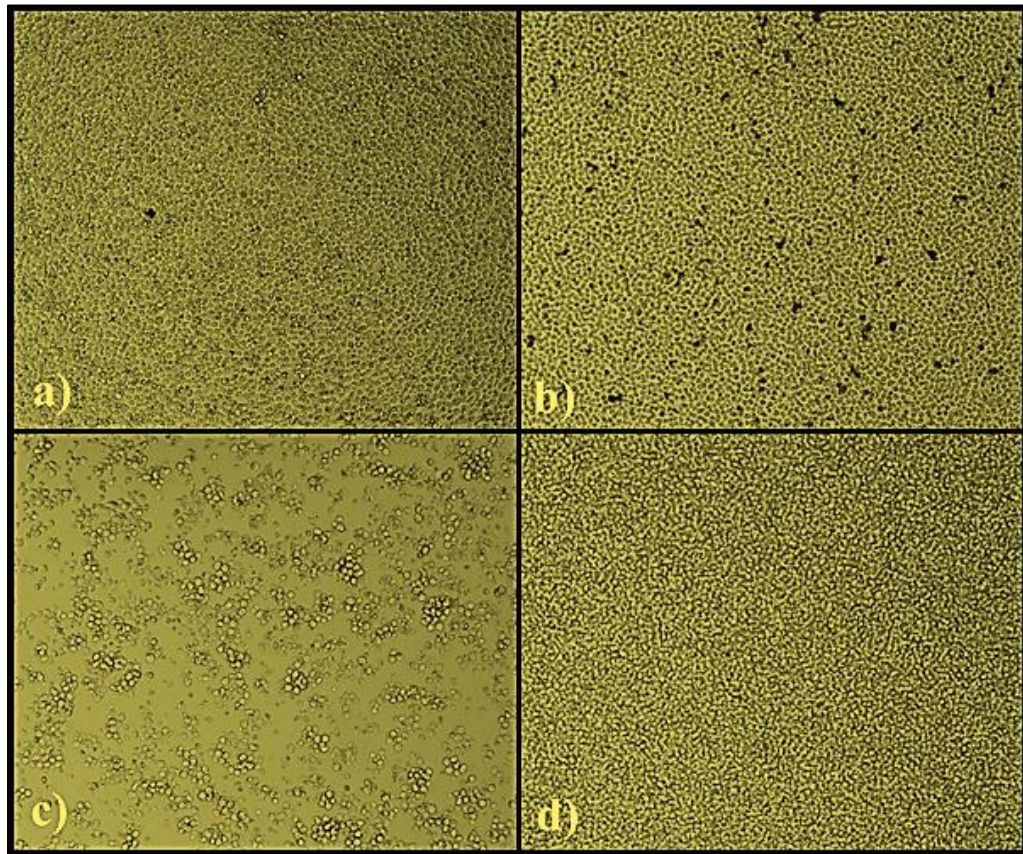


**Figure 4. 1: Cell lines a) NB4 b) FKH-1 c) K562 d) U937.** Cells were cultured in liquid medium (RPMI + 10% & 20%
FBS+1% L-Glutamate and 1% Pencillin and Streptomycin).

**4.2 AXL-RTK gene expression analysis**

**4.2.1 RNA Extraction for gene expression studies**

For the gene expression study of AXL-RTK in cell lines, RNA was extracted followed by its

quality and quantity check by running on 2% agarose gel and visually examining the 28S and

18S bands.
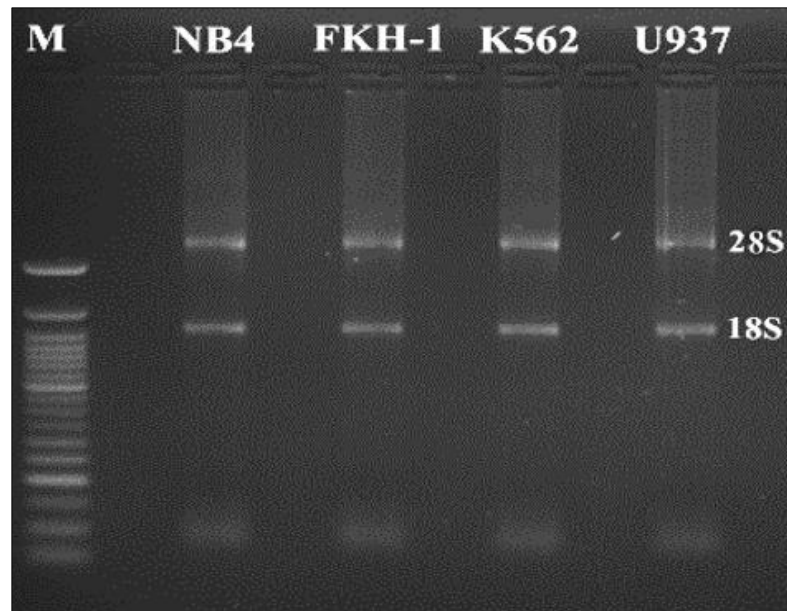


**Figure 4. 2: Gel image of RNA extracted from NB4, FKH-1, K562 and U937.** Cells were cultured in liquid medium (RPMI + 10% & 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). RNA was extracted using TRIZOL method

**4.2.2 cDNA quality check and AXL primer optimization by conventional PCR**

After the cDNA synthesis from the extracted RNA, primers for *GAPDH* and *AXL* were

optimized by using conventional PCR.
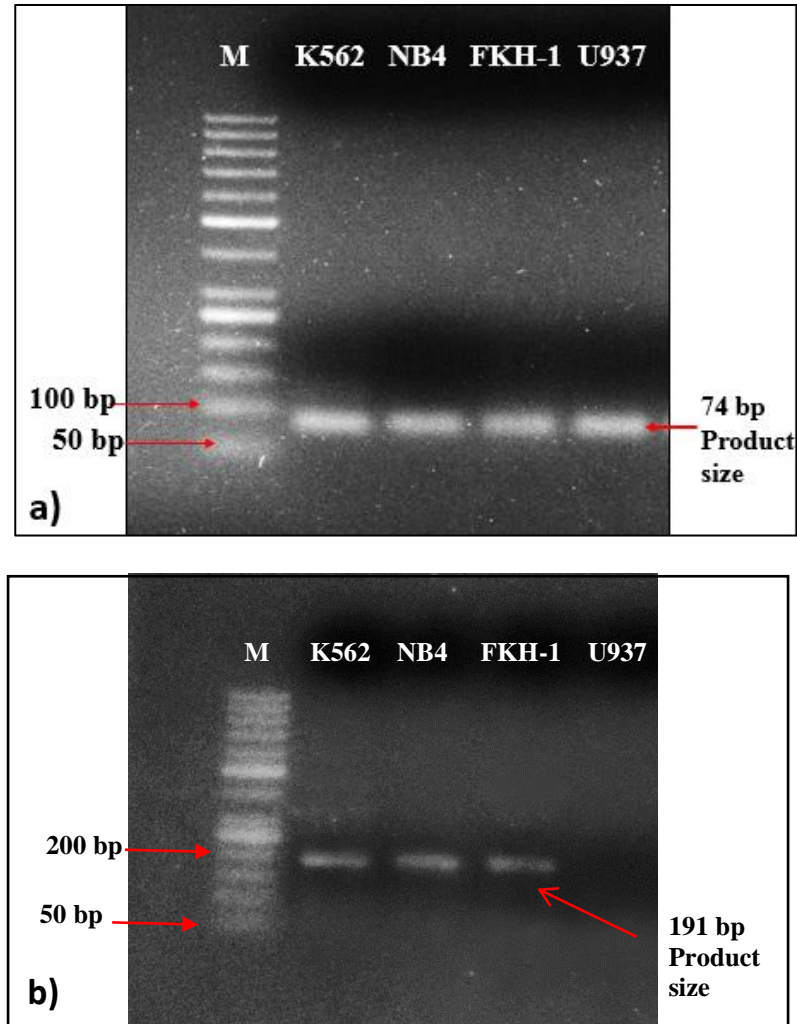
**Figure 4. 3: PCR amplification of primers optimization a) GAPDH b) AXL-RTK.** Cells were cultured in liquid medium (RPMI + 10% & 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). RNA was extracted and cDNA was prepared using standard protocols. Primers were optimized through gradient PCR

## 4.2.3 Gene expression analysis for AXL-RTK by real time PCR (qPCR)

After the primer optimization, real time PCR was performed using SYBR Green method to analyze the gene expression of AXL-RTK in NB4 and FKH-1 cells. K562 was taken as a positive control for AXL-RTK expression (Y. Jin *et al.*, 2017) and U937 was taken as a negative control for AXL-RTK expression. Data was analyzed using ABI 7300 system SDS software. *GAPDH* primer was used as a housekeeping gene against AXL-RTK.
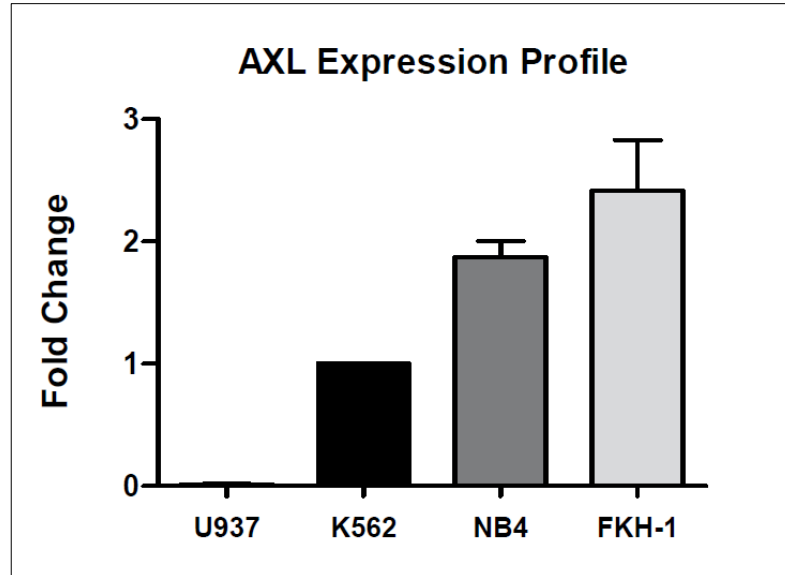
**Figure 4. 4: Expression analysis of AXL-RTK in U937, K562, NB4 and FKH-1.** Cells were cultured in liquid medium (RPMI + 10% & 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). K562 used as positive control and U937 used as negative control RNA was extracted and cDNA was prepared. Expression analysis of AXL-RTK was done through real time PCR.

Figure 4.4 shows significant expression of AXL-RTK in NB4 and FKH-1. K562 was taken as a positive control for AXL-RTK expression whereas U937 was taken as negative control so no AXL-RTK expression was seen. Hence AXL-RTK was observed to be expressing in PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells.

## 4.3 Pharmacological Targeting of AXL-RTK in NB4 and FKH-1 by R428

### 4.3.1 R428 interferes with the proliferation potential of PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells

AXL is a receptor tyrosine kinase that is seen to be overexpressed in many cancers including AML (O'Bryan *et al.*, 1991). Activation of AXL-RTK leads to the activation of different downstream signalling cascades including PI3K, MAPK, PKC and JAK/STAT and these pathways in turn leads to the activation of transcription factors that are involved in controlling the cellular proliferation e.g. transcription of anti-apoptotic genes, *c-myc, survivin, cyclin D1*

etc. (Scaltriti *et al.*, 2016). So after the confirmation of AXL-RTK expression in NB4 and FKH-1 cell lines, pharmacological targeting of AXL-RTK was performed with R428 at increasing concentrations in μM and proliferation was assessed through MTT assay after 72 hours in NB4 and after 96 hours in FKH-1 cells. R428 is known to be the first small AXL-RTK inhibitor that entered into the clinical trials in 2014 (F. Chen *et al.*, 2018) and is known to inhibit proliferation and induce apoptosis in cancer cells with special mention to AML (Huey *et al.*, 2016). Our result indicates a significant reduction in the proliferation potential of NB4 and FKH-1 cells in a dose dependent manner as shown in figure 4.5c and 4.5d respectively.
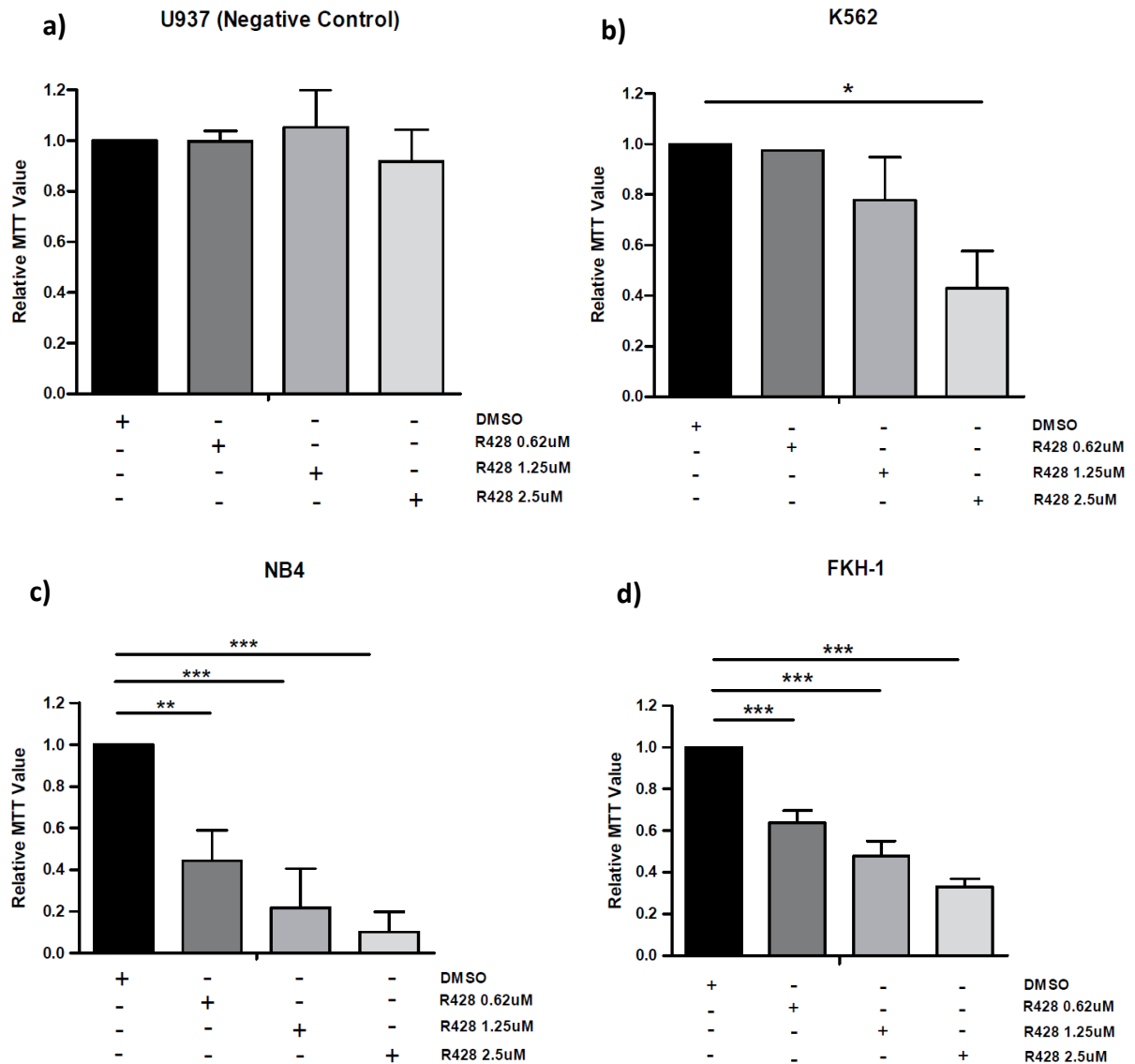
**Figure 4. 5: Effect of R428 on the proliferation potential of a) U937 (negative control) b) K562 (positive control) c) NB4 d) FKH-1 through MTT assay.** Cells were cultured in liquid medium (RPMI + 10% & 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NB4 and FKH-1 cells in the presence of 0.01% DMSO and indicated concentrations of R428. Statistical significance was tested using One-Way ANOVA (*p*-values <0.05 are statistically significant). IC-50 for NB4 was calculated to be 0.62μM whereas IC-50 for FKH-1 was calculated to be 1.25μM. Bars show mean ± SEM.

**4.3.2 Comparison between the anti-proliferative effect of R428 and ATRA on NB4 cells**

As discussed that ATRA is the current standard of care for PML/RARα-positive APL as the inception of ATRA as a treatment option for APL for more than three decades ago now has improved the clinical outcome of this lethal disease (Ng *et al.*, 2017). Though when ATRA along with chemotherapy was administered, a cure rate of more than 80% was achieved (Lo-Coco *et al.*, 2014). But ATRA is known to cause differentiation syndrome in APL patients 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 4.5) that R428 is able to interfere with the proliferation of NB4. So, we compared the effects of ATRA and R428 on the cell proliferation potential of NB4 at different concentrations in μM and proliferation was assessed through MTT assay after 72 hours. A significant reduction in the proliferation ability of NB4 by R428 was seen in a dose dependent manner when compared to ATRA. Hence R428 was found to be more potent than ATRA, showing more anti-proliferative effect.
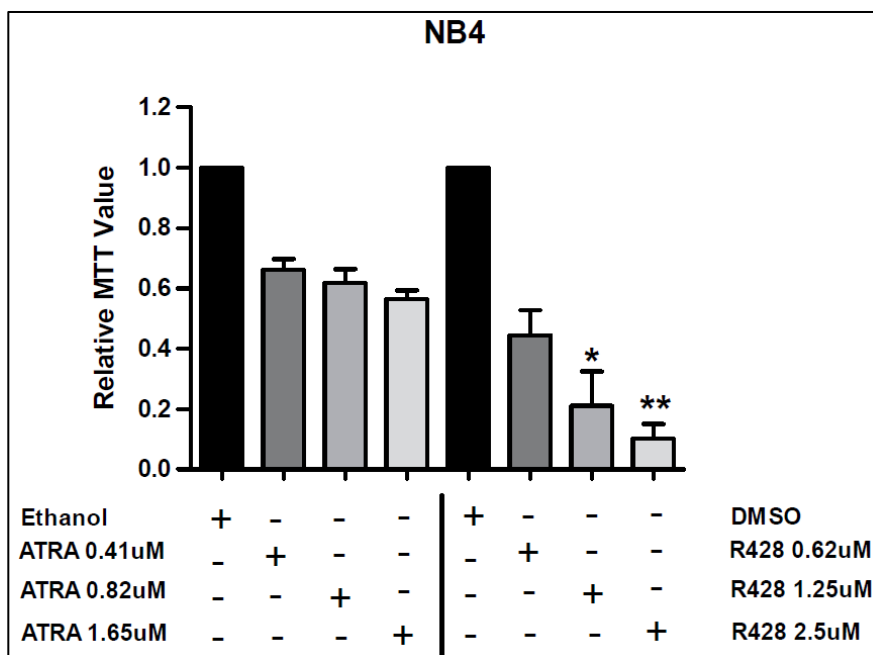
**Figure 4. 6: Comparison between ATRA and R428.** ATRA and R428 were compared for their effect on the cell proliferation potential of PML/RARα-positive NB4 cells. Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NB4 cells in the presence of 0.01% DMSO and indicated concentrations of R428 and ATRA. Cell proliferation was assessed through MTT assay after 72 hours. (*p*-values <0.05 are statistically significant). Bars show mean ± SEM

### 4.3.3 Anti-proliferative effect of R428 vs Imatinib on FKH-1 cells

It has been shown that ABL is active in DEK/CAN-positive FKH-1 cells (unpublished data). Our result showed that AXL is strongly active in FKH-1 cells. Therefore we were interested to disclose the role of AXL in the proliferation of FKH-1 cells and compare with ABL. We targeted FKH-1 cells with Imatinib as well as R428 at different concentrations in μM and proliferation was assessed through MTT assay after 96 hours for comparing the anti-proliferative activity of the two treatments (Figure 4.7, Left: R428 and Right: Imatinib). No significant difference between the anti-proliferative ability of the two treatments was seen. Both the treatments show almost equal effect on the cell proliferation ability of FKH-1 cells in a dose dependent manner. Hence both, R428 and Imatinib, might be an effective therapeutic option in the future through more research and validations.
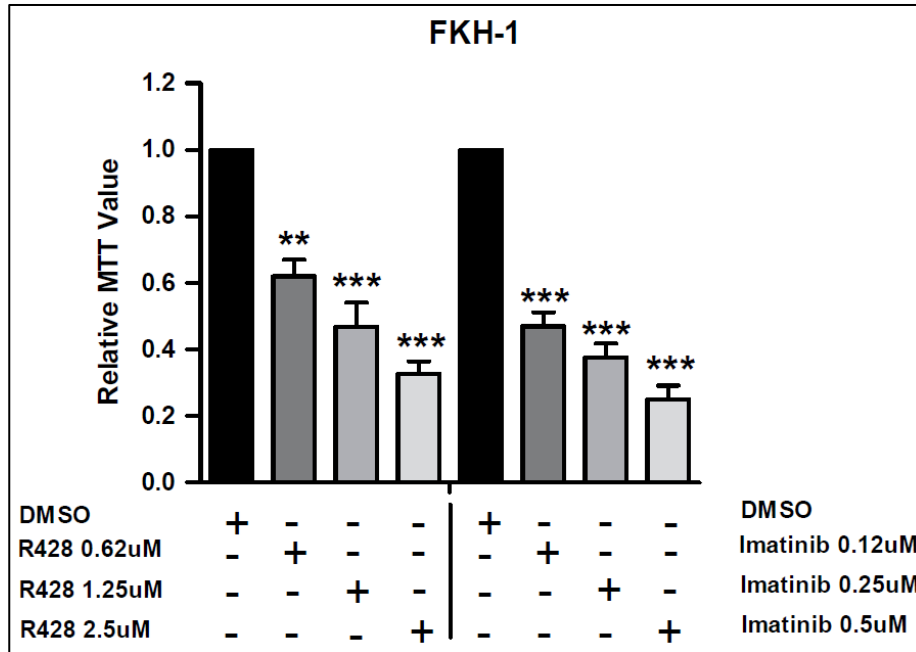
**Figure 4. 7: R428 (Left) vs Imatinib (Right).** The two drugs were compared for their anti- proliferative effect on DEK/CAN-positive FKH-1 cells. Cells were cultured in liquid medium (RPMI + 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NFKH-1 cells in the presence of 0.01% DMSO and indicated concentrations of R428 and Imatinib. Proliferation was assessed by MTT assay after 96 hours. ($p$-values <0.05 are statistically significant). Bars show mean ± SEM

### 4.3.4 Effect of combined treatment on the proliferation potential of NB4 and FKH-1 cells

ATRA along with chemotherapy is known to be the standard of treatment for PML/RARα-positive APL patients due to its promising results but as discussed above ATRA is known to cause differentiation syndrome in APL patients 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 the significant reduction in the proliferation ability of NB4 cells after treatment with R428 as compared to ATRA, we investigated the effect of combined treatment (R428 + ATRA) to see if there is any additive effect 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. Taken together our data showed (Figure 4.8) that combined treatment had a little significant effect on the cell proliferation of PML/RARα-positive NB4 by keeping concentrations of ATRA low as compared to the single treatments i.e. combination treatment with R428 and ATRA does showed additive anti-proliferative effects by targeting AXL and RARα respectively.
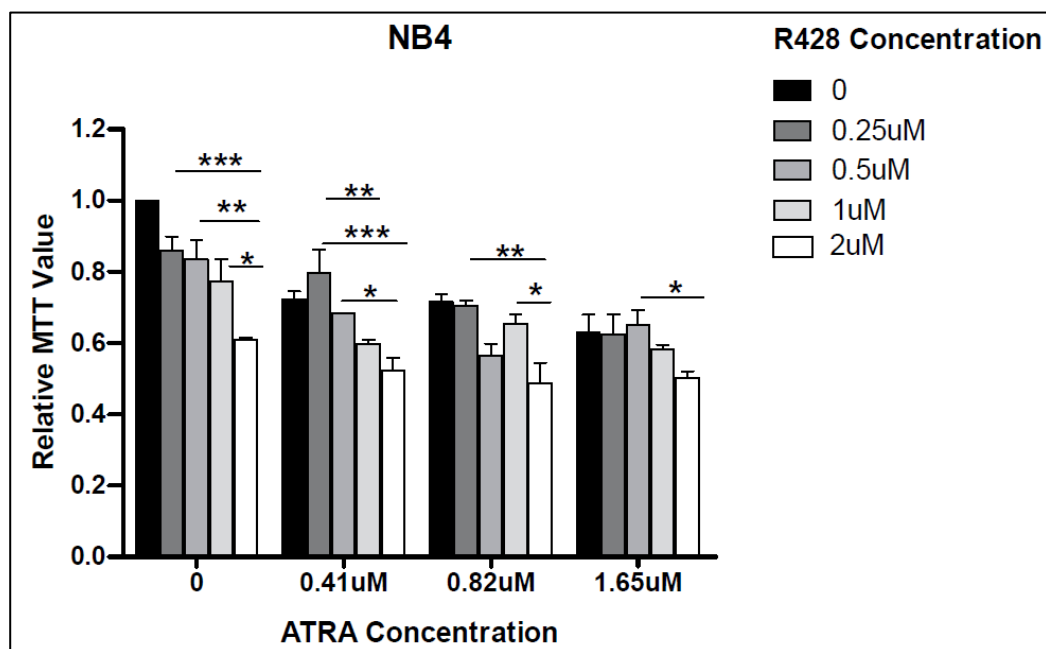


**Figure 4. 8: Effect of combination treatment (R428 + ATRA) on the proliferation potential of PML/RARα positive NB4 cells.** Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NB4 cells in the presence of 0.01% DMSO and indicated concentrations of R428 and ATRA. Cell proliferation was assessed through MTT assay after 72 hours. (*p*-values <0.05 are statistically significant). Bars show mean ± SEM

As already shown that R428 as well as Imatinib was able to reduce the proliferation of FKH-1 cells by targeting AXL and ABL respectively. So next we investigated whether there is an additive effect of the combined treatment (R428 + Imatinib) in comparison to the effect of single treatment on the proliferation ability of DEK/CAN-positive FKH-1 cells since AXL and ABL both are activated in DEK/CAN-positive FKH-1 cells. Proliferation was assessed

by MTT assay after 96 hours. Altogether the data from figure 4.7 shows that treating either with R428 or Imatinib alone reduce the proliferation of FKH-1 cells but combined treatment with R428 and Imatinib shows an additive effect on the cell proliferation ability of FKH-1 (Figure 4.9) by targeting AXL and ABL respectively and can aid in some regime development.
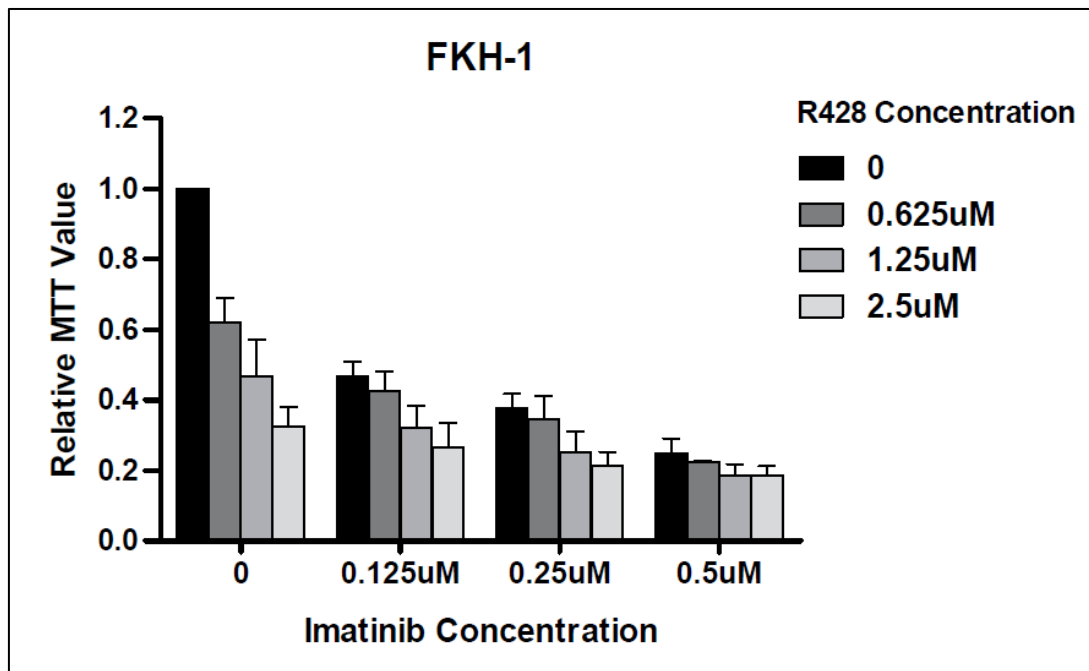


**Figure 4. 9: Additive effects of the combination treatment (R428 + Imatinib) on the proliferation potential of DEK/CAN-positive FKH-1 cells.** Cells were cultured in liquid medium (RPMI + 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NFKH-1 cells in the presence of 0.01% DMSO and indicated concentrations of R428 and Imatinib. Proliferation was assessed by MTT assay after 96 hours Bars show mean ± SEM

## 4.4 Apoptosis detection in NB4 and FKH-1 due to pharmacological targeting by R428

After testing the effect of R428 on the proliferation of NB4 and FKH-1 cells, we next investigated whether the reduction in cell proliferation is due to the apoptosis induction or some other mechanism is involved. For this, we employed DNA fragmentation assay since DNA degradation and fragmentation is one of the basic hallmark of cellular apoptosis (Rahbar

Saadat *et al.*, 2015). Cells were treated with different concentrations of R428 in µM and DNA

was extracted after 48-72 hours. Extracted DNA was then run on 1.5% agarose gel through

electrophoresis.
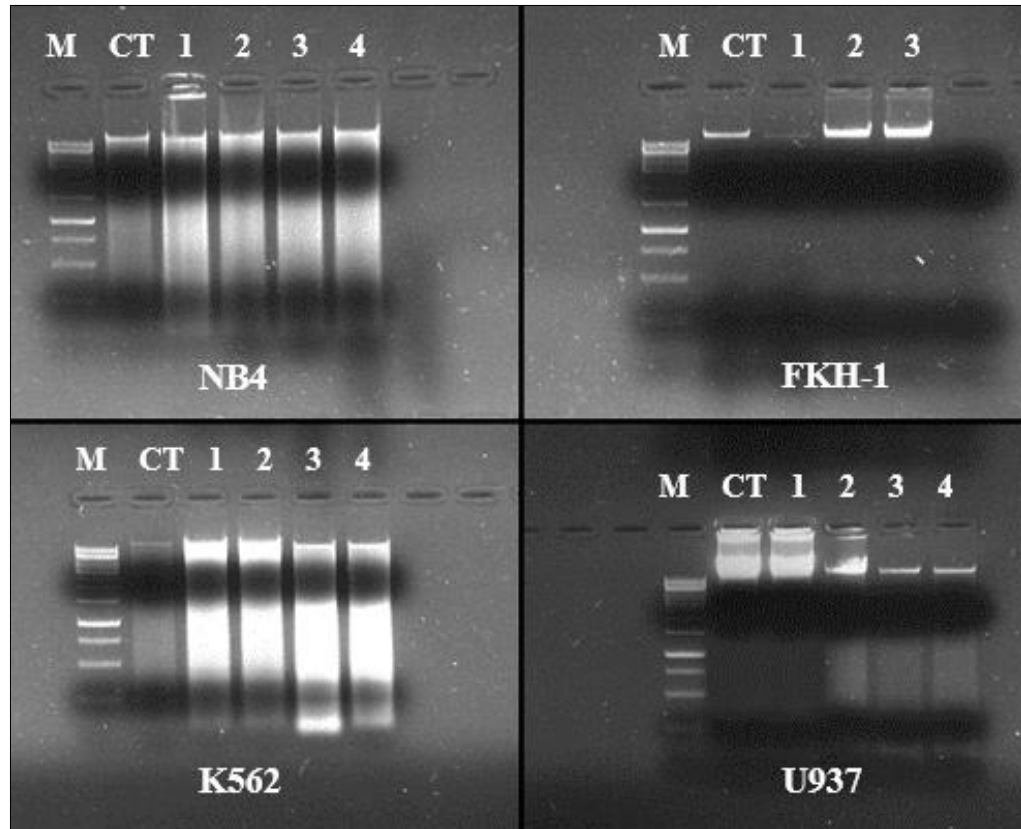


**Figure 4. 10: Gel images for DNA fragmentation assay for NB4, FKH-1, K562 and U937.** Cells were cultured in liquid medium (RPMI +10/20% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and different concentrations of R428 (M= 1kb ladder, CT= DMSO, 1= 0.62µM, 2= 1.25µM 3= 2.5µM and 4= 5µM) for 48-72 hours. DNA was then extracted and run on 1.5% agarose gel for visualization

In figure 4.10, degradation and fragmentation of DNA can be seen in NB4 and K562 cells

treated with R428 whereas intact DNA bands can be observed in FKH-1 and U937. Hence we

can say that reduction in cell proliferation ability of NB4 by R428 is due to the induction of

apoptosis whereas apoptosis induction by R428 is not related to the reduced cell proliferation

of FKH-1 instead it may be related to the cell cycle arrest which need further analysis thorough FACS.

## 4.5 Development of ATRA resistant NB4 cells

As mentioned that the consistent activation and overexpression of AXL is involved in the resistance development in cancerous cells to the chemotherapy as shown in primary samples from AML patients as well as in secondary AML cell lines (Huey *et al.*, 2016). So we wanted to investigate whether there is the involvement of AXL-RTK in developing resistance to therapy in APL. So we developed ATRA resistant NB4 cells (R-NB4) (Nason-Burchenal *et al.*, 1997). Approximately $2 \times 10^5$ NB4 cells/ml were seeded in a 6 well plate. Cells were treated with 1μM, 2μM and 3μM of ATRA each for one month. In another 6 well plate, same amount of NB4 cells were seeded and were treated with ethanol in order to make cells ATRA sensitive (S-NB4). MTT assay of both R-NB4 and S-NB4 cells was done against different concentrations of ATRA in μM side by side at the end of each month so as to determine whether resistance to ATRA is developed in R-NB4.
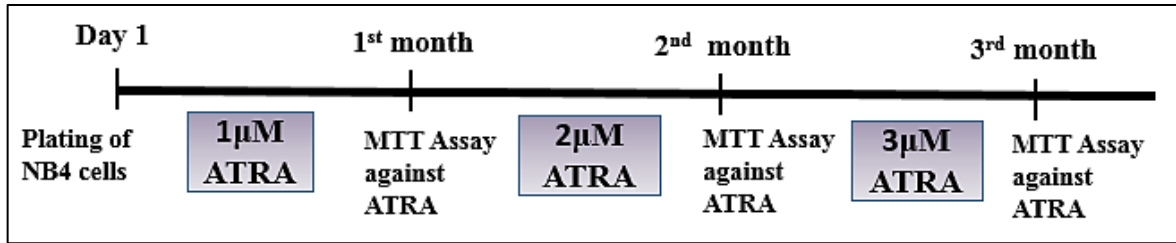
**Figure 4. 11a: Methodology for the development of ATRA resistant NB4 cells (R-NB4) (Nason-Burchenal *et al.*, 1997)**
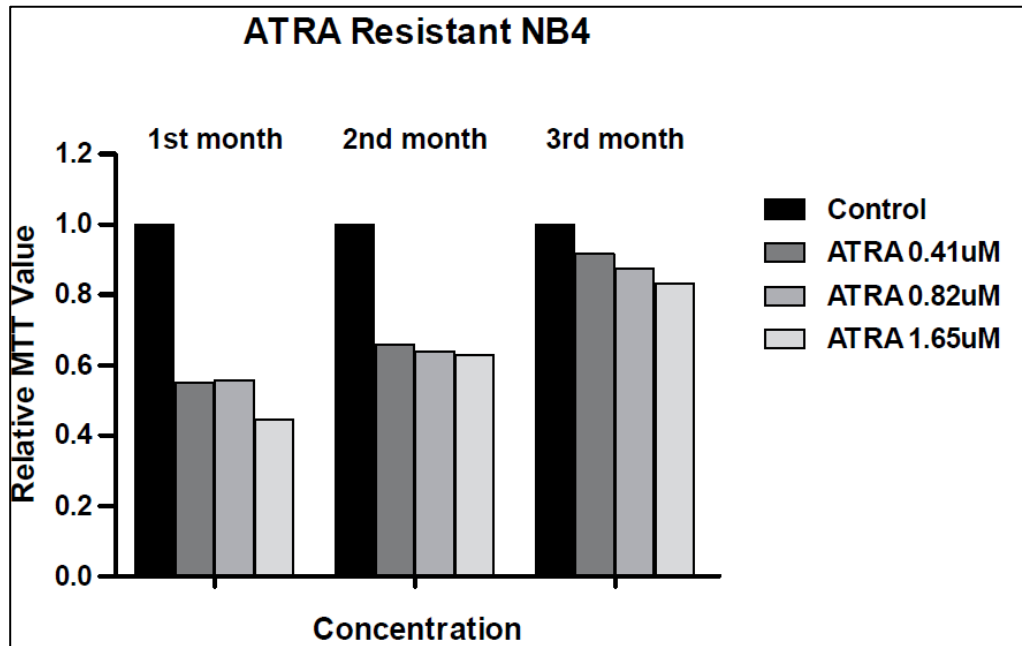


**Figure 4.11b: Development of ATRA resistant NB4 cells.** Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) to determine the proliferation potential of NB4 cells in the presence of indicated concentrations of ATRA. Cell proliferation was assessed through MTT assay at the end of each month to analyze the effect of ATRA

Figure 4.11b shows that how resistance to ATRA might have developed gradually after 3 months of treating NB4 cells with 1μM, 2μM and 3μM of ATRA each for one month and MTT assay was done to determine the resistance development. Next we used candidate gene approach in order to determine that whether the resistance development in R-NB4 is due to the increased AXL-RTK expression as compared to S-NB4. RNA was extracted from both R-

NB4 and S-NB4 and cDNA was prepared as described previously. Real time PCR was done

to determine the expression of AXL-RTK in R-NB4 and S-NB4.
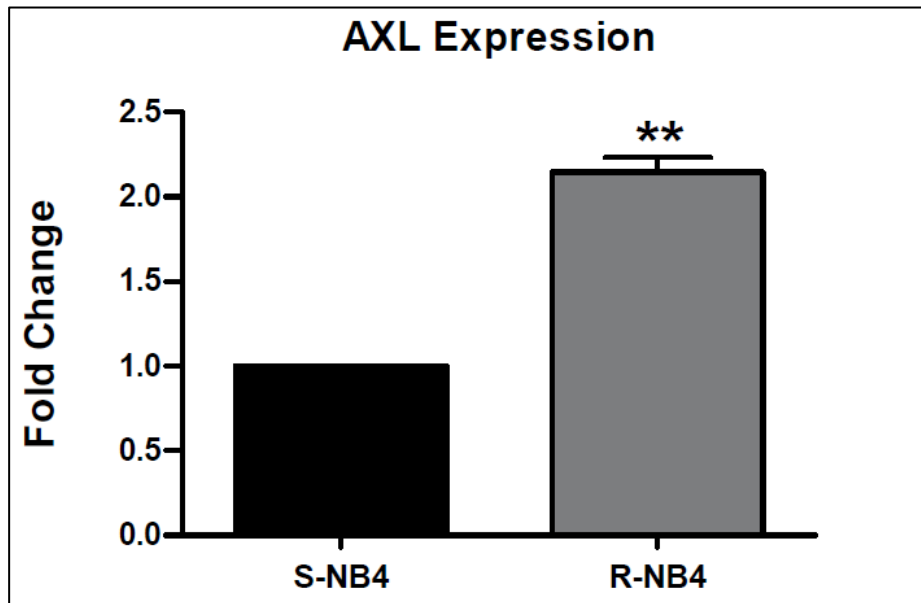


**Figure 4. 12: AXL-RTK expression profile in R-NB4 and S-NB4.** Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) and in the presence of 1μM, 2μM and 3μM of ATRA each for one month. S-NB4 were developed by treating NB4 cells with ethanol. RNA was then extracted after 3 months and cDNA was prepared. Expression analysis was done through real time PCR. Statistical significance was tested using Student's t-test ($p$-values <0.05 are statistically significant). Bars show mean ± SEM

As per Figure 4.12, R-NB4 showed significant increase in AXL-RTK expression as compared

to S-NB4. This shows that upregulation of AXL-RTK might play a role in developing

resistance to ATRA in APL.

## 4.5.1 R428 might have potential to overcome the developed resistance to ATRA in R-NB4 cells due to upregulation of AXL-RTK

A study on CML cells resistant to BCR-ABL small molecule inhibitor showed that how R428

inhibited these resistant cells by targeting the upregulated AXL-RTK (Vouri *et al.*, 2017).

Similarly after observing increased AXL-RTK expression in R-NB cells as compared to S-

NB4, we next aimed to target the AXL-RTK with the inhibitor R428 to see whether it can overcome the developed ATRA resistance. R-NB4 and S-NB4 cells were treated with different concentrations of R428 in μM. MTT assay was done to assess the cell proliferation after 72 hours.
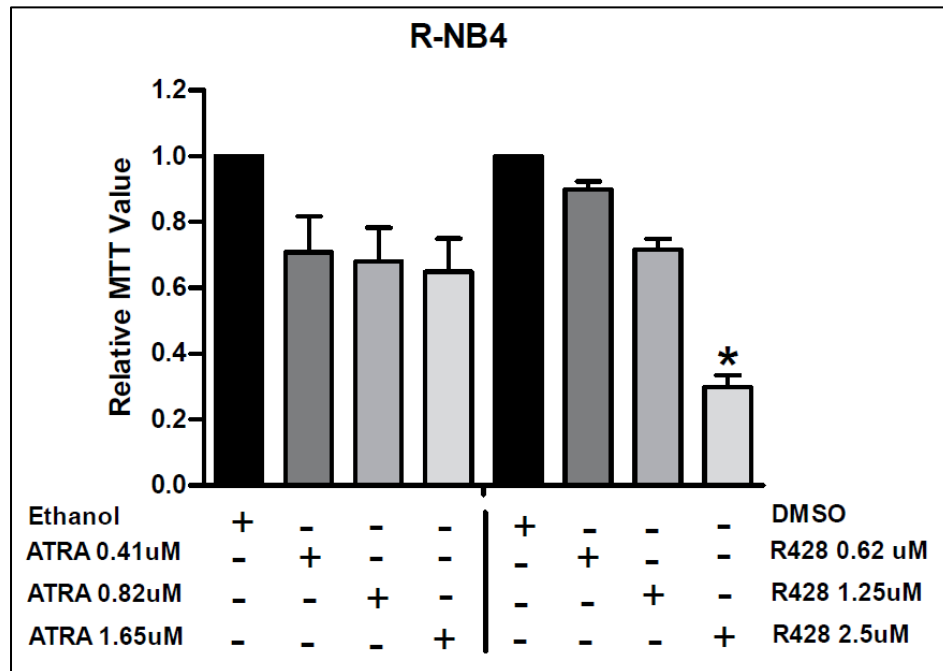


**Figure 4. 13: Effect of R428 on ATRA resistant NB4 cells.** Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin) in the presence of 1μM, 2μM and 3μM of ATRA each for one month. MTT was done against ATRA and R428. to determine the proliferation potential of NB4 cells in the presence of 0.01% DMSO and indicated concentrations of R428 and ATRA. Cell proliferation was assessed through MTT assay after 72 hours. ($p$-values <0.05 are statistically significant). Bars show mean ± SEM

A significant effect of R428 in overcoming the ATRA resistance in R-NB4 cells can be seen at higher concentration (Figure 4.13). Our results indicate that R428 might have the potential to overcome the developed therapeutic resistance to ATRA by targeting AXL-RTK in APL.

**4.6 Effect of targeting AXL on downstream signaling in PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells**

**4.6.1 Targeting AXL-RTK in PML/RARα-positive NB4 cells effect β-catenin target genes**

We observed a strong anti-proliferation effect of R428 on both PML/RARα-positive NB4 cells. As discussed previously that the activation of AXL-RTK stimulates pathways like MAPK/ERK, PI3K/Akt, JAK/STAT and Wnt/β-catenin pathway (Cristina *et al.*, 2016). Also, fusion protein PML/RARα is involved in the stabilization of β-catenin in CML, hence involved in the activation of Wnt-target genes like *c-myc*, *Axin2* etc.(Mü̈ller-Tidow, 2004). So we determined whether activation of PML/RARα is AXL dependent through stabilization of β-catenin in PML/RARα-positive NB4 cells and the effect of targeting AXL-RTK on downstream signaling of β-catenin. For this, genes *c-myc* and *Axin2* were selected through literature review and primers were optimized through gradient PCR and expression analysis was done in NB4 cells treated with R428 at different concentrations in μM, through real time PCR.
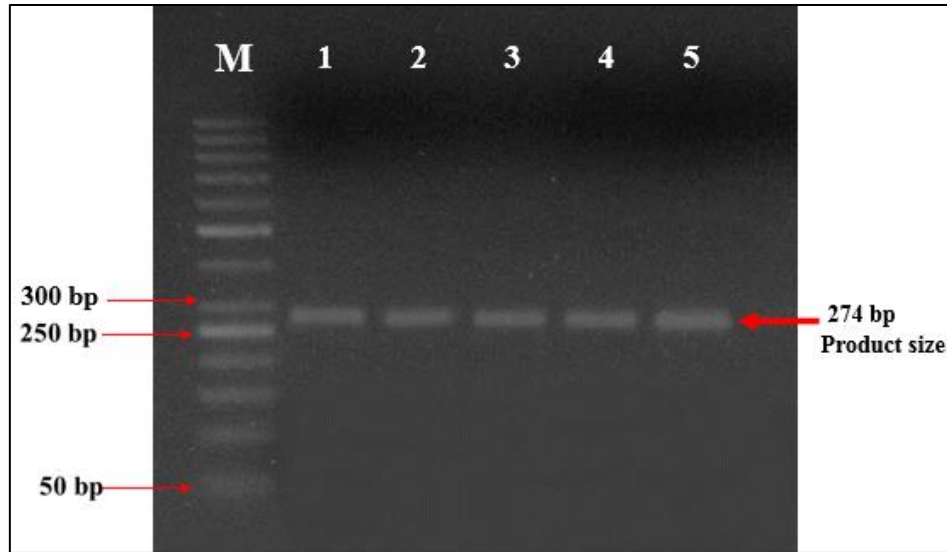
**Figure 4. 14a: PCR amplification of c-myc primer optimization.** Cells were cultured in liquid medium (RPMI + 10% & 20% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). RNA was extracted and cDNA was prepared. Primer optimization was done through gradient PCR.
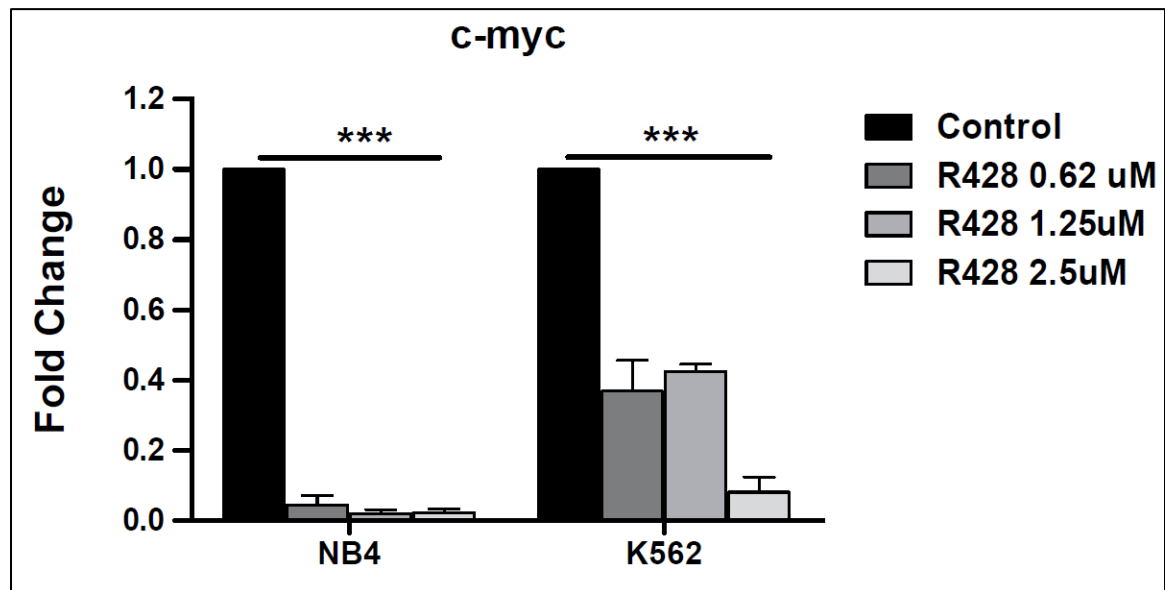


**4.14 b: Effect of targeting AXL-RTK on the expression of c-myc in PML/RARα-positive NB4 cells.** Cells were cultured in liquid medium (RPMI +10% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and indicated concentrations of R428. Expression analysis was done through real time PCR Statistical significance was tested using Two-Way ANOVA ($p$-values <0.05 are statistically significant). Bars show mean ± SEM. K562 was used as a positive control.
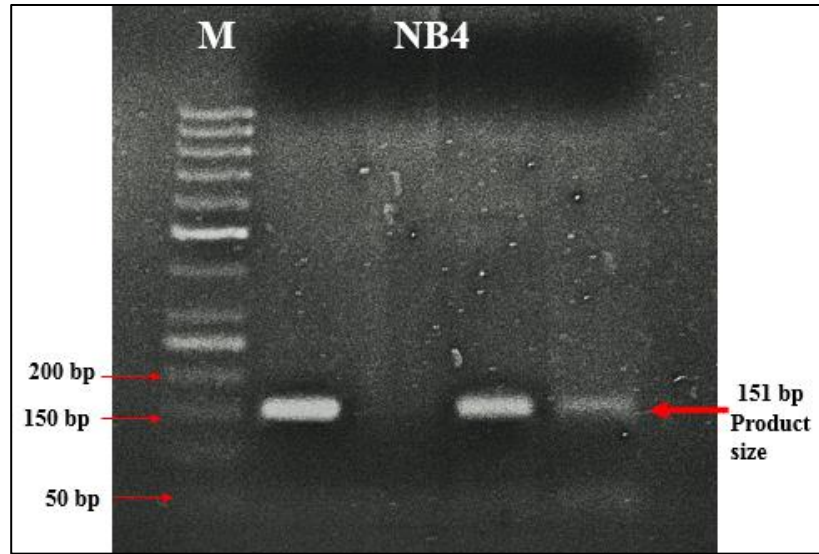
**Figure 4. 15a: PCR amplification of Axin2 primer optimization.** Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). RNA was extracted and cDNA was prepared. Primer optimization was done through gradient PCR.
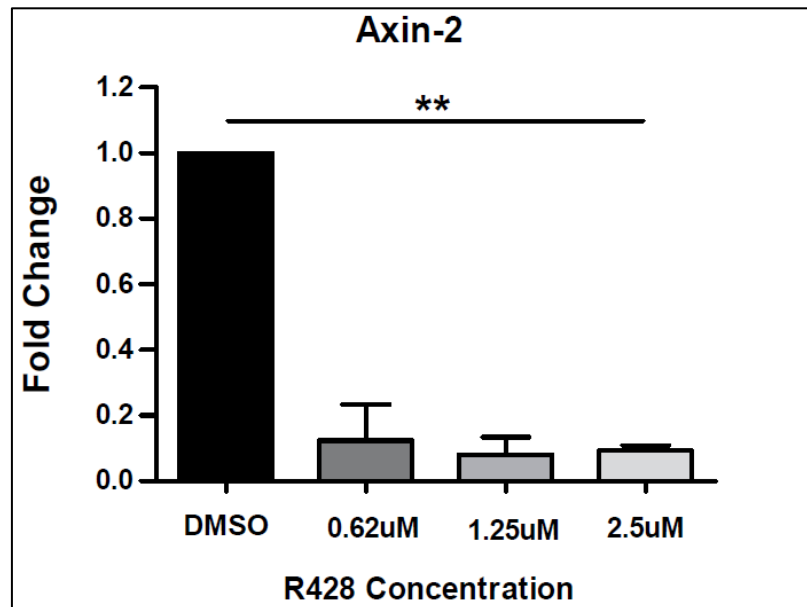


**4.15 b: Effect of targeting AXL-RTK on the expression of Axin2 in PML/RARα-positive NB4 cells.** Cells were cultured in liquid medium (RPMI +10% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and indicated concentrations of R428. Expression analysis was done through real time PCR Statistical significance was tested using One-Way ANOVA (*p*-values <0.05 are statistically significant). Bars show mean ± SEM

As shown by many studies that how different anti-cancer agents in different cancers are able to target Wnt signaling, resulting in the down regulation of β-catenin target genes like *c-myc* and *Axin2* (Y. Chen *et al.*, 2017; Jardé *et al.*, 2013; McCubrey *et al.*, 2014; Oronsky *et al.*, 2018). So it can be seen in Figure.4.15 and 4.17 that targeting AXL-RTK by R428 might be able to effect β-catenin target genes in NB4 cells i.e. a significant downregulation of *c-myc* and *Axin2* was observed in treated cells as compared to the control cells since R428 was able to interfere with the proliferation potential of NB4 cells and *c-myc* and *Axin2* function in cell proliferation. *Axin2* is considered as an indication of Wnt/β-catenin pathway activation (McCubrey *et al.*, 2014). Hence it can be concluded from these findings that AXL-RTK might be involved in the stabilization of β-catenin in PML/RARα-positive APL.

Hypoxia is a common condition during cancer growth and spread including AML. As a result of hypoxic condition, modulation in the expression of certain genes is evident as cellular response to hypoxia like hypoxia inducible factors (HIFs) i.e. Hif-α and Hif-β. These proteins as transcription factors are involved in the regulation of cellular processes like cell survival, metabolism, angiogenesis, motility, hematopoiesis etc. (Keith *et al.*, 2007). Also Hif-1α plays role in the maintenance and survival of CML stem cells and other LSCs (H. Zhang *et al.*, 2012). Also during hypoxia, some interaction between β-catenin and Hif-1α was found that results in the increased HIF-1-dependent transcription (Kaidi *et al.*, 2007; McCubrey *et al.*, 2014). Another study showed increased *Hif-1α* expression to be associated with high AXL expression in cancers like breast cancer (Nalwoga *et al.*, 2016). So, targeting AXL-RTK might have some effect on the expression of *Hif-1α* in Wnt/β-catenin dependent manner in PML/RARα-positive APL. For this purpose, we treated NB4 cells with R428 at different

concentrations in μM and real time PCR was performed for the expression analysis of *Hif-1α*.

Primer optimization was done using gradient PCR.
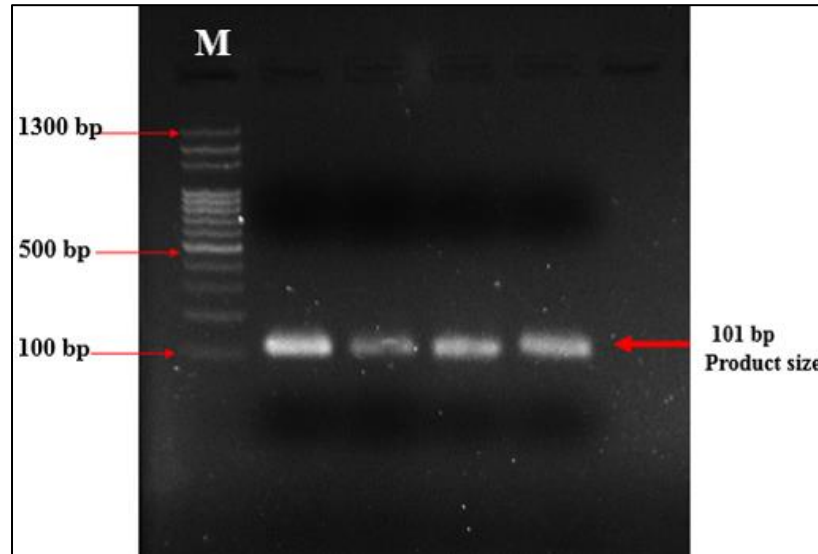


**Figure 4. 16a: PCR amplification of Hif-1a primer optimization**. Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Pencillin and Streptomycin). RNA was extracted and cDNA was prepared. Primer optimization was done through gradient PCR.



**4.16 b: Effect of targeting AXL-RTK on the expression of Hif-1α in NB4.** Cells were cultured in liquid medium (RPMI +10% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and indicated concentrations of R428. Expression analysis was done through real time PCR Statistical significance was tested using Two-Way ANOVA (*p*-values <0.05 are statistically significant). Bars show mean ± SEM. K562 was used as a positive control.

According to Fig.4.19, there is downregulation in the *Hif-1α* expression in NB4 cells when treated with R428. As shown previously that AXL-RTK is involved in the stabilization of β-catenin and APL leukemogenesis is β-catenin dependent, so it can be concluded from the results of figure 4.19 that targeting AXL-RTK with R428 in NB4 cells might downregulate *Hif-1α* expression in Wnt/β-catenin dependent manner.

## 4.6.2 Targeting AXL-RTK in DEK/CAN-positive FKH-1 cells effect downstream target genes

Though very little is known about the signaling in DEK/CAN positive AML, it was shown by Oancea et al that DEK/CAN is involved in leukemia induction in a mouse model from LT-SCs (C. Oancea *et al.*, 2010). It was reported that DEK/CAN fusion protein is involved i) in the promotion of eukaryotic translation initiation factor 4E (eIF4E) phosphorylation on Ser209, leading to its activation and ultimately increasing the protein formation in myeloid cells (Ageberg *et al.*, 2008), ii) upregulating mTORC1 leading to increased cell proliferation in human monocytic cell line U937 (Sandén *et al.*, 2013) iii) and activation of JAK/STAT5 pathway (Claudia Oancea *et al.*, 2014). These findings suggest that DEK/CAN is involved in the activation of pathways like PI3K/AKT, MAPK and JAK/STAT. As mentioned that the activation of AXL-RTK also leads to the activation of pathways like PI3K/AKT, MAPK and JAK/STAT and *c-myc* being the target gene of these pathways might also be regulated by AXL through these pathways but this needs to be confirmed. So we studied the effect of targeting AXL-RTK on the downstream signaling in FKH-1 cells through expression analysis of *c-myc*. Real time PCR was performed for the expression analysis of *c-myc* in FKH-1 cells treated with R428 at different concentrations in μM.

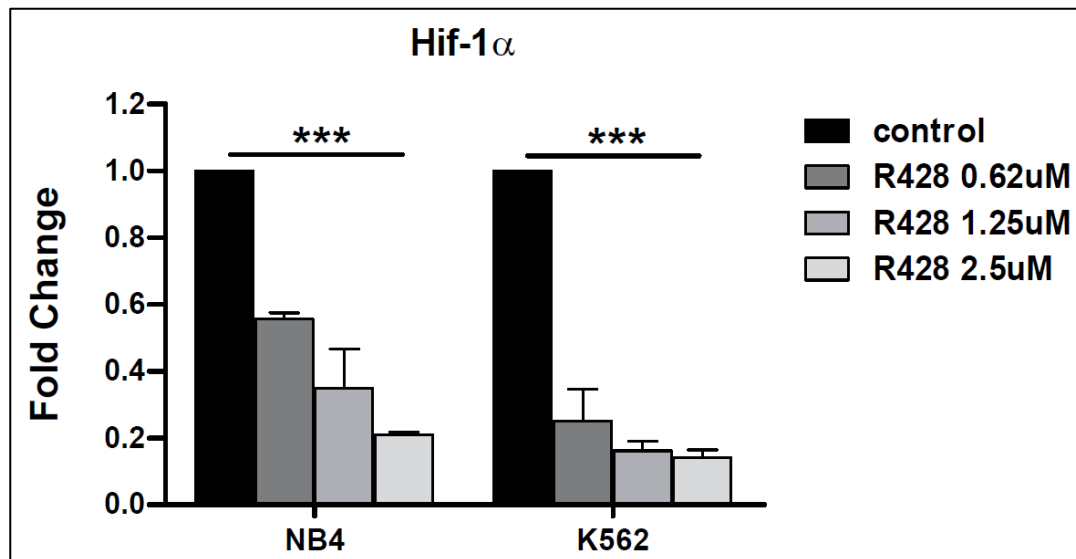**Figure 4. 17: Effect of targeting AXL on the expression of *c-myc* in DEK/CAN-positive FKH-1 cells.** Cells were cultured in liquid medium (RPMI +20% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and indicated concentrations of R428. Expression analysis was done through real time PCR. Statistical significance was tested using Two-Way ANOVA (P-values <0.05 are statistically significant). K562 was used as a positive control.

As shown that R428 interferes with the cell proliferation of FKH-1 cells and *c-myc* being an active regulator of cell proliferation was downregulated (Fig.4.17). So, targeting AXL-RTK with R428 affect the expression level of *c-myc* in FKH-1 cells i.e. a significant decrease in the expression of *c-myc* is observed after treating FKH-1 cells with different concentrations of R428 in µM as compared to the control. Hence we can conclude that targeting AXL-RTK in FKH-1 cells have an effect on the downstream signaling (PI3K/AKT, MAPK and JAK/STAT) and their target genes i.e. *c-myc* as *c-myc* activation in DEK/CAN-positive AML maybe STAT5 dependent but needs further validation.

Pathways involved in the progression of cancer also control the levels of *Hif-1α* (Masoud *et al.*, 2015). So, targeting AXL-RTK might have some effect on the expression of *Hif-1α*. For

this purpose, we treated FKH-1 cells with R428 concentration in μM and real time PCR was performed for the expression analysis of *Hif-1α*.
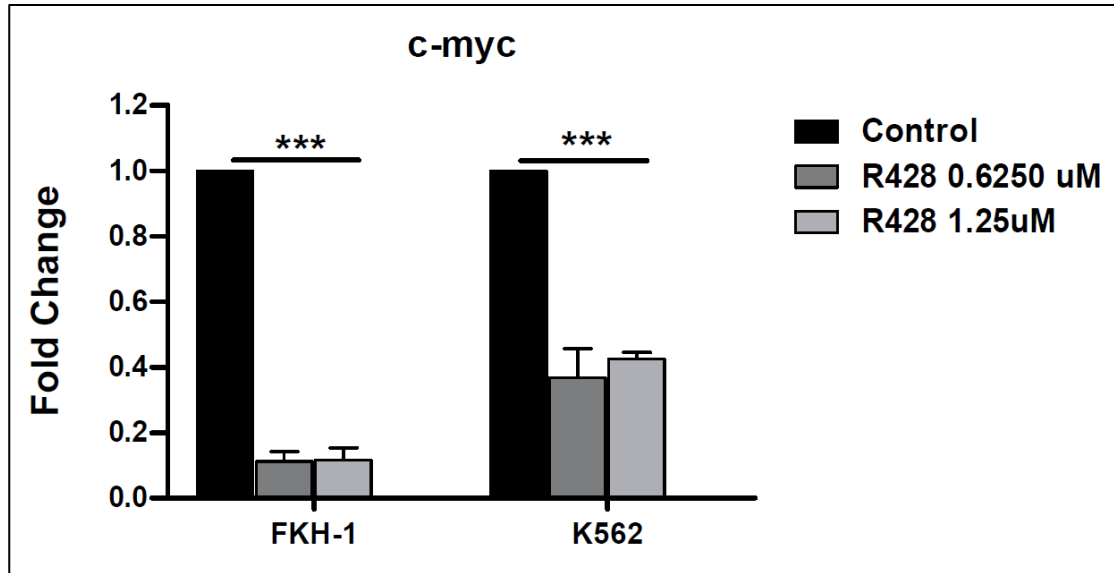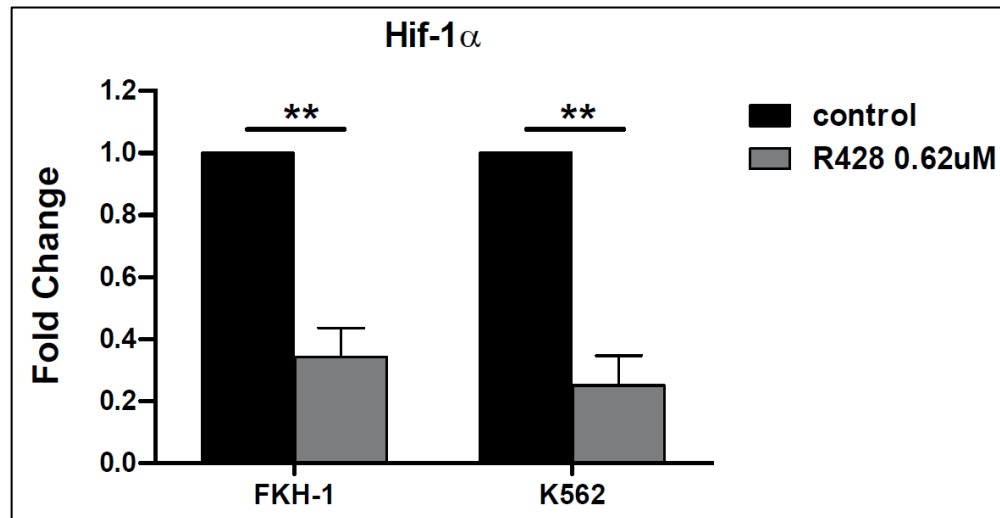


**Figure 4. 18: Effect of targeting AXL-RTK on the expression of Hif-1α in FKH-1.** Cells were cultured in liquid medium (RPMI +20% FBS+1% L-Glutamate and 1% Penstrep) and in the presence of 0.01% DMSO and indicated concentrations of R428. Expression analysis was done through real time PCR. Statistical significance was tested using Two-Way ANOVA (P-values <0.05 are statistically significant). K562 was used as a positive control.

According to figure 4.18, there is a downregulation in the expression level of *Hif-1α* in FKH-1 on treatment with R428. As discussed that AXL-RTK activates downstream pathways like PI3K/Akt/mTOR and MAPK and both pathways are involved in the leukemogenesis of DEK/CAN-positive AML due to AXL-RTK activation and *Hif-1α* activation is dependent on these pathways, hence targeting AXL-RTK might downregulate the expression of *Hif-1α* in PI3K/Akt/mTOR dependent manner in DEK/CAN-positive FKH-1 cells but needs further validation.

# CHAPTER 5

# DISCUSSION

The present study aimed to explore the role of AXL-receptor tyrosine kinase as a potential therapeutic target in the two subtypes of AML i.e. PML/RARα-positive APL and DEK/CAN-positive high risk AML, pharmacological targeting of this receptor by R428, comparison of AXL inhibitor R428 to the current therapy, an effect of the combined treatment in inhibiting the cell proliferation of NB4 and FKH-1 cells. AXL-RTK was found to be expressing in PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells. We found that R428 was able to interfere with the proliferation potential of PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells more effectively as compared to the current therapeutics i.e. ATRA for APL. It was also found that the combined treatment (R428 + ATRA) and (R428 + Imatinib) had an additive effect on the proliferation of NB4 and FKH-1 cells respectively. Due to the differentiation induction by ATRA and also differentiation syndrome (Asou, 2017), R428 might be a better targeted therapy than the current therapy ATRA. 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 hallmark of apoptosis (Rahbar Saadat *et al.*, 2015) whereas it is not in the case of DEK/CAN-positive AML, instead it might be related to cycle arrest in FKH-1 cells due to the role of *c-myc* in the cell proliferation and we have shown the downregulation of *c-myc* in FKH-1 cells upon treatment with R428. So it can be validated through FACS analysis. The role of AXL-RTK in developing resistance to the therapies is well known and well-studied in different cancers. So we explored the role of AXL-RTK in developing resistance to the current therapy ATRA in APL by developing ATRA resistant PML/RARα-positive NB4 cells. The expression of

AXL-RTK was found to be upregulated in ATRA resistant R-NB4 as compared to the S-NB4. Next we investigated the role of R428 in overcoming the therapeutic resistance and found that inhibitor R428 might have the potential to overcome the developed resistance to ATRA in PML/RARα-positive APL by targeting AXL-RTK.

Our results showed R428 to be strongly interfering with the proliferation of PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells by targeting AXL-RTK. So next we investigated the effect of targeting AXL-RTK on the downstream signaling in PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells. Wnt signaling plays a vital role in the pathogenesis of AML and CML and fusion proteins associated with leukemia like PML/RARα, are involved in the stabilization and upregulation of β-catenin through the activation of Wnt pathway in CML (Müller-Tidow *et al.*, 2004), hence involved in the activation of Wnt-target genes like *c-myc, Axin2, Hif-1α* etc. So our results showed a downregulation in the expression of *c-myc, Axin2* and *Hif-1α* upon targeting of AXL-RTK in PML/RARα-positive NB4 cells, suggesting a role of AXL-RTK in β-catenin regulation in APL. Since R428 strongly inhibited the proliferation of NB4 cells, so *c-myc* being an important regulator of cell proliferation (Miller *et al.*, 2012) was seen to be downregulated. Hypoxic environment is common during cancer growth and spread including AML. As a result of this, modulation in the expression of certain genes is evident as cellular response to hypoxia. Of these genes and transcription factors, hypoxia inducible factors (HIFs) i.e. Hif-α and Hif-β, are the most common and important ones. These proteins as transcription factors are involved in the regulation of cellular processes like cell survival, metabolism, angiogenesis, motility, hematopoiesis etc. (Keith *et al.*, 2007). Also some interaction of β-catenin and *Hif-1α* dependent transcription is also reported (Kaidi *et al.*, 2007). So keeping in

view the role of AXL in the regulation of β-catenin and *Hif-1α* being the target gene for β-catenin, there was a downregulation in the expression of *Hif-1α* in R428 treated PML/RARα-positive NB4 cells. Hence we can say that APL leukemogenesis is β-catenin dependent. Wnt pathway being the main pathway in regulating the self-renewal of normal as well as CSCs (Clevers, 2006), so further studies on the role of AXL-RTK in PML/RARα-positive CSCs can aid in eradicating CSCs and hence avoiding the cancer relapse.

Although a lot of research has been carried out in exploring the role of leukemia associated fusion proteins, the biology of DEK/CAN fusion protein is still not well understood and needs further exploration. So far it is reported that DEK/CAN is involved in the activation of Akt/mTOR and STAT5 signaling in DEK/CAN-positive AML (Carl Sandén, 2013) (Chiriches *et al.*, 2016). Our results showed a down regulation in the expression of *c-myc* and *Hif-1α* in DEK/CAN positive FKH-1 cells upon targeting AXL with R428 but whether this downregulation in the expression of *c-myc* and *Hif-1α* is Akt/mTOR and STAT5 dependent and β-independent, needs further exploration as *c-myc* and *Hif*-1α are the target genes for different pathways activated by AXL including Akt/mTOR and β-catenin. The common pathways activated by AXL-RTK in both PML/RARα-positive and DEK/CAN-positive AML are the Akt/mTOR and JAK/STAT pathway. It was shown that DEK/CAN fusion protein is unable to block the differentiation in AML as compared to PML/RARα fusion protein and also self-renewal capacity of DEK/CAN in comparison to PML/RARα is not significant (C. Oancea *et al.*, 2010). As PML/RARα regulates β-catenin that controls self-renewal of cells and we have shown in PML/RARα positive-NB4 cells that targeting AXL in NB4 cells down regulate the β-catenin target genes and involvement of DEK/CAN fusion protein in the regulation of β-catenin has not been explored yet, so the down regulation in the expression of

*c-myc* and *Hif-1α* might be Akt/mTOR and STAT5 dependent and β-independent but needs validation through targeting of JAK/STAT and β-catenin pathways.

## 5.1 CONCLUSION

Taken together the above discussion, it can be concluded that AXL-receptor tyrosine kinase might serve as a potential therapeutic target in PML/RAR-positive APL and DEK/CAN-positive AML and its targeting by R428 have a strong anti-proliferative effect on PML/RARα-positive NB4 and DEK/CAN-positive FKH-1 cells, suggesting R428 to be a better therapeutic option than the current one i.e. ATRA for APL. Additive effects on the proliferation potential of NB4 and FKH-1 cells can be achieved through combination treatment of R428 with ATRA for APL and with Imatinib for high risk AML. Since we explored the role of AXL-RTK in the stabilization of β-catenin in PML/RARα-positive APL by analyzing the expression of β-catenin target genes and β-catenin being the main player in regulating the self-renewal of CSCs, so this study may pave way in eradicating CSCs, eventually avoiding the cancer relapse. ATRA resistant NB4 cells were developed and the role of AXL-RTK in resistance development was explored and the potential of R428 in overcoming the resistance by targeting AXL-RTK was found. Effects of targeting AXL-RTK with R428 on the downstream signaling and on the biology of PML/RARα and DEK/CAN fusion proteins were explored, suggesting AXL-RTK to be a reliable novel therapeutic option in PML/RARα-positive APL and DEK/CAN-positive AML in the future.

*CHAPTER 6*

# REFERENCES

Adamietz, I. A., Bremer, M., Hoelzer, D., & Strumberg, D. (2009). Leukämien des Erwachsenen. *Der Onkologe, 15*(3), 311-332. doi: 10.1007/s00761-008-1498-7

Ageberg, M., Drott, K., Olofsson, T., Gullberg, U., & Lindmark, A. (2008). Identification of a novel and myeloid specific role of the leukemia-associated fusion protein DEK-NUP214 leading to increased protein synthesis. *Genes, Chromosomes and Cancer, 47*(4), 276-287. doi: 10.1002/gcc.20531

Anna Laurenzana, F. P., Wilson H. Miller. (2006). Role of PML/RARα in the pathogenesis of APL. *Drug Discovery Today: Disease Mechanisms, 3*(4), 499-505. doi: //doi.org/10.1016/j.ddmec.2006.11.008

Ashihara, E., Takada, T., & Maekawa, T. (2015). Targeting the canonical Wnt/β-catenin pathway in hematological malignancies. *Cancer science, 106*(6), 665-671. doi: 10.1111/cas.12655

Asou, N. (2017). Retinoic Acid, All-trans Retinoic Acid (ATRA), and Tamibarotene. In T. Ueda (Ed.), *Chemotherapy for Leukemia: Novel Drugs and Treatment* (pp. 183-211). Singapore: Springer Singapore.

Ben-Batalla, I.*, et al.* (2013). Axl, a prognostic and therapeutic target in acute myeloid leukemia mediates paracrine crosstalk of leukemia cells with bone marrow stroma. *Blood, 122*(14), 2443-2452. doi: 10.1182/blood-2013-03-491431

Borrow, J., Goddard, A., Sheer, D., & Solomon, E. (1990). Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science, 249*(4976), 1577-1580. doi: 10.1126/science.2218500

Cao, S., Wang, Z., Gao, X., He, W., Cai, Y., Chen, H., & Xu, R. (2018). FOXC1 induces cancer stem cell-like properties through upregulation of beta-catenin in NSCLC. *Journal of Experimental & Clinical Cancer Research, 37*(1), 220. doi: 10.1186/s13046-018-0894-0

Carl Sandén, M. A., Jessica Petersson, Andreas Lennartsson and Urban Gullberg. (2013). Forced expression of the DEK-NUP214 fusion protein promotes proliferation dependent on upregulation of mTOR. *BMC Cancer*.

Chang, H. R., Munkhjargal, A., Kim, M.-J., Park, S. Y., Jung, E., Ryu, J.-H., Yang, Y., Lim, J.-S., & Kim, Y. (2018). The functional roles of PML nuclear bodies in genome maintenance.

*Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 809*, 99-107. doi: https://doi.org/10.1016/j.mrfmmm.2017.05.002

Chen, F., Song, Q., & Yu, Q. (2018). Axl inhibitor R428 induces apoptosis of cancer cells by blocking lysosomal acidification and recycling independent of Axl inhibition. *American journal of cancer research, 8*(8), 1466-1482.

Chen, Y., Rao, X., Huang, K., Jiang, X., Wang, H., & Teng, L. (2017). FH535 Inhibits Proliferation and Motility of Colon Cancer Cells by Targeting Wnt/β-catenin Signaling Pathway. *Journal of Cancer, 8*(16), 3142-3153. doi: 10.7150/jca.19273

Chi, Y., Lindgren, V., Quigley, S., & Gaitonde, S. (2008). Acute Myelogenous Leukemia With t(6;9)(p23;q34) and Marrow Basophilia: An Overview. *Archives of Pathology & Laboratory Medicine, 132*(11), 1835-1837. doi: 10.1043/1543-2165-132.11.1835

Chiriches, C., Khan, D., Guillen, N., Rokicki, M., Guy, C., Mian, A., Ottmann, O. G., & Ruthardt, M. (2016). A Network of Activated Cancer Signaling Pathways in a High Risk Subgroup of Acute Myeloid Leukemia Targetable By SRC-Kinase Inhibitors. *Blood, 128*(22), 2746-2746.

Chung, E. J., Hwang, S.-G., Nguyen, P., Lee, S., Kim, J.-S., Kim, J. W., Henkart, P. A., Bottaro, D. P., Soon, L., Bonvini, P., Lee, S.-J., Karp, J. E., Oh, H. J., Rubin, J. S., & Trepel, J. B. (2002). Regulation of leukemic cell adhesion, proliferation, and survival by β-catenin. *Blood, 100*(3), 982-990. doi: 10.1182/blood.V100.3.982

Claudia Scholl, D. G. G., and Stefan Fröhling. (2008). Deregulation of Signaling Pathways in Acute Myeloid Leukemia. *Seminars in Oncology*, 336-345.

Clevers, H. (2006). Wnt/β-Catenin Signaling in Development and Disease. *Cell, 127*(3), 469-480. doi: https://doi.org/10.1016/j.cell.2006.10.018

Coombs, C. C., Tavakkoli, M., & Tallman, M. S. (2015). Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood cancer journal, 5*(4), e304-e304. doi: 10.1038/bcj.2015.25

Cristina, C., Laura, G., Cinzia, L., Nadia, Z., Diego, C., & Paola, P. (2016). Role of the Receptor Tyrosine Kinase Axl and its Targeting in Cancer Cells. *Current Medicinal Chemistry, 23*(15), 1496-1512. doi: http://dx.doi.org/10.2174/0929867323666160405112954

Daniel A. Pollyea, M., MS. (2017). The Case for Abandoning Induction Chemotherapy. America: University of Colorado School of Medicine, Aurora, CO.

Daniel V.T. Catenacci, G. J. S. (2005). Myelodysplasic syndromes: A comprehensive review. *Blood Reviews, 19*(6), 301-319. doi: //doi.org/10.1016/j.blre.2005.01.004.

Degos, L., & Wang, Z. Y. (2001). All trans retinoic acid in acute promyelocytic leukemia. *Oncogene, 20*(49), 7140-7145. doi: 10.1038/sj.onc.1204763

Döhner, H.*, et al.* (2017). Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood, 129*(4), 424-447. doi: 10.1182/blood-2016-08-733196

Embury, S. H., Elias, L., Heller, P. H., Hood, C. E., Greenberg, P. L., & Schrier, S. L. (1977). Remission maintenance therapy in acute myelogenous leukemia. *The Western journal of medicine, 126*(4), 267-272.

Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G. B., Kobayashi, R., Hunter, T., & Lu, Z. (2007). Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *The Journal of biological chemistry, 282*(15), 11221-11229. doi: 10.1074/jbc.M611871200

Fernandez, H. F., Sun, Z., Yao, X., Litzow, M. R., Luger, S. M., Paietta, E. M., Racevskis, J., Dewald, G. W., Ketterling, R. P., Bennett, J. M., Rowe, J. M., Lazarus, H. M., & Tallman, M. S. (2009). Anthracycline Dose Intensification in Acute Myeloid Leukemia. *New England Journal of Medicine, 361*(13), 1249-1259. doi: 10.1056/NEJMoa0904544

Ferrara, F. (2010). Acute promyelocytic leukemia: what are the treatment options? *Expert Opinion on Pharmacotherapy, 11*(4), 587-596. doi: 10.1517/14656560903505115

Ferreira, R., Ohneda, K., Yamamoto, M., & Philipsen, S. (2005). GATA1 Function, a Paradigm for Transcription Factors in Hematopoiesis. *Molecular and Cellular Biology, 25*(4), 1215-1227. doi: 10.1128/mcb.25.4.1215-1227.2005

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., Fransen, J., & Grosveld, G. (1997). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. *The EMBO journal, 16*(4), 807-816. doi: 10.1093/emboj/16.4.807

Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., & Orkin, S. H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proceedings of the National Academy of Sciences, 93*(22), 12355-12358. doi: 10.1073/pnas.93.22.12355

Gajendra, S. (2016). Flowcytometry in Acute Leukemia. *Clinics in Oncology, 1*.

Ganesan, S., Alex, A. A., Chendamarai, E., Balasundaram, N., Palani, H. K., David, S., Kulkarni, U., Aiyaz, M., Mugasimangalam, R., Korula, A., Abraham, A., Srivastava, A., Padua, R. A., Chomienne, C., George, B., Balasubramanian, P., & Mathews, V. (2016). Rationale and efficacy of proteasome inhibitor combined with arsenic trioxide in the treatment of acute promyelocytic leukemia. *Leukemia, 30*(11), 2169-2178. doi: 10.1038/leu.2016.227

Garçon, L., Libura, M., Delabesse, E., Valensi, F., Asnafi, V., Berger, C., Schmitt, C., Leblanc, T., Buzyn, A., & Macintyre, E. (2005). DEK-CAN molecular monitoring of myeloid malignancies could aid therapeutic stratification. *Leukemia, 19*(8), 1338-1344. doi: 10.1038/sj.leu.2403835

Goldin, L. R., Kristinsson, S. Y., Liang, X. S., Derolf, Å. R., Landgren, O., & Björkholm, M. (2012). Familial Aggregation of Acute Myeloid Leukemia and Myelodysplastic Syndromes. *Journal of Clinical Oncology, 30*(2), 179-183. doi: 10.1200/jco.2011.37.1203

Harris, N. L., Jaffe, E. S., Diebold, J., Flandrin, G., Muller-Hermelink, H. K., Vardiman, J., Lister, T. A., & Bloomfield, C. D. (1999). World Health Organization Classification of Neoplastic Diseases of the Hematopoietic and Lymphoid Tissues: Report of the Clinical Advisory Committee Meeting—Airlie House, Virginia, November 1997. *Journal of Clinical Oncology, 17*(12), 3835-3849. doi: 10.1200/jco.1999.17.12.3835

Hematology, A. S. o. (Producer). (2019). For Patients, American Society of Hematology. *American Society of Hematology*. Retrieved from https://www.hematology.org/Patients/Cancers/Leukemia.aspx

Ho, T.-C., LaMere, M., Stevens, B. M., Ashton, J. M., Myers, J. R., O'Dwyer, K. M., Liesveld, J. L., Mendler, J. H., Guzman, M., Morrissette, J. D., Zhao, J., Wang, E. S., Wetzler, M., Jordan, C. T., & Becker, M. W. (2016). Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. *Blood, 128*(13), 1671-1678. doi: 10.1182/blood-2016-02-695312

Hong, C.-C., Lay, J.-D., Huang, J.-S., Cheng, A.-L., Tang, J.-L., Lin, M.-T., Lai, G.-M., & Chuang, S.-E. (2008). Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. *Cancer Letters, 268*(2), 314-324. doi: https://doi.org/10.1016/j.canlet.2008.04.017

Huey, M. G., Minson, K. A., Earp, H. S., DeRyckere, D., & Graham, D. K. (2016). Targeting the TAM Receptors in Leukemia. *Cancers, 8*(11), 101.

Hughes, M. (2017). The Global Incidence And Prevalence Of Acute Myeloid Leukemia Over The Next Ten Years (2017-2027). *Journal of Cancer Research & Therapeutics*.

Ishiyama, K., Takami, A., Kanda, Y., Nakao, S., Hidaka, M., Maeda, T., Naoe, T., Taniguchi, S., Kawa, K., Nagamura, T., Atsuta, Y., & Sakamaki, H. (2011). Allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia with t(6;9)(p23;q34) dramatically improves the patient prognosis: a matched-pair analysis. *Leukemia, 26*, 461. doi: 10.1038/leu.2011.229

Jaeckel, S., Kaller, M., Jackstadt, R., Götz, U., Müller, S., Boos, S., Horst, D., Jung, P., & Hermeking, H. (2018). Ap4 is rate limiting for intestinal tumor formation by controlling the homeostasis of intestinal stem cells. *Nature Communications, 9*(1), 3573. doi: 10.1038/s41467-018-06001-x

Jardé, T., Evans, R. J., McQuillan, K. L., Parry, L., Feng, G. J., Alvares, B., Clarke, A. R., & Dale, T. C. (2013). In vivo and in vitro models for the therapeutic targeting of Wnt signaling using a Tet-OΔN89β-catenin system. *Oncogene, 32*(7), 883-893. doi: 10.1038/onc.2012.103

Jin, L., & Wu, L. (2018). *Recent Advances in Characterization of Pre-Leukemic/Leukemic Stem Cells in Acute Myeloid Leukemia*.

Jin, Y., Nie, D., Li, J., Du, X., Lu, Y., Li, Y., Liu, C., Zhou, J., & Pan, J. (2017). Gas6/AXL Signaling Regulates Self-Renewal of Chronic Myelogenous Leukemia Stem Cells by Stabilizing β-Catenin. *Clinical Cancer Research, 23*(11), 2842-2855. doi: 10.1158/1078-0432.ccr-16-1298

Juliusson, G., Antunovic, P., Derolf, Å., Lehmann, S., Möllgård, L., Stockelberg, D., Tidefelt, U., Wahlin, A., & Höglund, M. (2009). Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood, 113*(18), 4179-4187. doi: 10.1182/blood-2008-07-172007

Kadia, T. M., Ravandi, F., Cortes, J., & Kantarjian, H. (2016). New drugs in acute myeloid leukemia. *Annals of Oncology, 27*(5), 770-778. doi: 10.1093/annonc/mdw015

Kaidi, A., Williams, A. C., & Paraskeva, C. (2007). Interaction between β-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nature Cell Biology, 9*, 210. doi: 10.1038/ncb1534

Kappes, F., Burger, K., Baack, M., Fackelmayer, F. O., & Gruss, C. (2001). Subcellular Localization of the Human Proto-oncogene Protein DEK. *Journal of Biological Chemistry, 276*(28), 26317-26323. doi: 10.1074/jbc.M100162200

Kavanagh, S., Murphy, T., Law, A., Yehudai, D., Ho, J. M., Chan, S., & Schimmer, A. D. (2017). Emerging therapies for acute myeloid leukemia: translating biology into the clinic. *JCI Insight, 2*(18). doi: 10.1172/jci.insight.95679

Keith, B., & Simon, M. C. (2007). Hypoxia-inducible factors, stem cells, and cancer. *Cell, 129*(3), 465-472. doi: 10.1016/j.cell.2007.04.019

Khan S, M. A., Khattak BR, Rehman A, Zeb A  (2017). Childhood Leukemias in Khyber Pakhtunkhwa and Afghan Children Visiting to Hayatabad Medical Complex Hospital. *Archieves in Cancer Research, 5:No.3:149*. doi: 10.21767/2254-6081.1000149

Khwaja, A., Bjorkholm, M., Gale, R. E., Levine, R. L., Jordan, C. T., Ehninger, G., Bloomfield, C. D., Estey, E., Burnett, A., Cornelissen, J. J., Scheinberg, D. A., Bouscary, D., & Linch, D. C. (2016). Acute myeloid leukaemia. *Nature Reviews Disease Primers, 2*, 16010. doi: 10.1038/nrdp.2016.10

Ko, S.-I., Lee, I.-S., Kim, J.-Y., Kim, S.-M., Kim, D.-W., Lee, K.-S., Woo, K.-M., Baek, J.-H., Choo, J.-K., & Seo, S.-B. (2006). Regulation of histone acetyltransferase activity of p300 and PCAF by proto-oncogene protein DEK. *FEBS Letters, 580*(13), 3217-3222. doi: 10.1016/j.febslet.2006.04.081

Kodappully Sivaraman Siveen, S. U. a. R. M. M. (2017). Targeting acute myeloid leukemia stem cell signaling by natural products. *Molecular Cancer*.

Köhler, A., & Hurt, E. (2010). Gene Regulation by Nucleoporins and Links to Cancer. *Molecular cell, 38*, 6-15. doi: 10.1016/j.molcel.2010.01.040

Korshunov, Vyacheslav A. (2012). Axl-dependent signalling: a clinical update. *Clinical Science, 122*(8), 361-368. doi: 10.1042/cs20110411

Koshy, S. R. C. N. V. (2019). Cancer, Acute Promyelocytic Leukemia (APL, APML) StatPearls Publishing.

Kumar, C. C. (2011). Genetic Abnormalities and Challenges in the Treatment of Acute Myeloid Leukemia. *Genes & Cancer, 2*(2), 95-107. doi: 10.1177/1947601911408076

Lallemand-Breitenbach, V., Zhu, J., Chen, Z., & de Thé, H. (2011). Curing Apl through Pml/Rara degradation by As2O3. *Trends in molecular medicine, 18*, 36-42. doi: 10.1016/j.molmed.2011.10.001

Lane, A. A., & Ley, T. J. (2003). Neutrophil Elastase Cleaves PML-RARα and Is Important for the Development of Acute Promyelocytic Leukemia in Mice. *Cell, 115*(3), 305-318. doi: https://doi.org/10.1016/S0092-8674(03)00852-3

Lemmon, M. A., & Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell, 141*(7), 1117-1134. doi: 10.1016/j.cell.2010.06.011

Lento, W., Congdon, K., Voermans, C., Kritzik, M., & Reya, T. (2013). Wnt Signaling in Normal and Malignant Hematopoiesis. *Cold Spring Harbor Perspectives in Biology, 5*(2). doi: 10.1101/cshperspect.a008011

Licht, J. D. (2001). AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. *Oncogene, 20*(40), 5660-5679. doi: 10.1038/sj.onc.1204593

Licht, J. D. (2006). Reconstructing a disease: What essential features of the retinoic acid receptor fusion oncoproteins generate acute promyelocytic leukemia? *Cancer Cell, 9*(2), 73-74. doi: https://doi.org/10.1016/j.ccr.2006.01.024

Licht, J. D., & Sternberg, D. W. (2005). The Molecular Pathology of Acute Myeloid Leukemia. *ASH Education Program Book, 2005*(1), 137-142. doi: 10.1182/asheducation-2005.1.137

Lin, T. L., & Levy, M. Y. (2012). Acute Myeloid Leukemia: Focus on novel Therapeutic Strategies. *Clinical Medicine Insights: Oncology, 6*, CMO.S7244. doi: 10.4137/cmo.s7244

Lo-Coco, F., & Hasan, S. K. (2014). Understanding the molecular pathogenesis of acute promyelocytic leukemia. *Best Practice & Research Clinical Haematology, 27*(1), 3-9. doi: https://doi.org/10.1016/j.beha.2014.04.006

Lourenço, A. R., & Coffer, P. J. (2017). A tumor suppressor role for C/EBPα in solid tumors: more than fat and blood. *Oncogene, 36*, 5221. doi: 10.1038/onc.2017.151

Lowenberg, B., Downing, J. R., & Burnett, A. (1999). Acute Myeloid Leukemia. *New England Journal of Medicine, 341*(14), 1051-1062. doi: 10.1056/nejm199909303411407

Malanchi, I., Peinado, H., Kassen, D., Hussenet, T., Metzger, D., Chambon, P., Huber, M., Hohl, D., Cano, A., Birchmeier, W., & Huelsken, J. (2008). Cutaneous cancer stem cell maintenance is dependent on β-catenin signalling. *Nature, 452*, 650. doi: 10.1038/nature06835

Mandelli, F.*, et al.* (2009). Daunorubicin Versus Mitoxantrone Versus Idarubicin As Induction and Consolidation Chemotherapy for Adults With Acute Myeloid Leukemia: The EORTC and GIMEMA Groups Study AML-10. *Journal of Clinical Oncology, 27*(32), 5397-5403. doi: 10.1200/jco.2008.20.6490

Marlétaz, F., Holland, L. Z., Laudet, V., & Schubert, M. (2006). Retinoic acid signaling and the evolution of chordates. *International journal of biological sciences, 2*(2), 38-47. doi: 10.7150/ijbs.2.38

Masoud, G. N., & Li, W. (2015). HIF-1α pathway: role, regulation and intervention for cancer therapy. *Acta pharmaceutica Sinica. B, 5*(5), 378-389. doi: 10.1016/j.apsb.2015.05.007

Matsui, W. H. (2016). Cancer stem cell signaling pathways. *Medicine, 95*(1 Suppl 1), S8-S19. doi: 10.1097/MD.0000000000004765

Mayer, R., B. Davis, R., A. Schiffer, C., T. Berg, D., L. Powell, B., Schulman, P., A. Omura, G., Moore, J., Ross McIntyre, O., & Frei, E. (1994). Intensive Postremission Chemotherapy in Adults with Acute Myeloid Leukemia. *New England Journal of Medicine - N ENGL J MED, 331*, 896-903. doi: 10.1056/NEJM199410063311402

McCubrey, J. A., Steelman, L. S., Bertrand, F. E., Davis, N. M., Abrams, S. L., Montalto, G., D'Assoro, A. B., Libra, M., Nicoletti, F., Maestro, R., Basecke, J., Cocco, L., Cervello, M., & Martelli, A. M. (2014). Multifaceted roles of GSK-3 and Wnt/β-catenin in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention. *Leukemia, 28*(1), 15-33. doi: 10.1038/leu.2013.184

Mei, Y., Liu, Y.-B., Cao, S., Tian, Z.-W., & Zhou, H.-H. (2018). RIF1 promotes tumor growth and cancer stem cell-like traits in NSCLC by protein phosphatase 1-mediated activation of Wnt/β-catenin signaling. *Cell Death & Disease, 9*(10), 942. doi: 10.1038/s41419-018-0972-4

Mikesch, J. H., Steffen, B., Berdel, W. E., Serve, H., & Müller-Tidow, C. (2007). The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia. *Leukemia, 21*(8), 1638-1647. doi: 10.1038/sj.leu.2404732

Miller, D. M., Thomas, S. D., Islam, A., Muench, D., & Sedoris, K. (2012). c-Myc and cancer metabolism. *Clinical cancer research : an official journal of the American Association for Cancer Research, 18*(20), 5546-5553. doi: 10.1158/1078-0432.CCR-12-0977

Morgan, R. G., Ridsdale, J., Payne, M., Heesom, K. J., Wilson, M. C., Davidson, A., Greenhough, A., Davies, S., Williams, A. C., Blair, A., Waterman, M. L., Tonks, A., & Darley, R. L. (2019). LEF-1 drives aberrant β-catenin nuclear localization in myeloid leukemia cells. *Haematologica, 104*(7), 1365-1377. doi: 10.3324/haematol.2018.202846

Mu¨ller-Tidow, B. r. S., Thomas Cauvet, Lara Tickenbrock, Ping Ji, Sven Diederichs, Bülent Sargin, Gabriele Köhler, Matthias Stelljes, Elena Puccetti, Martin Ruthardt, Sven deVos, Scott W. Hiebert, H. Phillip Koeffler, Wolfgang E.Berdel. (2004). Translocation Products in Acute Myeloid Leukemia Activate the Wnt Signaling Pathway in Hematopoietic Cells. *Molecular and Cellular Biology*, 2890–2904.

Müller-Tidow, C., Steffen, B., Cauvet, T., Tickenbrock, L., Ji, P., Diederichs, S., Sargin, B., Köhler, G., Stelljes, M., Puccetti, E., Ruthardt, M., deVos, S., Hiebert, S. W., Koeffler, H. P.,

Berdel, W. E., & Serve, H. (2004). Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. *Molecular and Cellular Biology, 24*(7), 2890-2904. doi: 10.1128/mcb.24.7.2890-2904.2004

Myers, S. H., Brunton, V. G., & Unciti-Broceta, A. (2016). AXL Inhibitors in Cancer: A Medicinal Chemistry Perspective. *Journal of Medicinal Chemistry, 59*(8), 3593-3608. doi: 10.1021/acs.jmedchem.5b01273

Najafi, M., Farhood, B., & Mortezaee, K. (2019). Cancer stem cells (CSCs) in cancer progression and therapy. *Journal of Cellular Physiology, 234*(6), 8381-8395. doi: 10.1002/jcp.27740

Nalwoga, H., Ahmed, L., Arnes, J. B., Wabinga, H., & Akslen, L. A. (2016). Strong Expression of Hypoxia-Inducible Factor-1α (HIF-1α) Is Associated with Axl Expression and Features of Aggressive Tumors in African Breast Cancer. *PLOS ONE, 11*(1), e0146823. doi: 10.1371/journal.pone.0146823

Nason-Burchenal, K., Maerz, W., Allopenna, J., Martin, P., Dmitrovsky, E., Albanell, J., & Moore, M. A. S. (1997). Common defects of different retinoic acid resistant promyelocytic leukemia cells are persistent telomerase activity and nuclear body disorganization. *Differentiation, 61*(5), 321-331. doi: https://doi.org/10.1046/j.1432-0436.1997.6150321.x

Nasr, R., Guillemin, M.-C., Ferhi, O., Soilihi, H., Peres, L., Berthier, C., Rousselot, P., Robledo-Sarmiento, M., Lallemand-Breitenbach, V., Gourmel, B., Vitoux, D., Pandolfi, P. P., Rochette-Egly, C., Zhu, J., & de Thé, H. (2008). Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nature Medicine, 14*, 1333. doi: 10.1038/nm.1891

Nervi, C., Ferrara, F. F., Fanelli, M., Rippo, M. R., Tomassini, B., Ferrucci, P. F., Ruthardt, M., Gelmetti, V., Gambacorti-Passerini, C., Diverio, D., Grignani, F., Pelicci, P. G., & Testi, R. (1998). Caspases Mediate Retinoic Acid–Induced Degradation of the Acute Promyelocytic Leukemia PML/RARα Fusion Protein. *Blood, 92*(7), 2244-2251.

Ng, C., & Chng, W. (2017). Recent advances in acute promyelocytic leukaemia [version 1; peer review: 3 approved]. *F1000Research, 6*(1273). doi: 10.12688/f1000research.10736.1

O'Bryan, J. P., Frye, R. A., Cogswell, P. C., Neubauer, A., Kitch, B., Prokop, C., Espinosa, R., Le Beau, M. M., Earp, H. S., & Liu, E. T. (1991). axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Molecular and Cellular Biology, 11*(10), 5016-5031. doi: 10.1128/mcb.11.10.5016

Oancea, C., Rüster, B., Brill, B., Roos, J., Heinssmann, M., Bug, G., Mian, A. A., Guillen, N. A., Kornblau, S. M., Henschler, R., & Ruthardt, M. (2014). STAT activation status

differentiates leukemogenic from non-leukemogenic stem cells in AML and is suppressed by arsenic in t(6;9)-positive AML. *Genes & Cancer, 5*(11-12), 378-392. doi: 10.18632/genesandcancer.39

Oancea, C., Rüster, B., Henschler, R., Puccetti, E., & Ruthardt, M. (2010). The t(6;9) associated DEK/CAN fusion protein targets a population of long-term repopulating hematopoietic stem cells for leukemogenic transformation. *Leukemia, 24*, 1910. doi: 10.1038/leu.2010.180

Olofsson, T. B. (1991). Growth Regulation of Hematopoietic Cells :An overview. *Acta Oncologica, 30*(8), 889-902. doi: 10.3109/02841869109088241

Oronsky, B., Reid, T. R., Oronsky, A., Caroen, S., Carter, C. A., & Cabrales, P. (2018). Brief report: RRx-001 is a c-Myc inhibitor that targets cancer stem cells. *Oncotarget, 9*(34), 23439-23442. doi: 10.18632/oncotarget.25211

Oyarzo, M. P., Lin, P. J., Glassman, A. B., Bueso-Ramos, C. E., Luthra, R., & Medeiros, L. J. (2004). Acute myeloid leukemia with t(6;9)(p23;q34) is associated with dysplasia and a high frequency of flt3 gene mutations. *American journal of clinical pathology, 122*(3), 348-358.

Park, I.-K., Mishra, A., Chandler, J., Whitman, S. P., Marcucci, G., & Caligiuri, M. A. (2013). Inhibition of the receptor tyrosine kinase Axl impedes activation of the FLT3 internal tandem duplication in human acute myeloid leukemia: implications for Axl as a potential therapeutic target. *Blood, 121*(11), 2064-2073. doi: 10.1182/blood-2012-07-444018

Privette Vinnedge, L. M., Kappes, F., Nassar, N., & Wells, S. I. (2013). Stacking the DEK: From chromatin topology to cancer stem cells. *Cell Cycle, 12*(1), 51-66. doi: 10.4161/cc.23121

Rahbar Saadat, Y., Saeidi, N., Zununi Vahed, S., Barzegari, A., & Barar, J. (2015). An update to DNA ladder assay for apoptosis detection. *BioImpacts : BI, 5*(1), 25-28. doi: 10.15171/bi.2015.01

Raziq, F. S. a. F. (2014). Clinical Presentations of Acute Leukemia *Journal of the College of Physicians and Surgeons Pakista, 24*(7), 472-476. doi: 07.2014/JCPSP.472476.

Riss TL, M. R., Niles AL, et al. (2013). Cell Viability Assays. In G. A. Sittampalam GS, Brimacombe K, et al (Ed.): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Rowe, J. M. (2009). Optimal induction and post-remission therapy for AML in first remission. *ASH Education Program Book, 2009*(1), 396-405. doi: 10.1182/asheducation-2009.1.396

Rowley, J., & Potter, D. (1976). Chromosomal banding patterns in acute nonlymphocytic leukemia. *Blood, 47*(5), 705-721.

Sandén, C., Ageberg, M., Petersson, J., Lennartsson, A., & Gullberg, U. (2013). Forced expression of the DEK-NUP214 fusion protein promotes proliferation dependent on upregulation of mTOR. *BMC Cancer, 13*(1), 440. doi: 10.1186/1471-2407-13-440

Scaltriti, M., Elkabets, M., & Baselga, J. (2016). Molecular Pathways: AXL, a Membrane Receptor Mediator of Resistance to Therapy. *Clinical Cancer Research, 22*(6), 1313-1317. doi: 10.1158/1078-0432.ccr-15-1458

Scholl, C., Gilliland, D. G., & Fröhling, S. (2008). Deregulation of Signaling Pathways in Acute Myeloid Leukemia. *Seminars in Oncology, 35*(4), 336-345. doi: https://doi.org/10.1053/j.seminoncol.2008.04.004

Seif, A. E. (2011). Pediatric leukemia predisposition syndromes: clues to understanding leukemogenesis. *Cancer genetics, 204* (5), 227-244

Seipelt, G., Hofmann, W.-K., Martin, H., Wassmann, B., Boehme, A., Ottmann, O. G., & Hoelzer, D. (1998). Comparison of toxicity and outcome in patients with acute myeloid leukemia treated with high-dose cytosine arabinoside consolidation after induction with a regimen containing idarubicin or daunorubicin. *Annals of Hematology, 76*(3), 145-151. doi: 10.1007/s002770050379

Shearer, B. M., Knudson, R. A., Flynn, H. C., & Ketterling, R. P. (2005). Development of a D-FISH method to detect DEK/CAN fusion resulting from t(6;9)(p23;q34) in patients with acute myelogenous leukemia. *Leukemia, 19*(1), 126-131. doi: 10.1038/sj.leu.2403557

Shimizu, Y., Schull, W. J., & Kato, H. (1990). Cancer Risk Among Atomic Bomb Survivors: The RERF Life Span Study. *JAMA, 264*(5), 601-604. doi: 10.1001/jama.1990.03450050059028

Shinagawa, K.*, et al.* (2014). Tamibarotene As Maintenance Therapy for Acute Promyelocytic Leukemia: Results From a Randomized Controlled Trial. *Journal of Clinical Oncology, 32*(33), 3729-3735. doi: 10.1200/jco.2013.53.3570

Shivdasani, R., & Orkin, S. (1996). The transcriptional control of hematopoiesis [see comments]. *Blood, 87*(10), 4025-4039.

Shlush, L. I.*, et al.* (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature, 506*, 328. doi: 10.1038/nature13038

Siveen, K. S., Uddin, S., & Mohammad, R. M. (2017). Targeting acute myeloid leukemia stem cell signaling by natural products. *Molecular Cancer, 16*(1), 13. doi: 10.1186/s12943-016-0571-x

Slovak, M. L., Gundacker, H., Bloomfield, C. D., Dewald, G., Appelbaum, F. R., Larson, R. A., Tallman, M. S., Bennett, J. M., Stirewalt, D. L., Meshinchi, S., Willman, C. L., Ravindranath, Y., Alonzo, T. A., Carroll, A. J., Raimondi, S. C., & Heerema, N. A. (2006). A retrospective study of 69 patients with t(6;9)(p23;q34) AML emphasizes the need for a prospective, multicenter initiative for rare 'poor prognosis' myeloid malignancies. *Leukemia, 20*, 1295. doi: 10.1038/sj.leu.2404233

Society, T. A. C. (2018, January 8, 2019). Key Statistics for Acute Myeloid Leukemia (AML). *About Acute Myeloid Leukemia (AML).* from https://www.cancer.org/cancer/acute-myeloid-leukemia/about/key-statistics.html

Soekarman, D., von Lindern, M., Daenen, S., de Jong, B., Fonatsch, C., Heinze, B., Bartram, C., Hagemeijer, A., & Grosveld, G. (1992). The translocation (6;9) (p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. *Blood, 79*(11), 2990-2997.

Springuel, L., Renauld, J.-C., & Knoops, L. (2015). JAK kinase targeting in hematologic malignancies: a sinuous pathway from identification of genetic alterations towards clinical indications. *Haematologica, 100*(10), 1240-1253. doi: 10.3324/haematol.2015.132142

Steffen, B., Müller-Tidow, C., Schwäble, J., Berdel, W. E., & Serve, H. (2005). The molecular pathogenesis of acute myeloid leukemia. *Critical Reviews in Oncology/Hematology, 56*(2), 195-221. doi: https://doi.org/10.1016/j.critrevonc.2004.10.012

Takei, H., & Kobayashi, S. S. (2019). Targeting transcription factors in acute myeloid leukemia. *International Journal of Hematology, 109*(1), 28-34. doi: 10.1007/s12185-018-2488-1

Tenen, D. G., Hromas, R., Licht, J. D., & Zhang, D.-E. (1997). Transcription Factors, Normal Myeloid Development, and Leukemia. *Blood, 90*(2), 489-519.

Toffalini, F., & Demoulin, J.-B. (2010). New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases. *Blood, 116*(14), 2429-2437. doi: 10.1182/blood-2010-04-279752

Touw, I. P., De Koning, J. P., Ward, A. C., & Hermans, M. H. A. (2000). Signaling mechanisms of cytokine receptors and their perturbances in disease. *Molecular and Cellular Endocrinology, 160*(1), 1-9. doi: https://doi.org/10.1016/S0303-7207(99)00206-3

Vardiman, J. W. (2010). The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: An overview with emphasis on the myeloid neoplasms. *Chemico-Biological Interactions, 184*(1), 16-20. doi: https://doi.org/10.1016/j.cbi.2009.10.009

Vellenga, E., van Putten, W., Ossenkoppele, G. J., Verdonck, L. F., Theobald, M., Cornelissen, J. J., Huijgens, P. C., Maertens, J., Gratwohl, A., Schaafsma, R., Schanz, U., Graux, C., Schouten, H. C., Ferrant, A., Bargetzi, M., Fey, M. F., & Löwenberg, B. (2011). Autologous peripheral blood stem cell transplantation for acute myeloid leukemia. *Blood, 118*(23), 6037-6042. doi: 10.1182/blood-2011-07-370247

Vouri, M., & Hafizi, S. (2017). TAM Receptor Tyrosine Kinases in Cancer Drug Resistance. *Cancer Research, 77*(11), 2775-2778. doi: 10.1158/0008-5472.can-16-2675

Waldmann, T., Scholten, I., Kappes, F., Hu, H. G., & Knippers, R. (2004). The DEK protein—an abundant and ubiquitous constituent of mammalian chromatin. *Gene, 343*(1), 1-9. doi: https://doi.org/10.1016/j.gene.2004.08.029

Wang, C., Jin, H., Wang, N., Fan, S., Wang, Y., Zhang, Y., Wei, L., Tao, X., Gu, D., Zhao, F., Fang, J., Yao, M., & Qin, W. (2016). Gas6/Axl Axis Contributes to Chemoresistance and Metastasis in Breast Cancer through Akt/GSK-3β/β-catenin Signaling. *Theranostics, 6*(8), 1205-1219. doi: 10.7150/thno.15083

Wang, Z.-Y., & Chen, Z. (2008). Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood, 111*(5), 2505-2515. doi: 10.1182/blood-2007-07-102798

Warrell, R. P., Frankel, S. R., Miller, W. H., Scheinberg, D. A., Itri, L. M., Hittelman, W. N., Vyas, R., Andreeff, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M. S., & Dmitrovsky, E. (1991). Differentiation Therapy of Acute Promyelocytic Leukemia with Tretinoin (All-trans-Retinoic Acid). *New England Journal of Medicine, 324*(20), 1385-1393. doi: 10.1056/nejm199105163242002

WHO. (2019). Pakistan Fact sheets. *Globocan.* from http://gco.iarc.fr/today/data/factsheets/populations/586-pakistan-fact-sheets.pdf

Yang, M.-H. Z. a. Q.-M. (2014). NUP214 fusion genes in acute leukemia (Review). *Oncology Letters*, 959-962.

Zhang, D.-E., Zhang, P., Wang, N.-d., Hetherington, C. J., Darlington, G. J., & Tenen, D. G. (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α-deficient mice. *Proceedings of the National Academy of Sciences, 94*(2), 569-574. doi: 10.1073/pnas.94.2.569

Zhang, H., Li, H., Xi, H. S., & Li, S. (2012). HIF1α is required for survival maintenance of chronic myeloid leukemia stem cells. *Blood, 119*(11), 2595-2607. doi: 10.1182/blood-2011-10-387381

Zheng, X., Seshire, A., Rüster, B., Bug, G., Beissert, T., Puccetti, E., Hoelzer, D., Henschler, R., & Ruthardt, M. (2007). Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARα-positive leukemic stem cells. *Haematologica, 92*(3), 323-331. doi: 10.3324/haematol.10541

Zhu, H.-H., Qin, Y.-Z., & Huang, X.-J. (2014). Resistance to Arsenic Therapy in Acute Promyelocytic Leukemia. *New England Journal of Medicine, 370*(19), 1864-1866. doi: 10.1056/NEJMc1316382

Zhu, J., Zhou, J., Peres, L., Riaucoux, F., Honoré, N., Kogan, S., & de Thé, H. (2005). A sumoylation site in PML/RARA is essential for leukemic transformation. *Cancer Cell, 7*(2), 143-153. doi: https://doi.org/10.1016/j.ccr.2005.01.005