Evaluation of Anti-Proliferative Activity of Imidazole Derivatives in BCR/ABL1 Positive Chronic Myeloid Leukemia

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Dedicated to

My Purest Love on the Globe, My Most Affectionate Abu and Ami

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Aysha Bibi

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List of Acronyms

A

ABL	Abelson murine leukemia virus homology gene
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia

B

BCR	Breakpoint cluster region
BM	Bone marrow
bp	Base pairs

C

cDNA	Complementary DNA
CLL	Chronic lymphatic leukemia
CML	Chronic myeloid leukemia
CR	Complete remission
CSCs	Cancer stem cells

D

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates

F

FISH	Fluorescence in situ hybridization

H

HSCT	Hematopoietic stem cell transplantation

I

IL	Interleukin
J	
JAK	Janus Kinase
L	
LSCs	Leukemic stem cells
M	
M	Molar
MAPK	Mitogen activated protein kinase.
mM	Millimolar
µl	Microliter
µM	Micromolar
MTT	Tetrazolium salt
N	
NF water	Nuclease free water NUP214 Nucleoporin 214
P	
PCR	Polymerase chain reaction
Ph+	Philadelphia positive
R	
RBCs	Red blood cells
RNase	Ribonuclease
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RTK	Receptor tyrosine kinases
T	
t (9;22)	Translocation of chromosome 9 and 22
TAE	Tris acetate EDTA

U

UV	Ultraviolet

W

WBCs	White blood cells
Wnt	Wingless-related integration site

+	Positive
-	Negative
%	Percent
°C	Degree Celsius

ABSTRACT

Chronic myeloid leukemia (CML) is a myelo-proliferative disease characterized by a reciprocal translocation between chromosome 9 and chromosome 22 leading to increased proliferation of granulocytic cell lineage leads to high number of granulocytes and their immature precursors in blood profile. Present therapeutic strategies like chemotherapies along with targeted therapies for leukemia proved insufficient as disease remission and adaptive resistance along with cancer stem cells are chief factors behind plasticity as a treatment response in leukemia. Intrinsic and acquired mutations in BCR/ABL1 positive CML are salient reason of resistance development to tyrosine kinase inhibitors, the frontline therapeutic option. Range of imidazole derivatives have already exhibited anticancer potential in various cancers. The current research investigated four novel imidazole derivatives L7, L4, R-35, and R-NIM04 for their anti-leukemic potential and possible mechanism of action in BCR/ABL1 positive CML cells through *In-silico* and *In-vitro* approaches. Molecular docking was performed to shortlist the possible target candidates for these compounds followed by in-vitro cytotoxicity investigation using MTT assay. Only L7 was able to interfere with proliferation of CML cells *in-vitro*. Our *In-silico* findings shows, Breakpoint Cluster Region protein (BCR) as a potential drug target for L7 (B.E -7.25 kcal/mol). Subsequently, treatment with L7 at different concentrations interferes with the proliferation potential of BCR/ABL1 positive CML cells with $p\text{-values}<0.05$. Following MTT assay, RT-PCR findings indicated the downregulation of AXL-RTK and *c-Myc* suggesting that L7 interferes with Wnt/□-Catenin signaling pathway and reduces cellular proliferation by targeting fusion gene BCR/ABL1 in CML cells. Hence this research proffers L7 imidazole derivative as a potential therapeutic option for BCR/ABL1 positive CML.

1. INTRODUCTION

Leukemia is known as amalgam of cancers found in blood and bone marrow that arises due to the incomplete proliferation of white blood cells called blasts or leukemic cells which are unable to fight the infections and impair the potential of bone marrow to make normal WBC's. One or more mutations at genetic level leads to a differentiation halt and thus immature blast or leukemic cells do not mature. The blasts grows more rapidly than normal cells, replace them and may spread to the lymph nodes and other body organs.(Chapalamadugu, Ojochenemi, & Chatakonda). Anaemia, thrombocytopenia, leukopenia and leukocytosis are specifically associated with the development of leukemia while bone pain, weight loss and fever are non-specific symptoms (Davis, Viera, & Mead, 2014).

It is included among most familiar malignant disorder affecting the population globally. In Pakistan, it ranked as 5th most common reason of mortality by WHO, 2019. (Ahmad, Yusuf, & Burney, 2015) have reported that leukemia affects males (5.2%) more than females and AML is the most commonly diagnosed leukemia followed by CML, ALL and CLL. Globally, in 2018, leukemia is ranked as the 15th most commonly diagnosed cancer with 309,006 mortalities, amounting to the 11th major cause of death due to cancer. Leukemia have universal geographic distribution with high occurrence and mortality in developed and developing countries.(Tebbi, 2021).

Acute leukemia is a clonal malignancy of blood forming organs characterized by undifferentiated and immature hematopoietic blast cells. It progresses rapidly and results in decrease of normal erythrocytes and platelets in the blood. Acute leukemia can be acute lymphoid leukemia (ALL) or acute myeloid leukemia (AML). On the basis of hematopoietic cell lineage involved, like myeloid,

lymphoid, undifferentiated or mixed, these disorders are classified accordingly. While chronic leukemia is marked by uncontrolled expansion and proliferation of mature and differentiated hematopoietic cells thus can be classified accordingly as chronic lymphocytic leukemia (CLL) or chronic myeloid leukemia (CML) and it is generally harder to cure than acute leukemia.

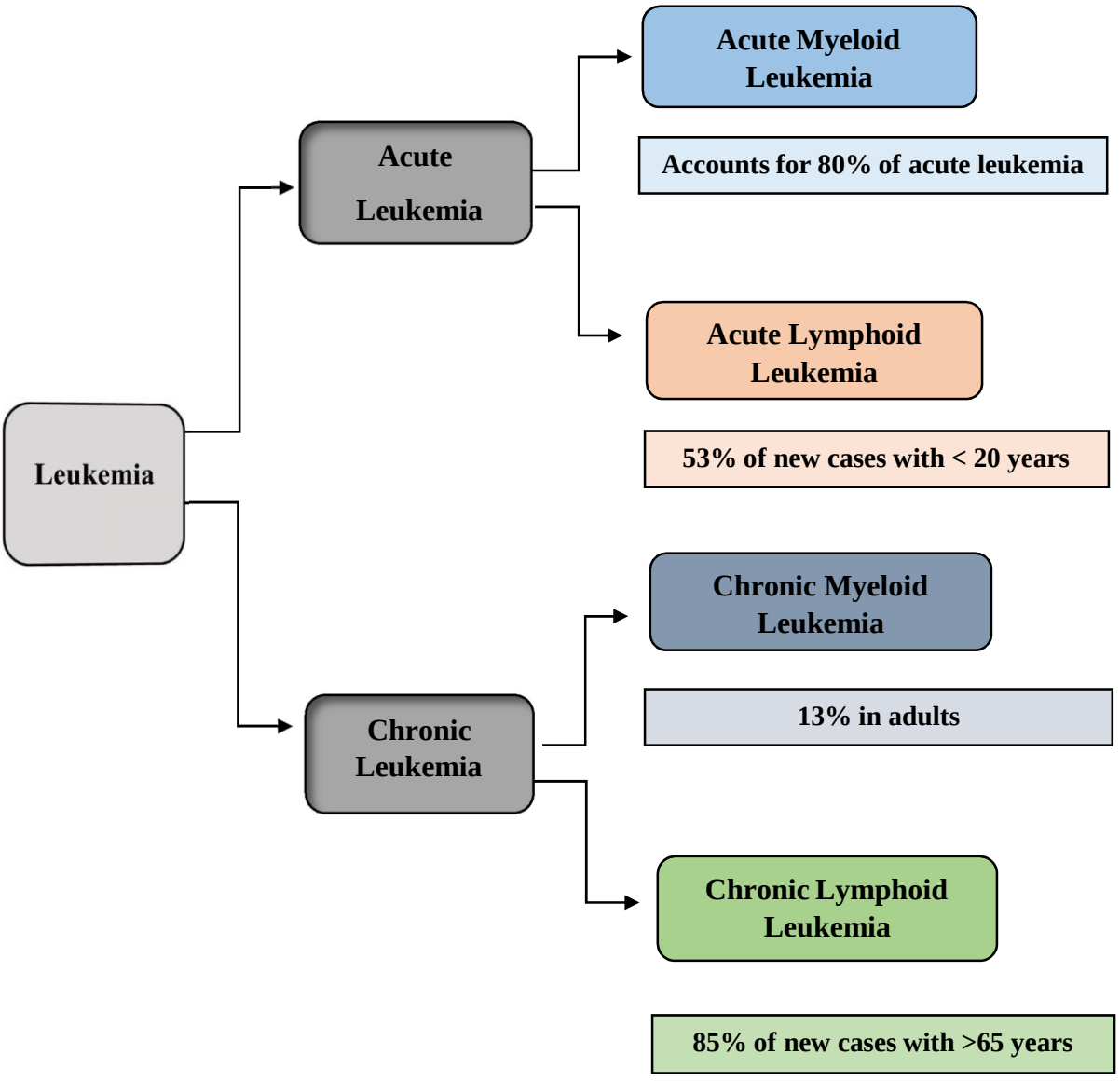

Figure 1.1: Leukemia types and their prevalence (Davis et al., 2014)

Chronic myeloid leukemia is known as a myeloproliferative disorder characterised by increased granulocytic cell lineage proliferation, resulting in a high count of granulocytes as well as their premature precursors in the blood profile. CML is distinguished by the translocation t(9;22)(q34;q11.2) known as the Philadelphia chromosome (Ph), which results in the reciprocal exchange of the Abelson murine leukaemia oncogene (ABL1) from chromosome 9q34 to chromosome 22q11, resulting in BCR/ABL1 gene fusion. This fusion commodity encodes an oncogene (P210, more seldomly P230 or P190), which causes tyrosine kinase upregulation and aberrant down - stream signalling, likely to result in differentiation arrest, increased proliferation, resistance to apoptosis, as well as interfering with normal myelopoiesis.(Acar & Uz, 2018). Many domains from both BCR and ABL1 are found in the BCR/ABL1 fusion protein. A coiled-coil domain at the N-terminus, a Ser/Thr kinase domain with a docking site for GRB2, and a ras domain are part of BCR. BCR portion also contains the homolog gene family/Guanine nucleotide exchange factors (Rho/GEF) kinase domain, while the ABL1 portion contains the src homology (SH) domain, a proline-rich domain, and DNA- and actin-binding domain. All of the hybrid proteins play a role in increasing tyrosine kinase activity..(Kang et al., 2016)

Normally, role of BCR protein is not well revealed but some studies reported its involvement in regulation of movement of cells and communication within cells by acting as a GTPase as well as kinase protein. Depending upon normal cellular conditions, ABL proto-oncogene1 non-receptor tyrosine kinase protein is involved in various cellular signalling including division, cell growth, differentiation, migration, adhesion with other cells and apoptosis.

It is a critical factor in the development of both solid tumors and leukemias. Genomic instability leads to atypical fusion products like BCR/ABL1, TEL/JAK2 and TEL(ETV6)/ABL1 which act as potential source of hematologic malignancies(Turner & Alexander, 2005). The cells harboring

BCR/ABL1 fusion also have high number of double-strand breaks (DSBs) in DNA and exhibit activation of single-strand annealing (SSA) repair process. BCR/ABL1 fusion product expand the nuclear translocation and expression of WRN protein which encodes a helicase required during the DSB repair mechanisms ultimately promoting the cell survival and genetic instability(Slupianek et al., 2011). The upregulated BCR/ABL1 exerts post-transcriptional, translational and post-translational effects on genes having anti-apoptotic, anti-differentiation and mitogenic activity like MYC, YES-1, MAPK(ERK12), LYN, STAT5, BCL2 etc and also inhibits the tumor supressors (p53, PP2A, CCAAT/C/EBPa)(Jamieson, 2008).

Generally targeted therapies are used for treatment of CML like tyrosine kinase inhibitors (TKI's) have resulted in improved long-term survival as compared to chemotherapies. CML accounts for 15-25% of all adult leukemia's and 14% of leukemia's overall.(Chisti & Leukemias). Tyrosine kinase inhibitors such as Imatinib (first generation-TKI), dasatinib, bosutinib and nilotinib (second generation TKI's) are used as a standard treatment against chronic phase chronic myeloid leukemia. If resistance develops against first and second generation tyrosine kinase inhibitors, Ponatinib, a third generation tyrosine kinase inhibitor targeting T315I mutation is a therapeutic option in chronic myeloid leukemia despite of its side effects(M. W. Deininger, O'brien, Ford, & Druker, 2003).

Intolerance to therapeutic strategies and resistance are the key player in faliure of Imatinib. A diverse range of heterogenous factors are associated with resistance development like point mutations in the kinase domain of hybrid BCR/ABL protein as well as upregulation of multiple drug-resistance genes resulting in reduced inhibitor level and making leukemic cells resistant to ongoing treatment(Rossari, Minutolo, & Orciuolo, 2018). Pleural effusion, pulmonary atrial hypertension, cardiovascular diseases, metabolic diseases, diarrhea and rarely renal faliure are the

major side effects of all five TKIs available for treatment of CML(García-Gutiérrez & Hernández-Boluda, 2019).

Imadizoles are basically organic alkaloid five cornered aromatic ring structures acting as a chief pharmacophore in the field of drug development since its discovery in 1840's. Naturally it is present in essential amino acids, biotin, histamine, pilocarpine alkaloids and histadine while synthetically it can be found in losartan fungicides, cimetidine, plant growth regulators, herbicides and therapeutic agents(Lo et al., 1992). Various studies have reported broad spectrum biological activities of imadizoles and its derivatives such as anticancer(Cai, Li, Zhou, & Wu, 2009), antiviral(D. Sharma et al., 2009), antifungal(Pierard, Vroome, Borgers, Cauwenbergh, & Pierard-Franchimont, 2006), antibacterial(Khabnadideh, Rezaei, Motazedian, & Eskandari, 2007), antiparasitic(Sánchez-Moreno et al., 2012), and many more medicinal effects. Effectual anticancer properties have led to the development of many clinically approved imadizoles including nilotinib, tipifarnib, temzolomide, mercaptopurine, decarbazine etc against various types of cancers. The intriguing anticancer properties of imadizoles prompted the widespread development of their derivatives in the hope of improving efficiency and reducing side effects.

1.1 Hypothesis

Adaptive resistance, adverse side effects and presence of cancer stem cells are the major hurdles in curing leukemia. The demand of hour is to find out the potential therapies that can lower disease progression and mortality rates. Imidazole derivatives have shown remarkable pharmaceutical potencies to control cancer and can be a safe alternative to anticancer chemotherapy. The current study was designed to find out the potential drug targetsof a novel imadizole derivative L7 in BCR/ABL1 positive CML cells through In-silico approaches and investigate the anti-proliferative potential of L7 against BCR/ABL1 positive CML cells through In-vitro testing. The novel

imadizole derivative L7 was predicted to target the key player, the fusion gene BCR/ABL1 in CML and known downstream signalling pathways involved in disease progression and leukemogenesis.

1.2 Aims & Objectives

- To find out potential candidate targets of a novel imidazole derivative L7 using *insilico* approaches in BCR-ABL positive K562 cells.
- To evaluate antiproliferative properties of L7 using vet lab experiments and leukemic cell lines.
- To evaluate mechanism of anticancer potential of L7 by analyzing downstream cancer signaling pathways.

2. LITERATURE REVIEW

2.1 Hematopoiesis

A solely supervisod process of producing cellular components of the blood occurs during developmental period and all round adulthood in spleen, lymph nodes and bone marrow to make and replenish the complete blood profile. Basically it starts from a haemaopoitic stem cell and differentiate it into lymphoid and myeloid cell lineages for further differentiation in hierarchy of haematopoisis. In 1938, Downey presented the notion of hierarchies of pluripotent cells. Before that, it was thought that there are only one kind of progenitors capable of producing all blood cells. Many origin specific transcriptional and growth related factors along with their receptors are tangled in the process of haematopoisis for differentiation of stem cells through specific gene expression. Any disruption in this framework forms the basis for leukemic pathways(Kim, Stachura, & Traver, 2014).

2.1.1 Hematopoietic Hierarchy and HSC's Regulation

Hematopoiesis starts from hematopoietic stem cells (HSCs) which are pluripotent cells having self-renewal capacity as well as ability to differentiate into all kinds of blood cells and begins a multistep process of hematopoiesis. HSC differentiates to form multipotent progenitor cells (MPP), its immediate descendant which then differentiates into lymphoid and myeloid progenitors that broadly differentiate further. MMP cells rapidly differentiate into any HSC type with non-self-renewal property. RBC's, granulocytes, macrophages/monocytes and platelets are included in myeloid lineage while lymphoid lineage make up B and T-cells, natural killer cells and dendritic cells(Iwasaki & Akashi, 2007). The mature blood cells have finite life span therefore; their numbers are constantly restocked.

During normal homeostasis, approximately 90% of HSC's in bone marrow are modulated by attaining quiescence state at G0 stage of cell cycle. This is the basic reason of preservation of genetic integrity of HSC's and prevents any kind of damage related to cell replication. A limited number of HSC's are continuously involved in the normal hematopoiesis while a huge number remains inactive which enter cell cycle for proliferation only in case of bleeding, stress, inflammation and infection. HSC receptors for chemokines, cytokines and danger related molecular mechanisms are responsible for efficient response to such demand-adapted hematopoiesis(Jagannathan-Bogdan & Zon, 2013).

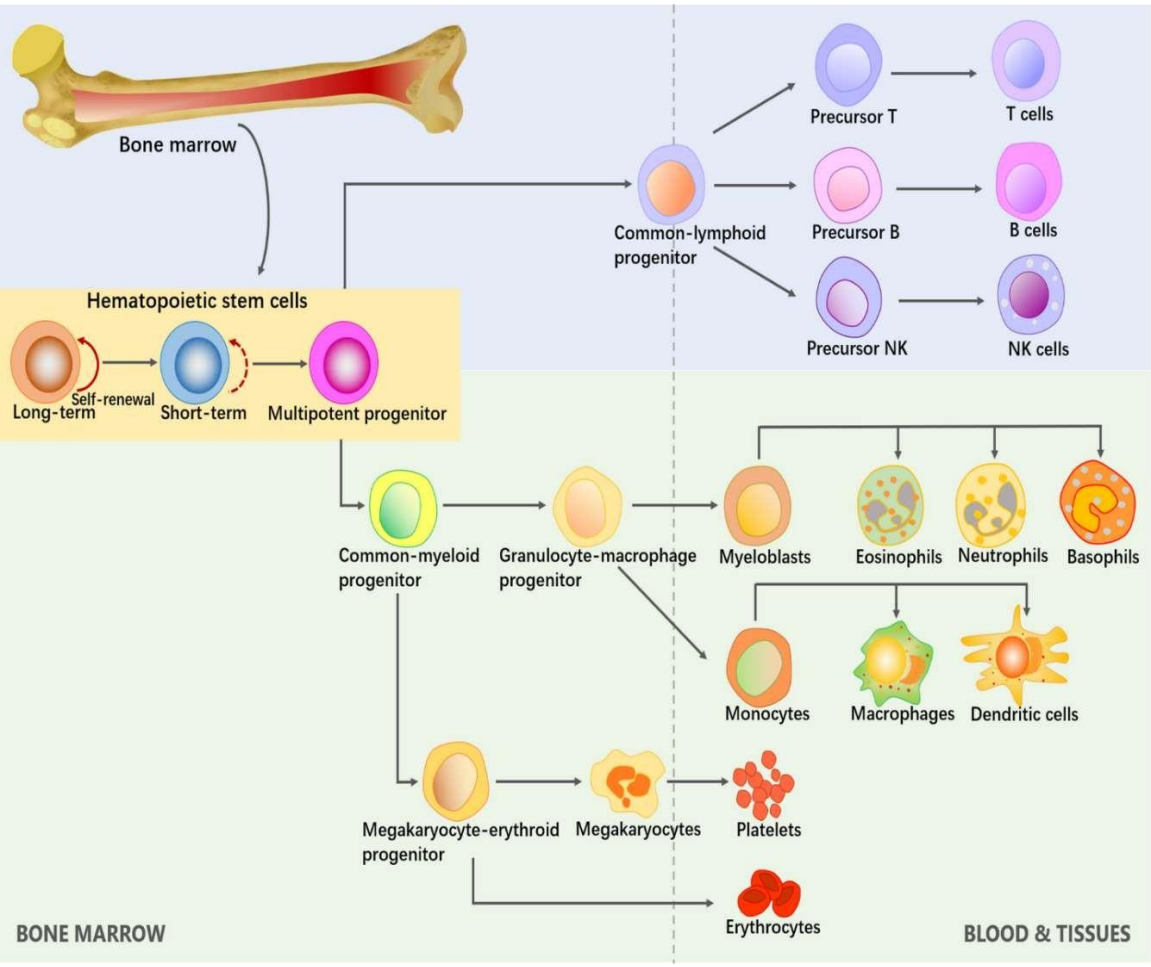

Figure 2.1: The Haematopoietic Hierarchy. HSCs located at the top of hierarchy with ability to self-renew and differentiate into mature blood cells (Pan Zhang et al., 2019)

2.2 Leukemia and Its Types

Leukemia is known as amalgam of cancers found in blood and bone marrow that arises due to the incomplete proliferation of white blood cells called blasts or leukemic cells which are unable to fight the infections and affect the capacity of bone marrow to make normal WBC's. One or more mutations at genetic level leads to a differentiation halt and thus immature blast or leukemic cells do not mature. The blasts grow more rapidly than normal cells, replace them and may spread to the lymph nodes and other body organs. Leukemias can be grouped as acute and chronic leukemia based on the disease history

- Acute myeloid leukemia (AML)
- Acute lymphoid leukemia (ALL)
- Chronic myeloid leukemia (CML)
- Chronic lymphoid leukemia (CLL)

It is a proliferative neoplasm of hematopoietic cells caused by a variety of factors that cause somatic mutations in pluripotent stem and progenitor cells. Mutated neoplastic cells function similarly to hematopoietic stem cells in that they can self-replicate, differentiate, and nourish progenitor cells into different hematopoietic clades. These leukemic, unipotent stem cells can mature to phenocopies of mature blood cells to varying degrees.

2.3 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is known as myelo-proliferative disease characterised by increased granulocytic cell lineage proliferation resulting in a high count of granulocytes and their immature precursors in blood profile. CML is described by the reciprocal translocation t(9;22)(q34;q11.2) known as Philadelphia chromosome(Ph) resulting in reciprocal transfer of

Abelson murine leukemia oncogene (ABL1) at chromosome 9q34 to breakpoint cluster region (BCR) at chromosome 22q11 leading to BCR/ABL1 fusion gene. The major cytogenetic aberration associated with CML is this Philadelphia chromosome since many years. Apart from CML, Philadelphia chromosome is also associated with many hematological malignancies

(Haider & Anwer, 2021).

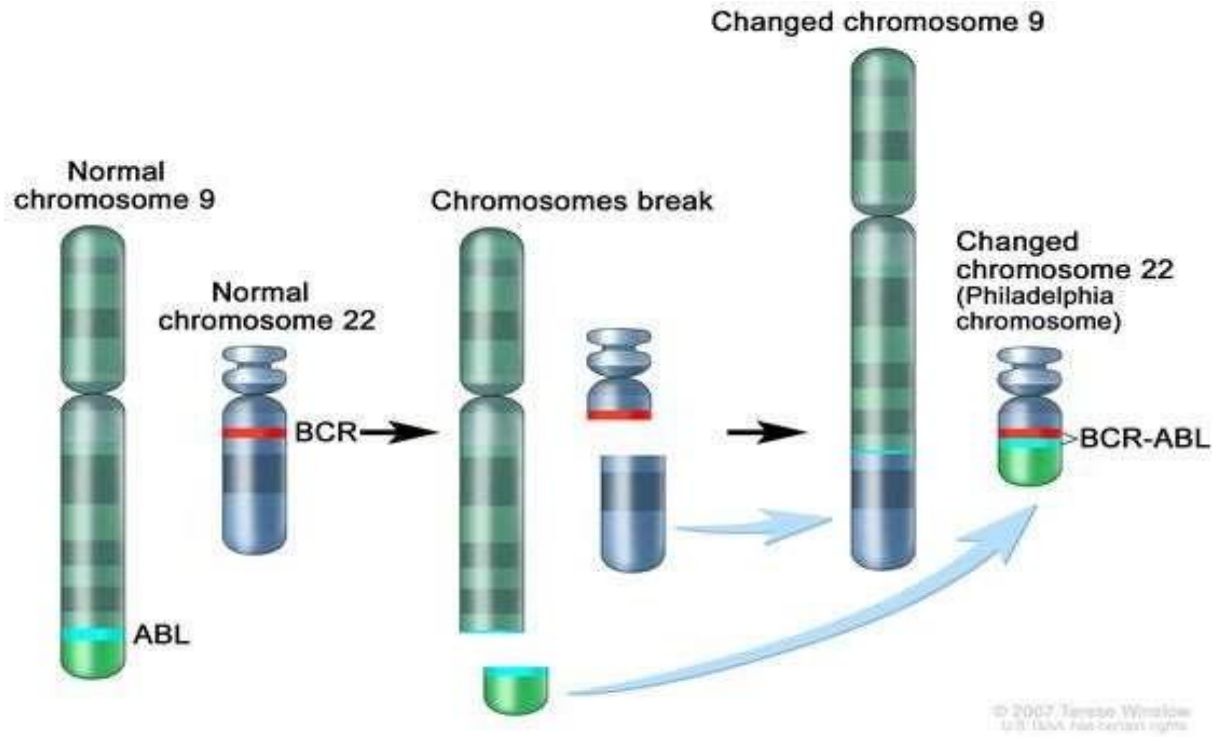

Figure 2.2: Formation of Philadelphia Chromosome. Reciprocal translocation between chromosome 9 and 22.
https://www.cancer.gov/publications/dictionaries/cancer-terms/def/philadelphia-chromosome

After the discovery of this reciprocal translocation, many researches were conducted to investigate the molecular pathways associated with CML. Further studies of BCR/ABL hybrid genes present in different leukemias portrayed different poses of coupling between BCR and ABL genes proving it as an obligatory event in leukemic pathogenesis.

Tyrosine kinase activity was discovered for targeted therapy of CML, leading to the development of the tyrosine kinase inhibitor (TKI) imatinib. (Woessner, 2013). After the discovery of first and second generation tyrosine kinase inhibitors, they were used as first-line treatment option of CML (M. Deininger et al., 2009; Kantarjian et al., 2010; Nicolini et al., 2012; Saglio et al., 2010) leading to drastic improvement of patient's survival rates. But targeting the stem cell niche is required for its eradication.

2.3.1 Incidence, Prevalence and Survival

Annual incidence rate of 0.87 people/100,000 people, worldwide, is recorded for chronic myeloid leukaemia. An obvious spike in this rate is seen due to increase in age, individuals aged 70 or above show increase in rate up to 1.52 while 56 years old is regarded as the median age of diagnosis. A slight male predominance is seen as pattern of diagnosis show higher frequency in males across all age groups in comparison to females. A range of male to female ratio shown between 1.2 and 1.8 (Lin et al., 2020). Exposure to ionizing radiations is likely to increase the potential risk to develop CML manifold, representing almost 17% of all the radiation induced leukaemia per year. Ever since the accident of Chernobyl nuclear power plant, an increase in CML incidence among power plant cleanup workers is noted (Rohrbacher & Hasford, 2018).

Access to the targeted therapy for CML treatment has relatively improved the survival rate as described recently, 89% of overall survival of 8 years, especially in patients compliant to the TKI therapy. Though, survival rate in patients with more than 70 years of age is still reducing. It can be deduced that the better life expectancy and prolonged survival particularly in countries with better healthcare system and medical resources in CML leads to an increase in the prevalence rates globally estimated around 10-12 out of 100,000 inhabitants. In another report, within the next 30

years, CML is expected to be the most common type of leukemia. (Castagnetti et al., 2015) (Höglund, Sandin, & Simonsson, 2015).

2.3.2. Histopathology of CML

Chronic myeloid leukaemia can either be bi-phasic or tri-phasic, disease progresses through three distinct phases. Overtime accumulation of additional cytogenetic abnormalities other than BCR-ABL might be the contributing factor in worsening of each phase.

Blast cells contribute for less than 10% of white blood cells in the chronic phase, with elevated levels of eosinophils and basophils in blood circulation. In such case, an inspection of peripheral blood smear shows granulocytes matured at different stages that is a cause of leukocytosis. Additionally, biopsy reveals distinguishable granulocyte proliferation in all chronic phase cases while half of such cases also present with megakaryocytic proliferation.

An increase in blast cells may be observed of about 15% but not more than 30% in peripheral blood smear, accelerated phase is usually not well defined and unstable. Biopsy results define the accelerated phase with visibly similar changes relative to the chronic phase however, an addition of dysplastic changes is described. As compared to chronic phase where dysplasia in granulocyte population was absent, a significant dysplasia is noticeable in granulocytes of accelerated phase (Arber et al., 2016). Bone marrow displays an increase in reticulin, fibrosis, a persistent thrombocytopenia, and splenomegaly is recorded. Patients with accelerated phase do not answer well to the treatment as compared to a generally better response shown by chronic phase patients (Kolenova, Maloney, & Hunger, 2016).

Regarded as the terminal phase, most chronic phase patients advance towards acute like blast phase marked by 20% of blast cells as shown in peripheral smear. Large clusters of blast cells are

accumulated in bone marrow and eventually proliferate into the extramedullary, mostly detectable in skin, bone, central nervous system and lymph nodes. These blast cells usually differentiate from cells of myeloid origin (Song, Liu, Pinilla-Ibarz, & Zhang, 2020). Nevertheless, at some instances other lineages also contribute as to case of lymphoblasts. The aggressive blast phase is highly resistant towards treatment with chemotherapeutic agents with a response rate of less than 20% while median survival rate of 3-6 months. Less than 5% of all patients are diagnosed in blast phase (Bonifacio, Stagno, Scaffidi, Krampera, & Di Raimondo, 2019) (DeFilipp & Khoury, 2015).

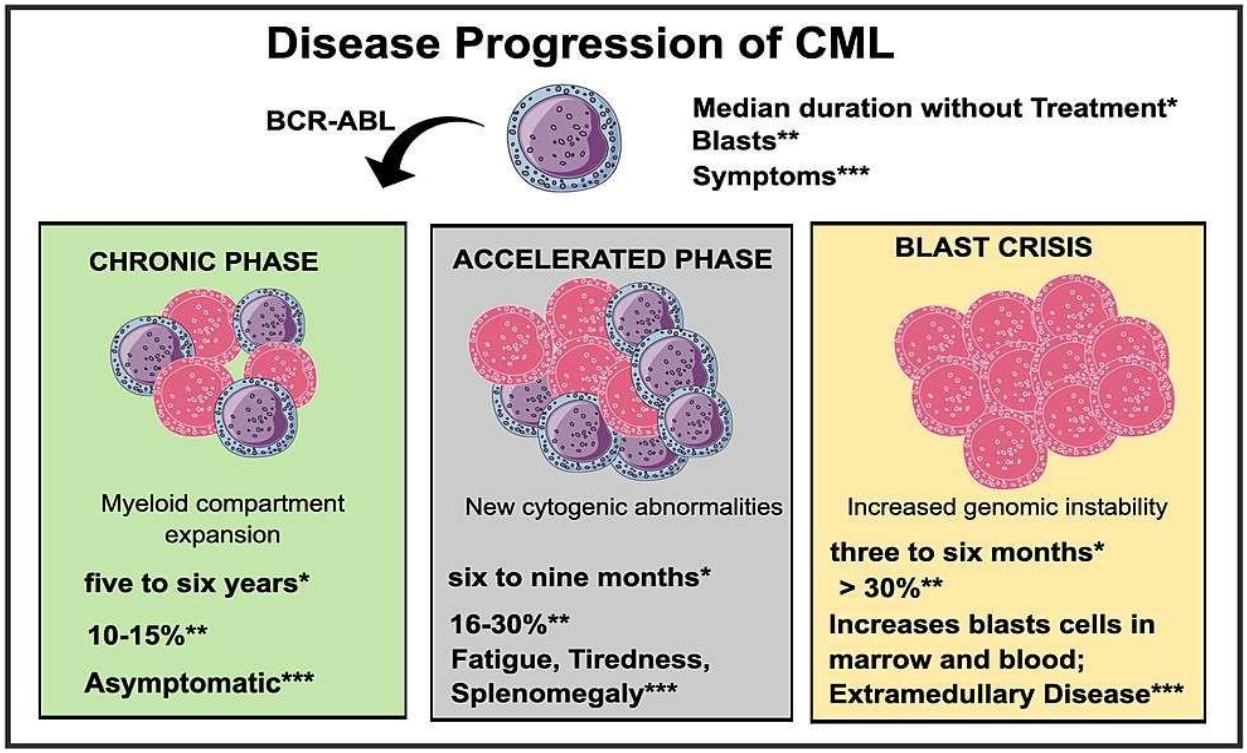

Figure 2.3: Characteristics of chronic, accelerated and blast phase in CML disease progression (Clarke & Holyoake, 2017)

2.3.3. BCR Gene and its Structural Domains

In contrast to ABL, BCR was first recognized due to the emergence of novel hybrid fusion gene and is named after its break-point cluster region, present on chromosome 22, BCR is mapped to a region of 135 kb length. A cytoplasmic 160 kDa BCR protein is unanimously expressed encoded by both 4.5 kb and 6.7 kb transcripts (Brown & Betz, 2017).

BCR protein elucidates its vital cellular functions through several structurally important domains including a serine-threonine kinase, encoded by first large exon, its substrate is part of 14-3-3 family of proteins called Bap-1. Dbl-like-pleck-strin homology domain PH is another functionally essential domain positioned in the central part of BCR protein (Peiris, Li, & Donoghue, 2019). A GDP-GTP guanine diphosphate-triphosphate exchange factor, Rho, is stimulated by this PH domain consequently activating downstream transcription factors and NF-KB. BCR protein also enables the function of Ras family of proteins which controls oxidation of NADPH in phagocytic cells. This ability of BCR is due to the existence, in the C terminal region of protein, of GTPase activity domain of a Ras family member, (Hai, Kizilbash, Zaidi, Alruwaili, & Shahzad, 2014) Rac-triphosphate exchange factor, Rho, is stimulated by this PH domain consequently activating downstream transcription factors and NF-KB. BCR protein also enables the function of Ras family of proteins which controls oxidation of NADPH in phagocytic cells. This ability of BCR is due to the existence, in the C terminal region of protein, of GTPase activity domain of a Ras family member. Role of dimer formation is achieved by the presence of coil like looped domain. Activation of the hybrid protein BCR-ABL is achieved through phosphorylation at tyrosine residue (position 177) of BCR protein. Once this tyrosine residue is phosphorylated it can then

bind to an adaptor molecule Grb2 which then further activates downstream signalling involving Ras pathway (P. Gupta, Ashar, Ashby, Lin, & Chen).

2.3.4 ABL Proto-oncogene and its Domains

The proto-oncogene, ABL, was first recognized for its similarity with v-Abl (Abelson murine leukemia viral onco-gene) which is a viral proto-oncogene with ability to produce lymphoid tumors in mouse. ABL proto-oncogene is part of a non-receptor tyrosine kinases (NTKs) family which are mainly described for involvement in controlling cellular proliferation, cellular differentiation, apoptosis, and cell adhesion (Wang, 2014) (Colicelli, 2010). Various tissues have shown ubiquitous expression of ABL gene, which encode proteins from two different transcripts based on early alternative exons namely 1b and 1a. The C-terminal portion of ABL contains main domains such as actin, 3 nuclear localizing signals along with a nuclear transport signal, and only non-receptor tyrosine kinase with a DNA binding sites. These elements enable ABL protein to act as a shuttle between cytoplasm and nucleus playing various roles (Kiani et al., 2016).

Other than containing a C terminal portion unlike to the Src, other significant domains that relate with respective Src domains include: the Src homologous SH1 domain, which is characterized by its tyrosine kinase activity, a SH2 domain that functions by binding to sequences containing phosphotyrosine, and a SH3 domain with its ability to bind to specific sequences rich in proline (Corbi-Verge et al., 2013).

Strictly regulated by numerous physiological mechanisms, alteration in function of these tyrosine kinases are frequently reported in several cases of malignancies and is also implied a common primarily occurring defect in chronic myeloid leukemia.

ABL is tightly regulated by several mechanisms involving SH3 and SH2 domains along with certain segments of N terminal region. These mechanisms aim at either downregulating tyrosine kinase activity of ABL or maintaining it in an inactive conformation (Panjarian, Iacob, Chen, Engen, & Smithgall, 2013) (Corbi-Verge et al., 2013).

Cytoplasm bound ABL is part of pathways regulating proliferation induced by growth factors. One such signalling cascade involves PDGFR and non-receptor TKs acting as a contributor in mitogenic signalling that further stimulates Myc expression through Ras/Erk pathway (Khatri, Wang, & Pendergast, 2016). Phosphorylation of ABL on Thr 735 which is required for its binding to 14-3-3 protein causes sequestering of ABL in cytoplasm. As a result of damage to DNA, phosphorylation of 14-3-3 protein occurs induced due to the activated Jun N-terminal kinase (JNK). This leads to release of ABL from its sequestered position in cytoplasm with its subsequent transfer to the nucleus (Nihira, Taira, Miki, & Yoshida, 2008).

2.3.5 BCR/ABL Hybrid Gene

Even though different BCR-ABL hybrid fusion genes show certain molecular variability, and each create distinct transcripts, nonetheless, all BCR-ABL hybrid genes have a region acquired from its BCR gene at the 5' as well as a region constituted of lengthy ABL sequence at its 3' side. This bulky ABL section lacks alternative exon 1b mostly rather than alternative exon 1a which is present quite often. In all the incidences of BCR-ABL except those with sporadic cases breakpoint region falls upstream to the exon 2 within BCL gene (H.-Q. Zhu & Gao, 2019).

In CML, breakpoints primarily occur on a specific region of BCR gene located at its center on chromosome 22 known as major break-point cluster region (M-BCR). In some other

cases, breakpoint junction is between exon 1 of BCR situated on a site called as minor break-point cluster region m-BCR and exon 2 of abl. Also, in some small number of CML cases, a micro breakpoint cluster region located towards very 3' end of BCR is involved thus establishing a juncture between BCR exon-19 and ABL exon-2. Consequently, a longer transcript is generated as compared to a relatively shorter one produced from junction including m-BCR and ABL exon 2 (Soverini, Mancini, Bavaro, Cavo, & Martinelli, 2018).

2.3.6 BCR/ABL1 Downstream Signalling Pathways

Multiple pathways and the crosstalk between them are implicated by the hybrid BCRABL1 that appear to be indispensable in the neoplastic transformation of cells. Such perturbations in signalling pathways dictate the cell fate in three most significant areas of concern including apoptosis, cell adhesion, and cell proliferation. Activation of these pathways is greatly associated with progression of leukaemogenesis, and many pathways are linked with more than one functions. One such example is of PI3K/AKT signalling that is intricated in both cell apoptosis as well as proliferation. Several pathways also differ in their functions regarding the specific lineage of cells positive with BCR-ABL1 (Bernt & Hunger, 2014).

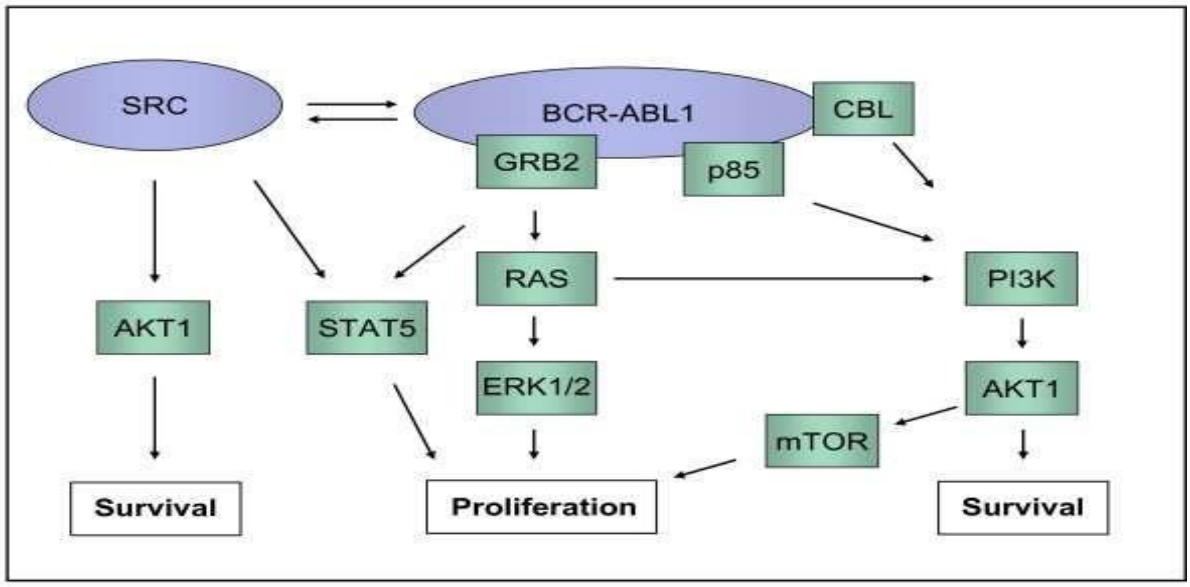

Figure 2.5 Down-stream signalling of BCR/ABL1 Hybrid

2.3.7 RAS- PI3K/AKT Signaling

Signalling pathway controlled by Ras proteins along with its other family-members plays pivotal duties in CML disease progression. As soon as BCR-ABL1 is activated, it interacts with cellular adaptor molecules such as Grb2 thus, starting a complex chain of signalling between various proteins. Bound Grb2 and hybrid protein complex then constitutively interacts with SOS protein through SH3 domain of Grb2 where it begins its role as Ras guanine nucleotide releasing protein (GNRP). Consequently, inactive GDP bound Ras protein is then activated by binding with GTP. Furthermore, Ras signalling can be escalated more by another complex involving SHC gene product, Shc-Grb2 (Bertacchini et al., 2015).

Another crucial adaptor protein, Crkl, acts as an intermediate molecule containing SH2 and SH3 domains necessary for required binding. Once Crkl is tyrosine phosphorylated, it ends up associating BCR-ABL1 signalling to activating PI3K/AKT signalling thus attributing

deregulation in multiple cellular functions encompassing apoptosis and proliferation under the constitutive activation of BCR-ABL (Jankowski et al., 2012).

Ras signalling is intensely relevant to disease progression in CML through promoting cellular proliferation along with its significant link with PI3K/AKT pathway which in turn implicates inhibition of apoptosis, both cell characteristics are hallmark of CML. In order to sustain the proliferative properties of BCR-ABL positive cells, activated PI3K and AKT are required (Braun, Eide, & Druker, 2020).

All in all, constitutive upregulation in BCR-ABL1 activity further initiates Ras signalling that activates cell signalling pathways including kinases such as RAF1, MEK1, MEK2, and ERK1/2. Stimulation in the activity of these kinases enable certain events in nucleus prior to their needed time and ultimately lead to the transactivation of trancription factors c-FOS *and c-MYC*. This ensues the activation of such genes responsible for cell proliferation (Szeto, 2017).

2.3.8 MYC Activation

MYC, a protooncogene with an increased expression level in transformed cells in CML,is another necessary nuclear target for the deregulated BCR-ABL1 signalling pathway. Other than indirectly upregulating MYC gene expression mediating through signalling pathways such as RAS, BCR-ABL1 itself directly interacts in governing expression mechanism enabled through SH2 domain and the C-terminus region located at ABL portion of the hybrid. Upregulation in MYC expression proved its association with CML disease progression and specially the transformation of haematopoietic cells due to its involvement in inducing transcriptional activation of specific genes by MYC transcription factor. Changes in the transcription of these specific genes are crucial for

the cell fate since they are connected with cell cycle regulation and apoptosis (N. Sharma et al., 2015).

2.3.9 JAK/STAT Pathway

BCR-ABL1 stimulation of MYC activity has its greater significant because of its implication on the activation of another protein, JAK2. It has already been established that JAK2 activation is essentially showed in high percentage of cases with various myeloproliferative disorders. Hence, it is deduced that JAK2 activation through MYC is highly relevant to the CML pathogenesis. JAK2 binds at C-terminus portion of BCR/ABL1 which then phosphorylated by Src homology domain of hybrid protein. Activated JAK2 is involved in number of downstream activities including blockage of tyrosine protein phosphatase 2A activity thereby, promoting growth and survival of transformed leukaemic cells (Valent, 2014). Under normal cellular conditions, cytokines binding with thier receptor followed by receptor linked JAK kinases activation. Activated JAK kinases play the role of mediator in the nuclear translocation of various STATs (Gleixner et al., 2017). Both STAT3 as well as STAT5 are active, constitutively, in the BCR/ABL1 CML. However, the route of activation of STATs demonstrated in BCR-ABL1 positive cells considerably differ from the normal cells. Apart from employing JAK kinases to activate STATs, tyrosine residues phosphorylated on BCR-ABL1 directly interact with STAT protein, allowing the activation of STAT.

Alternatively, Hck kinase from the Src family of kinases is also used for the activation of STAT. Continuous activation of STAT5 helps to maintain the abnormal cell in its transformed state by promoting cell cycle progression and inhibition of apoptosis through upregulated expression levels of Cyclin D1 as well as Bcl-xl, respectively. Apart from these pathways, various other signalling pathways are notably altered in BCR-ABL1 positive CML cases. NF-KB

transcription factor is activated by BCR-ABL1 while alterations in p38 MEK pathway signalling are showed in BCR-ABL1 positive CML cells (Bar-Natan, Nelson, Xiang, & Frank, 2012).

2.3.10 Wnt/β-Catenin Signaling

Role of activated canonical Wnt/β-Catenin signaling entails influence over genes expression involved in differentiation, apoptosis, and cell proliferation. Under normal conditions Wnt pathway stays inactivated and β-Catenin trapped by destruction complex. Formed in cytosol, destruction complex is comprised of axin, glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), casein kinase alpha (CK1α) surrounding β-Catenin. Under no stimulation, β-Catenin undergoes proteasomal degradation and ubiquitination thus controlling its signalling and nuclear translocation. Wnt proteins act as ligand and bind at frizzled (Fzd) receptor along with its coreceptor lipoprotein-receptor related protein 5 and 6 (LRP 5,6) thereby, activating disheveled(Dvl) protein. This event finally dissociates destruction complex freeing β-Catenin thus stabilizing its free pool in cytosol. Once translocated to nucleus, β-Catenin cause activation of LEF-TCF transcription factors. Eventually, β-Catenin-LEF-TCF signalling pathway triggers transcription of genes necessary for cell growth like c-Myc and cyclin D1 (Thanendrarajan, Kim, & Schmidt-Wolf, 2011).

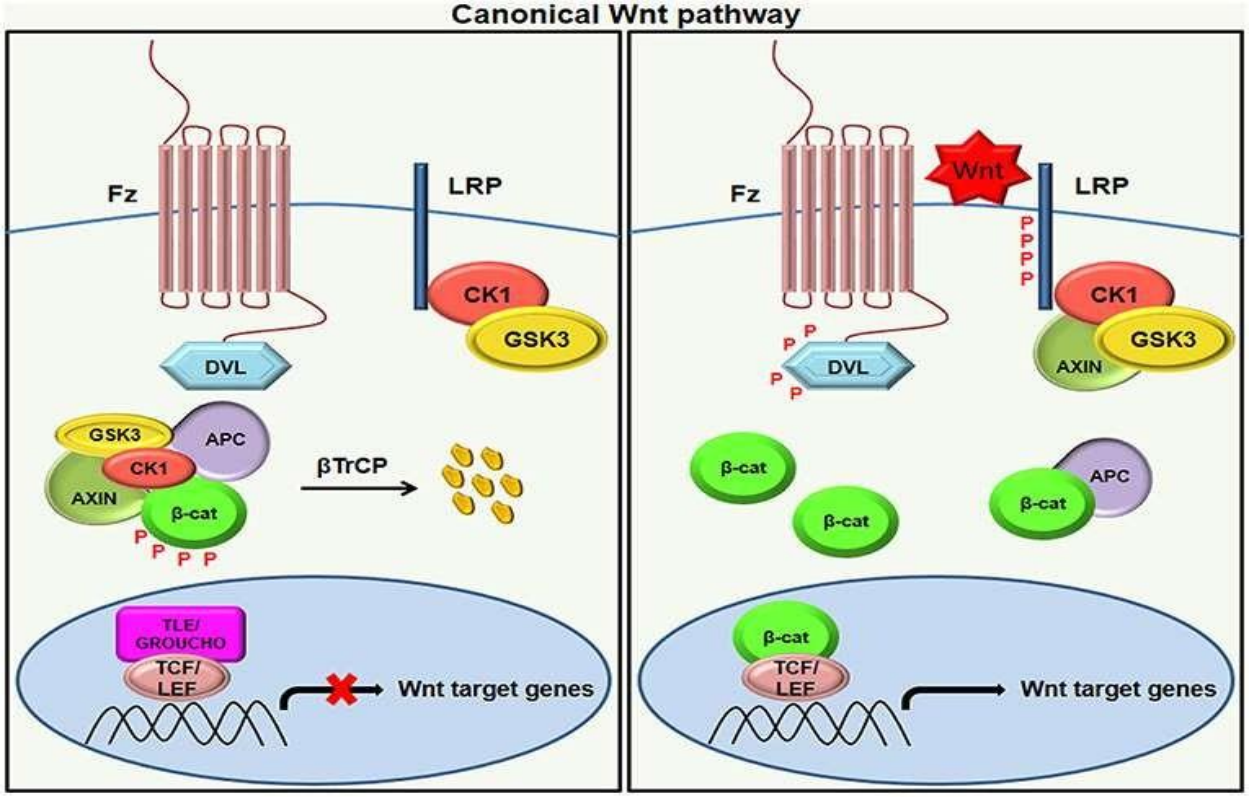

Figure 2.6 Canonical Wnt Signalling Pathway

2.3.11 BCR-ABL1 Leukaemogenesis

BCR-ABL1 is known to be involved in neoplastic transformation of Philadelphia positive cells along with induction of leukemogenic potential. As a result of this hybrid rearrangement, subsequent increase in kinase activity of ABL portion of BCR-ABL1 is noted.

However, for the BCR-ABL stimulated leukaemogenesis, ABL activity alone might not be sufficient and certain domains and critical sequences outside the ABL region of the hybrid act as determining factors for the severity and specificity for lineage. Oncogenic potential is triggered when the otherwise normally regulated kinase activity of ABL is deregulated subsequently, leading to continuously activated form of ABL protein. Intersection with the N terminal region of BCR contains sequences sequences that leads to

dimerization of the BCR-ABL1 protein, constitutively. This, in turn, leads to transphosphorylation process that constitutively activates kinase activity of ABL (Hantschel et al., 2012).

Consequently, this deregulated process causes an uncontrolled tyrosine kinase function of BCR/ABL1 that vigorously triggers downstream effector proteins. Ultimately, loss of sensitivity to apoptotic stimuli, uncontrolled proliferation of cells, and reduced adherence of leukaemic cells to bone marrow stromal surface, are the hallmark consequences of CML phenotype (Valent et al., 2019).

Typically, kinase domain of ABL is under strict regulation with lack of any oncogenic activity even in its over expressed form. Additionally, numerous inhibitory proteins bind to the ABL and regulate its activity, also stabilize the autoinhibition which is implicated through strict control of both inter and intra molecular interactions of ABL. Kinase domain of ABL is bound to SH3 and SH2 to maintain inactive conformation of protein. This conformation remains stable by the contact between hydrophobic portion in the C-terminal of the protein and a myristoyl group present in the N terminal portion of the protein. Thus, any abnormality in these regions and sequences of protein leads to activation of catalytic domain of the protein (Hossain, Dubielecka, Sikorski, Birge, & Kotula, 2012) (Dölker et al., 2014).

On the flip side, fusion of BCR/ABL replaces initial few residues of ABL with a set of sequences from the N terminal region of BCR. Dimerization is then caused by coiled CC motif sited on the BCR region which leads to upregulation in tyrosine kinase activity of protein. This dimerization allows the transphosphorylation of Tyr residue in kinase domain at position 1294. Subsequently, another Tyr residue phosphorylation takes place at position-177 in BCR protein thus, creating high affinity Grb2 binding site where Grb2 attaches through its SH2 domain. Grb2 then forms a complex by binding to SOS and GAB2 through its

SH3 domain activating RAS in its downstream that further activates downstream effectors.Moreover, it is noted that any mutation in Tyr residue at 177 position in BCR leads to an abrogation of BCR-ABL1 leukaemogenic potential thus eliminating its role in inducing myeloid leukaemia (Dixon, Constance, Tanaka, Rabbitts, & Lim, 2012). Adding up to this, the imbalance of tyrosine phosphatases is also shown in leukaemic cells, their downregulation causative factor in transformation of BCR-ABL1 positive cells (Gehrke, Gandhirajan, & Kreuzer, 2009).

2.3.12 BCR/ABL1 Hybrid and Apoptosis

Typically, for a regular cell homeostasis process, proteins with pro-apoptotic function hold significance. While the chimeric BCR-ABL1 kinase has exhibited anti-apoptotic function, normal ABL protein has a potent pro-apoptotic role in contrast to the oncogenic BCR-ABL. This may be explained by the fact that both ABL and BCR-ABL inhabit different sub cellular localization. Due to nuclear export and localization domains in a regular ABL protein, it is possible for ABL to shuttle between cytoplasm and nucleus, thus, can enter nucleus when needed to perform pro-apoptotic function. However, the emergence of ABL in the form of BCR-ABL makes it unable to enter the nucleus and favour apoptosis as BCR-ABL1 is exclusively retained in the cytoplasm (Danisz & Blasiak, 2013) . This reason aside, furthermore, BCR-ABL1 can act as an inhibitor for apoptosis by initiating a signalling pathway. As already described, BCR-ABL1 interacts with and activates PI3K/AKT pathways. This pathway is shown to be impacted by several other pathways including Ras, and Crkl. Once the Gab2/Grb2 complex enables the activation of PI3K pathway through BCRABL1, the AKT kinase is activated through a series of PI3K phosphorylation products. In its activated form, AKT kinase acts as a downstream effector of BCR-ABL1 stimulated PI3K signalling pathway. A

step-by-step chain of phosphorylation of various downstream substrates is initiated by AKT kinase as soon as it is activated. Thus, AKT fulfills its cellular responsibilities by activating number of proteins including mTOR, bad, caspase9, MDM2, ask1, and Fkhrl. Akt phosphorylates pro-apoptotic bad at serine 136 which is then trapped in a complex with 14-3-3 protein thus, is unable to interact with anti-apoptotic proteins. Antiapoptotic bax is free to bind with pro-apoptotic bax halting the development of bax homodimers in the outer membrane of mitochondria. Consequently, there seems to be no cytochrome c release from mitochondria into cytosol so that the caspases stay inactivated. Besides, akt can directly phosphorylate caspase 9 at serine 196 preventing activation of apaf-1/cytochrome c. It is appropriate to deduce that BCR-ABL efficiently deregulates apoptosis since it not only governs AKT performance but also directly activates the antiapoptotic Bcl_2 and Bcl_xl expression (Găman et al., 2021).

Consequently, these deregulated cellular events started by onco-fusion BCR-ABL1 leads to the disruption of the controlled process of apoptosis. So, ultimately, inhibition of apoptosis by BCR-ABL1 facilitates the prolonged survival of abnormal clones, further then proliferation causes expansion of such abnormal clones and eventually, outnumbers the normal cell population to increase disease severity(Carter et al., 2016) (Zheng et al., 2016).

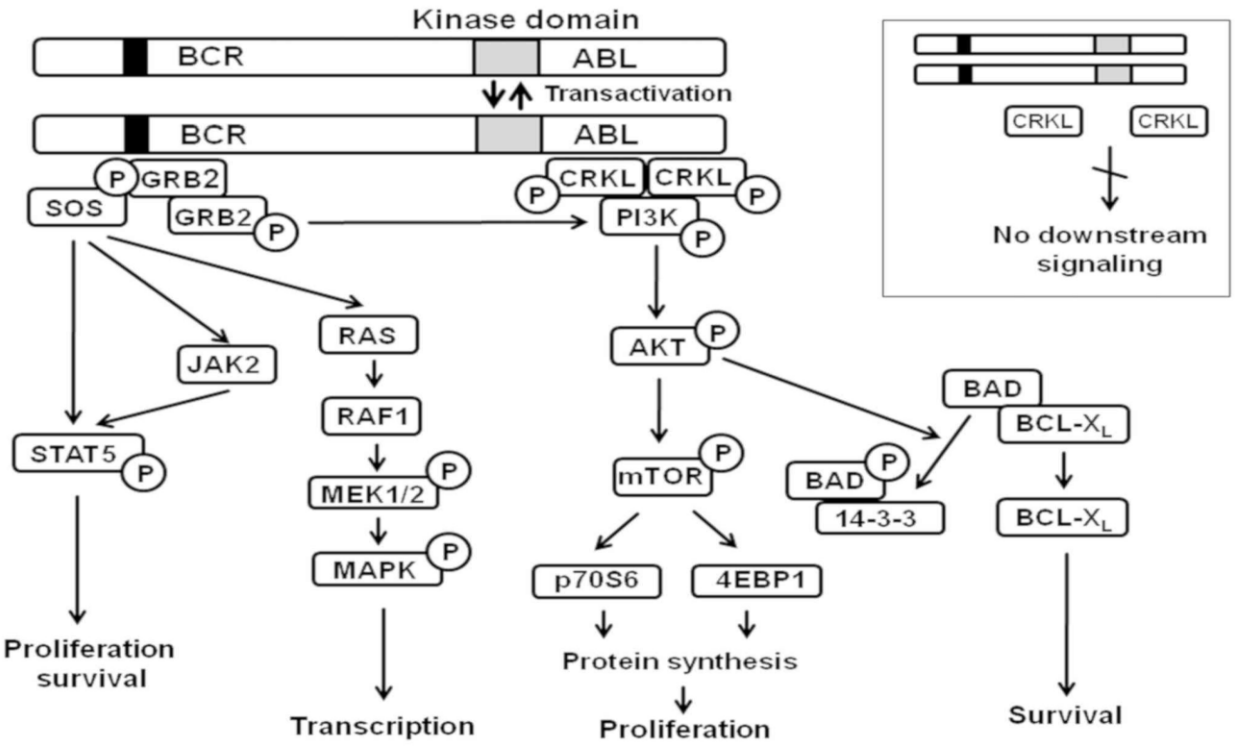

Figure 2.7 Effect Of BCR-ABL1 Signalling. Implications On Various Cellular Mechanisms (Bavaro et al., 2019)

2.4 Therapeutic Strategies and their Shortcomings

The earliest form of CML management included the treatment using arsenic and splenic irradiation. Until 1953 this served as a palliative medication rather than targeting the basic characteristics behind disease advancement and delivered only temporary effect (Jabbour & Kantarjian, 2018).

In 1953, modification of an alkylating agent produced busulfan, which when introduced as a treatment for CML patients considerably regulated disease by controlling its clinical features. Investigations suggested that busulfan limited the number of leukocytes and reversed the splenomegaly specially in newly diagnosed CML patients. Hydroxyurea also called as hydrea is a drug used in initial stages of disease to lower the count of white blood cells in CML patients.

Administration of hydroxyurea for the treatment of CML patients started in 1960 and gradually replaced busulfan in widespread usage as a first-line cytotoxic drug.

Following these objections, further research described interferon alpha as the new candidate for the preferred treatment. A specific number of patients with CML when treated with interferon alpha clearly showed ph-negative haematopoiesis to some extent. Another set of patients became entirely ph-negative while also a dose-dependent ph-negativity was observed. Eventually, following 20 years became the era of interferon alpha owing to its effectiveness in extending life by 1-2 years (Yung, Lee, Chu, Yip, & Gill, 2021).

2.4.1. Haematopoietic Stem Cell Transplantation (HSCT)

Throughout 1970s attempts were made to cure leukaemic patients through haematopoietic stem cell transplantation preferably use of HLA_identical sibling donors' bone marrow in an allogenic stem cell transplant. Basic understanding behind the process is to first kill the existing leukaemic cells through chemoradiotherapy followed by the transplantation of healthy blood forming stem cells, meanwhile, graft versus leukaemia effect was presented as an answer to any future complications in recipients (Eskazan & Tiribelli, 2018) (A. Gupta & Khattry, 2014). First remarkable milestone in this regard was achieved in 1979 when four CML patients underwent allogenic stem cell transplantation after being treated with high doses of chemotherapy and radiotherapy. Bone marrow does not contain ph-positive cells was shown after median-follow up of 24 months in the all of four patients. Further successful transplantations of chronic phase patients with genetically HLA identical sibling as a donor helped in putting forward the notion of allogenic stem cell transplantation as a cure for CML patients since survival outcome improved significantly (Oyekunle et al., 2011).

The paradigm shifted towards tyrosine kinase inhibitors as first preferable treatment thus, limiting HSCT use as a standard therapy. Nonetheless, over the course of time, issues of imatinib resistance and intolerance emerged in chronic phase patients and inadequate imatinib response in CML patients of accelerated and blast phase, ultimately, proved a definite role of HSCT in CML treatment (Oyekunle et al., 2011).

2.4.2. First Generation TKI's

Imatinib, with its approval in 2001 transformed the treatment of CML by converting a fatal disease into a manageable condition. Referred to as signal transduction inhibitor, imatinib, is termed as a magic bullet for its illustration as an inhibitor of BCR-ABL which governs the main oncogenic pathway in CML. Imatinib successfully attaches with the BCR/ABL kinase domain, a conformation that acts as an ATP binding site, and blocks phosphate transfer to the tyrosine residue on oncogenic protein. This prevents the autophosphorylation of protein, inhibiting the constitutive activity of tyrosine kinase and BCR/ABL no longer exerts its role in the oncogenesis of CML (Sacha, 2014). Consequently, this process acts as a switch-off for the downstream signalling pathways of BCR-ABL that promote leukemogenesis. Imatinib performs through a dose-dependent manner and characterized by its impressive total oral bioavailability of 98%. Since its approval by FDA, imatinib is used a front line of treatment for CML patients and has demonstrated complete cytogenetic response (CCyR) of 49% to 77% after a year of standard dosage and 18% to 58% of major molecular response (MMR) after one year of treatment. In 1/3rd of patients molecular relapse occurs who achieve MMR with in sixmonths of imiatinib withdrawal. Major side effects include nausea, bone ache, and myelosuppression. Myelosuppression tends to increase with the rise of dosage of imatinib from 400mg to 600mg per day (Annunziata et al., 2020).

Though, imatinib has an undeniable role in the therapeutic armamentarium as first of its kind molecularly targeted treatment for CML, it has its shortcomings. The process of imatinib resistance that increases with the course of disease cut short the effectivity of drug. kinase site mutations in BCR-ABL specially in the P-loop domain attributes to poor prognosis and influences the mechanism of resistance.

2.4.3. Second Generation TKI's

For such instances, to overcome the resistance, secondary treatment options are employed. In such cases, dose of imatinib is increased, second generation TKi are administered or the option of HSCT is utilized. Nilotinib (300 mg two times/day), dasatinib (100 mg daily), and bosutinib (500 mg daily) are the second generation TKi used in such cases and have proved better efficacy than imatinib and a rapid response. In reality, second-generation TKi, instead of imatinib, has a better prognosis in recently diagnosed chronic phase patients (Flis, Bratek, Chojnacki, Piskorek, & Skorski, 2019).

2.4.4. Third Generation TKI's

Ponatinib, which is a third_generation TKI, 45 mg/day is a third_line treatment option for patients in advanced phase of CML or those chronic phase patients where other tyrosine kinase inhibitors have not worked, especially those with T3151 mutation (Eden & Coviello, 2018).

In particular, patients with gate keeper mutation T315I, present greater challenge during treatment since both 1st and 2nd generation tyrosine kinase inhibitors are ineffective to deliever any substantial outcome in such patients. Detected in 15% of patients with imatinib resistance, T315I, represents a kinase domain mutation of threonine to isoleucine at aminoacid 315. Threonine at 315 position is a highly conserved residue, situated close to

ABL catalytic domain and controls the entrance to hydrophobic pocket of enzymatic active site, thus called gatekeeper. Mutation at this site changes the structural conformation of the binding site for inhibitors due to steric clashes (Baccarani et al., 2019).

2.5 Imidazole Derivatives for Cancer Treatment

Various imadizole complexes have been reported for significant anticancer activity. Imadizoles are basically organic alkaloid five cornered aromatic ring structures acting as a chief pharmacophore in the field of drug development since its discovery in 1840's. Naturally it is present in essential amino acids, biotin, histamine, pilocarpine alkaloids and histadine while synthetically it can be found in losartan fungicides, cimetidine, plant growth regulators, herbicides and therapeutic agents(Lo et al., 1992). Various studies have reported broad spectrum biological activities of imadizoles and its derivatives such as anticancer(Cai et al., 2009), antiviral(D. Sharma et al., 2009), antifungal(Pierard et al., 2006), antibacterial(Khabnadideh et al., 2007), antiparasitic(Sánchez-Moreno et al., 2012), and many more medicinal effects. Effectual anticancer properties have led to the development of many clinically approved imadizoles including nilotinib, tipifarnib, temzolomide, mercaptopurine, decarbazine etc against various types of cancers. The intriguing anticancer properties of imadizoles sparked widespread research into imadizole derivatives in the hopes of improving efficacy while minimising side effects.

Table: 2.1 Imidazole derived inhibitors used in study

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

3. MATERIALS AND METHODS

3.3. *In-silico* Study

3.1.1. Target Library Selection

Gene expression profile for each cell-line (K562, U937, NB4 and FKH1) was retrieved by using the databases such as National Center for Biotechnology Information (NCBI), GeneCards, Cancer Cell Line Encyclopedia (CCLE) and literature review for the selection of drug targets.

3.1.2. Selection of Domains

The FASTA sequences for all the selected genes were retrieved from Uniprot and subjected to Position-Specific Iterative Basic Local Alignment Tool (PSI-BLAST) to obtain the sequence similarity in protein structure and threshold score for percent identity was set above 98%. The protein structures with highest percent identity of 98%or above was selected.

3.1.3. 3D Crystallographic Protein Structures

Accession number for each target protein was taken from PSI-BLAST and subjected to Protein Data Bank (PDB) to obtain 3D Crystallographic structure of protein in PDB format.

3.1.4. Protein Structures Purification

Protein structures downloaded from PDB with multiple chains were purified by excluding the extra or identical chains, water molecules and non-standard amino acids by using UCSF Chimera 1.14.

3.1.5. Binding Site Prediction

An automated pocket detection and analysis tool DoGSiteScorer was used for the prediction of potential active drug binding pockets in relevant protein structure on the basis of hydrophobic interactions. Pockets with highest binding score were selected for all proteins.

3.1.6. Ligand Preparation

The ligand structure (2D) was retrieved using PubChem, a database of chemical molecules in SDF file format and converted into mol2 format using a chemical expert software Open Babel.

3.1.7. Molecular Docking

To further predict the favored binding of a ligand to its macromolecule receptor, molecular docking was performed using a molecular modelling simulation software Autodock 4.2. Ligand and macromolecule preparation was done by removing water molecules, addition of hydrogen atoms, computing charges etc. Autogrid was set by using previously calculated binding pocket scores and Lamarckian Genetic Algorithm with default parameters was selected for docking. The output file of docking simulations was converted into PDB file format using Cygwin commands and further visualized by Discovery Studio visualizer v4.

3.1.8. Visualization of Protein-Ligand Interaction

Ligand-Protein complexes with lowest free binding energies for each target were analyzed and visualized using Protein-Ligand Interaction Profiler (PLIP).

3.4. *In-vitro* Study

3.2.1. Cell Line

K562: CML model with Philadelphia (Ph) chromosome carrying BCR-ABL1 fusion gene.

3.2.2. Cell Lines Culture Media and Conditions

RPMI-1640 growth medium (Gibco Life Technologies) given with 10% Foetal Bovine Serum (FBS) (Gibco Life Technologies) for U937, NB4, K562 and 20% for FKH1 and 1% Penstrip (Gibco Life Technologies) was used for culturing the cell line models. Penstrip was added to avoid microorganism contamination. Cell lines were cultured in 6-well tissue culture plate and placed in a 5% CO2 humidified incubator at the temprature 37°C. Cell density less than 1.0 x 10^6 /ml was maintained throughout culture to avoid contact inhibition.

3.2.3. Cryopreservation

For cryopreservation of cells, freezing solution-I and freezing solution-II was prepared. Solution-I contains 70% RPMI (v/v) and 30% FBS (v/v) while solution-II contains 80% RPMI (v/v) and 20% DMSO (v/v). Cells were taken in falcon and centrifugation was done at 1200-1400rpmi for 5 minutes. After removing supernatant, pallet was washed with PBS followed by another centrifugation at 1200-1400rpmi for next 5 minutes. The pallet obtained was then resuspended in 2ml of freezing solution-I and followed by transfer of 1ml of this solution to each of two cryovials. Then 1ml of freezing solution-II was added into both cryovials dropwise. Cryovials were shifted in Mr.Frosty containing iso-propanol and placed at -80°C. Next day cryovials were placed in another box and stored at -80°C.

3.2.4. Thawing

For thawing, cryovials containing cryopreserved cells were taken out from liquid nitrogen freezer (-80°C) and quickly thawed by placing in incubator at 37°C for 1-2 minutes. Gently mixed until thawed properly and transferred into the falcon tube for washing with phosphate saline buffer (PBS) to remove dimethyl sulfoxide (DMSO). The suspension was centrifuged at 1600rpmi and 37°C for 5 minutes, discarded the supernatant and pallet was again suspended in 10% FBS media. The cells were plated in 6-well culture plate and placed at 37°C in CO2 incubator.

3.2.5. Trypan blue exclusion assay

Trypan blue exclusion assay was used to count the number of viable cells present in per ml of media. This is important in seeding the equal number of viable cells for achieving accurate results in MTT assay. A 1:1 cell suspension dilution with 0.4% trypan blue staining solution (Gibco Life Technologies) was prepared through proper vortex and 10ul of prepared dilution was loaded onto the haemocytometer chambers for visualization under microscope. Dead cells appeared blue while living cells do not and the viable cells were then counted in the haemocytometer's four grids. carefully as per following calculations;

$$\frac{\textbf{Number of cells in grid1 + grid2 + grid3 + grid4 x Dilution factor x } 10^4}{\textbf{cells/ml4}}$$

3.2.6. MTT cytotoxicity assay

MTT assay is a calorimetric assay which is based on reduction of yellow tetrazolium salt (MTT dye) to purple formazon crystals by NADPH dependent oxidoreductase in metabolically active cells. Cells were plated into 96-well flat-bottom culture plate at a number of 10,000 cells/50ul followed by the addition of 50ul of 10mM drug in different concentrations (µM) of (10, 5, 2.5,

1.25) in triplicates and incubated at 37°C for 72hours for U937, NB4 and K562 cell line, while 96hours for FKH1 cell line. 0.1% DMSO was added into solvent control to avoid its effect on cell proliferation. Upon completion of necessary incubation, 15µl of sterilized and filtered MTT dye (5mg/ml in PBS) was added into each well (already have cells + drug concentrations) and again allowed to incubate for 3-4 hours to allow crystal formation. Then without disturbing the crystals, 100µl of medium was removed and 90-100µl DMSO was added into wells for crystals dissolving the carefully by avoiding the bubble formation. Finally, upon complete solubilization of crystals, absorbance for each well was measured at 550nm using BIO-RAD spectrophotometric microplate reader. MTT assay was done at least three times for each cell line with biological triplicates.

3.2.7. Mechanistic Studies for gene expression profile analysis

3.2.7.1. Cells Treatment with Drug

In a six-well culture plate, 1million/ml cells were plated in 10% FBS media with 0.01% DMSO and 1.25µM concentration of drug diluted in DMSO and allow to incubate for 72-96 hours. After that RNA extraction followed by cDNA synthesis was done for analysis of gene expression.

3.2.7.2. RNA Extraction

After treatment, 1-2million cells per ml were taken in an Eppendorf (2ml) and centrifuged at 1500rpmi for 5 minutes. Supernatant was removed and pallet obtained was re-suspended in chilled PBS and again centrifuged at 1500rpmi for 5 minutes to obtain new pallet. Afterwards, pallet was resuspended in 1ml Trizol reagent (RNA Isolation Reagent) and homogenized it by gently up and down pipetting. After homogenization, incubation was done on ice for 5 minutes and it is important to ensure the separation of nucleoprotein complexes. Then added 200µl chloroform followed by vigorous shaking for next 15 seconds. Afterwards, Eppendorf was again incubatation was done on ice for 10-15 minutes, then centrifugation at 12000 x g at 2-4°C for 20 minutes. As a result, mixture

was separated in three distinct phases, an upper aqueous phase having required RNA, an interphase with DNA, and an organic pink phase having proteins. The aqueous upper phase was transferred very carefully into another Eppendorf followed by addition of 500µl of chilled isopropanol and then shaken for few seconds. At this point, incubation was done again on ice for next 15 minutes and then centrifugation at 12000 x g at 2-4°C for 20 minutes. Pallet obtained was then washed with 500ul 75% chilled ethanol and centrifugation was done at 7500 x g at 2-4°C for another 7 minutes. Supernatant was discarded and pallet was air dried inside the laminar flow hood for 15-20 minutes and then resuspended in 20µl of nuclease free (NF) water. RNA extracted was stored at -80°C immediately to avoid degradation until further processing.

3.2.7.3. RNA Quantification and Quality Check

Nanodrop 2000 (Thermoscientific, USA) was used for quantification of extracted RNA and purity was checked through 260/280ratio check. RNA quality was checked through gel electrophoresis. 1% agarose gel was prepared in 50ml of 1X TAE buffer stained with 4ul of ethidium bromide and 500ul of 1% bleach to eliminate secondary structures if any. Gel running conditions were 80V, 500A for 35minutes.

3.2.7.4. cDNA Synthesis

To synthesize complementary DNA, 1ul oligo dT20 primer (10µM), 1µl dNTPs (2.5mM), 0.5µl RNAase inhibitor (40U/ul), 1µl DTT (100mM), 2µl of 10x reaction buffer and 1µl of RTase were added in a 0.2ml PCR tube. Then 1000ng of extracted RNA was added in the same PCR tube for reverse transcription then nuclease free (NF) water was added to make the 20µl final volume. Afterwards, PCR tubes were placed in a thermocycler and the protocol was followed as per mentioned on cDNA synthesis kit (Wizbiosolutions). Then the cDNA was kept at -20°C until further processing.

3.2.7.5. cDNA Quantification and Primer Optimization

cDNA quantification and primer optimization was done using a conventional PCR. A reaction mixture was prepared in a 0.2ml Eppendorf containing 1000ng template cDNA (2µl), 10x PCR buffer (2.5µl), 10mM dNTPs (1.5µl), forward and reverse primers (1µl each), 25mM $MgCl_2$ (2µl, Thermoscientific), Taq DNA polymerase (0.5µl) (Wizbiosolutions) and NF water (9.5µl). Thermocycling conditions for conventional PCR was set at; initial denaturation for 5 minutes at 95°C followed by 35 PCR amplification cycles. Afterwards, each amplification cycle consisted of three phases, first denaturation for 1 minute at 95°C, second annealing for 45 seconds at 60°C and third extension for another 45 seconds at 72°C. Then last extension of cDNA template strand was set at 72°C for next 10 minutes. Afterwards, at -20°C PCR products were stored for later analyzation using 2% TAE agarose gel electrophoresis.

3.2.7.6. Gel electrophoresis

Gel electrophoresis was used to examine amplified PCR products in a 1 percent agarose gel. For 2% agarose gel, 1g of agarose was measured and dissolved in 50ml of 1X TAE buffer followed by its melting in microwave. Then stained with 4ul of ethidium bromide. Afterwards, gel was poured in a casting tray to get solidified for next 20minutes. 4ul of PCR products were loaded with 2ul of DNA loading dye in each well (Thermoscientific). Results were analyzed by comparing with Gene Ruler 100bp DNA ladder (Thermoscientific).

3.2.8. Real-Time PCR (qPCR)

The analyzation of primers for specified amplification of gene of interest at 10mM were done through Real-Time PCR (7500 RT qPCR System). 0.1ml PCR strips were used for the preparation of reaction mixture that contains 0.5ul cDNA, 2ul qPCR master (SYBR), 0.5ul of forward and reverse primers (10mM) each and 6.5ul NF water to make the total volume of 10ul.

	Stages	**Temperature**	**Time**
Stage 1	Initial incubation Initial denaturation	50°C 95°C	2min 10min
Stage 2	40 cycles 1st Denaturation 2nd Annealing 3rd Extension	 95°C 60°C 72°C	 15s 45s 30s
Dissociation stage	Step 1 Step 2 Step 3	95°C 60°C 95°C	30s 30s 30s

qPCR cycling parameters were used as above mentioned. In order to normalize the RNA level, beta-actin was utilised as a housekeeping gene. Dissociation stage was used for melt curve analysis and resultant data was collected at 72°C. The 2-ΔΔC method was used for relative quantification and analyzation of gene expression.

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

3.2.9. Cells Treatment for DNA Extraction

In a 6-well culture plate, 1million/ml cells of NB4 and K562 were plated in complete medium with 0.01% DMSO in control and 1.25µM concentration of drug diluted in DMSO in separate wells. Then incubated for 72 hours.

3.2.10. DNA Extraction for Fragmentation Assay

After 72 hours incubation, K562 and NB4 treated cells were taken in a 2ml Eppendorf for centrifugation at 2000rpmi for 5minutes. Supernatant was discarded and pallet was washed with 200ul chilled PBS and centrifuged at 1600rpmi for 5minutes. Again supernatant was discarded and pallet was dissolved in 1ml of solution A followed by invert mixing for several times. The at room temperature, sample incubation was done for 10minutes and then centrifuged at 13000rpmi for 1minute. Nuclear pallet was obtained and resuspended in 500ul of solution A followed by another centrifugation at 13000rpmi for next 1minute. Supernatant was removed and nuclear pallet was dissolved in 500ul of solution B, 20% SDS (12ul) and 5ul proteinase K. The sample was mixed and then placed at 37°C for overnight. Following the overnight incubation, 500ul of solution C and 500ul of solution D was added in same Eppendorf and centrifuged at 13000rpmi for 10minutes. This centrifugation was resulted in distinct phase layer separation. Upper aqueous phase layer was carefully transferred in a new Eppendorf, to which 500ul of solution D was again added, centrifugation was done at 13000rpmi for 10minutes. Again upper aqueous phase layer was obtained and transferred into another new Eppendorf. 500ul of chilled iso-propanol and 55ul of sodium acetate (3M, pH6) was added into sample and invert mixing was done several times to ensure the precipitation of DNA followed by centrifugation at 13000rpmi for 10minutes. After removing supernatant pallet was washed with 200ul of 70% ethanol by centrifugation at 13000rpmi for 7minutes. Ethanol was completely removed without disturbing the pallet and pallet

was air-dried in biosafety cabinet hood for 20-25minutes. DNA pallet was then dissolved in 50ul of TE.

3.2.11. DNA Quantification

After extraction, the DNA was quantified using NanoDrop2000 (Thermoscientific USA), purity was analyzed by 260/280 ratio check and DNA was placed at -20°C until further processing.

3.2.12. Apoptosis Analysis through Gel Electrophoresis

For 1.5% agarose gel preparation, 0.75g agar was dissolved in 50ml of 1X TAE buffer and microwaved for 1-2minutes until the agarose is completely dissolved (over-boiling will lead to the evaporation of buffer and alteration of the percentage of gel). 4ul of ethidium bromide was then added into the flask and let to cooled down for few minutes. Afterwards, gel was poured into casting tray carefully to avoid any bubble formation and let it to solidified for 20minutes. Gel was placed in gel tank, 3ul of DNA ladder was loaded in first well and 4ul of DNA sample along with 2ul of loading dye was added in next each well. The gel running conditions were 90V, 500A for 40minutes. Gel bands were analyzed using ChemiDoc Imaging System.

Solution A	Sucrose (0.32M), Tris (10mM pH 7.5), MgCl2 (5mM), Triton X100 1%(v/v)
Solution B	Tris (10mM pH 7.5), NaCl (400mM), EDTA (2mM pH 8.0)
Solution C	Phenol
Solution D	Chloroform + Isoamyl alcohol (24:1)

4. RESULTS

4.3. *In-silico* Study

4.1.1. Structure of Ligands

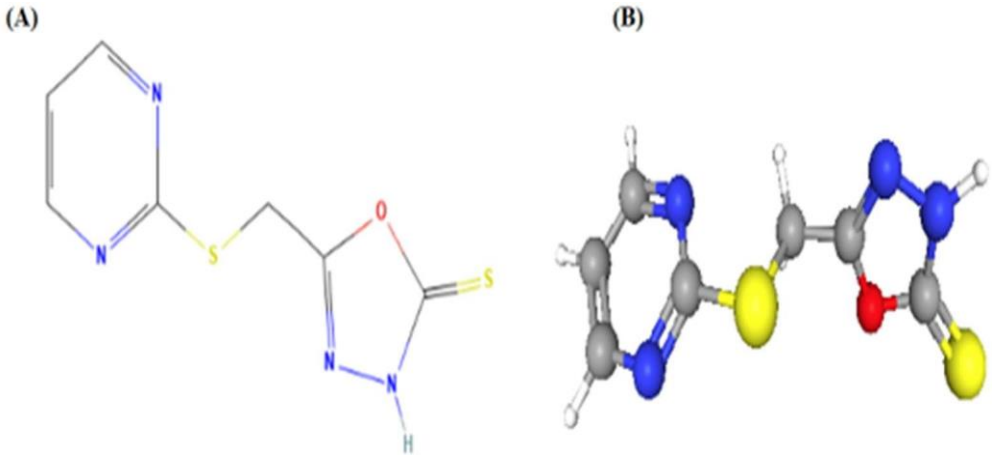

Figure 4.1: 2D and 3 D Structure of L7 used in in-silico study

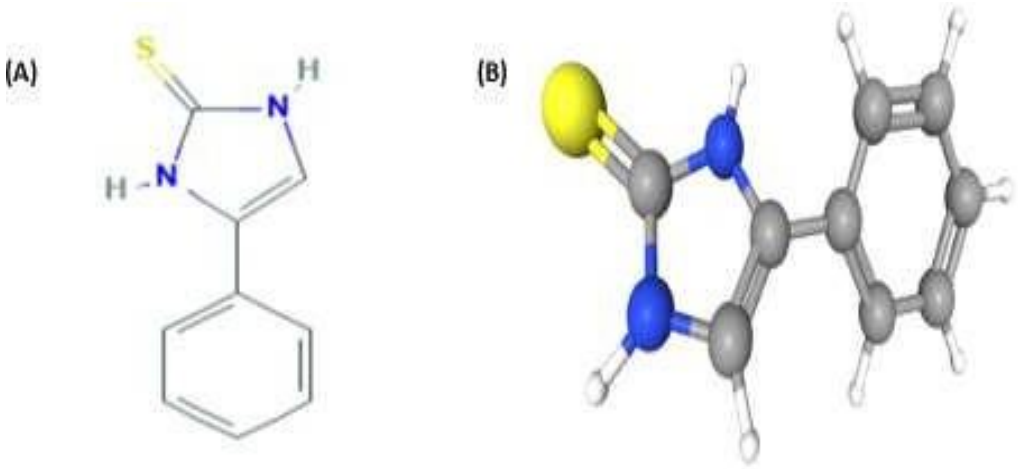

Figure 4.2: 2D and 3D Structure of L4 used in in-silico study

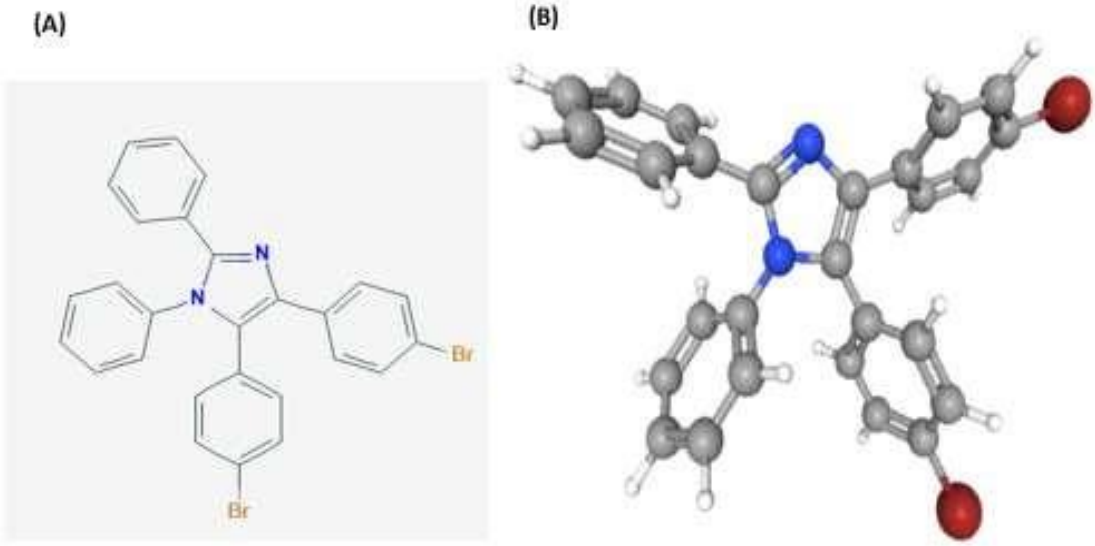

Figure 4.3: 2D and 3D Structure of R-35 in-silico study

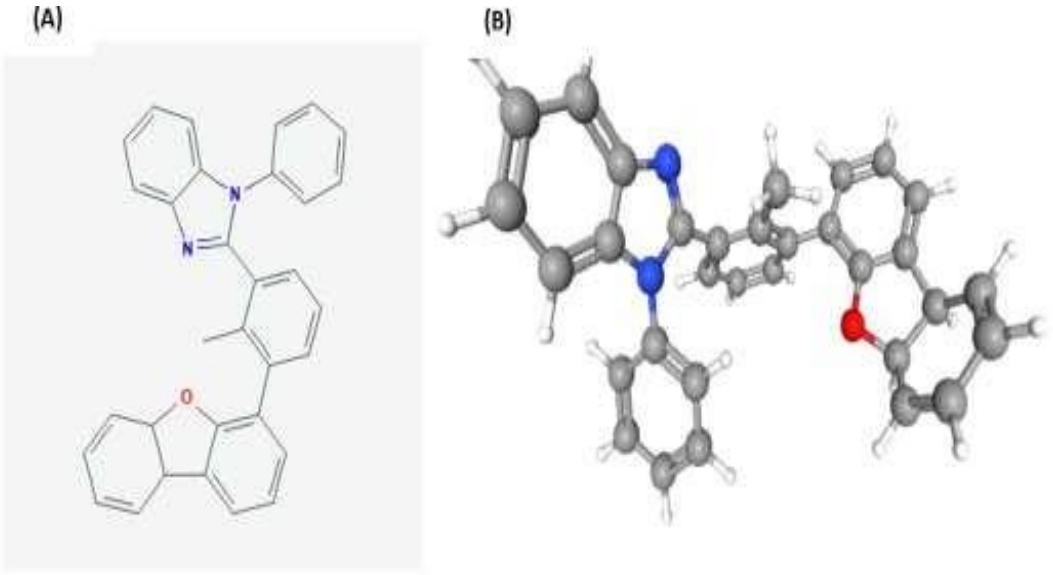

Figure 4.4: 2D and 3D Structure of R-NIM04 used in in-silico study

4.1.2. Molecular Docking of L7 with Drug Target Genes in *BCR-ABL1* Positive K562 Cells

For underlaying research study, candidate gene list were retrieved from Human Genes Database NCBI (National Centre for Biotechnology Information and Gene Card). Some of the genes were selected through litrature review. Then FASTA sequence were selected from UniProt database and Psi-Blast was performed through Blast in order to compare primary biological sequences like amino acids or nucleotides. Protein structure in PDB format were downloaded from PDB database for docking. For each protein docked with ligand (L7), 20 different poses along with their corresponding binding energies were generated. Protein ligand complex was visualised using Chimera and the best ligand pose was selected on the basis of binding affinities and the least binding energy value. For K562, top four least binding energies were generated for L7,L4,R35,RNIM04 complexed with BCR, MAPK3, BCR and MYC respectively.

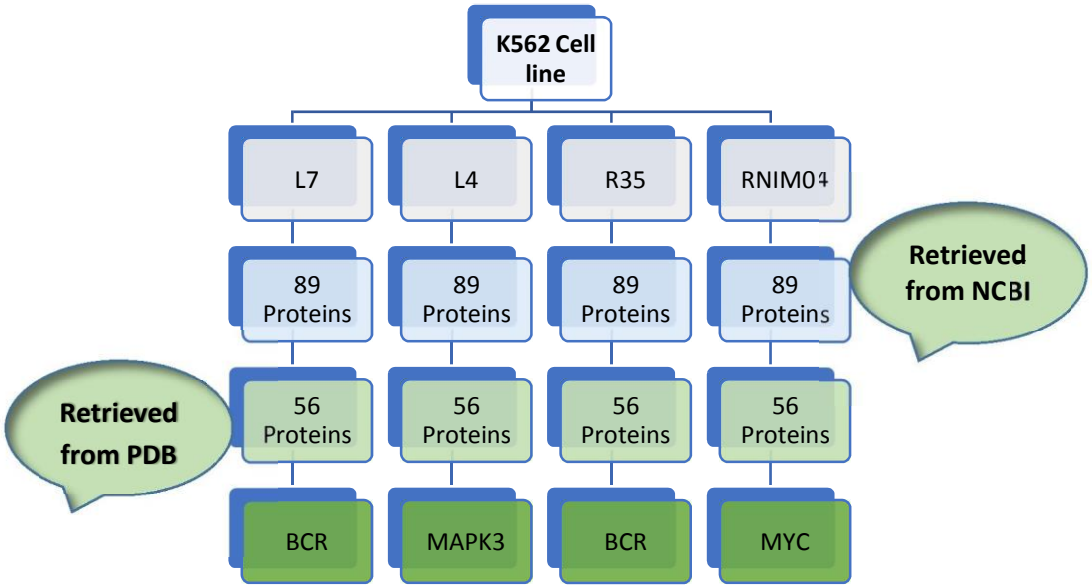

Figure 4.5: Hierarchical Paradigm of Proteins based on Inclusion Exclusion Criteria; Grey blocks represents the ligands, blue box shows total proteins taken from NCBI gene database, light green box shows shortlisted proteins on the basis of 3Dstructure available and dark green box represents shortlisted protein targets with lowest ligand-protein binding energies.

4.3.2. Shortlisted Potential Protein Targets

Table 4.1: K562 cell line specific targets and their estimated free binding energies determined via molecular docking **with L7, L4, R-35 and R-NIM04**

Ligands	**Shortlisted Proteins of K562 (With lowest binding energy)**			**Binding Energies (kcal/mol)**
	Gene ID	**Description**	**PDB**	
L7	BCR	Breakpoint Cluster Region	5n6r	-7.25
	MAPK3	Mitogen-activated protein kinase 3	4qtb	-6.59
	MAPK1	Mitogen-activated protein kinase 3	4qta	-5.75

	TNF	Tumor necrosis factor	5mu8	-5.07
L4	MAPK3	Mitogen-activated protein kinase 3	4qtb	-7.31
	TP53	Cellular tumor antigen p53	6rz3	-6.87
R-35	BCR	Breakpoint Cluster Region	5n6r	-9.83
	MTOR	Serine/threonine-protein kinase mTOR	5h64	-10.21
	JAK2	Erthropoiten receptor	6e2q	-10.67
R-NIM04	MYC	*Myc* Proto-Oncogene Protein	6g6j	-11.12
	BCR	Breakpoint Cluster Region	5n6r	-12.57
	ABL1	Tyrosine-protein kinase ABL1	5mo4	-12.45

4.1.3. Protein-Ligand Interaction Profiling and Bonding Interactions of Docked L7, L4, R-35 and R-NIM04 with Target Genes

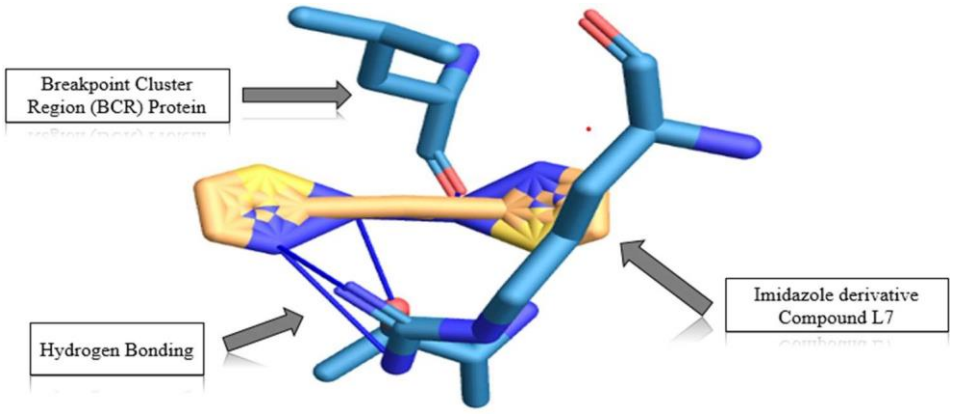

Figure 4.6 Best selected pose of L7 with BCR on the basis of free binding energy determined by molecular Docking.

Table 4.2: Bonding interactions of docked L7 with target protein

Hydrogen Bonds

Index	Residues	Amino acids	Distance H-A	Distance D-A	Donors Angle	Protein donor	Side chains	Donor Atoms	Acceptor Atoms
1	499A	ARG	2.85	3.47	120.19	yes	yes	219 [Ng+]	3505 [Nar]
2	499A	ARG	2.32	2.96	120.11	yes	yes	218 [Ng+]	3505 [Nar]
3	660A	LEU	2.19	2.77	155.92	no	no	3503 [Npl]	2807 [O2]
4	664A	THR	2.01	2.71	127.98	yes	yes	2887 [O3]	3504 [N2]

4.1.4. Protein-Ligand Interaction Profiling and Bonding Interactions of Docked L4 with Target Genes

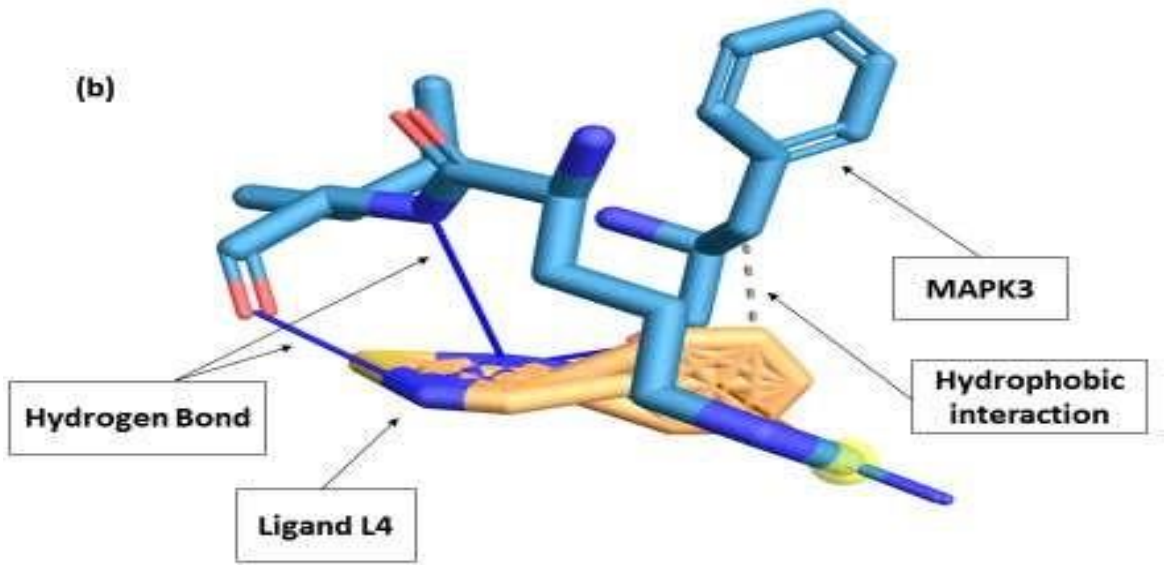

Figure 4.7 Protein-ligand interaction profiling of L4 with MAPK3, best selected pose.

Table 4.3: Bonding interactions of docked L4 with target proteins

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	189A	ARG	3.92	2982	1408
2	348A	PHE	3.25	2981	2724

Hydrogen Bonds

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	190A	ILE	2.45	2.82	120.96			2973 [Nam]	1423 [O2]
2	190A	ILE	2.98	3.85	147.08			1420 [Nam]	2972 [Nam]
3	348A	PHE	2.12	2.66	146.63			2972 [Nam]	2723 [O2]

π-Cation Interactions

Index	Residue	AA	Distance	Offset	Protein charged?	Ligand Group	Ligand Atoms
1	189A	ARG	4.72	1.28		Aromatic	2977, 2978, 2979, 2980, 2981, 2982

4.1.5. Protein-Ligand Interaction Profiling and Bonding Interactions of Docked R-35 with Target Genes

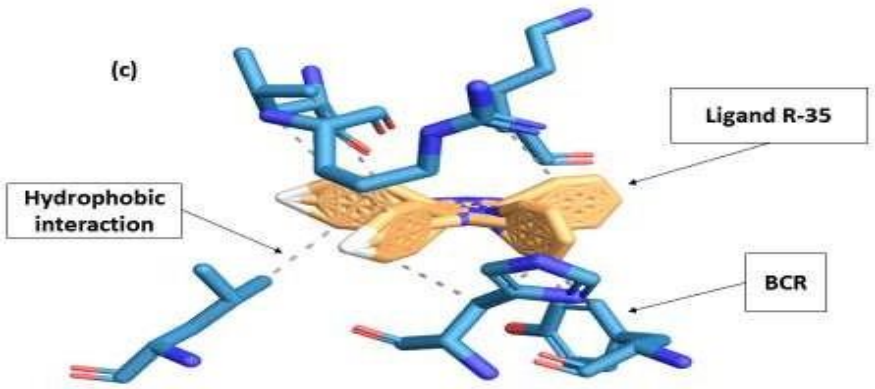

Figure 4.8 Interactions of R-35 with residues of BCR

Table 4.4: Bonding interactions of docked R-35 with target protein including hydrophobic interactions.

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	513A	TYR	3.74	3514	442
2	513A	TYR	3.26	3524	444
3	516A	HIS	3.69	3507	490
4	516A	HIS	3.4	3515	490
5	520A	LEU	3.14	3529	553
6	623A	ARG	3.9	3508	2201
7	642A	LEU	3.36	3527	2499
8	642A	LEU	3.99	3526	2501
9	645A	LYS	3.25	3520	2558

4.1.6. Protein-Ligand Interaction Profiling and Bonding Interactions of Docked R-35 with Target Genes

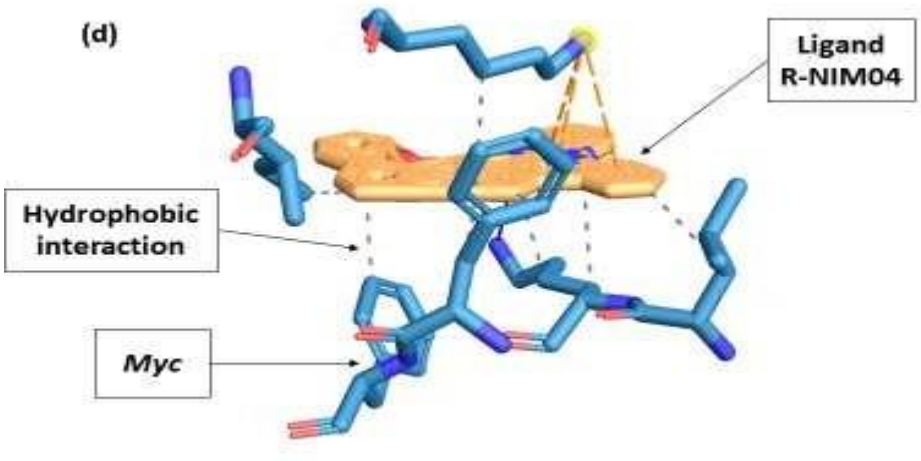

Figure 4.9 Best pose of R-NIM04 with *Myc* protein.

Table 4.5: Bonding interactions of docked R-NIM04 with target protein including hydrophobic interactions

Index	Residue	AA	Distance	Offset	Protein charged?	Ligand Group	Ligand Atoms
1	939A	LYS	3.95	0.86	**yes**	Aromatic	679, 695, 697, 700, 707, 708
2	939A	LYS	4.11	1.41	**yes**	Aromatic	679, 680, 700, 701, 702
3	939A	LYS	4.12	1.44	**yes**	Aromatic	707, 708, 709, 710, 711, 712

4.4. In-vitro Study

4.2.1. Culturing of Cell lines

K562 cell line were successfully revived, cultured and maintained at the conditions described previously with maximum viability.

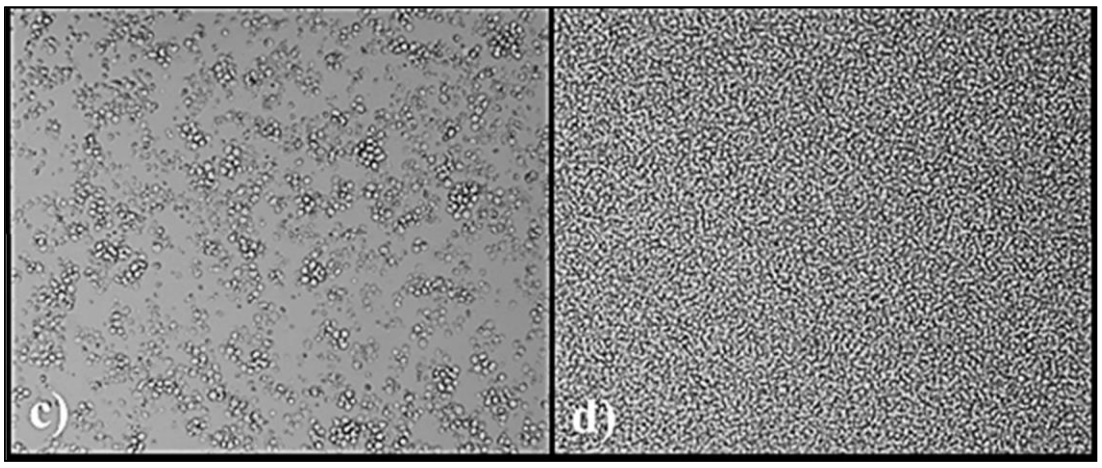

Figure 4.10: Cell line K562. Cells were grown in liquid media (RPMI + 10% +1% L-Glutamate and 1% Pen-Strep)

4.2.2. Effect of L7, L4, R-35 and R-NIM04 on Proliferation of K562 Cell Line

To evaluate the cytotoxic potential of novel compounds L7, L4, R-35 and R-NIM04 against human leukaemic cells, K562 cell line were incubated with different doses of L7, L4, R-35and R-NIM04. Cell proliferation estimated through MTT assay after incubation of 72 hours in K562 cells showed that only L7 was able to produce cytotoxicity in BCR-ABL positive CML cells.

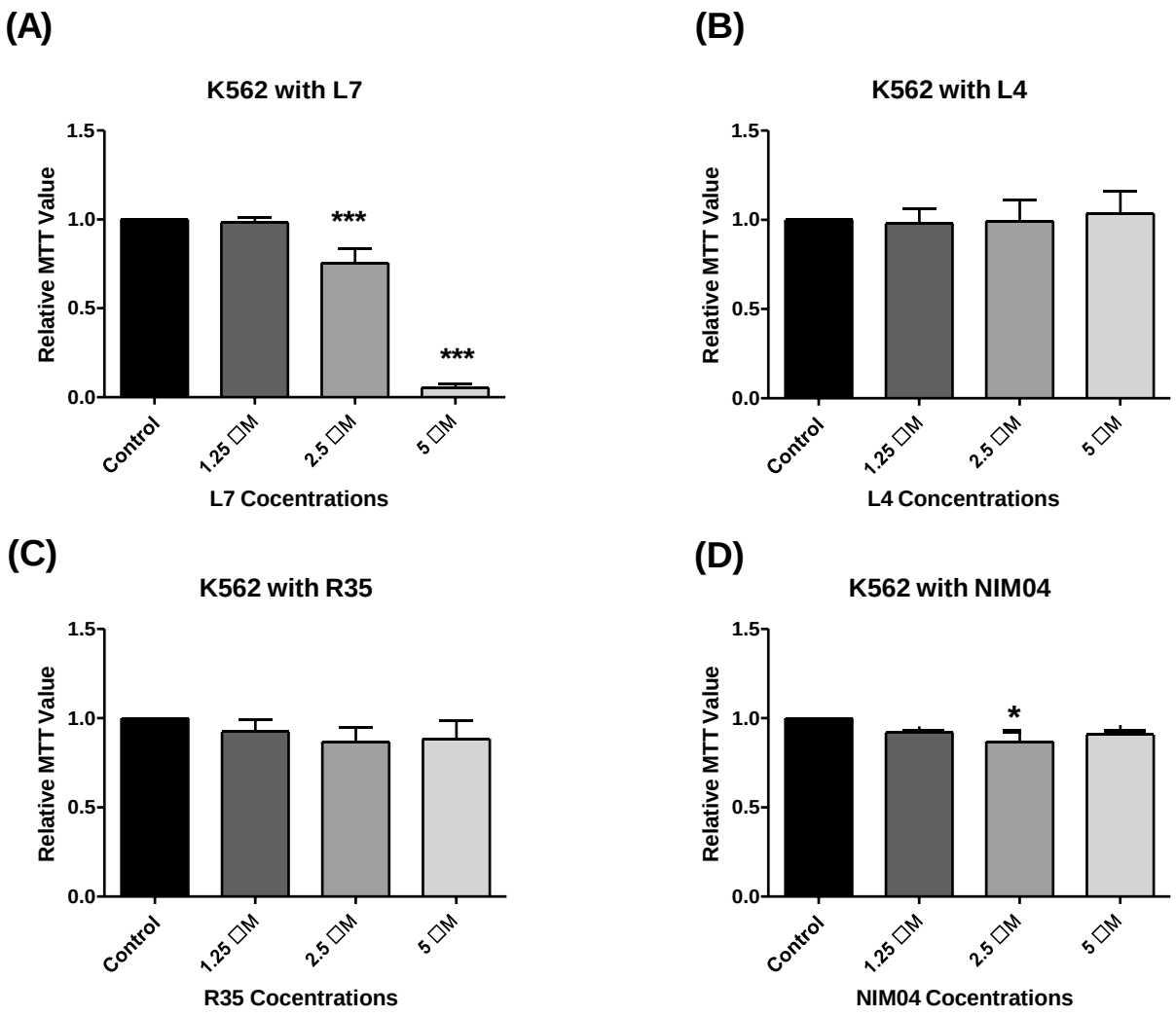

Figure 4.11: Effect of L7, L4, R-35 and R-NIM04 on the proliferation potential of K562 cells through MTT assay. Cells were grown in liquid media (RPMI + 10% FBS+1% L-Glutamate and 1% Pen-Strep) to assess the proliferation of K562 cells in the presence of Ethanol and DMSO (0.01%) and given concentrations of L7, L4 R-35 and R-NIM04. Statistical significance was tested using One-Way ANOVA (pvalues <0.05 were taken to be statistically significant). Bars show mean ± SEM. * p_value < 0.05, ** p_value < 0.01, *** p_value < 0.001.

4.2.3. Comparison between the Anti-Proliferative Effect of L7, L4, R-35, R-NIM04 and Imatinib on K562 Cells

Imatinib is a capable first line TKI used in CML treatment that effectively binds to the BCR-ABL at its ATP binding domain. As already shown in the figure, L7 acts as an anti-proliferative agent for K562 cells. Considering this, comparison of the effect of imatinib and L7, L4, R-35, R-NIM04 was made on the proliferation potential of K562 cells at different concentrations and proliferation was determined after 72 hours incubation through MTT assay. Both the treatments showed slightly dose-dependent manner effect on proliferation of K562 cells as depicted in figure 4.12.

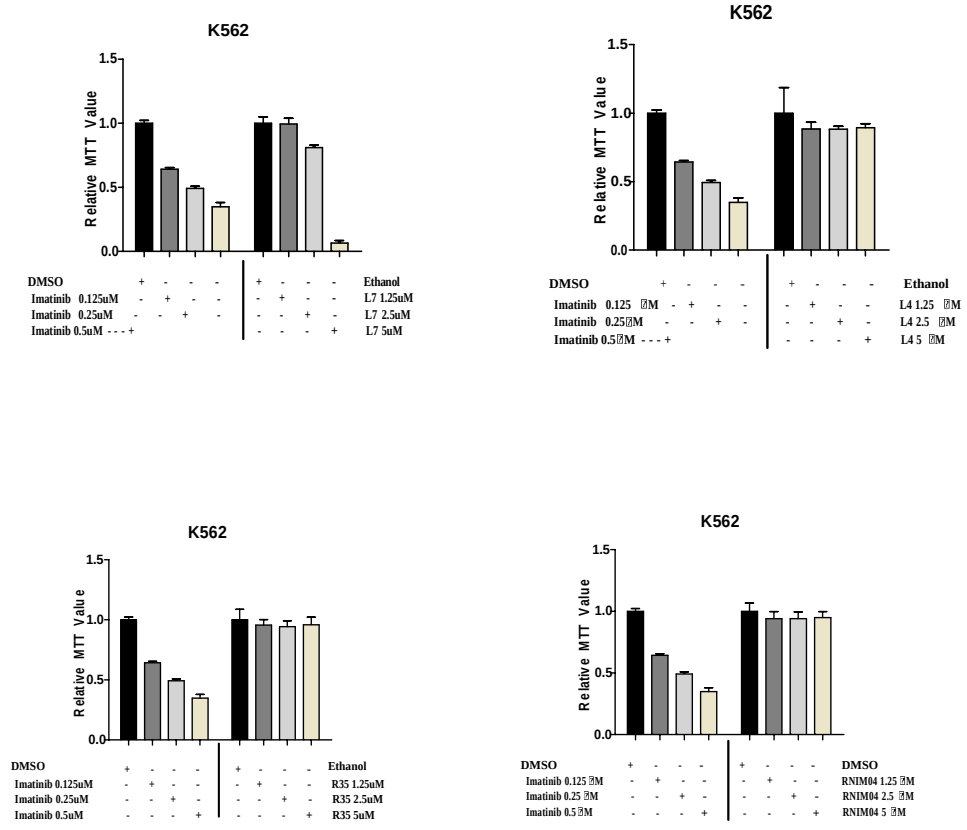

Figure 4.12: Comparison between L7, L4, R-35, R-NIM04 and Imatinib for their anti-proliferative effect on BCR-ABL1 positive K562 cells to assess the proliferation of K562 cells in the presence of DMSO and Ethanol (0.01%) and given concentrations of L7, L4, R-35, R-NIM04 and Imatinib.

4.2.4. Effect of L7 treatment on expression of AXL in BCR-ABL1 Positive K562 cells

In the light of previous results observed in this study, we further investigated that whether K562 cells treated with L7 has an effect on the expression level of AXL through real time PCR expression analyses and compared with the effect of Imatinib on the expression level of AXL receptor (figure 4.13). Results showed significant fold change of AXL expression in BCR-ABL1 positive K562 cells after treatment with 2.5µM of L7.

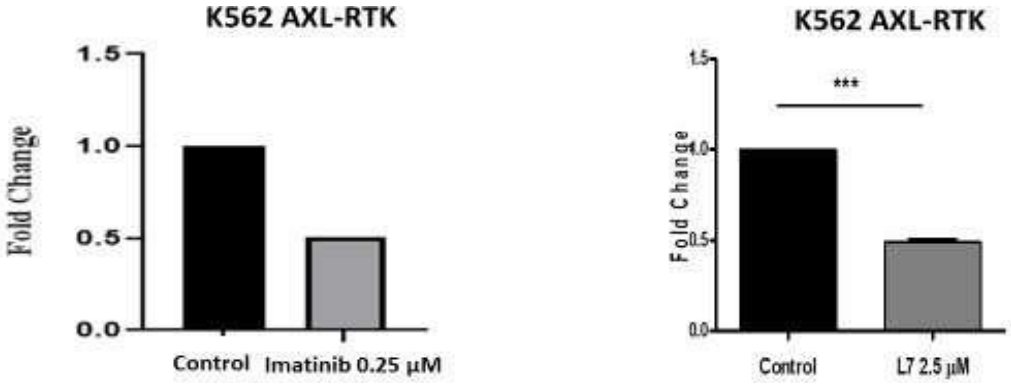

Figure 4.13: Effect of L7 treatment on AXL expression in BCR-ABL1 positive K562 cells. Cells were grown in liquid media (RPMI + 10% +1% L-Glutamate and 1% Pen-Strep) in the presence of DMSO (0.01%) and given concentration of L7. Extracted RNA from cultured cells and cDNA synthesized was further used in expression analyses done through real time PCR. Statistical significance of results was measured using Student's t-test and p-values <0.05 was considered as significant. Bars show mean ±SEM. *** p-value < 0.001

4.2.5. Effect of AXL-RTK targeting on downstream signalling pathways in K562 Cells

Effect of Targeting AXL Tyrosine Kinase in BCR-ABL1 Positive K562 Cells On β-Catenin Target Genes

The canonical Wnt/β-Catenin pathway is linked with cancer cell proliferation and chemotherapy resistance and translocation of stabilized β-Catenin in nucleus result in activation of Wnt target genes acting as a key process in cancer progression. Here, we investigated the effect of AXL targeting on Wnt β-Catenin signalling through expression analyses of Wnt-target genes like c-Myc using real time PCR. Results demonestrate significant reduction of cMyc expression in K562 cells treated with 2.5μM of L7 (figure 4.14)

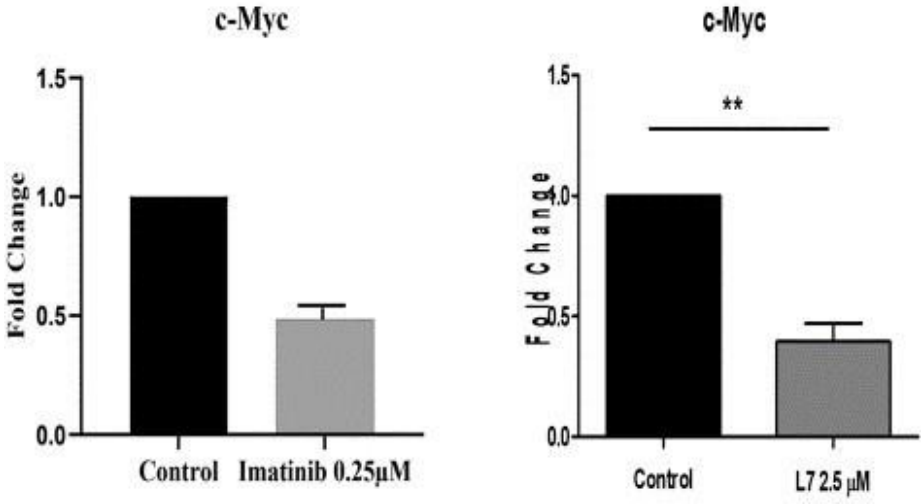

Figure 4.14: Effect of AXL targeting on the c-MYC expression in BCR/ABL1 positive K562 cells. Cells were grown in liquid media (RPMI + 10%FBS+1% L-Glutamate and 1% Pen-strep) in the presence of DMSO (0.01%) and given concentration of L7. RNA was extracted from cultured cells and cDNA synthesized was further used in expression analyses done through real time PCR. Statistical significance of the reults was measured using Student's t-test and p-values <0.05 were considered significant. Bars show mean ± SEM. * p-value < 0.05.

4.2.6. Apoptosis Detection in K562 Cells Due to Pharmacological Targeting by L7

After observing the anti-proliferative effect of L7 on K562 cells, next the reason behind reduction in cell proliferation was investigated as whether apoptosis induction or other mechanisms involving cell cycle arrest. For this purpose, DNA fragmentation assay was carried out as apoptotic DNA fragmentation is used as an indication of cellular apoptosis. Extracted DNA was run on 1.5% agarose gel electrophoresis. In the figure 4.15, fragmentation of DNA can be seen in K562 cells treated with L7 at 2.5µM concentration. According to the result, apoptosis is induced in K562 cells after treatment with L7.

Figure 4.15: Gel image of DNA fragmentation assay for K562 cells. Cells were grown in liquid media (RPMI +10% FBS+1% L-Glutamate and 1% Pen-strep) in the presence of DMSO (0.01%) and 2.5µM concentration of L7 (M= 1000bp ladder, C= Ethanol) for 48-72 hours. DNA was then extracted and run on 1.5% agarose gel for visualization.

4.2.7. Effect of Combination Treatment on Proliferation Potential of K562 Cells

The issue of resistance to therapeutics can be managed through strategies such as combination treatment that may lessen the risk of relapse. As already shown that L7 and imatinib were able to reduce the proliferation potential of K562 cells, effect of their combined treatment on K562 cells was investigated as whether an additive or synergistic effect in comparison to the effect of single treatment of imatinib and L7 on K562 cells. MTT assay was performed to assess the proliferation potential after 72 hours. Combined treatment showed little additive effect in selected concentrations of both imatinib and L7. Figure 4.16 shows effect of different concentrations of both imatinib and L7 treated in combination.

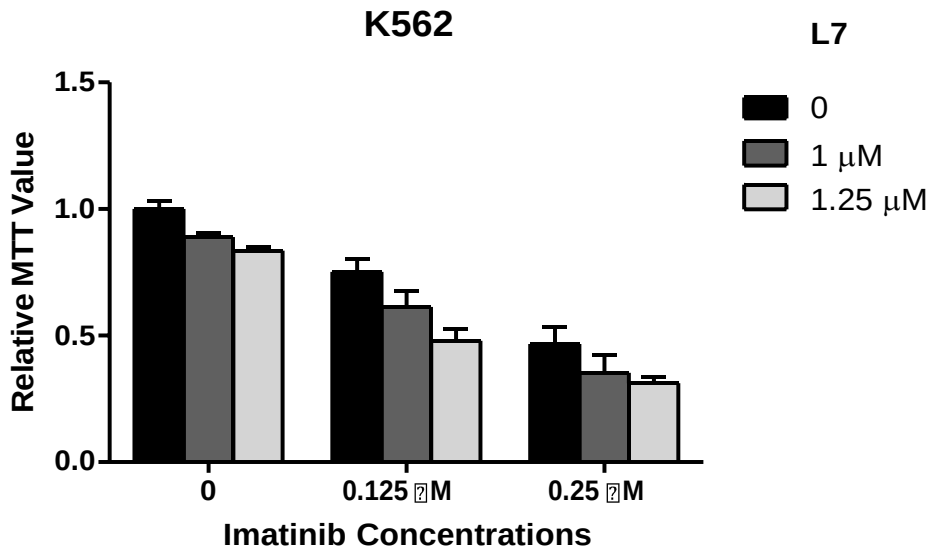

Figure 4.16: Effect of combination treatment (L7 + Imatinib) on the proliferation of BCR/ABL1 positive K562 cells. Cells were grown in liquid media (RPMI + 10%+1% L-Glutamate and 1% PenStrep) to assess the proliferation of K562 cells in the presence of Ethanol (0.01%) and given concentrations of L7 and Imatinib used in various combinations.

4.2.8. Effect of L7 in Combination with Imatinib on Expression of AXL-RTK and its Downstream signaling

By keeping in view the above described anti-proliferative potential of novel compound L7, we investigated the effect of L7 in combination with front-line therapeutic option Imatinib on expression of AXL receptor tyrosine kinase and its downstream signalling pathways including *c-myc* activity. To check the expression of thses genes real time PCR was performed. Results showed significant fold change of AXL and *c-Myc* expression in BCR-ABL1 positive K562 cells after treatment with L7 1μM + Imatinib 0.125μM.

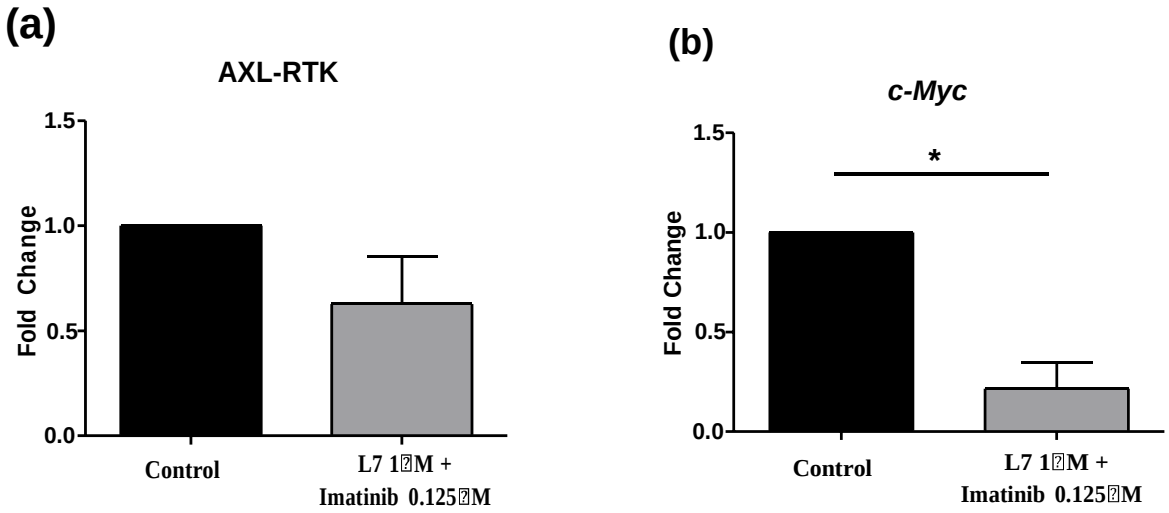

Figure 4.17: Effect of L7+Imatinib targeting on the expression of AXL and c-MYC in BCR/ABL1 positive K562 cells. Cells were grown in liquid media (RPMI + 10%FBS+1% L-Glutamate and 1% Penstrep) and in the presence of Ethanol (0.01%) and given concentration of L7. RNA was extracted from cultured cells and cDNA synthesized was further used in expression analyses done through real time PCR. Statistical significance was measured using Student's t-test and p-values <0.05 are considerd significant. Bars show mean ± SEM. * p-value < 0.05

4.2.9. Apoptosis Detection in K562 Cells Due to Co-Targeting by L7+Imatinib

After investigating the anti-proliferative effect of L7+Imatinib Co-targetting on K562 cells, DNA fragmentation assay was carried out as apoptotic DNA fragmentation is used as an indication of cellular apoptosis. Extracted DNA was run on 1.5% agarose gel electrophoresis. In the figure, fragmentation of DNA can be seen in K562 cells treated with L7 1µM+Imatinib 0.125µM concentration. According to the result, apoptosis is induced in K562 cells after treatment with L7+Imatinib.

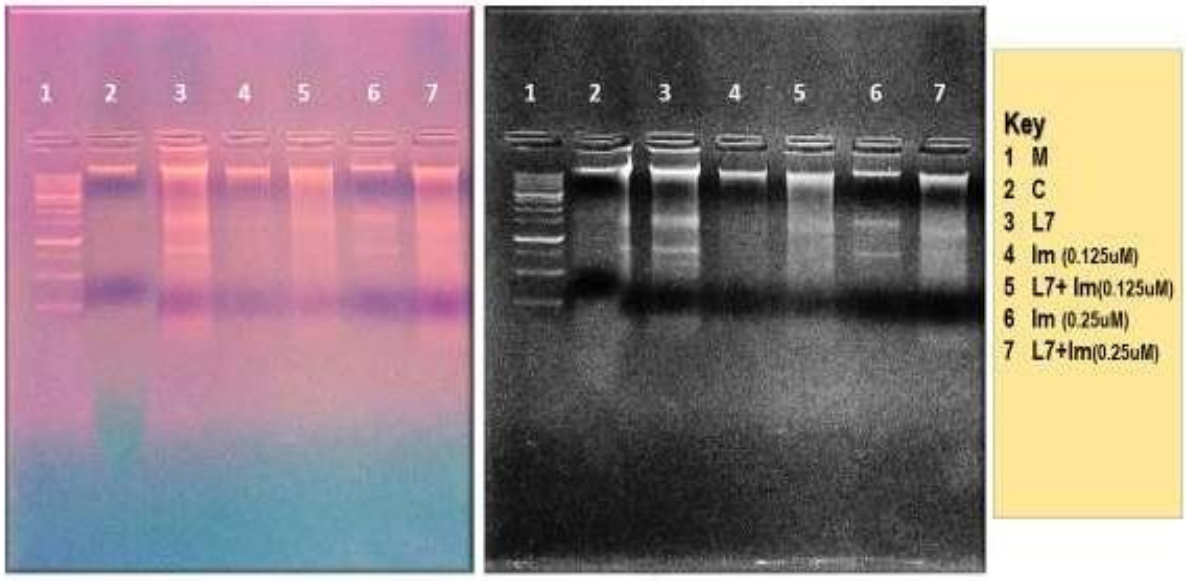

Figure 4.18: Gel image of DNA fragmentation assay for K562 cells. Cells were grown in liquid media (RPMI +10% FBS+1% L-Glutamate and 1% Penstrep) in the presence of DMSO (0.01%) and 2.5µM concentration of L7 (M= 1000bp ladder, C= DMSO) for 48-72 hours. DNA was then extracted and run on 1.5% agarose gel for visualization.

5. DISCUSSION

Interest in the therapeutic effects of imadizole derivatives on cancer treatment and cure has been increased greatly over the past decades. Various kinds of imadizole derivatives has been proved to have anti cancer potential activity against different types of cancer with remarkable outcomes for solid cancers like breast cancer (P. Sharma, LaRosa, Antwi, Govindarajan, & Werbovetz, 2021). As leukemia is among the major reason of cancer related deaths throughout the globe, chemotherapeutic strategies along with the tyrosine kinase inhibitors turn upside down the treatment array for leukemia more specifically chronic myeloid leukemia. However, the shortcomings (like disease relapse and development of resistance with the persistent activity of leukemic stem cells) of first, second and third generation TKIs (Bhatia, 2017) shifted the paradigm towards imadizole derivatives.

In the present study, we tested the anti cancer activity of imadizole derivative novel compounds L7 on BCR/ABL1 positive K562 cell line for the first time. This study was designed to identify the potential therapeutic targets of L7 using *In-silico* and *In-vitro* approaches. In the first aim of the study, interaction between potential candidates in K562 and our compound L7 shortlisted potential protein targets through molecular docking. The 2D structure of ligand was converted into 3D structure for docking purpose and free binding energy was the criteria used for determination of binding affinty between ligand and protein target. The negative and low free binding energy directly relates to a stronger binding affinity between ligand-protein complex and depicts the ligand in its most favourable conformation. In this regards, poses of our compound binding with various protein candidates of K562 cell line were analysed and best pose was extracted.

Drug Candidates with the least binding energies were shortlisted and protein-ligand interaction profiling of these shortlisted candidates revealed bonding interactions predominantly hydrogen bonds, hydrophobic interactions, pi-stacking, and pi-cations interactions with residues of the binding site. For K562, protein-ligand complex with least binding energy are shown in (Figure 4.2). The protein ligand complex with remarkable impact is with characteristically most crucial component of aberration exhibited by CML. Significant binding affinity was displayed by BCR component of hybrid BCR-ABL1 with our novel compound L7, indicating towards inhibitory action of L7 on the BCR-ABL1 activity (Table 4.1)

In the light of *In-silico* results, we further hypothesized in order to confirm the inhibition of BCR-ABL1 and its downstream signalling by pharmacological targeting with L7, we compared the effect of imatinib and L7 on the downstream signalling in K562 cells *in-vitro* for its similar or different inhibitory route. It was investigated that L7 decrease the proliferation of BCR/ABL-positive CML cells. Furthermore, comparative analysis between L7 and Imatinib mesylate, an already approved FDA drug for CML, showed significant inhibitory effect of L7 on K562 cells in accordance with Imatinib. K562 cells express the p210 form of the BCR-ABL fusion gene, which is the predominant form found in CML.

Following the comparative analyses, combination treatment utilising L7 and Imatinib (L7 + Imatinib) showed additive effect in K562 cells in comparison to individual anti-proliferative effect of both (Figure 4.8). Considering this scenario, further to find out the mechanism of action behind the anti proliferative effect of L7 on K562 cells, DNA fragmentation assay was performed. Since fragmented DNA is hallmark of apoptosis, it was seen that L7 activity is inducing a significant level of DNA apoptosis alone and in combination with L7 (Figure 4.15, 4.18). Typically, conventional drugs induce apoptosis in cancer cells at higher concentrations which is eventually

diminished due to the development of multi-drug resistance (MDR) as reported by (Belloc et al., 2007). Targeting cancer cell through non-apoptotic routes is one way to evade such resistance.

Furthermore, RT-PCR results clearly showed that L7 downregulated the expression of AXL, a therapeutic target for CML (Figure 4.5). According to studies AXL is over-expressed in primary CD34+ leukaemic stem cells and its expression level is reliant on BCR/ABL1 protein levels in CML cells. It has been concluded that AXL downregulation decreases the survival ability and self-renewal capacity of CD34+ CML cells. Also, AXL is associated with drug resistance as its levels significantly rises in imatinib resistant CML cells (Dufies et al., 2011). It is observed that AXL activates Wnt/β Catenin pathway that plays a vital role in CML pathogenesis. High expression of AXL stabilises protein level of β-catenin which in turn is the key regulator of self-renewal in CML cells. Stabilised β-catenin translocates then in nucleus where it actively performs its transcriptional activity and triggers the expression of downstream Wnt target genes like c-Myc, Axin2 etc (Ruan, Kim, Ogana, & Kim, 2020). It is important to be noted here that BCR-ABL1 can stimulate β-catenin as well through PI3K/AKT pathway.

Previously, *in-vitro* and *in-vivo* studies showed that inhibition of this BCR/ABL1 downstream pathways ultimately leads to β-catenin degradation, subsequently, achieving downregulation of its target genes in CML (Dinner & Platanias, 2016). Our novel compound L7 successfully downregulated the expression Wnt/β-Catenin target genes like c-Myc in K562 cells (Figure 4.6) emphasising that L7 efficiently interferes with Wnt/β-Catenin signalling pathway. c-Myc is widely prevalent in CML cells and facilitates the transformation of chronic phase to blast phase and regulates proliferation, differentiation, and apoptosis of haematopoietic cells (J. Zhu, Sunohara, Benyoucef, & Brand, 2018).

Similarly, expression analyses of AXL and c-Myc performed by targeting K562 cells with imatinib showed downregulation of the genes (Figure 4.17). Thus, affirming our hypothesis about the inhibitory role of L7 on BCR-ABL1 through interaction with BCR part of BCR/ABL1 hybrid. Therefore, the interference seems to be coherent with imatinib with respect to downstream signalling. These findings are significant clinically, as BCR-ABL1 inhibition by L7 is dependent on interaction with BCR region rather than ABL as demonstrated by imatinib. This mechanism might be helpful in eliminating resistance originating due to mutations in ABL domain thus making current therapies ineffective as they bind with ABL for their inhibitory action.

The present study is the first one to successfully underscore the anti-leukaemic effect of the novel compound, L7, and its inhibitory route of action. Validation mechanisms like FACS is requisite to further highlight the phenomenon of L7 preventing proliferation. Expression analysis of downstream signalling proteins needs to be determined through reliable methods like immunohistochemistry and western blotting. Further studies should be performed on the inhibitory role of L7 in therapy resistant cells and against leukaemic stem cells and their eradication with regard to disease relapse. Since L7 is an imadizole derivative compound, toxicity assessment needs to be done to evaluate its role as a safe anti-leukaemic agent with considerable therapeutic index.

6. CONCLUSION

During the past decades, various therapeutic options were proposed for chronic myeloid leukemia but the complications emerging overtime, limited their efficacy. Immense research on targeted therapies for leukemia shifted the paradigm of treatment towards imadizole derivatives. Keeping in veiw the findings of current study, it can be concluded that novel imadizole derivative compound L7 serve as a potential therapeutic option in BCR-ABL1 positive CML. Since our *In-silico* results indicated BCR component of BCR/ABL1 hybrid as key target of our ligand L7, further investigation revealed that L7 downregulated target genes (including AXL-RTK and c-myc) involved in progression of disease, resistance development and maintainance of LSC's. Therefor,current study in a long term outlook may pave in the direction of future validatin and investigation into the CSC's eradication. However, more research is required to elaborate the efficacy and effectiveness of novel imadizole derivative compound L7.

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