To Evaluate the Role of Antibiotic (Gramicidin A) in Myeloid Leukemia

By

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MS-Healthcare Biotechnology

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A thesis submitted as a final year project in partial fulfillment of the requirement for the degree of Master of Science

In

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Syeda Maham

Dedicated To

My Father (Late)

(The loss of my father will always sting. But now, everything that I do is in honor of him and celebrates his life.)

And

My Mother for all her sacrifices and prayers.

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Syeda Maham

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LIST OF ACRONYMS

A

APL Acute promyelocytic leukemia

ATRA All trans retinoic acid

ATO Arsenic trioxide

B

BM Bone marrow

Bp Base pairs

BCR Breakpoint Cluster Region

C

cDNA Complementary DNA

CML Chronic myeloid leukemia

CR Complete remission

CSCs Cancer stem cells

D

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTPs Deoxyribonucleotide triphosphates DTP Drug-tolerant persisted

F

FISH Fluorescence in situ hybridization

G

GrA Gramicidin A

H

HSCT Hematopoietic stem cell transplantation

I

IL Interleukin

J

JAK Janus Kinase

L

LICs Leukemia initiating cells.

LSCs Leukemic stem cells

M

M Molar

mM Millimolar

µlMicroliter

µM Micromolar

MRD Measurable residual disease

MTT Tetrazolium salt

MPPs Multipotent progenitors

N

NF water Nuclease free water NUP214 Nucleoporin 214

O

OS Overall survival

P

PCR Polymerase chain reaction

PLZF Promyelocytic leukemia zinc finger PML/RARα

PLRA Promyelocytic leukemia/retinoic acid receptor α

R

RA Retinoic acid

RBCs Red blood cells

RNase Ribonuclease

rpm Revolution per minute

RPMI Roswell Park Memorial Institute RT Reverse transcriptase

RTK Receptor tyrosine kinases

T

t (15;17) Translocation of chromosome 15 and 17 TAE Tris acetate EDTA

TRM Treatment-related mortality

U

UV	Ultraviolet
W	
WBCs	White blood cells
Wnt	Wingless-related integration site
+	**Positive**
-	**Negative**
%	**Percent**
°C	**Degree Celsius**

ABSTRACT

Gramicidin A is a well-known antibiotic and recently was reported to induced tumour cell death, however, little is understood about the molecular mechanism of gramicidin A as a therapeutic agent for solid tumours. Similarly, its role in liquid cancer is unknown.Here, we investigated the role of gramicidin A in leukemic cells. Leukemia is the cancer of white blood cells and is of two origin i-e myeloid and lymphoid origin. Gramicidin A upon testing in vitro interferes with the proliferation potential of PML/RARα-positive APL cells and BCR-ABL positive CML cells. The current study evaluated the cell cytotoxicity of gramicidin A and its anti proliferative activity on NB4 and K562 cell lines. Generalized toxicity is a significant challenge to the development of ionophores as therapies for human cancer. But we identified a concentration of Gramicidin A showing no hemolysis. Furthermore, we investigated the combine effect of already approved treatments of CML and APL with Gramicidin A and is has shown additive effect in both cases. Similarly, the expression analysis indicated that Gramicidin A interferes with the β-catenin dependent leukemogenesis and downregulates AXL-RTK in APL cells with the expected downregulation of downstream c-Myc, Eya3 and Axin 2 related to the Wnt/β-catenin signalling. Taken together, these results demonstrate a new role for gramicidin A as a potent inhibitor in case of myeloid leukemia.

1) INTRODUCTION

A healthy process called hematopoiesis occurs when stem cells in the body multiply and develop into several types of blood cells. Hematopoiesis primarily occurs in the bone marrow. Multipotent hematopoietic stem cells can develop into lymphoid and myeloid progenitor cells under physiologically appropriate conditions. B cells, T cells, and natural killer cells, which are all lymphoid progenitor cells that undergo further differentiation, each play a crucial part in maintaining a healthy body. On the other hand, myeloid progenitor cells that differentiate into myelocytes help to generate dendritic cells, neutrophils, eosinophils, basophils, monocytes (macrophages), and neutrophils. Each of these cells is crucial to the body's regular immunological process.

The mast cells that play a role in allergic reactions and the reticulocytes that differentiate into erythrocytes and megakaryocytes are the other important cells of the myeloid progenitor origin. The red blood cells, platelets, and white blood cells that make up blood each have a vital role in maintaining normal biological function. A slight dysregulation of these cells can cause a variety of clinical disorders, including dysfunctional bone marrow syndromes and cancers like leukemia. Since the normal process of hematopoiesis is controlled by a number of variables.

White blood cell cancer called leukemia typically starts in the bone marrow. This type of cancer starts in the hematopoietic stem cells, where it produces aberrant white blood cells. As these WBCs build up, leukemic blasts are created in the bone marrow. Every year, 300,000 new cases of leukemia are predicted to be diagnosed worldwide. According to the American Cancer Society, there are currently 61,090 new cases of leukemia in the country. Leukemia is the fourth most common kind of cancer in Pakistan, while it ranks as the 14th most usual form of cancer worldwide. Pakistan experiences an annual diagnosis rate of 5.6% of leukemia cases.

Classification of leukemia

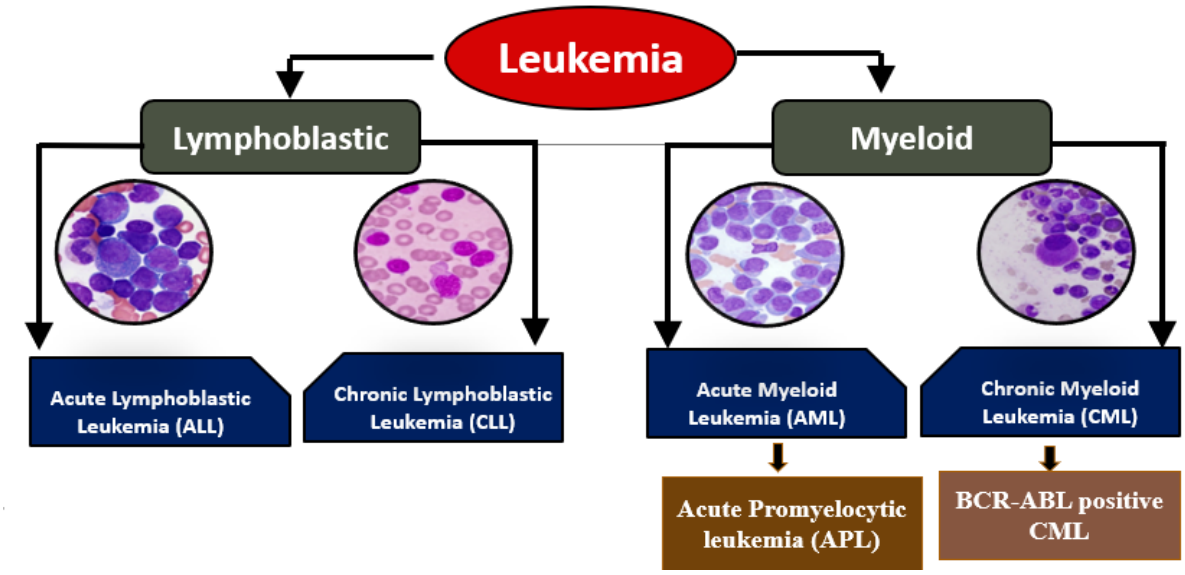


Figure1: Classification of leukemia.

1.1 Acute Promyelocytic Leukemia

A variant of acute myeloid leukemia called acute promyelocytic leukemia (APL) is characterized by a lack of differentiation during the promyelocytic stage. This barrier leads to the accumulation of immature white blood cells in the bone marrow. APL is an acute myeloid leukemia (AML) (Jimenez, Chale, Abad, & Schally, 2020). 10-15% of all AML cases involve APL. (Tallman & Altman, 2008). According to the French, American, and British (FAB) categorization system, the disorder is recognized as AML-M3. APL was initially reported by LK Hillestad (a Norwegian hematologist) in 1957. In 1959, J. Bernard identified 20 APL patients and described the growth of promyelocytes in detail (Francesco Lo-Coco & Cicconi, 2011). A translocation between chromosomes 15 and 17 (t (15; 17) (q24; q21), which results in the creation of a PML-RARA fusion protein, characterizes most APL cases (Kakizuka et al., 1991). The fusion protein PML-RARA results in a dominant negative mutation because it suppresses apoptosis, promotes the growth of leukemic progenitors, and impairs differentiation (Rogaia, Grignani, Nicoletti, & Pelicci, 1995). The fusion protein PML-RAR disrupts PML's homeostatic function and deregulates the transcription of RAR target genes. RAR is a transcription factor that plays a crucial part in the transcription of genes essential for cellular growth, survival, differentiation, and cell death, among other physiological functions (Di Masi et al., 2015). An unusual molecular device known as the

nuclear body is recruited by PML. These PML-NBs conducted a variety of cellular tasks, such as tumour control, homeostasis, response to viral infections, senescence, differentiation, angiogenesis, and maintenance of genomic stability (Hadjimichael et al., 2017). The fusion protein PML-RAR causes the PML nuclear bodies to become disorganised and inhibits the transcription of RAR target genes, which causes myeloid progenitor proliferation and a stop in promyelocytic maturation (Grignani et al., 1998) (K. Wang et al., 2010). Patients with APL are divided into low-, intermediate-, and high-risk groups based on risk and WBC level. Patients with WBC counts greater than 10,000/L are considered high-risk APL patients, whereas those with WBC counts less than 10,000/L are considered low and intermediate risk patients (Sanz et al., 2000). Both arsenic trioxide (ATO) and all-trans retinoic acid (ATRA) have excellent cure rates and are essential in the treatment of APL. As a differentiation agent, ATRA targets the PML-RAR complex, degrades it, and transmits signals that drive promyelocytes to differentiate into adult granulocytes (Breitman, Selonick, & Collins, 1980; de Thé & Chen, 2010). By causing apoptosis and boosting APL cells' differentiation by focusing on the PML-RAR fusion gene, ATO mediates its treatment response. Patients who have just received an APL diagnosis often only take ATRA and ATO, not chemotherapy (H.-H. Zhu, 2020). The APL subtype of adult AML is now thought to be the most curable form of the disease because of recent therapy advancements (Thomas, 2019). Treatment resistance, recurrence, and early mortality still exist despite the availability of first-line treatments for issues like differentiation syndrome. Differentiation Syndrome, commonly known as the ATRA syndrome, is a potentially fatal side effect of treatment that often shows up early on. The following symptoms make up the differentiation syndrome: fever, weight gain, hypotension, pleural effusion, peripheral edoema, and acute renal failure (Yilmaz, Kantarjian, & Ravandi, 2021). Relapse and early death remain two of the biggest challenges of treating high-risk APL patients (Stahl & Tallman, 2019).

1.2 Chronic myeloid leukemia:

A change in the genome in the hematopoietic stem cell compartment is the cause of the clonal myeloproliferative illness chronic myeloid leukemia. Following this modification, the bone marrow produces an excessive number of granulocytes, which leads to splenomegaly and hyperleukocytosis. More than 90% of CML cases have the Philadelphia chromosome, which results from a balanced reciprocal translocation t (9; 22) (q34; q11), as it defines cytogenetic feature.

The breakpoint cluster region (BCR) of chromosome 22 and the Abelson (ABL) proto-oncogene on chromosome 9 fuse because of this translocation. A protein with strong tyrosine kinase activity that is encoded by the chimeric gene functions as a tumor-promoting factor. There is little knowledge of the etiological variables worldwide. Several epidemiological research have shown the impact of ionizing radiation on the beginning of CML in those exposed, while other investigations raise the possibility that benzene exposure plays a role in the disease's development. The affected population's median age is 56 years old, and it accounts for about 15–20% of adult leukemia cases. The incidence of CML fluctuates between 0.6 and 2.8/100,000 people worldwide each year.

In a short amount of time, there has been significant advancement in the treatment of chronic myeloid leukemia in patients with Ph+, beginning with allogeneic hematopoietic stem cell transplantation, recombinant interferon alfa (rIFN), or medications used as initial treatment like hydroxyurea, cytarabine, or busulfan, and more recently, tyrosine kinase inhibitors (TKIs). These inhibitors have a direct impact on the BCR-ABL oncoprotein's kinase activity, drastically altering the prognosis for remission and survival in patients with CML. When coupled with other anticancer medications (like interferon-α), approved TKIs may help to increase the effectiveness of the therapy.

Relapse has been seen in the clinical environment, despite the promising results. Cancer stem cells and adaptive resistance are to blame for this relapse. According to research, antibiotics can encourage the death of cancer cells (apoptosis), stop cancer growth, and stop cancer spread. Due to these factors, the use of antibiotics in the treatment of cancer is rising. These antibiotics, known as ionophore antibiotics, can disrupt cellular ionic equilibrium by creating channels across the cell membrane, which results in malfunction and cell death. Salinomycin and Gramicidin are still in use. An antibiotic has demonstrated encouraging outcomes in a variety of malignancies. Most of the molecules in the naturally occurring gramicidin generated by Bacillus brevis are gramicidin. A. Gramicidin’s are solely monovalent cation-selective, with potassium predominating over sodium. The ionophore has been widely used as an additional experimental tool to cause alterations in the plasma membrane potential in various biological systems, primarily as a depolarizing agent, because of its capabilities as an ionic channel. The extracellular ionic content affects the effect of gramicidin on the plasma membrane potential (PMP).

1.3 Hypothesis

The goal of the current study was to examine gramicidin A's ability to fight both chronic myeloid leukemia in the K562 cell line and acute promyelocytic leukemia in the NB4 cell line. The creation of more potent targeted therapies is of tremendous interest because the key issues with treating APL and CML are differentiation syndrome, relapse, drug toxicity, and resistance to current medicines. Antibiotics are currently being employed to eradicate cancer stem cells. Gramicidin A, an antibiotic used for this purpose, has shown encouraging outcomes in cases of solid malignancies, but we are interested in employing Gramicidin A to target leukemic cancer cells to determine its involvement in liquid cancer.

1.4 Aims and Objectives

1. To check the potential targets of Gramicidin A in CML and APL cell lines by using Insilco approaches.
2. In vitro validation of Gramicidin A in AML and CML cell lines.
3. To know about the mechanism of action of Gramicidin A in APL and CML cancer stem cells.

2) LITERATURE REVIEW

2.1 Hematopoiesis

Hematopoiesis is a controlled process that produces mature blood cells and involves a high throughput of cells via cell division and differentiation from self-renewing hematopoietic cells. Starting from the main haematopoietic stem cell at the higher level, cells differentiate into myeloid and lymphoid lineages (Kim, Stachura, & Traver, 2014). (Fig. 2.1). This process involves several early and origin specific growth factors and different transcription factors, responsible for differentiation of those hematopoietic stem cells via expression of origin specific genes. AML pathogenesis is initiated by any disruption in this setup (Steffen, Müller-Tidow, Schwäble, Berdel, & Serve, 2005).

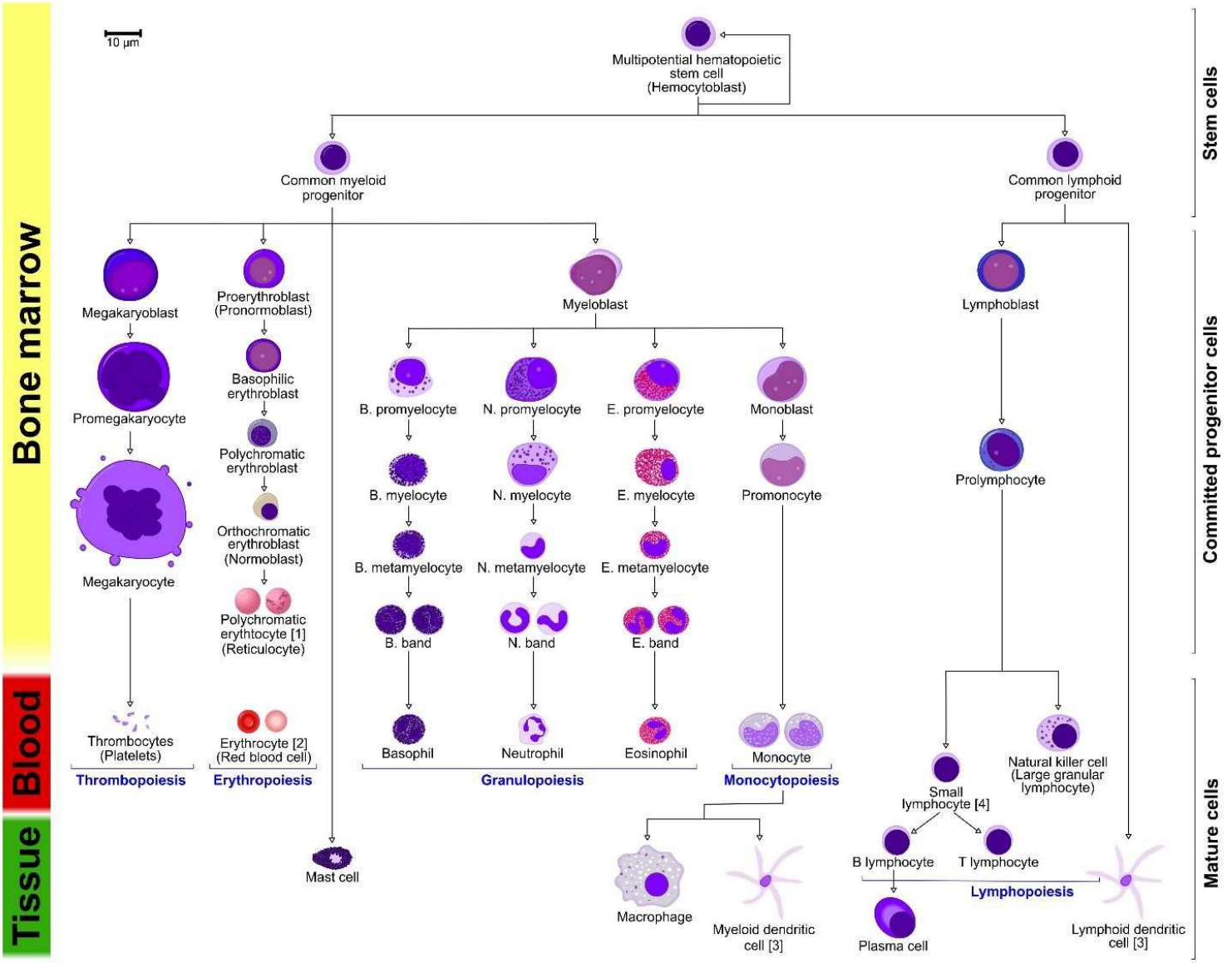


Figure 2.1: The process of Hematopoiesis (Iskander, 2021)

Hematopoietic stem cells (HSCs) are pluripotent stem cells that can differentiate and self-renew asymmetrically into all blood cells, initiating the multistep process of haematopoiesis. HSC differentiates into multipotent progenitor cells (MPP), which then differentiate into lineage committed myeloid and lymphoid progenitor cells that proliferate and differentiate extensively (Iwasaki & Akashi, 2007). Multipotent progenitor cells rapidly multiply and retain the ability of differentiation to any haematopoietic cell type but lose the ability to self-renew. Myeloid blood cells include granulocytes, platelets, red blood cells and macrophages/monocytes, whereas lymphoid cells include natural killer cells, B cells, T cells and dendritic cells. Because mature blood cells have a finite life span, their numbers are constantly replenished. As committed blood cells continue to form, self-renewal ability gradually declines (M. Kondo, 2010).Human diseases including anemia and leukemia occur due to defect in hematopoiesis process (Kumar & Evans, 2015). Abrupt gene expression in hematopoietic stem cells or multipotent progenitor cells leads to chromosomal alterations which bring about a variety of hematopoietic malignancies.

Leukemogenesis is the process by which leukaemia develops, and it is one of the three major classes of Haematopoiesis cancers/malignancies. Leukemias are cancers caused by the transformation of hematopoietic cells in blood or bone marrow. For bone marrow, transformed cells from cancerous progeny typically enter the bloodstream. As a result, leukemias frequently manifest as "liquid tumors," with markedly increased lymphoid, myeloid, or, in rare cases, erythroid lineage cells in bone marrow or blood. Myelomas are tumors that develop from fully differentiated plasma cells and can be found in the bone marrow, tissue, or blood as dispersed clones or as solid masses. Unlike normal plasma cells, which stop dividing after differentiation, myeloma cells continue to proliferate and synthesise many Ig chains. HCs are formed when a target cell aids in different genetic changes to repair genes of DNA, tumor suppressor genes, or oncogenes, and are clonal in nature. (TSGs) (Hsieh, 2014). Researchers discovered chronic and acute myeloid leukemias as an abnormality of HSCs in the 1980s, in which the cells that start these leukemias are innately stem cell-like in that they can self-renew but cannot differentiate properly.

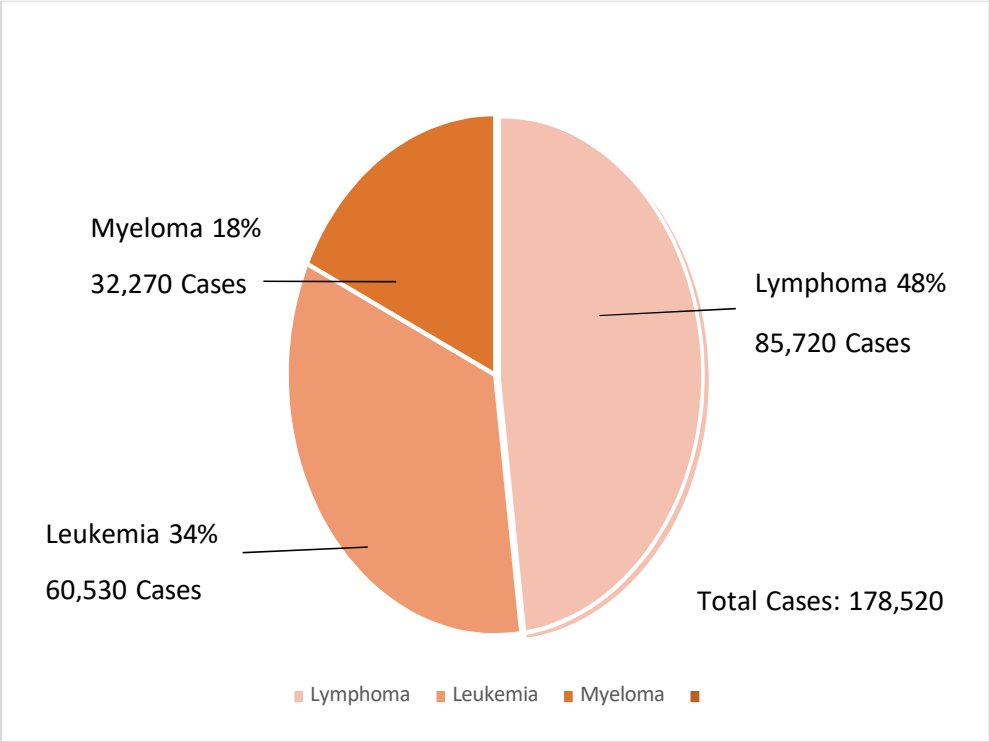


Fig.2.2 Estimated % new cases of leukemia Source: *Cancer Facts & Figures, 2020*. American Cancer Society; 2020.

2.2 Prevalence of Leukemia in Pakistan

Pakistani Prevalence Leukemia has become a global health concern due to its fatality, with morbidity and mortality increasing over time. Leukemia affects both genders in Pakistan, with males being affected at a higher rate (5.2%). In 2018, leukemia accounted for 4.1% of all cancer-related cases and ranked fifth in cancer-related mortalities in Pakistan (WHO, 2019), while another study found AML to be the most common type of leukemia in Pakistan, followed by CML (Ahmad, Yusuf, & Burney, 2015).

2.3 ACUTE PROMYLEOCYTIC LEUKEMIA

2.3.1 Introduction to APL

LK Hillestad, a Norwegian haematologist, was the first to report and describe Acute Promyelocytic Leukemia in 1957. J.Bernard provides a more detailed description of APL two years later by describing promeylocyte proliferation. Cicconi and Francesco Lo-Coco (2011)

Acute myeloid leukemia (AML) is classified into several subtypes, one of which is APL, which accounts for 10-15% of all AML cases (Tallman & Altman, 2008). In promyelocytes, APL is recognized by maturation arrest and differentiation block. Acute Promyelocytic Leukemia is caused by the expression of the oncogenic fusion protein PML-RAR; a product of the PML-RAR gene, which is a fusion product of a reciprocal and balanced translocation between the PML gene on chromosome 15 and the RARA retinoic acid receptor gene on chromosome 17.

PML protein is involved in many cellular functions such as tumor suppression, antiviral functions, homeostasis, differentiation, angiogenesis, and DNA repair. PML-NBs perform these functions (Lallemand-Breitenbach, 2010). When the retinoic X receptor binds to the ligand-dependent transcription factor RAR, it forms transcriptionally active heterodimers. RAR's primary function is to regulate gene expression. RARE-containing genes are involved in a variety of physiological activities in cells, including cell growth, survival, differentiation, and death. Retinoid receptors also activate kinase signaling pathways (Di Masi et al., 2015). The PML-RAR fusion protein inhibits the transcription of RAR target genes, causing promyelocyte proliferation and disrupting PML-NBs (Grignani et al., 1998). ATRA (All trans retinoic acid) and ATO (arsenic trioxide) are critical in the treatment of APL, with excellent cure rates.

The activity of ARTA is responsible for the maturation of promyelocytes into granulocytes (Breitman et al., 1980). APL patients are divided into three groups based on their risk. Intermediate and low WBC counts are sometimes combined and reported as 10,000/ul. Patients with a WBC count greater than 10,000/ul are considered high risk (Sanz et al., 2000). Patients with intermediate and low risk can be treated with ATRA + ATO, but patients with high risk require cytotoxic chemotherapy as ATRA + ATO alone are insufficient (Osman et al., 2018). Relapse is more likely in APL patients who are considered high risk; however, regardless of risk status, 10-20% of APL patients relapse (Vitaliano-Prunier et al., 2014).

2.3.2 Molecular Pathogenesis:

The unique properties of the PML-RAR fusion product have proven to be critical in the molecular studies of APL. The PML-RARA fusion product disrupts the gene expression of hematopoietic progenitor cells, myeloid differentiation, and stem cell self-renewal. 2014; Vitaliano-Prunier et al. In a normal situation, the receptor alpha of retinoic acid, also known as RARa, will form heterodimer with the receptor of nuclear hormone proteins retinoid X receptors (Chambon, 2005). The heterodimer of RARa-RXR binds to the elements of retinoic acid response and regulates expression of numerous genes (RAREs, are segments of DNA).

These DNA sequences control differentiation and self-renewal. If the retinoic acid (ligand) is not present, the heterodimer complex will bind corepressors such as nuclear receptor corepressors (NCoR) and thyroid and retinoid hormone receptor silencing mediators (SMRT) (Kishimoto et al., 2006) Histone deacetylases play a role in transcriptional repression as well. These corepressors are released because of a conformational change caused by retinoic acid and RARa binding; co-activators are then recruited, which regulates the Gene expression and chromatin remodeling Shihimoto et al. (2006) PML-RARa fusion proteins disrupt coactivator recruitment and inhibit transcription of response elements of retinoic acid (RAREs).

Figure 2.3 Molecular mechanisms involved in APL

The fusion protein PML- RARA (19) also interferes the formation of PML nuclear bodies (Vitaliano-Prunier et al., 2014). Sphere-shaped PML nuclear bodies are attached to the nuclear matrix. They regulate a broad range of nuclear functions, like epigenetic silencing, translation, replication, and senescence, as well as p53 signaling, possibly by controlling proteolysis and sumoylation (de Thé, Pandolfi, & Chen, 2017; Vale′ rie Lallemand & de Thé, 2010).

The key regulator of these domains of PML gene, and its recruits several other proteins, including DAXX, a key modulator of apoptosis and translation repressor. Posttranslational modifications to the PML protein, such as phosphorylation and sumoylation, are important for the recruitment of functionally associated proteins (Vale′ rie Lallemand & de Thé, 2010). The PML-RARA-RXR complex presence has been linked to pathogenesis of APL. J. Zhu and colleagues (2007) RXRA sumoylation is an important step in the transformation of APL cell.

2.3.3 Translocations in APL

Other than t(15;17), new cytogenetic translocations have been discovered in one to two percent of APL cases on average.

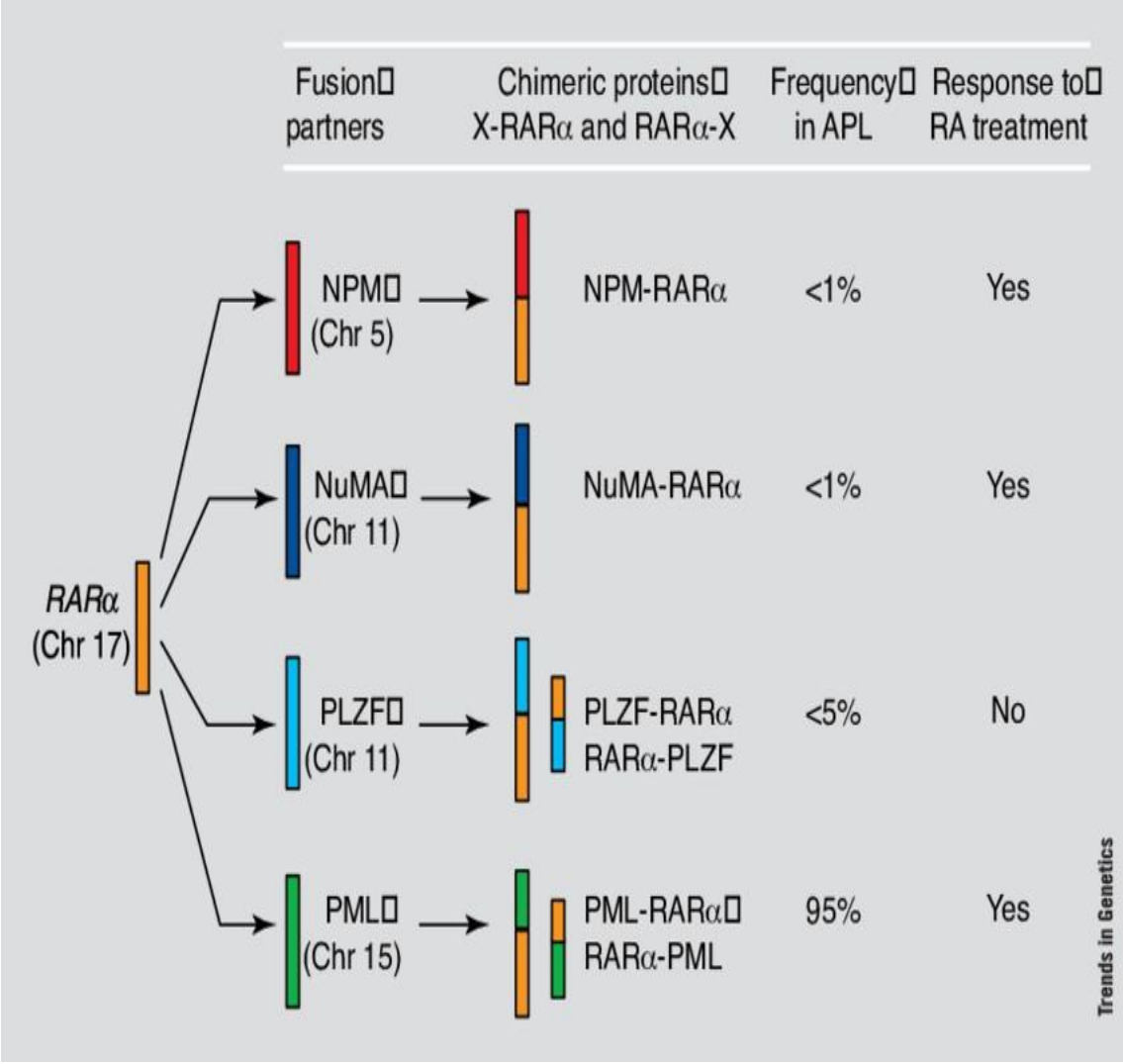


Figure 2.3.3 APL translocations involving RARα (Lin, Egan, & Evans, 1999)

So far, 12 APL fusion variants have been reported, all of which are linked to the RARA gene (Baba, Pandith, Shah, & Baba, 2019). More than 30 patients have been identified with the most common APL variant, ZBTB16 (previously PLZF)- RARA translocation, acquired from the rearrangement t(11;17)(q23;q21) (Z. Chen et al., 1993). Several other identified translocations result in RAR fusion with the following genes.

Table 2.3. Additional genes involved in APL

SR.No	Gene	Reference
1.	NPMI	(Corey et al., 1994)
2.	NUMAI	(Wells, Catzavelos, & Kamel-Reid, 1997)
3.	STAT5B	(Arnould et al., 1999)
4.	PRKAR1A	(Catalano et al., 2007)
5.	FIP1L1	(T. Kondo et al., 2008)
6.	BCOR	(Yamamoto et al., 2010)
s7.	NABP1 (once OBFC2A)	(Won et al., 2013)
8.	TBL1XR1 (once TBLR1)	(Y. Chen et al., 2014)
9.	GTF2I	(Li et al., 2015)
10.	IRF2BP2	(Yin et al., 2015)
11.	FNDC3B	(Cheng et al., 2017)

APL is distinguished by the absence of transmembrane glycoproteins such as CD7, CD11a, CD11b, CD14, and CD18 expression, as well as infrequent HLA-DR expression and downregulation of CD34, whereas APL cells exhibit upregulation of CD13 and CD117, as well as atypical expression of CD2 (T-cell linked antigen) involved in high leukocyte count and microgranular variant

morphology. CD56 expression has been linked to ATRA and chemotherapy resistance. Noguera and colleagues (2019).

2.3.4 PML and Its Functions:

The PML gene, which is found on chromosome 15q24 and has nine exons, generates several spliced transcripts. Splicing causes differences in the C-terminal exon portions of the PML gene's isoforms. All PML isoforms share the N-terminal region of the PML gene, which is encoded by exons 1-3 and consists of a RING-B-Box-Coiled-coil/tripartite motif domain. These isotypes can be found in both the cytoplasm and the nucleus of a cell (Jensen, Shiels, & Freemont, 2001). PML's two primary functions are genomic instability and tumor suppression, and these activities are carried out via temporary or constitutive interactions with over 170 proteins (Lallemand-Breitenbach, 2010). PML isoform-specific domains primarily mediate these connections, which govern a variety of processes such as stem cell self-renewal, transcription, and HSC epigenetic alterations (Chen et al., 2019), as well as p53-dependent and p53-independent apoptosis (Matt & Hofmann, 2018). Some of the most important PML functions are as follows:

- PML is a tumor suppressor protein that is required for the formation of promyelocytic leukaemia nuclear bodies, which are nuclear macromolecular complexes. It is typically found in the nuclear bodies of promyelocytic leukemia (PML-NBs).PML-NBs are distinct nuclear foci that can be found in the nuclei of most mammalian cells and range in size from 0.2 to 1.0 micrometers. They typically have one to thirty bodies per nucleus, but this varies depending on the type of cell, as well as the cell cycle phase and differentiation stage.

- PML-NBs are dynamic structures that change in quantity, size, and position during the cell cycle and in response to cellular stressors such as senescence induction and DNA damage (Bernardi & Pandolfi, 2007).

- PML-NBs have been discovered near other nuclear organelles, such as Cajal bodies, and have been linked to genomic regions involved in transcriptional processes. Furthermore, a specific relationship with specific chromosomal locations has been demonstrated.

- PML-NBs contain many proteins, both temporarily and permanently. As a result, PML-NBs have been linked to the regulation of numerous cellular processes, including the induction of apoptosis and senescence, the inhibition of proliferation, the maintenance of genomic stability, and antiviral responses.

- Recent evidence suggests that PML-NBs are made up of a variety of distinct structures, and that different PML-NBs may govern different cellular processes depending on their protein composition, location in the nucleus, and mobility (Bernardi & Pandolfi, 2007).

2.3.5 RARA and its Functions:

RARA1 and RARA2 are encoded by the RARA gene, which is located on chromosome 17q21. These isotypes have distinct Activation Function 1 domains (AF-1) at their N-termini because they use different promoters and exons (Zelent, Guidez, Melnick, Waxman, & Licht, 2001). The nuclear receptor superfamily includes the RARA protein, which shares up to 90% homology with the RARB and RARG proteins. When ligand is present, the retinoid X receptor (RXR) and the retinoic acid receptor A (RARA) form a heterodimer that binds to the retinoic acid responsive elements (RARE) in the promoter of the target gene. These RAREs contain tandem 5'-AGGTCA-3' sites known as DR1-DR5, which regulate the transcription of numerous transcripts (Collins, 2002).

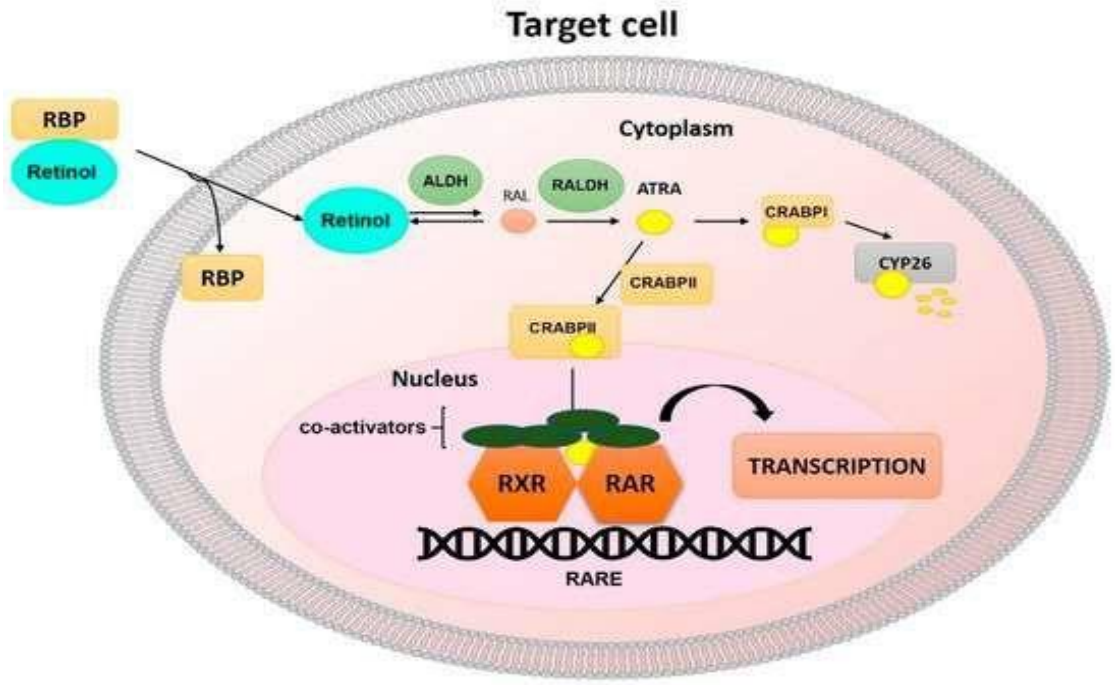


Figure 2.3.6 Mechanism of Retinoic Acid Receptor signaling (Conserva, Anelli, Zagaria, Specchia, & Albano, 2019)

RARA and RXR, on the other hand, interact with a multiprotein complex containing corepressors in the absence of ligand. Through nuclear receptor corepressors (N-CoR) and thyroid and retinoic hormone receptor silencing mediators, this interaction causes histone deacetylation, chromatin condensation, and transcriptional suppression (SMRT). RARA is a nuclear transcription factor that not only plays a role in myeloid haematopoietic cell differentiation. RARA, along with HDAC3, HDAC5, and HDAC7, regulates microRNA-10a suppression, which enhances an inflammatory response (Liquori et al., 2020).

2.3.6 PML-RARA fusion Gene:

The PML gene produces several mRNA transcripts of varying lengths due to breakpoints in three exonic regions known as bcr. A break in the PML gene's intron 6 (bcr1) results in the formation of the long (L-) isoform mRNA, which then results in the production of PML6-RARA3 mRNA, which accounts for approximately 70% of APL cases. A breech in the short (S-) isoform mRNA is produced by PML's intron 3 (bcr3). This break causes the production of PML3-RARA3 mRNA, which accounts for approximately 20% of APL cases. A breakpoint within exon 6 (bcr2) results in the formation of variable (V-) isoform mRNA, which is then joined to RARA exon 3. This type of APL is responsible for 10% of all incidents. Variations in the occurrence of the three isoforms, on the other hand, have been described based on geography or ethnicity Liquori and colleagues (2020).

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

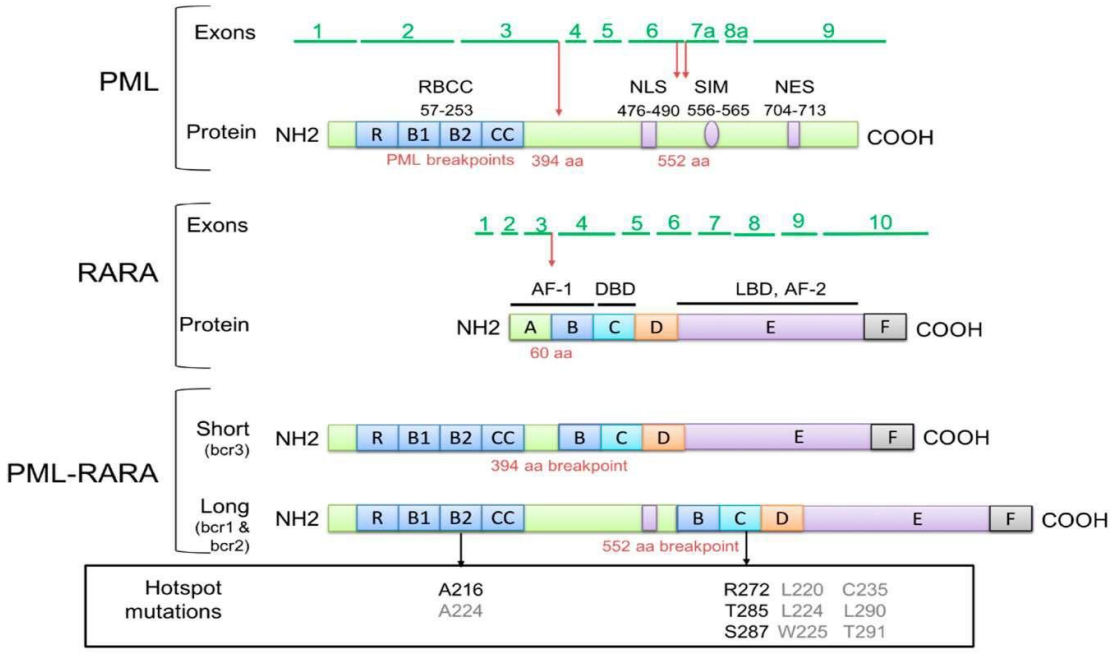


Fig.2.3.7 Structure of the APL primary event

(Proteins PML and RARA, as well as their related fusion protein PML-RARA, with breakpoints (indicated in red) and hot - spot mutations (in the box at the bottom of the figure; in black are presented mostly mutated positions, and in rarer grey changes). PML contains the following structures: RING finger (R), B boxes (B1 and B2), coiled-coil domain (CC), nuclear localization signal (NLS), SUMO-interacting motif (SIM), and others nuclear export notification (NES). The N-terminal domain (A,B) of RARA contains the activation function domain 1 (AF-1) as well as the DNA-binding domain (C), the hormone-binding domain (E), and other regulatory domains (D and F). (Liquori and colleagues, 2020)

2.3.7 Leukemiogenesis of PML/RARα:

3 Microspeckles form because of the disordering or disarray caused by the PML-RAR fusion in acute promyelocytic leukaemia, which alters the structure of nuclear bodies. 2010 (Lallemand-Breitenbach). This phenomenon occurs because the PML portion of the PML-RAR fusion gene lacks a SUMO-binding motif. Because PML-RAR has a proclivity to multimerize with a diverse range of protein classes, this fusion gene has the potential to cause cancer through both gain of function and dominant-negative effects (Pandolfi, 2001). On the one hand, PML-RAR inhibits myeloid differentiation during the promyelocyte stage. This occurs because it inhibits the transcription of genes required for myeloid development, such as those required for granulocyte differentiation. PML-RAR, on the other hand, promotes leukemic cell proliferation and survival, that results in the timely accumulation of promyelocytes in bone

marrow of APL patients (Kamashev, Vitoux, & De Thé, 2004).

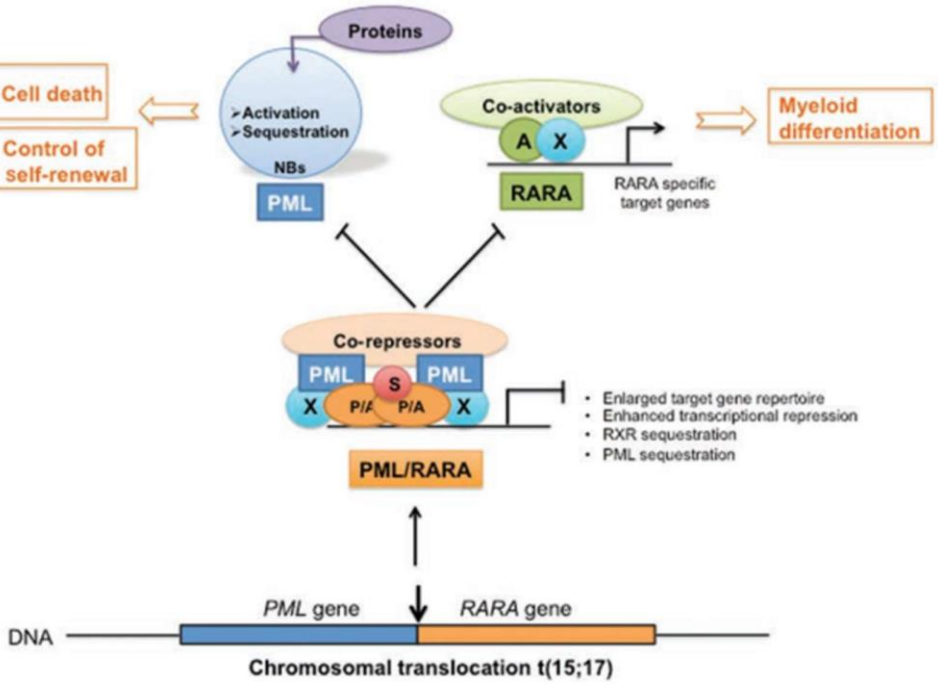


Fig.2.3.8 The Leukemogenesis of fusion product PMLRARA

The PML/RARA fusion can disrupt PML nuclear bodies as well as acts as a transcriptional repressor. PML/RARA is sumoylated in addition to binding RXR (X) and PML (S). Target gene repression is accomplished through PML/RARA can be suppressed by recruiting co-repressors. This inhibits the action of RARA (A) targets, which is important for myeloid differentiation. This also prevents the PML nuclear bodies formation, which are domains that attract many partner proteins to increase posttranslational modifications, allowing those proteins to be activated or sequestered. Defects in apoptosis control or stem cell self-renewal capacity have been linked to defective nuclear bodies. (J. Zhu, Nasr, Ablain, & Lallemand-Breittenbach, 2015).

Recent research paints a complicated picture of the APL pathogenesis process. PML-RAR tetramers can form binding interactions with a wide range of DNA locations that the RAR-RXR receptor does not normally recognize. The PML-RAR protein can recognize a non-canonical DNA sequence that may be involved in widespread transcriptional dysregulation (Martens et al., 2010). Furthermore, the PML-RAR fusion product contains significant beneficial domains involved in leukemiogenesis and are susceptible to retinoic corrosive and arsenic trioxide (de Thé et al, 1990). The RAR gene contains RXR-binding domains, DNA binding domains, hormone binding domains, whereas the PML gene contains both coiled-coil and RING finger sites (Poddighe & Weghuis, 2016).

2.3.8 Treatment Strategies

Acute promyelocytic leukaemia was once thought to be the most lethal form of acute leukaemia; however, recent treatment advances have made this subtype of acute myeloid leukaemia extremely curable in adults (Thomas, 2019). Patients with APL, whether at low or high risk of developing the disease, can now benefit from the concept of chemo-free treatment. The combination of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), without the use of chemotherapy, is now considered the first line and standard treatment for newly diagnosed cases of APL. Since the turn of the century, medical researchers have been working on non-chemotherapeutic approaches to the treatment of newly identified cases of APL (H.-H. Zhu, 2020).

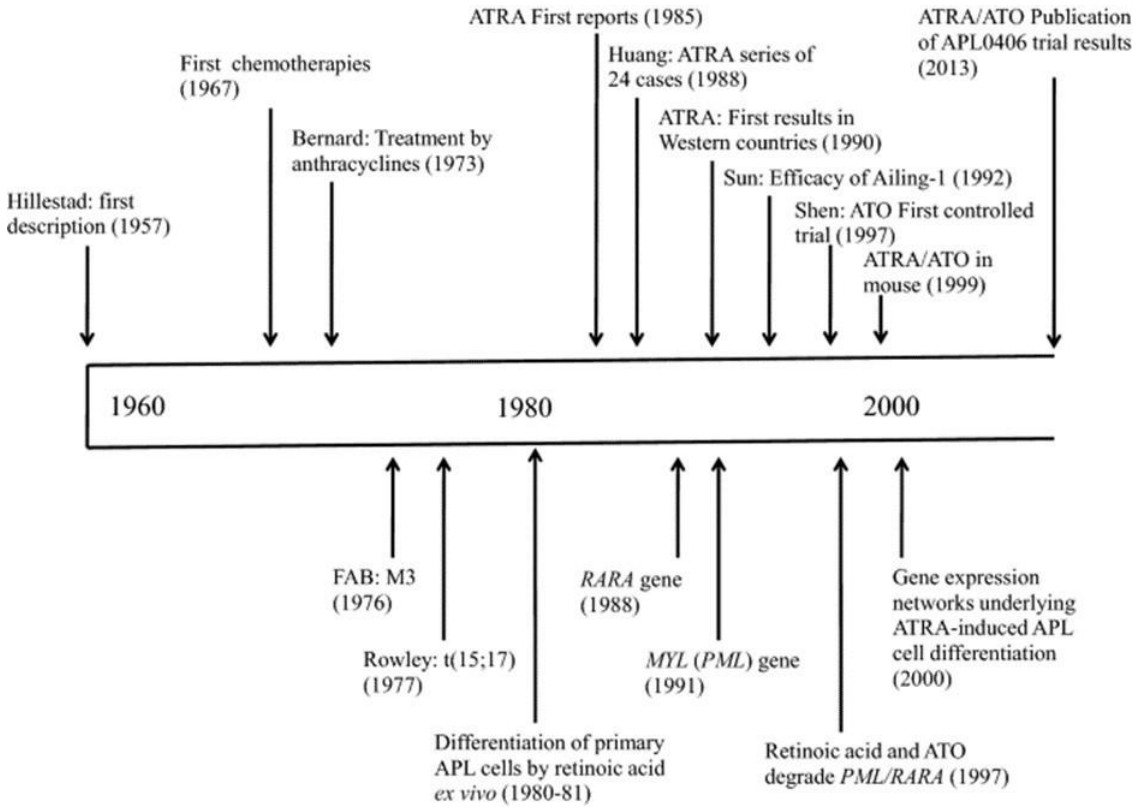


Fig.2.3.9 Highlights in the history of APL Treatment (Thomas, 2019)

2.3.9 All Trans Retinoic Acid Receptor (ATRA)

FDA approved ATRA as the first drug to treat APL. It works by recognizing the PML-RARa complex and degrading it via a proteasome-ubiquitin or caspase-dependent pathway. This activates the RAR/Retinoid X receptor, which mediates granulocyte differentiation signals (de Thé & Chen, 2010). RARa's transcriptional co-activators are recruited to the mutant because of the changes in PML-conformation, resulting in the activation of differentiation genes and, as a result, promyelocyte maturation (Liu et al., 2000).

In 1987, a Chinese journal reported that giving ATRA alone to four newly diagnosed APL patients and two chemotherapy-resistant APL patients resulted in complete remission for all of them (Huang et al., 1987). The Shanghai Institute of Haematology reported remarkable success in 24 APL patients a year later. While ARTA alone resulted in CR in 23 of these patients, one patient required low-dose cytosine arabinoside in addition to ATRA (Z.-Y. Wang & Chen, 2008).

20

2.3.10 Treatment with ATRA alone

Following the Shanghai group's initial report (Huang et al., 1987), many other institutions around the world, including the Hospital Saint-Louis in Paris (Degos et al., 1990) and the Memorial Sloan Kettering Cancer Center in New York (Warrell Jr et al., 1991), conducted confirmatory studies on the efficacy of ATRA alone. These studies describe high rates of CR in APL patients as well as rapid coagulopathy improvement. A notable feature was the terminal differentiation of malignant promyelocytes in bone marrow. Despite receiving continuous ATRA treatment, patients who achieved CR experienced relapse within a few months.

2.3.11 ATRA+ Chemotherapy

Several non-randomized studies (Fenaux et al., 1992; Kanamaru et al., 1995; G. Sun et al., 1994) and a randomized study (Fenaux et al., 1993) were conducted in the early 1990s in which significant better results in terms of CR and relapse were observed in APL patients who were treated with ATRA and then followed by chemotherapy rather than those treated alone with ATRA or chemotherapy. Later, the European APL group conducted a randomized study (Fenaux et al., 1999) that demonstrated the superiority of ATRA in combination with concurrent chemotherapy to ATRA followed by chemotherapy. This was reinforced during the trials. Mandelli et al. (1997), Sanz et al. (2004), and Sanz et al. (1999). Based on these findings, the European LeukemiaNet defined the standard of care for newly diagnosed APL patients as ATRA + Chemotherapy induction, followed by 2-3 courses of chemotherapy as consolidation. 2009 (Sanz et al.)

2.3.12 Arsenic Trioxide (1974-2002)

According to preliminary research, ATO mediates its response by removing the PML-RARa fusion protein. In the treatment of APL, the rate of complete remission induced by ATO is high (87%). ATO's action mechanism involves either promoting differentiation of APL cells at low levels or inducing apoptosis at high levels. PML- RARa degradation occurs during ATO-induced APL cell differentiation, allowing promyelocytes to overcome the maturation block. ATO also induces apoptosis via mechanisms that appear to be independent of the fusion protein, including cytochrome C release from mitochondria into the cytosol, which activates caspases. Miller, Jr. (2002).

A group from Harbin Medical University in China identified ATO as a potential anti-cancer drug in the early 1970s. This group reported in 1992 that 21 of 32 APL patients treated with Ailing-1 (an anticancer solution containing 1% ATO) achieved CR. After ten years, there is a 30% chance of survival Sun, H., Ma, Hu, and Zhang (1992). The Shanghai group later

confirmed these findings (G.-Q. Chen et al., 1997). ATO was given to relapsed patients after treatment with ATRA + Chemotherapy in a multicenter trial in the United States (Soignet et al., 2001). With ATO alone, 85% of patients achieved CR. These studies established ATO as an effective therapy for relapsed APL patients, and the FDA approved it in 2000.

Between 1974 and 1985, 32 patients with newly diagnosed APL received one ATO injection, and long-term follow-up results were studied. Ohno, Asou, and Ohnishi (2003) The findings revealed a 50% 5-year overall survival rate, 50% complete remission rate, and 19% partial remission rate. The finding was later confirmed in a study involving 124 patients from the same group who were given pure ATO. P. Zhang and colleagues (2000) Lu et al. were the first to report an excellent 100% CR rate and 76.6% 3-year disease free survival rate in 19 patients treated with oral As4S4 (tetra-arsenic tetra sulphide) (Lu et al., 2002). The total arsenic course, which lasted more than three years in both studies, had an impact on the patients' quality of life. This resulted in the creation of ATO+ATRA chemo-free model.

2.3.13 ATO + ATRA (2002- now)

Estey et al. were the first to investigate a chemo-free ATO +ATRA model during induction and post-remission treatment (Estey et al., 2006). This study included 82 patients, and their post-induction treatment included four courses of ATO+ATRA. The findings revealed a 92% complete remission rate, an 85% 3-year overall survival rate, and a 9% early death rate. A long-term follow-up on the same group later confirmed this result, providing the foundation for the APL0406 study.

Lo-Coco et al. conducted APL0406, a randomized trial for low-risk APL patients (newly diagnosed) using ATRA+idarubicin vs. ATRA+ATO. Platzbecker et al., 2017; F Lo-Coco et al., 2013). With a median follow-up of 34.4 months, the ATRA+ATO group achieved 100% CR and 99% 2-year OS. Another trial, NCRI AML17, showed 94% CR and 92% 5-year OS regardless of patient risk status. Burnette et al. (2015)

This study's findings supported ATRA+ATO as the standard treatment for APL patients with high or low risk. APL is distinguished by retinoic acid receptor target gene epigenetic and transcriptional repression. TGM2 and RAR are both involved in differentiation and are methylated heavily during lukemogenesis. Huynh et al. recently proposed that ATRA+ATO treatment induces TGM2 and RAR expression, followed by terminal differentiation of NB4 promyelocytes. Huynh and colleagues (2019).

2.3.14 Oral ATO+ATRA

A group from Hong Kong was the first to administer oral ATO, and they conducted a series of clinical trials. Gill et al., 2019; Gill et al., 2018; Foa et al., 2011 Grill et al. studied 73 patients with relapsed APL over a 15-year period and found that the 5-year survival rate was 79.5% and the 10-year survival rate was 67.3%. Gill and colleagues (2018) ORH-2014 is an oral arsenic that has completed phase 1 testing and found that 15mg is safe. (Ravandi and colleagues, 2020) A 10mg dose is recommended in future phase 2 and 3 trials. An oral ATO developed in Australia is also being evaluated in the ALLG phase 1 study.

2.4 Problems in existing Treatments

2.4.1 Early Death Rate

Early death is defined as death occurring during induction or within 30 days of diagnosis. Lo-Coco et al. (2013) Early death is a significant barrier to curing APL patients. According to the US SEER database, the ATRA+chemotherapy era has improved early death rates over time. Dinmohamed and colleagues (2016) It is unclear whether ATRA+ATO will further reduce the ED rate. Zhu et al. reported a 5.5% ED rate in an ATRA+ATO group study with 758 participants. Murthy and colleagues (2020). Future research is needed to determine whether the ED rates in the ATRA+ATO and ATRA+ Chemotherapy models differ.

2.4.2 ATRA+ATO Toxicity

Headache, GI toxicity, and liver damage are all common side effects. Leucocytosis (WBS count greater than 10 10'9 /L) is the fatal side effect of ATRA+ATO. In APL patients, hyperleukocytosis can be a predictor of ED and subsequent relapse, differentiation syndrome. Yoon and colleagues (2019).

2.4.3 Resistance

Treatment resistance is a significant barrier in the treatment of APL patients. Mutations in the retinoic acid receptor's ligand binding domain cause ATRA resistance. Roussel and Lanotte (2001) In vitro studies using NB4 resistant cell lines revealed that PML-RARA becomes unresponsive to retinoic acid because of these mutations, but it can bind to RARE and inhibit transcription. (Mozziconacci and colleagues, 2002) PML/RARA degradation has also been linked to retinoid acid resistance. Fanelli and colleagues (1999). ATO resistance has also been observed in vitro and in vivo, and this has been linked to clustered mutations in the B2 domain of the PML moiety, which is a direct arsenic binding site. Roussel and Lanotte (2001).

Table 2.3.9 Treatment strategies for APL and their shortcomings

Sr.No	Treatment	Shortcomings	Reference
1.	ATRA alone	Relapse within 5 months despite continuous ATRA treatment	(Sanz & Barragán, 2021)
2.	ATRA + Chemotherapy	Differentiation Syndrome	(Yilmaz et al., 2021)
3.	ATO alone	Relapse, ATO related toxicities	(Ghavamzadeh et al., 2011)
4.	ATRA+ ATO	Early Death rate, Overloaded fluid even in the absence of Differentiation Syndrome	(Yilmaz, Naqvi, & Ravandi, 2019)
5.	Tamibarotene	Disease free survival is still short	(Sanford et al., 2015)

2.5 CHRONIC MYELOID LEUKEMIA

2.5.1 Introduction

Dr. Rudolf Virchow, and Dr. John Hughes Bennett in 1845 were the two pathologists, described CML for first time (Bennett JH, 1845: Virchow R et al, 1845). Although there was some debate about who described leukemia first, Virchow acknowledged in a public seminar that Bennett "observed a case of undeniable leukemia some months before I saw my first case." It is worth noting that the first reports of CML were written before blood staining methods were developed in the late 1800s. CML is now classified as a clonal hematopoietic stem cell disorder, with a yearly occurrence of one to two cases per 100,000 people. The chronic, or stable, phase of CML is distinguished by an overabundance of differentiated and functional myeloid cells. 90% to 95% of patients will be diagnosed at this stage. Historically, the disease progressed in 4 to 6 years on average from an "accelerated phase" to an invariably fatal acute leukemia, also known as blast crisis. The accumulation of molecular abnormalities most likely causes disease progression, resulting in a progressive loss of the leukemic clone's capacity for terminal differentiation (Calabretta B, 2004). With current therapies, however, median survival may be extended to 30 years, and patients may never transform and die of their leukemia. CML therapy has historically been empirically based. In the late 1800s, the Fowler solution, developed in mid-1700s by Dr. Thomas Fowler, was the mainstay of CML therapy. The ingredient most active in Fowler solution was almost certainly potassium arsenite, and there has recently been renewed interest in using arsenic preparations to treat CML (Geary CG et al, 2000; Calabretta B, 2004; . Ito K, Bernardi R, Morotti A, et al, 2008). During the 1900s, radiation, busulfan, hydroxyurea, interferon- (IFN-), and stem cell transplantation were developed for other indications, extensively tested, and discovered to be effective in CML (Geary CG et al, 2000). Allogeneic stem cell transplantation is the only proven curative therapy, but it comes with significant mortality and morbidity.

2.5.2 Molecular pathogenesis of CML

The transition from empiric to rationally designed therapy necessitates an accurate understanding of molecular pathogenesis of disease and unravelling the CML molecular pathogenesis began shortly after formation of ASH. In 1960, Peter Nowell and David Hungerford described a chromosomal abnormality in CML patients, an acrocentric chromosome that was thought to be a chromosomal deletion, while working in Philadelphia (Nowell PC et al, 1960). This was the first time a chromosomal abnormality was linked to a specific disease, cancer. The authors stated

in their seminal paper that "the findings suggest a causal relationship between the chromosomal abnormality observed and chronic granulocytic leukemia" (Nowell PC et al, 1960). This foresight was met with doubt because the chromosome abnormality was thought to be an associated rather than a causative process. The chromosome abnormality was discovered to be a shortened chromosome 22 as chromosomal banding techniques advanced. In 1973, Dr. Janet Rowley discovered that the shortened chromosome 22, known as the Philadelphia (Ph) chromosome, resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9:22) (q34;q11)9 . (Figure 2.4)

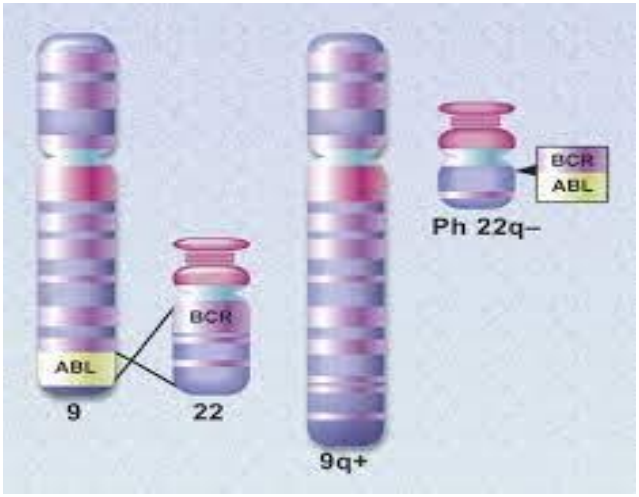


Figure 2.4 Schematic diagram of the translocation that creates the Philadelphia chromosome. The ABL and BCR genes reside on the long arms of chromosomes 9 and 22, respectively. As a result of the (9;22) translocation, a BCR-ABL gene is formed on the derivative chromosome 22 (Philadelphia chromosome). Illustration by A. Y. Chen.

Investigation of transforming was carried out in the 1970s and 1980s. Retroviruses have resulted in significant advances in the comprehension of Cancers in humans. Investigations showed that mutations in normal cellular genes are carcinogenic. As an example, in 1970 the The Abelson murine leukemia virus was explained (Abelson HT, 1970) that caused the discovery of its transforming gene, v-ABL and Cloning of natural cellular homolog, c-ABL (Goff SP et al, 1980). Then was found that c-ABL, which normally resides on chromosome 9, had been translocated to chromosome 22 in patients of CML.14 Because on chromosome 22 the breakpoints clustered in a relatively small region spanning 5.3 kilobases, it was than named the BCR (breakpoint cluster region) (Deininger MW et al, 2000; Groffen J, 1984).

Researchers were able to pinpoint the precise molecular consequences of the (9;22) chromosomal translocation thanks to the combination of chromosomal abnormalities and transforming retroviruses (Figure 2.4). Northern blots with ABL probes revealed that ABL mRNA was larger than normal in CML patients (Collins SJ et al,1984; Gale RP et al,1984) It was later discovered that this was a chimeric mRNA composed of BCR and ABL sequences (Shtivelman E et al, 1985). Similarly, a larger-than-normal ABL protein with tyrosine kinase activity was discovered in CML cells and identified as the product of the BCR-ABL mRNA. 20-22 Because v-ABL possessed a novel kinase activity, the ability to phosphorylate tyrosine residues, these findings provided an important relation between the fields of oncogenes and protein kinase biochemistry (Witte ON et al, 1980; Lugo TG et al,1990).

Because it was previously known that v-ABL possessed a novel kinase activity, the ability to phosphorylate tyrosine residues, these findings provided an important link between the fields of oncogenes and protein kinase biochemistry. BCR-ABL have higher tyrosine kinase activity than c-ABL, which is essential for BCR-ABL to transform cells. In 1990, John Groffen's laboratory, as well as George Daley and Richard van Etten in David Baltimore's laboratory, confirmed the BCR-leukemogenicity ABLs. They expressed BCR-ABL in animal models and presented that BCR-ABL alone caused leukemia, (Daley GQ et al, 1990; Heisterkamp N et al,1990) establishing BCR-ABL as a leukemic oncogene.

Following the identification of BCR-ABL as the molecular pathogenetic event in CML, considerable effort was expended in understanding the BCR-ABL’s molecular mechanism action via the identification of signaling pathways impacted by BCR-ABL tyrosine kinase activity. Many BCR-ABL binding partners and substrates have been discovered, and current research is focusing on linking these to the specific defects that characterize CML (Melo JV et al, 2004). These defects include increased or decreased proliferation and apoptosis respectively, of a hematopoietic stem cell or progenitor cell, resulting in a huge increase in myeloid cell numbers; premature induction of immature myeloid cells into the circulation, thought to be due to a defect in myeloid progenitor attachment to stroma of marrow; and genetic instability, which leads to progress of disease. The RAS pathway activation is one example of a cellular pathway connected to high proliferative rate.

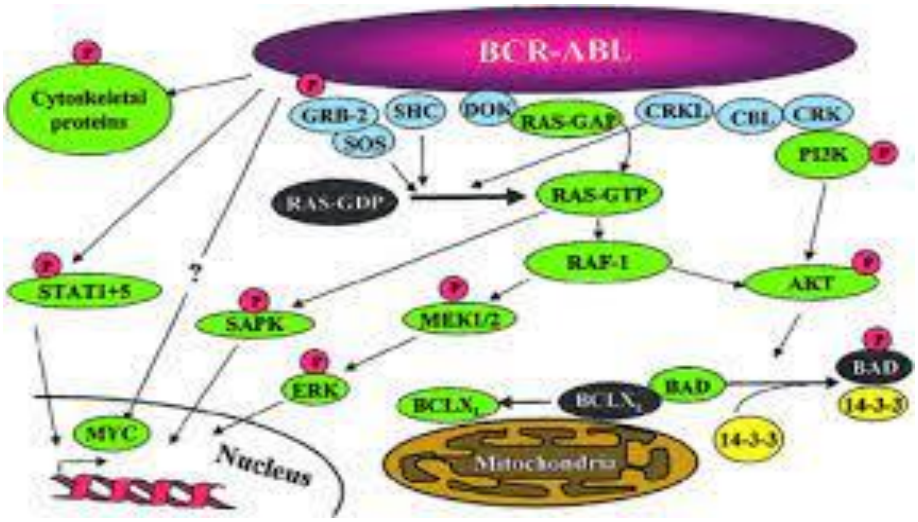


Figure 2.4.1 Signaling pathways impacted by BCR-ABL expression. Note that this is a simplified diagram and that many more associations between BCR-ABL and signaling proteins have been reported. Reprinted from Deininger et al.15

STAT5-mediated upregulation of the antiapoptotic molecule BCLXL and AKT-mediated phosphorylation and inactivation of the proapoptotic molecule BAD may both contribute to protection against programmed cell death (Melo JV et al, 2004). CML cells have lower fibronectin adhesion, which could be due to CRKL phosphorylation. Apart of infinite number of pathways activated by BCR-ABL and the higher complexity revealed in these pathways, all functions of BCR-transforming ABL are dependent on its TK activity (Lugo TG et al,1990).

Thus, BCR-ABL was identified as an ideal therapeutic target in CML between 1960 and 1990. It is found in all CML patients and has been proven to be the cause of the disease. Mutagenic analysis has revealed that BCR-ABL functions as an activated tyrosine kinase, and that this activity is essential for protein's function of transforming. For these reasons, a BCR-ABL kinase inhibitor is thought to be a powerful and selective CML treatment.

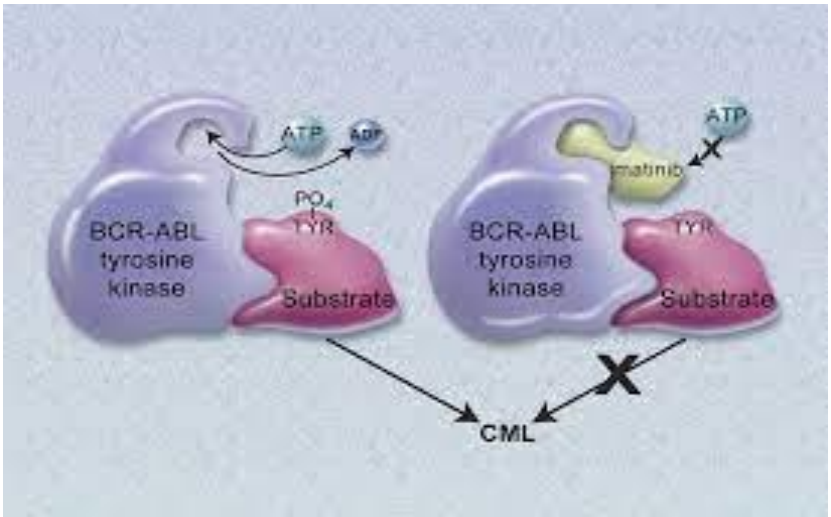


Figure 2.4.2 Mechanism of action of imatinib. (A) The constitutively active BCR-ABL tyrosine kinase functions by transferring phosphate from ATP to tyrosine residues on various substrates to cause excess proliferation of myeloid cells characteristic of CML. (B) Imatinib blocks the binding of ATP to the BCR-ABL tyrosine kinase, thus inhibiting kinase activity. Illustration by A. Y. Chen.

2.5.3 Tyrosine Kinase Inhibitors (TKIs)

Following the discovery of the Philadelphia (Ph) chromosome in 1960, CML was the first human malignancy to be associated with a consistent chromosomal abnormality (Jabbour, E et al, 2016). A reciprocal translocation between the chromosomes 9 and 22 results in a smaller chromosome 22, known as chromosome Philadelphia, found in 96% of CML patients (Heisterkamp N et al, 1983). This genetic material transfer results in the fusion gene of BCR-ABL1. The ABL1 non-receptor tyrosine kinase gene on chromosome 9 is fused with the BCR gene on chromosome 22, which is an activator of RhoGEF and GTPase (Kang, Z.-J et al, 2016). This oncogene encodes the BCR-ABL1 oncoprotein, that has constitutive tyrosine kinase activity and is required for transformation of HSC and initiation of leukemia (Laurent, E et al, 2001). The small molecule discovery that inhibited BCR-ABL1 activity dramatically altered the course of the disease, and with time CML became a chronic disease (Reff, M.J et al,2019; Savage, D.G et al, 2002). This TKI inhibits the interchange of BCR-ABL1 and ATP, preventing cell signals, decreasing cell proliferation, and causing cell death in clones of CML.. Imatinib, dubbed "magic bullet" in 2001 by Time magazine, was the foremost TKI approved by FDA and European Medicines Agency (EMA) for the treatment of resistant CML patients (Manley, P.W et al, 2002) and after two years, in newly diagnosed patients (Soverini S. et al,2018).

The currently allowed TKIs are five for the treatment of CML. Imatinib, Dasatinib, Nilotinib, Bosutinib, and Ponatinib are the TKIs. Imatinib, dasatinib, nilotinib, and bosutinib are allowed by FDA and EMA for first-line treatments of CML (Jabbour, E et al, 2020). Past the last two decades, the development of above drugs to treat CML has been quite achieving in a never-ending battle against resistance. A second-generation TKI, Radotinib is currently only approved by Korea, and Flumatinib was allowed for CML treatment in China at the end of 2019. Nonetheless, despite significant advances in CML treatment in past few years with the invention of TKIs, some patients (20-30%) develop acquired resistance to treatment during CML (Jabbour E. et al, 2016; Kantarjian et al, 2012).

TKI treatment effectiveness is dependent on proper BCR-ABL1-drug interaction (Apperley, J.F et al, 2007) and the mechanisms responsible for BCR-ABL1 kinase reactivation are the most studied (Zhao, H et al, 2020). Overexpression of BCR-ABL1 and fusion gene mutations impairs TKI binding to targeted kinase that can cause resistance and are labelled as BCR-ABL1 dependent mechanisms (Patel, A.B et al, 2017). Most common mechanism of TKI resistance is the incident of point mutations on the ABL1 KD, which is more usual in acquired resistance cases than primary resistance cases and is linked with a poor prognosis (Soverini S. et al,2014; Kaehler, M et al, 2021; Deininger et al, 2020). Other generation TKIs capable of overcoming and inhibiting mutated BCR-ABL1 have been developed because of this resistant mechanism.. The approval of second-generation TKIs as CML treatment, fewer mutations are expected to emerge than with Imatinib because these TKIs can work even when some mutations are present (Hochhaus, A et al, 2011). Those patients who fail Imatinib or second-generation chemotherapy, gets BCR-ABL1 mutational analysis.

A similar comparison of studies showing resistant Imatinib CML patients, found that patients resistant to therapy had higher levels of expression of DNA damage repair genes such as RAD51L1, FANCA, and ERCC5 (De Lavallade et al, 2010; Villuendas R. et al, 2006). These findings lend credence to the idea that DNA damage repair impairment of CML is a direct cause of TKI resistance and CML evolution. Discovery of simultaneous decrease in OCT1 and OCTN2 expression in Imatinib-resistant cell lines (Alves et al. 2019), indicated that more than one influx transporter contributes to resistance (Alves, R et al, 2015). Switching to Dasatinib, which crosses the cell membrane via diffusion, may help in taking few TKI (Hiwase D.K. et al, 2008; Giannoudis, A et al, 2008). Furthermore, survivin levels are higher in TKI-resistant CML patients and cell lines resistant to Imatinib than in sensitive ones (Carter, B.Z et al, 2006). For apoptosis protein (IAP), Survivin act as inhibitor, that regulates the apoptosis and cell cycle, promoting

cancer cell survival by restricting cell death and causing cell division (Bernardo P.S et al, 2020). For BCR-ABL1 KD mutation screening, the gold standard is Sanger sequencing, but its sensitivity is low (10-20%). The reason is that for first, second, and even third generation TKIs the compound mutations are extremely resistant to in some cases (Zabriskie M.S.et al, 2014; Mughal, T.I et al, 2021; Cumbo C. et al, 2020), BCR-ABL1 clonal configuration is critical.

For CML treatment the TKIs approved are orally administered and they compete for binding at the ATP-binding site, to the BCR-ABL1 TK. The second-generation TKIs are Dasatinib, Nilotinib, Bosutinib, and Radotinib, while the third generation TKI is Ponatinib. In the event of resistance, according to ELN guidelines, the BCR-ABL1 KD mutation profile should be investigated to plan the second line of treatment selection. Each TKI has a different profile of sensitivity to the various mutations that have been identified, with TKI (Imatinib) firstly designed have less potency in resistance events (Meenakshi Sundaram et al, 2020; Redaelli et al, 2012; Soverini et al, 2011). The next-generation TKIs approved were created with the goal of reversing the resistance and intolerance seen in Imatinib-treated patients, particularly for TKI efficacy the point mutations were identified as risky. Nilotinib, on the other hand, is a result of Imatinib chemical modification and has approximately 30 times the potency of first-generation TKI in vitro. Just like Imatinib, Nilotinib, attaches to inactive BCR-ABL1 and it also earmarks PDGFR and c-KIT (Sacha et al, 2018).

This therapy protocol does not provide cure even in the clinical trials of second and third generation TKI, despite the significant success of BCR-ABL1 inhibitors in CML. Imatinib being the first TKI to be developed for CML treatment have failed to provide cure for CML. The above-mentioned shortcomings and drawbacks of imatinib makes it less efficient for CML treatment as compared to second and third generation of TKIs. In terms of potency, imatinib is known to have lesser potency than other generations of TKIs as well as several mutations in BCR-ABL1 molecule of CML patients shows resistance to Imatinib TKI.

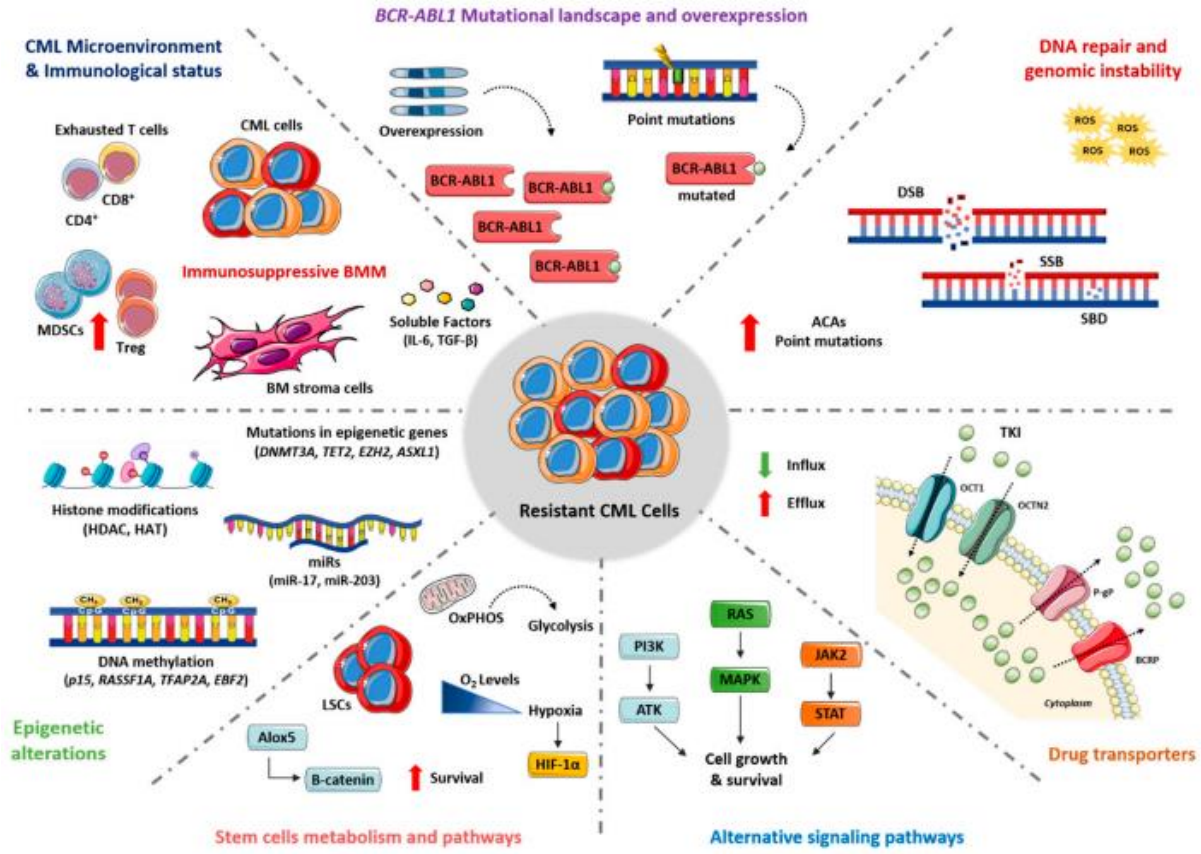


Figure 1. Molecular mechanisms of resistance to TKIs in CML. The molecular mechanisms responsible for TKI resistance in CML include: BCR-ABL1 mutations and BCR-ABL1 overexpression; alteration of DNA damage repair and genomic instability (increasing the additional chromosome abnormalities (ACAs) and point mutations); changes in drug transporters activity (e.g., increased efflux and decreased influx); activation of alternative signaling pathways (e.g., PI3K/AKT, JAK/STAT, and RAS/MAPK); changes in leukemia stem-cell metabolism and pathways (e.g., metabolic shift, Hypoxia/HIF-1α, and Alox5/β-catenin); epigenetic alterations (e.g., mutations on epigenetic regulating genes such as DNMT3A and/or increased methylation of p15 and EBF2 genes); altered expression of microRNAs (e.g., miR-17 and miR-203); changes in the microenvironment and immunological status (e.g., immunosuppressive bone marrow microenvironment (BMM) with increased MDSCs and Treg, plus exhausted T cells).

2.5.4 Antibiotics and Cancers

2.5.4.1 Salinomycin

In the early 1970s, Miyazaki and colleagues from Kaken Chemicals Co., Ltd.'s research division in Tokyo, Japan from the strain no. 80614 of Streptomyces albus culture broth (Y. Miyazaki et al, 1974) isolated salinomycin. It was isolated in the form sodium salt prism which was colorless using this method (Y. Miyazaki et al, 1974). Salinomycin was later shown against Gram-positive bacteria to have antimicrobial activity like Bacillus subtilis, Staphylococcus aureus, Micrococcus flavus and Sarcina lutea (Y. Miyazaki et al, 1974; H. D. Danforth et al,1977; H. Mehlhorn et al, 1984). Salinomycin was a new member of the monocarboxylic polyether antibiotic family, X-ray crystallographic analysis revealed that (H. Kinashi et al, 1973). (Figure 2.4.4.1)

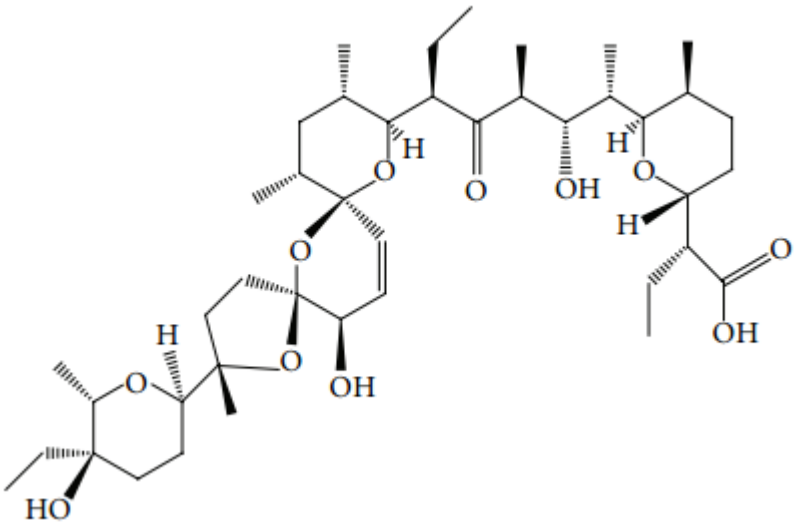


Figure 2.4.4.1 Structural formula of salinomycin. The pentacyclic molecule with a unique tricyclic spiroketal ring system has a mass of 751 Da, a molecular formula of C42H70O11, a melting point of 113◦C, and a UV absorption at 285 nm. Adapted from [102, 103], with permission from Elsevier B.V

Furthermore, salinomycin has been shown to function as a monovalent cation ionophore in various biological membranes, including membranes cytoplasm and mitochondria, with strong preference for K+ (M. Mitani et al, 1975), inhibiting oxidative phosphorylation and causing K+ efflux in mitochondria and cytoplasm (M. Mitani et al, 1976; B. C. Pressman et al, 1978). Salinomycin, however, has never been approved as a treatment for human diseases for a variety of reasons. Many reports and studies published over the last three decades have proved that salinomycin is toxic to mammals after oral or inhalative ingestion accidentally (J. Rollinson et al, 1987; M. Aleman et al, 2007).

It was revealed that human breast CSCs in mice are selectively eliminated by salinomycin, in Gupta and colleagues' seminal 2009 study (P. B. Gupta et al, 2008). A subsequent study found that major apoptosis in human cancer cells is caused by salinomycin (D. Fuchs et al, 2009). Because of these findings, for the first time in a clinical trial with a small group of patients with metastatic breast, head, and neck cancers in 2010, salinomycin was used (R. Steinhart et al, 2018). Every second day for three weeks, salinomycin intravenous administration of 200-250 gkg1 resulted in partial regression of tumor metastasis with only minor side effects, compared to

chemotherapeutic drugs that causes severe acute and long-term side effects (R. Steinhart et al, 2018).

Salinomycin selectively kills human breast CSCs, according to Gupta and colleagues (P. B. Gupta et al, 2009). In a sophisticated experimental approach, the authors used immortalised human mammary epithelial cells (called HMLER) to generate cells undergoing epithelial-mesenchymal transition (EMT), that can induce cancer cells with self-renewal and drug resistance capabilities, known as a latent embryonic programme (B. Elenbaas et al, 2001; K. Polyak et al, 2006). These HMLER-shEcad exhibited CSC-like characteristics such as the ability to form tumorspheres in suspension cultures (M. F. Clarke et al, 2006), high and low expression of CD44 and CD24, respectively, and resistance to chemotherapeutic and cytotoxic agents (P. B. Gupta et al, 2009).

It was also shown that, unlike Paclitaxel, an anti-breast cancer drug, CD44high/CD24low CSCs proportions were also reduced by salinomycin in cultures of HMLER-shEcad and control cells that have not went through EMT. Furthermore, salinomycin pretreatment of HMLER-shEcad cells inhibited tumorsphere formation, which was not observed after paclitaxel pretreatment (P. B. Gupta et al, 2009). These findings unarguably reveal that breast CSCs and AML SCs can be targeted by salinomycin, and several recent studies show that salinomycin has similar effects in other CSCs. Salinomycin also causes apoptosis and death of CSCs as well as causes interference with ABC transporters.

Previous research looked at the effect of salinomycin on the viability of UM cells. Salinomycin effectively reduced cell viability in 92.1, Mel270, Omm1, and Omm2.3 cells, respectively. Salinomycin, on the other hand, showed less cytotoxicity in normal retinal pigmented epithelium cells (ARPE-19). Meanwhile, salinomycin inhibited clonogenicity in UM cells in agarose-containing culture. These results show that inhibition of the growth of UM cells in vitro and cytotoxicity of UM cells is caused by salinomycin effectively.

The major cause of death in UM patients is Hepatic metastasis, which is a significant malignant feature (M. F. Clarke et al, 2006). In previous research, the migration and invasion of UM cells through salinomycin was assessed in vitro. With salinomycin treatment, Wound healing scratch test showed a significant reduction in cell migration. Comparable to transwell assay, lesser number of UM cells migrated into the bottom

chamber in contrast with the controlled one. Moreover, using the transwell chamber assay the invasiveness of UM cells was highly declined in the salinomycin-treated group. All these findings show that on migration and invasion, salinomycin has an observable suppressive effect.

Being a selective inhibitor of CSCs in multiple cancers, salinomycin (Gupta PB et al, 2009), it was observed that if it could remove CSCs in UM. Using the formation of melanosphere, self-renewal capability and serial plating assays were investigated. The results revealed the melanospheres size and number as well as the capacity for re-plating were reduced in salinomycin treated UM cells. Furthermore, Aldefluor positive cells in Mel270 percentage was reduced through salinomycin treatment. These results show that salinomycin is also effective against cancer stem cells in UM cells.

The scientists identified stemness-related transcriptional factors after elucidating the underlying mechanism by which salinomycin effectively suppresses CSC phenotypes. Twist1 and SOX2 levels were found in salinomycin-treated Mel270 cells to be lower. Further investigation revealed the metastasis-free survival and overall survival in patients with UM correlated with levels of TWIST1 rather than SOX2, using two publicly available sets of GSE22138 and the TCGA database. Furthermore, salinomycin-treated mice tumor xenografts had reduced Twist1 proteins through IHC staining and Western blot assays. Furthermore, qRT-PCR revealed that salinomycin reduced TWIST1 transcription in 92.1 cells. These findings suggest that aggressive characteristics on UM cells can be conferred through increased TWIST1 expression. To assess the toxicity of salinomycin on CSCs, previous researchers focused on TWIST1 in subsequent experiments. Salinomycin also induces apoptosis in UM cells as well as it prevents the hepatic metastasis of UM cells.

2.5.4.2 Gramicidin A: A Channel Forming Ionophore

Ionophores are drugs that have this effect by disrupting transmembrane ion concentration gradients. Many ionophores are naturally produced by microorganisms and these drugs have broad-spectrum antibiotic properties against Gram-positive bacteria, fungi, parasites, and viruses to defend themselves against competing microbes, (Kevin II DA, 2009). In veterinary medicine and as growth-promoting drugs in agriculture Ionophores have traditionally been used as antibiotics (Kart A. et al, 2008), but new research has revealed that they have exceptional anticancer properties. Most of this investigation has focused on the mobile carriers monensin and salinomycin as

compared to channel formers' potential anticancer properties have received little attention..

Gramicidin A is the most basic and well-studied ionophore. It was the first antibiotic to be isolated and used in a clinical setting, and its early success paved the way for the clinical development (Kelkar DA, 2007). GA is a short linear peptide and is highly hydrophobic, and within biological membranes a unique helix nanopore is formed that spans the membrane, when two GA monomers dimerize end-to-end (Wang F. et al, 2017) In biological systems, through the channel formed by GA dimers, water and inorganic monovalent cations can freely diffuse, causing Na+ influx/K+ efflux, membrane depolarization, osmotic swelling, and cell lysis (Wang F. et al, 2017; Otten-Kuipers MA. Et al, 1996).

A broad-spectrum antibiotic activity is associated with GA (Dubos RJ, 1939; Moll GN et al, 1991), and it can now be confirmed that unlike other mobile carrier ionophores, it also has compelling anticancer properties that are both like and distinct. Derived from the microbe Bacillus brevis, Gramicidin A is an ionophore antibiotic like Salinomycin and is a hydrophobic linear Penta decapeptide. In human breast adenocarcinoma cells, GrA combined with curcumin was recently shown to induce cell apoptosis and overcome multidrug resistance (Verdoodt B, 2012). In renal cell carcinoma, GrA may have cytotoxic and antiangiogenic properties in vitro and in vivo (David JM. Et al, 2013; David JM. et al, 2014). Moreover, in a breast cancer cell line GrA-inspired peptides for cancer nanotherapeutics were discovered to induce apoptosis (Chakraborty K. et al, 2018)

In previous research studies (David et al., 2015), GA is proven to be cytotoxic for RCC as well as it inhibits angiogenesis and HIF in RCC. In preliminary study (David JM. Et al, 2013), the GA resulted to be cytotoxic against human cancer cell lines derived from renal cell carcinoma (RCC). Resistant to both chemotherapy and radiation, Renal cell carcinoma is a rare but deadly disease that is histologically diverse. With only 10% of survival chances, Invasive RCC is a 5-year disease-specific cancer (Baldewijns MM. et al, 2008; Patard JJ, 2005). The six RCC cell lines tested discovered that GA treatment reduced the viability. This finding suggests that GA may be effective in various RCC subtypes, but still there were no established treatments for the rarer RCC subtypes. In previous research, Gramicidin A was compared to the ionophore monensin, tested in different cell lines, and it was discovered that GA reduced cell viability as much as

monensin. However, monensin induces apoptosis cell lines, while GA induced cell death via a necrotic mechanis (ATP depletion by blocking of the oxidative phosphorylation and glycolytic metabolic pathways). GA was also discovered, in vivo, to cause inhibition of tumor growth.

GrA is known to have inhibited growth of pancreatic cancer cells (Wang et al., 2019). GrA is also known to have inhibited cell proliferation and apoptosis as well as have cause structural changes on cell surface of cancer (Wang et al., 2019). GrA and Sal were tested for cytotoxicity using a proliferation assay. BxPC-3 and MIA PaCa-2 inhibition was caused by GrA. GrA was extremely effective in causing the proliferation of pancreatic cancer cell lines. SEM clearly revealed pancreatic cancer cells morphological changes on the cell surface. In BxPC-3 and MIA PaCa-2 cells, over the cell membrane there were numerous microvilli-like protrusions surface. Slender and bent microvilli-like protrusions were appeared. These microvilli-like protrusions were reduced through GrA treatment in pancreatic cell lines. In contrast, with salinomycin no discernible differences were observed in two cell lines. Morphological changes were revealed in SEM clearly on the pancreatic cancer cells surface. Almost all their microvilli-like protrusions were lost through treatment with Gramicidin A. As compared to salinomycin the protrusions remained unchanged. In pancreatic cancer cell membranes, Gramicidin A induced exceptional ultrastructural changes.

GrA shows cytotoxicity in human gastric cancer cells (Chen at el., 2019). Figure 2.4.42 depicts the chemical structure of gramicidin. The human gastric cancer SGC-7901 and BGC-823 cells, were treated with Gramcidin A for 24 hours to observe any cytotoxic effect. This treatment notably reduced the percentage of living cells, and gramicidin A caused the proliferation of two different gastric cancer cells. Furthermore, from results it was observed that SGC-7901 cells were more subtle to Gramicidin A treatment.

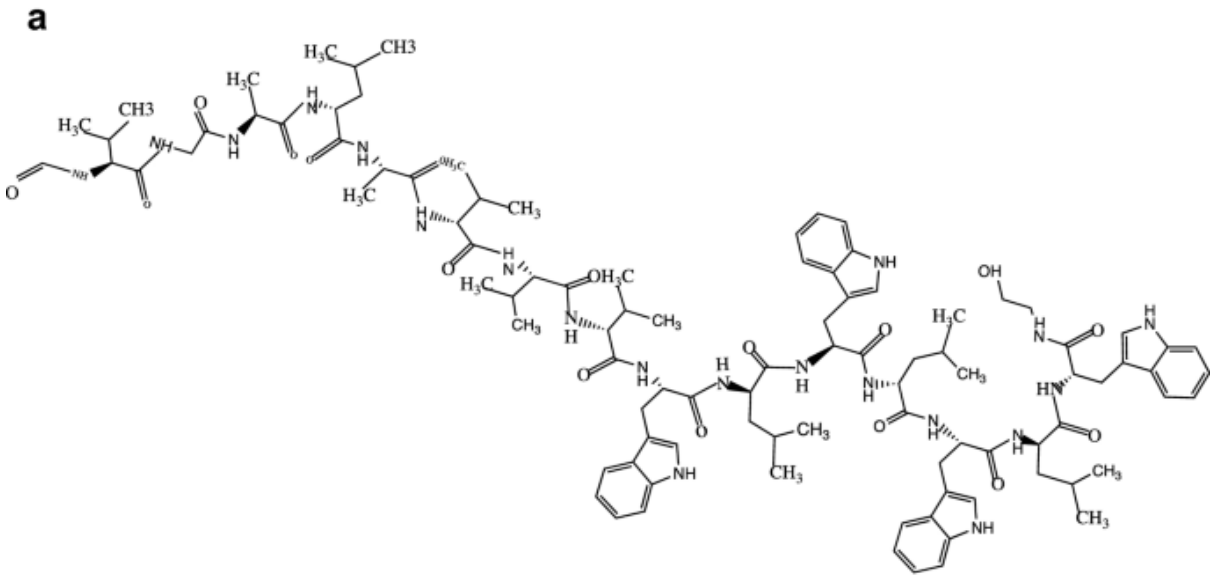


Fig.2.4.4.2 The chemical structure of gramicidin .

RBE and HuCCT1, are cholangiocarcinoma cells in which cell proliferation was investigated by Gramicidin A. Scientists discovered that these cell lines were sensitive to Gramicidin A treatment, with IC50 values as low as 50 Nm. Furthermore, floating cellular debris showed the necrotic cell death in both types of cholangiocarcinoma cell lines. To investigate the cytotoxicity of gramicidin further, Calcein AM (green, live cells) were used and ethidium homodimer-1 (red, necrotic dead cells) staining to detect necrotic cell death. With the treatment of Gramicidin, both the cell lines exhibited necrotic dead cells.

RNA sequencing reveals that GrA downregulates EGR4 in cholangiocarcinoma cell growth. GrA also reduces cell proliferation in cholangiocarcinoma cell lines (Gong et al., 2019). In RBE cells treated with gramicidin, a total of 265 DEGs were identified with PBS and gramicidin treatment. Between PBS and gramicidin treatment, many cholesterol biosynthesis pathways were enriched, indicating that the lipid metabolism pathway may play a key role in cholangiocarcinoma cell growth. Following this, scientists discovered one of the most down-regulated genes in DEGs to be the early growth response protein 4 (EGR4). In previous research, qPCR was used to verify the RNA-seq data and discovered that EGR4 mRNA was significantly down-regulated in both cholangiocarcinoma cells. As a result, after gramicidin treatment, the protein level of EGR4 was notably lower in both cells. It appears cholangiocarcinoma cell growth is inhabited by Gramicidin through downregulating EGR4.

According to the above literature on Gramicidin A and salinomycin, GrA has been extensively researched in solid cancers like breast cancer, human gastric cancer and so

on. Its effect on liquid cancers such as AML and CML has yet to be investigated. Salinomycin has been studied for its effect on both solid and liquid cancers. Salinomycin has recently been shown to target CSCs in different types of human cancers, including gastric cancer (Q. M. Zhi et al, 2011), lung adenocarcinoma (Y. Wang, 2011), osteosarcoma (Q. L. Tang et al, 2011), colorectal cancer (T. T. Dong et al, 2011), squamous cell carcinoma (SCC) (Y. Wang, 2011), and prostate CSCs (K. Ketola. 2012), suggesting that salinomycin may be effective in CSCs of many, if not all, types of human cancers. In this research study, the effect of Gramicidin A will be studied on AML and CML (Liquid Cancers).

3) MATERIALS AND METHOD

3.1 In silico study

3.1.1 Target genes selection

The National Centre for Biotechnology Information (NCBI), Cancer Cell Line Encyclopedia (CCLE), and Gene Cards databases were used to choose the targeted genes.

3.1.2 Therapeutic Target Library

The targeted genes' FASTA sequences were retrieved from Uniprot, and the Position-Specific Iterative Basic Local Alignment Tool (PSI-BLAST) was used to compare the sequences to the protein structures. Proteins that shared a high percentage of identity were chosen for additional research.

3.1.3 Protein 3D Crystallographic Structure

The accession number for 3D protein structures was collected from PSI-BLAST, and the structures were produced using the protein databank (pdb). The PDB format of the protein structures was downloaded

3.1.4 Purification of Protein Structure

UCSF Chimera 1.14 was used for the purification of proteins and this purification involved the removal of water molecules anvd other nonstandard amino acids.

3.1.5 Binding Pockets Identification

Finding the active drug binding sites in the target protein structures was done using DoGSiteScorer, an online automated pocket recognition and analysis programme that predicts binding sites based on hydrophobic interactions. Pockets with binding scores close to 1 were chosen.

3.1.6 Ligand preparation

Pubchem was the programme used to retrieve the ligand structure in SDF format. Open Babel, another piece of software, was used to convert this format to Mol2 format.

3.1.7 Molecular docking

The preferred interaction of a ligand with its protein receptor was predicted using molecular docking using the molecular modelling simulation software Autodock 4.2. By incorporating hydrogen atoms, calculating charges, and removing water molecules, the

ligand and macromolecule were created. The Auto grid was configured using binding pocket scores that had previously been computed. Lamarckian Genetic Algorithm was chosen with default parameters to dock ligand with protein

3.1.8 Analysis and Visualization of Binding Pose

Cygwin instructions were used to convert the docked file into PDB format for analysis and visualization. By choosing the protein ligand complexes with the lowest binding energies, the predicted binding energies of these poses were further examined.

3.1.9 Protein-Ligand Interaction Profiler

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

3.2.0 Ligand used in study

Gramicidin A, a ligand with the chemical formula C99H140N20O17 and a molecular weight of 1882.29 g/mol, was employed in this investigation. This ligand's solvent was DMSO.

3.2 In vitro study

3.2.1 Cell lines in the study

In this investigation, the cell lines used for AML and CML were NB4 and K562, respectively.

3.2.2 Culturing of cells

To prevent any contamination of the cells, both cell lines were maintained in sterile conditions using RPMI-1640 (Gibco Life Technologies) mix supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 1% Penstrip (Biowest). For the best growth of these cells, a water jacketed humidified incubator was used for maintenance at 5% CO2 and 37°C. 1106 cells/ml were kept in culture to prevent any type of contact inhibition.

3.2.3 Freezing of cells

Two freezing solutions were created to freeze or cryopreserve these cells. As opposed to solution II, which contained 80% RPMI 1640 and 20% DMSO, solution I had 70% RPMI 1640 and 30% FBS.

Separate eppendorf tubes containing cells were used for centrifuging them at 1400–1600 rpm. The acquired palette was then resuspended in 2 ml of solution I after being cleaned with PBS. Each cryovial received 1ml of resuspension following correct pipetting upside-down. It was dropped into the cryovial in the same manner as Solution II, which included DMSO. Since these cryovials contain isopropanol, which reduces the temperature by 1°C per minute, they were then placed in Mr. Frosty for an overnight period at -80. The cryovials were then placed in a cryobox the following day and kept at -80 to ensure longer-term preservation.

3.2.4 Thawing of cells

Cryovials from a -80 liquid nitrogen freezer were collected, and they were quickly thawed in an incubator for a period of 3 to 5 minutes. To prevent contamination, these cryovials were sprayed with ethanol before being placed inside a laminar flow hood. Transferred to epidendorffs, the cryovials suspension was centrifuged at 1600 rpm. The pallet was cleaned with PBS after the supernatant was discarded. After that, the pallet was reconstituted in recently prepared 10% Fbs media. After that, the cells were grown in a 6 well plate in a C02 incubator.

3.2.5 Trypan blue exclusion assay

Before plating the MTT assay each time, a trypan blue exclusion assay was conducted to count the live cells to plate/seed a consistent number of cells per well and obtain reliable data. The 6-well culture plate's cells from every well were removed, collected in a centrifuge tube, pelleted, and then resuspended in 1 ml of 10% culture medium. Reconstituted cells were vortexed before being put in 10ul portions onto a paraffin strip and then diluted with 10ul of trypan blue dye. With pipetting up and down, homogenize it. Finally, a haemocytometer was filled with 10ul of dilution. Due to a damaged plasma membrane, non-viable cells absorb the dye and look blue, whereas viable cells exclude the dye and appear white. The precise cell count per ml was determined by carefully counting all the viable cells in four grids of the chamber using the formula below:

$$\frac{\text{No of cells in grid 1+ grid 2+ grid 3+ grid 4}}{} \text{ X dilution factor X 10,000/ml}$$

3.2.6 Drug Dilutions

The compound Gramicidin A was first dissolved in its solvent DMSO to obtain 2mM of initial stock solution. From the stock solution, different drug concentrations were prepared in 10% fbs media. The dilution factor at each further step was constant, which resulted in a geometric progression of the concentration in a logarithmic fashion. The 2mM stock solution was serially diluted according to following method:

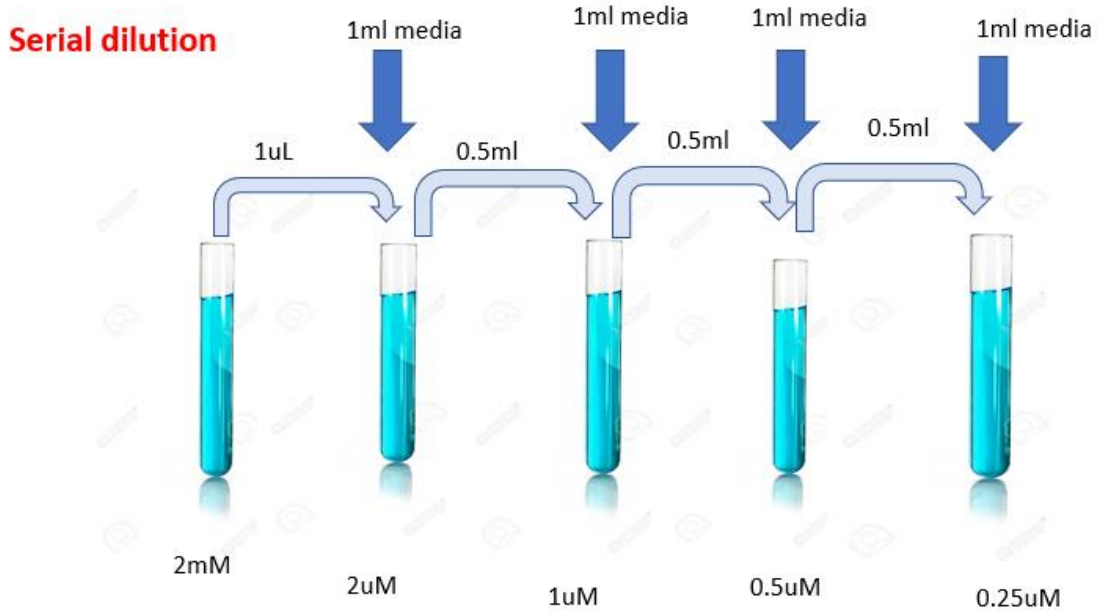


Fig 3.2.6 Serial Dilution of 2mM drug in 10% FBS media

3.2.7 MTT Assay

In a 96-well plate, 1 104 cells per 50 ul were plated. In duplicate wells, 50ul of the inhibitor gramicidin A at various doses (10uM, 5uM, 2.5uM, 1.25uM, and 0.625uM) were added. 50ul of cells were treated with 50ul with 0.01% DMS0 in the triplicate -ve control. Additionally, a duplicate plating of 100ul culture material (used to create drug dilutions) was performed. This was done to reduce, if any, the impact of the medication solvent and medium. The plate was then left in an incubator for 72 hours (the time it takes for the NB4 and K562 cell lines to double in size) at 37°C and 5% CO2. In each well of a 96-well plate containing cells and drug concentrations, 15ul of MTT dye (5mg/ml in PBS) was added following the completion of the doubling time. This was done to evaluate the vitality of the cells following drug treatment at various doses. The 96-well plate was placed back into the incubator for 3–4 hours to facilitate crystal formation after the MTT dye addition. This is a colorimetric assay, and the live cells turn the tetrazolium dye into purple-colored, insoluble formazan crystals based on their metabolic activity. The plate was removed from the incubator after three hours, and the 100ul medium in the wells was removed without damaging the crystals. To dissolve the formazan crystals, 100ul of DMSO was then added to the wells. A spectrophotometric

plate reader was used to determine the absorbance at 550 nm after the crystals had completely dissolved.

3.3 Mechanistic Studies

3.3.1 Treatment of cells with gramicidin A

NB4 cells and K562 cells were grown in a 6-well plate at a density of 1.0 106 cells/ml and subjected to a 72-hour inhibitor treatment at a dose of 0.25 uM for Nb4 cells and 0.5 uM for K562 cells, respectively. Following treatment, RNA was extracted from the treated cells, and cDNA was then synthesized for gene expression experiments.

3.3.2 RNA Extraction

A 2ml eppendorf was filled with 1-2 million treated cells, and the sample was spun for 5 minutes at 1500 rpm. The supernatant was discarded after which 500ul of cooled PBS was added and centrifuged one more at 1500 rpm for five minutes. The resulting pellet was resuspended in 1ml of TRIZOL after the supernatant was discarded (life technologies). Pipetting cells up and down to homogenise them, then incubating them in ice for five minutes. Then 200ul of chloroform was added, followed by 15 seconds of vigorous shaking, and 10 minutes of incubation on ice. The tube was then centrifuged for 20 minutes at 12000 x g and 4 °C. Three layers of the mixture were identified: an upper aqueous layer containing RNA, an interphase layer containing DNA that appeared as a thin white layer, and a lower organic pink layer containing proteins. Approximately 350ul of the upper aqueous layer of the RNA was carefully removed and transferred into another tube. 500ul of cold isopropanol was then added, and the tube was shaken before being incubated on ice for 15 minutes. the tube was centrifuged for 20 minutes at 12000xg and 4°C. The resulting pellet was rinsed with 75% cooled ethanol and centrifuged once more at 7500xg and 4°C for 5 minutes, discarding the supernatant in the process. Carefully removing the ethanol, the pellet was air dried for 20 minutes at room temperature inside the hood. The pellet was resuspended in 20ul of NF water in the tube, which was then set aside at -80°C for future cDNA synthesis.

3.3.3 RNA Quality and Quantity Check

Using Nanodrop 2000 (Thermoscientific, USA), the RNA was measured, and the 260/280 ratio was examined to validate its purity.

3.3.4 Complementary DNA (cDNA) Synthesis

1000ng of RNA was used as the reverse transcription template to create the cDNA. I took 1uL (10uM) of oligo dT20 in a 0.2mL microtube. It was supplemented with 1ul DTT (100mM), 1ul dNTPs (2.5mM), 2ul 10x Reaction Buffer, and 0.5ul RNase Inhibitor 40U/ul. After adding 2ul of template RNA, nuclease-free water was added to create a 20ul total volume. The reaction profile was then established in accordance with the kit protocol after placing the microtubes in the thermocycler (wizbio solutions).

3.3.5 Confirmation of cDNA Synthesis

20ul of cDNA, 2ul of 25mM MgCl2, 2.5ul of 10X reaction buffer, 1.5ul of 10mM dNTPs, 0.5ul of Taq DNA polymerase, 1ul of forward primer, 1ul of reverse primer (GAPDH), and 9.5ul of NF water were combined to make the PCR reaction mixture. The PCR profile was set up so that stage 1 involved initial denaturation at 95°C for 5 minutes, followed by stage 2 of 35 PCR cycles, each of which included denaturation at 95°C for 1 minute, annealing at 60°C for 45 seconds, and cDNA strand extension at 72°C for 45 seconds. Stage 3's last prolongation was granted for 10 minutes at 72°C. After that, the PCR product was stored at -20°C for later processing.

3.3.6 Gel Electrophoresis

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

3.3.7 Real-Time PCR for Gene Expression Analysis

Real-time PCR was used to assess the gene of interest's primers by specifically amplifying the product at 10 mM. (Applied Biosystems 7300). The entire process was conducted on ice. For each primer, a specific PCR strip was created. To produce the

reaction mixture, 5ul of cDNA, 1.5ul of forward and reverse primers (10 mM each), and 12.5ul of SYBR Green Master Mix (2 X) were added (solarbio). By adding 4.5ul of NF water, the reaction volume was increased to 25ul. There were three main steps in the reaction. The reaction mixture was first heated to 50 °C for 2 minutes, then heated to 95 °C for 10 minutes, and then 40 cycles of PCR were added. Three incubation phases make up each PCR cycle: 1) for 15 seconds at 95 °C, 2) for 45 seconds at 60 °C, and 3) for 30 seconds at 72 °C. For melt curve analysis, the dissociation stage was added. Three incubations were administered during the dissociation stage: one at 95°C for 30 seconds, one at 60°C for 30 seconds, and one at 95°C for 30 seconds. The information was gathered at 72 °C. SDS software for the ABI 7300 system was used for data analysis. As a housekeeping gene for normalization, GAPDH primer was used. The 2-C technique was employed to calculate the relative quantification of gene expression.

3.3.8 DNA Fragmentation Assay for Apoptosis Analysis

To determine if the drug under investigation is causing cell cycle arrest or triggering apoptosis, 1 million cells/ml were plated, treated with 0.25uM of the drug for Nb4 cells and 0.5uM for K562 cells, and then incubated at 37°C for 72 hours. Next, genomic DNA was recovered from the treated cells. To obtain a pellet, treated cells were placed in a 2 ml Eppendorf and centrifuged for 5 minutes at 1500 rpm. Next, 200ul of PBS was added, and centrifuged for 5 minutes at 2000 rpm. After discarding the supernatant, 1 ml of Sol A was added. It was vortexed and mixed inverted four or five times before being incubated at room temperature for 10 minutes. The tube was then centrifuged for one minute at 13000 rpm. After discarding the supernatant, 500 ul of Sol A was added once again. centrifuged for one minute at 13000 rpm. Supernatant was removed, 500 ul of Sol B was added, 20 ul of 20% SDS, 5 l of Proteinase K (Thermo ScientificTM), and it was vortexed. It was incubated at 37°C overnight. After incubation, 500 ul each of phenol (Sol C) and chloroform (Sol D) were added, and the mixture was centrifuged at 13000 rpm for 10 mins. Carefully transferring the aqueous phase into a new tube, 500 ul of Sol D was added, and it was centrifuged at 13000 rpm for 10 min. Once more, the aqueous phase was transferred into a second tube, to which 50–55 ul of sodium acetate and 500 ul of isopropanol were added. vortexed, inverted, and well mixed. 13000 rpm centrifuged for 10 minutes. The pellet was obtained when the supernatant was discarded. The resulting pellet was cleaned in 70% ethanol and allowed to air dry for an hour. The DNA was then mixed with 10–20 ul of TE 1X Buffer and kept at –20 °C.

Table 3.3

SOLUTION A	SOLUTION B	SOLUTION C	SOLUTION D
0.32M Sucrose 10mM Tris (pH 7.5) 5mM Mgcl2 Triton X-100 1% (v/v)	10mM Tris (pH 7.5) 400mM NaCl2 2mM EDTA (pH 8.0)	Phenol	Chloroform 24 volume + Isoamyl alcohol 1 volume

3.4 DNA Quantification

The amount of genomic DNA that was extracted was measured using Nanodrop 2000 from Thermo Scientific USA.

3.4.1 Apoptosis Analysis

Extracted DNA samples were combined in a 2:1 ratio with loading dye and ran on a 1.5% agarose gel for DNA fragmentation analysis. Thermo Scientific's 1 kb DNA ladder was used as the marker. The gel was run at 90 volts and 5000 amps for 45 minutes while being observed with a UV illuminator.

3.4.2 Hemolysis Assay:

To determine if Gramicidin A had a cytotoxic effect on healthy RBCs, a hemolysis experiment was conducted. Red blood cell death is referred to as hemolysis. Red blood cells and test materials were incubated at a particular PH during this testing. Whole human blood was collected in an EDTA tube, centrifuged at 500X g for five minutes at 4C, and then continuously washed in PBS. Blood cells were washed with PBS four to

five times to obtain packed RBCs, after which the supernatant was discarded. PBS and packed RBCs were combined to create 2% erythrocyte solution. PBS was serially diluted with gramicidin A (0.25uM, 0.5uM). For various concentrations, 550ul of blood solution and 30ul of drug were produced in a tube and incubated at 37C for an hour. PBS was used as the negative control, while 10% triton X was produced in erythrocyte suspension as the positive control. The tubes were centrifuged at 500X g for 5 min at 4C to produce blood pellets. The absorbance of these tubes was then measured between 405 and 550 nm, and the results were analysed in comparison to controls.

$$Hemolysis = \frac{abs\ sample - abs\ negative\ control}{abs\ positive\ control - abs\ negative\ control} X100\%$$

3.5 Statistical Analysis

Results are shown as the standard error of the mean (SEM) for all experiments (MTT tests) and Real-Time PCR reactions, respectively. One-way and two-way ANOVA, as well as the student's t-test, were used to analyse the data. P-values below 0.05 were considered significant. GraphPad Prism 8.01 was used to create the visual data.

4) RESULTS

4.1 In silico Analysis

4.1.1 Ligand Structure

The structure of Gramicidin A obtained from PubChem was as follow:

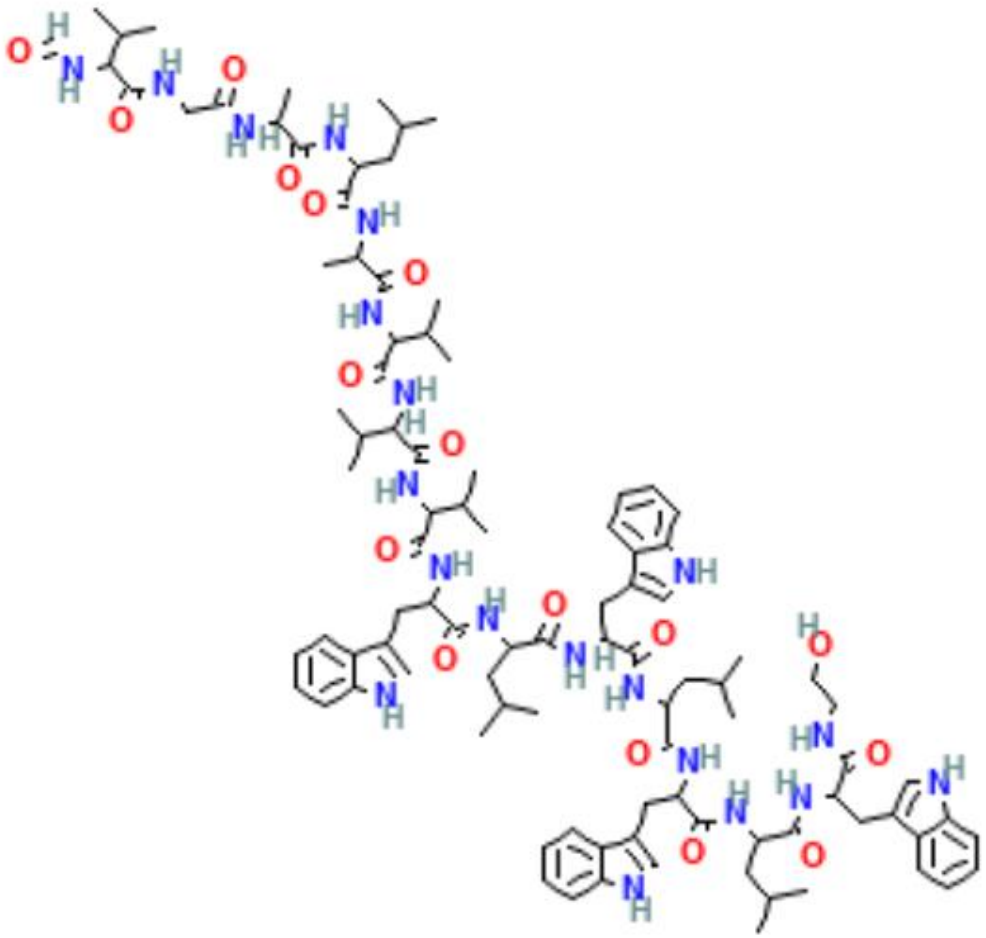


Figure 4.1.1: Structure of Gramicidin A

4.1.2 Target Proteins

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

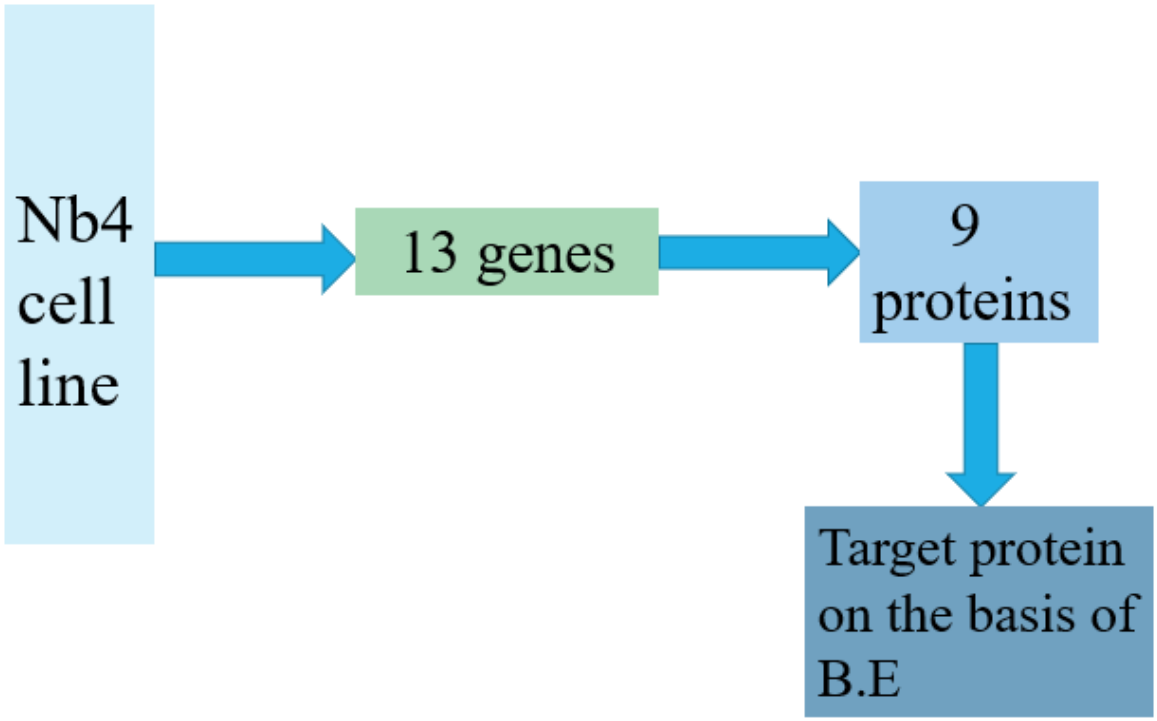


Figure 4.1.2 (a): Hierarchical Model of NB4 Proteins

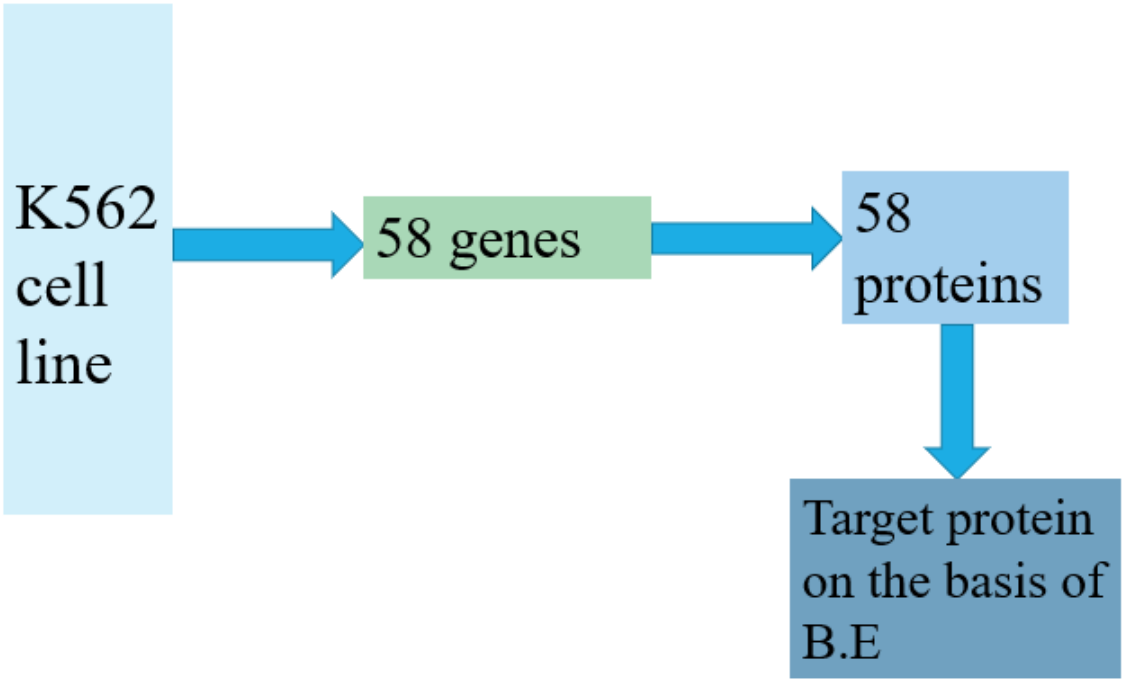


Figure 4.1.2 (b): Hierarchical Model of K562 Proteins

4.1.3 Molecular Docking Analysis

Computer-based drug design is the main objective of molecular docking; it primarily predicts the best possible interaction between the ligand (which may one day be a medication) and the targeted proteins. Automated molecular docking makes structurally and energetically based predictions about molecular recognition. According to this theory, docking allows for accurate calculation of binding strength and easy prediction of ligand structure within the confines of a receptor's binding site (Waszkowycz, Clark, & Gancia, 2011). The NB4 cell line and K562 cell line's targeted proteins were docked with the ligand Gramicidin A. Following molecular docking, the potential therapeutic targets were chosen based on their low binding energies.

Table4.1: Binding Energies of Proteins targets in NB4 cell line

LIGAND	Shortlisted Proteins of NB4 (With lowest binding energy)			Binding Energies (kcal/mol)
	Gene ID	Description	PDB	
Gramicidin A	PML	PML nuclear body scaffold	1bor	-4.5
	TGM2	Transglutaminase 2	4pyg	-5.8

Table4.2: Binding Energies of Proteins targets in K562 cell line

LIGAND	Shortlisted Proteins of K562 (With lowest binding energy)			Binding Energies (kcal/mol)
	Gene ID	Description	PDB	
Gramicidin A	**TGM2**	**Protein glutamine gamma glutamyl transferase 2**	**4PYG**	**-6.3**
	BCR	**Breakpoint cluster region protein**	**5N6R**	**-7.6**

4.1.4 Protein-Ligand Interaction Profile

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

Protein- ligand complex of PML-Gramicidin A in NB4

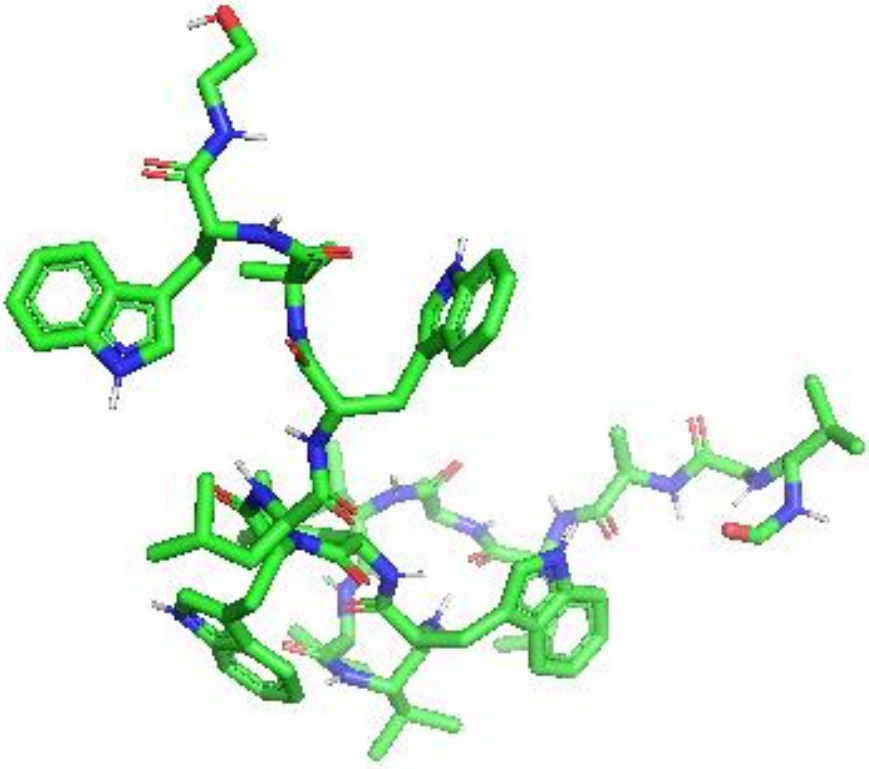


Figure 4.1.4 (a): Protein ligand interaction profile image indicating the binding pose of Gramicidin A with PMLprotein in NB4.

Protein- ligand complex of TGM2-Gramicidin A in NB4

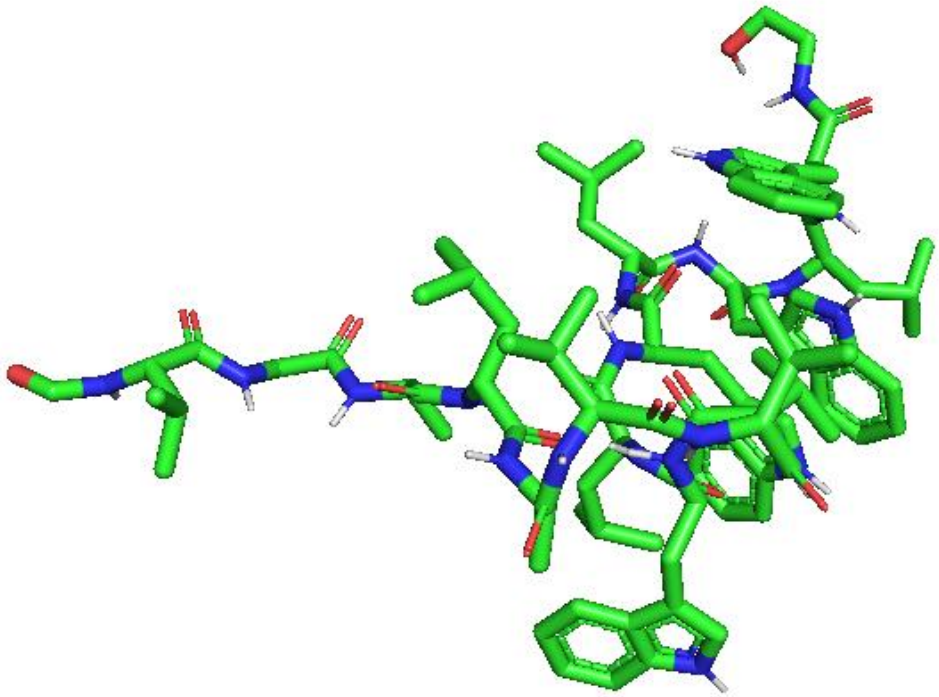


Figure 4.1.4 (b): Protein ligand interaction profile image indicating the binding pose of Gramicidin A with Tgm2 protein in NB4.

Protein- ligand complex of BCR-Gramicidin A in K562

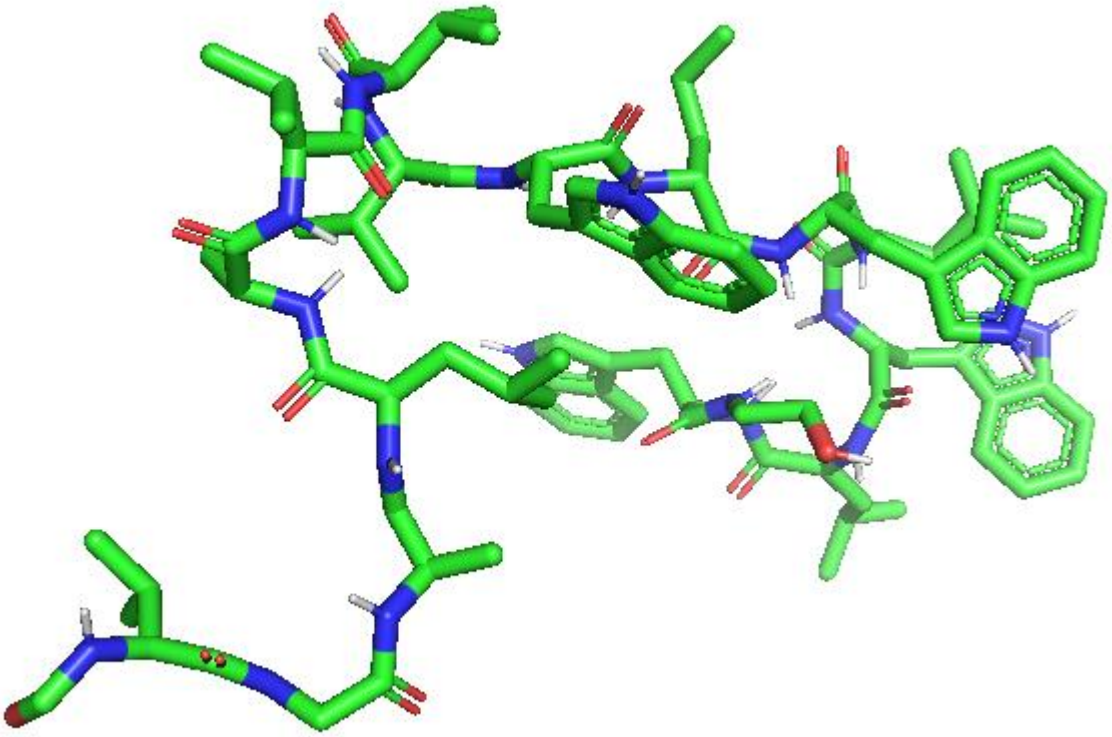


Figure 4.1.4.(c): Protein ligand interaction profile image indicating the binding pose of Gramicidin A with BCR protein in K562.

Protein- ligand complex of TGM2-Gramicidin A in K562

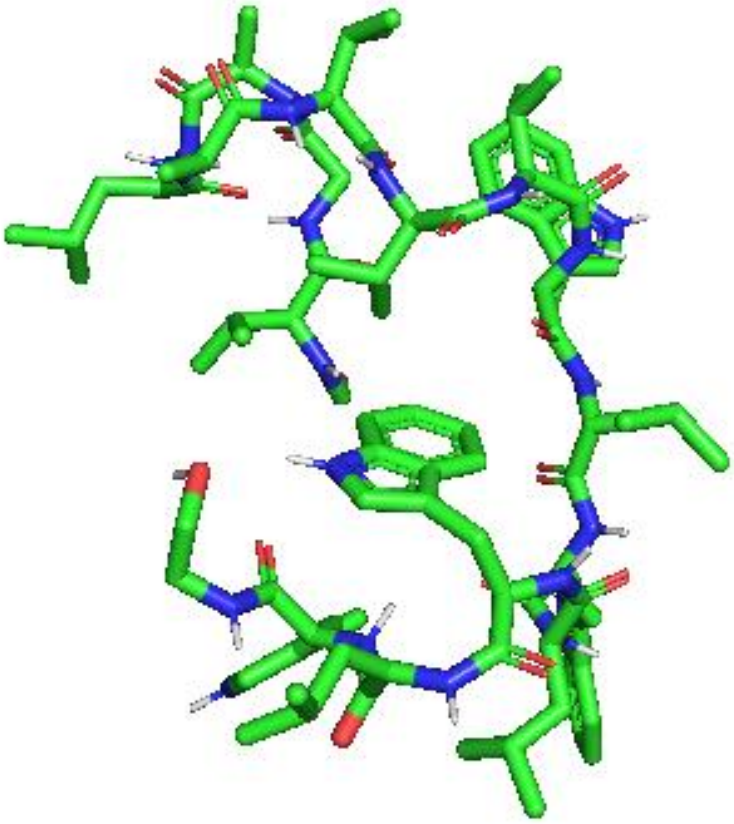


Figure 4.1.4 (d): Protein ligand interaction profile image indicating the binding pose of Gramicidin A with Tgm2 protein in K562.

4.1.5 Gramicidin A interferes with the proliferation potential of PML/RARα-positive APL cell line

In this study, action potential of gramicidin A was assessed against NB4 cell line, Cells were treated with different concentrations of gramicidin A and an MTT assay was performed after 72-hours to assess the proliferation. Figure ()

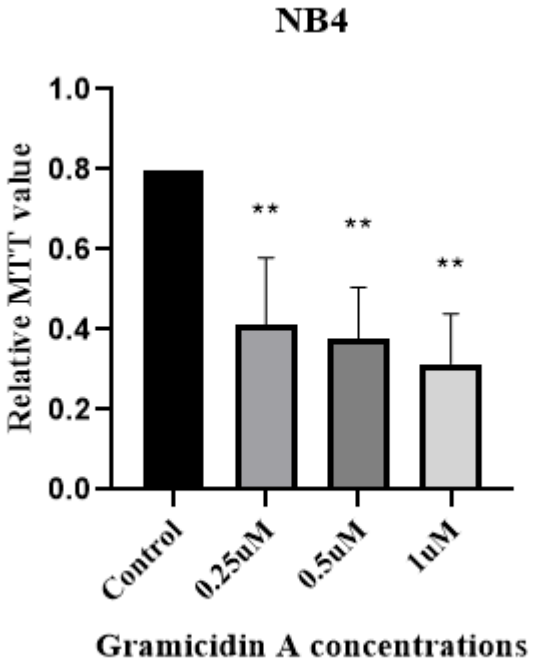


Figure 4.1.5: Effect of Gramicidin A on the proliferation potential of NB4 cells through MTT assay

Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Penicillin and Streptomycin) to determine the proliferation potential of NB4 cells in the presence of 0.01% DMSO and indicated concentrations of Gramicidin A. Statistical significance was evaluated using One-Way ANOVA (p-values <0.001 are statistically highly significant). Bars show mean ± SEM. The antibiotic gramicidin A interferes with the proliferation potential of PML/RARα-positive NB4 cells.

4.1.6 Comparison between the anti-proliferative effect of Gramicidin A and ATRA on NB4 cells

As discussed, ATRA is the current standard of care for PML/RARα-positive APL as the inception of ATRA as a treatment option for APL for more than three decades ago now has improved the clinical outcome of this lethal disease (Ng & Chng, 2017). Though when ATRA along with chemotherapy was administered, a cure rate of more than 80% was achieved (Francesco Lo-Coco & Cicconi, 2014). But ATRA is known to cause differentiation syndrome in APL patients with a swift rise in leukocytes number. So, the patients who receive ATRA only as APL treatment after attaining CR, are seen to undergo relapse (Asou, 2017). We have shown (Figure) that Gramicidin A is able to

interfere with the proliferation of NB4. So, we compared the effects of ATRA and gramicidin A on the cell proliferation potential of NB4 at different concentrations in μM and proliferation was assessed through MTT assay after 72 hours. A significant reduction in the proliferation ability of NB4 by gramicidin A was seen in a dose dependent manner when compared to ATRA.

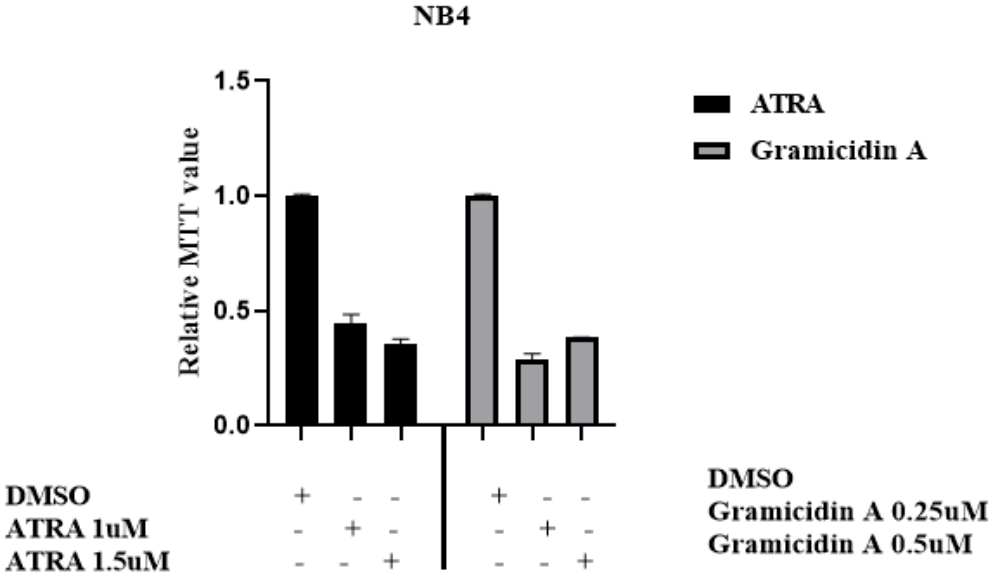


Figure 4.1.6: Comparison between Gramicidin A and ATRA

Gramicidin A and ATRA were contrasted in terms of how they affected the ability of PML/RAR-positive NB4 cells to proliferate. Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Penicillin and Streptomycin) to determine the proliferation potential of NB4 cells in the presence of 0.01% DMSO and indicated concentrations of Gramicidin A and ATRA. Cell proliferation was assessed through MTT assay after 72 hours.

4.1.7 Gramicidin A interferes with the proliferation potential of BCR-ABL positive CML cell line

In this study, action potential of gramicidin A was evaluated against k562 cell line, Cells were treated with different concentrations of gramicidin A and an MTT assay was performed after 72-hours to assess the proliferation. Figure ()

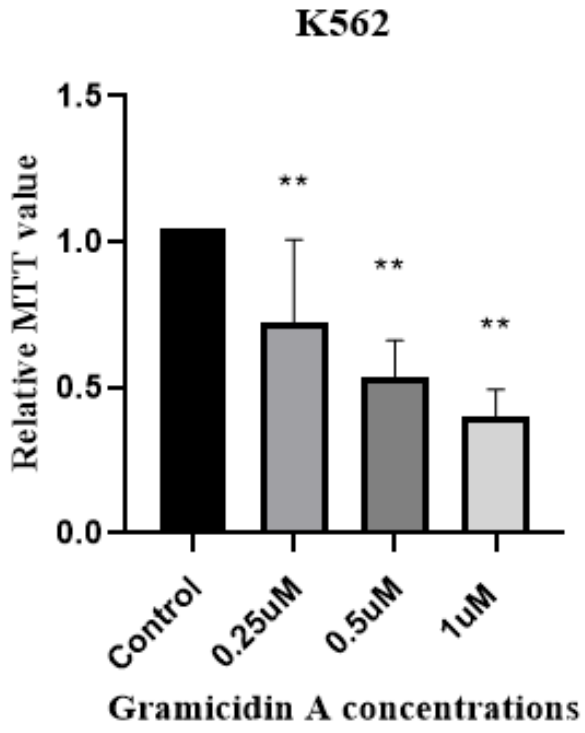


Figure 4.1.7: Effect of Gramicidin A on the proliferation potential of k562 cells through MTT assay

Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Penicillin and Streptomycin) to determine the proliferation potential of K562 cells in the presence of 0.01% DMSO and indicated concentrations of Gramicidin A. Statistical significance was assessed using One-Way ANOVA (p-values <0.001 are statistically highly significant). Bars show mean ± SEM.

4.1.8 Comparison between the anti-proliferative effect of Gramicidin A and imatinib on K562 Cells

Imatinib is the drug of choice for BCR ABL positive patients. The first signal transduction inhibitor (STI) applied in a therapeutic setting was imatinib. It stops a BCR-ABL protein from acting in the chronic myeloid leukemia oncogenic pathway (CML).

The constitutive tyrosine kinase activity is directly inhibited by imatinib. By blocking the transfer of a phosphate group to tyrosine on the protein substrate and the consequent activation of phosphorylated protein, imatinib binds to the BCR-ABL kinase domain. As a result, leukemic cell death is promoted, and proliferative signals are prevented from reaching the nucleus. (Imatinib in Chronic Myeloid Leukemia: An Overview) but imatinib after several doses causes resistance in patients. TKI resistance is defined as a patient's non-response after treatment. We have shown (Figure) that Gramicidin A is able to interfere with the proliferation of K562. So, we compared the effects of Imatinib and gramicidin A on the cell proliferation potential of K562 at different concentrations in μM and proliferation was assessed through MTT assay after 72 hours. A significant reduction in the proliferation ability of K562 by gramicidin A was seen in a dose dependent manner when compared to imatinib.

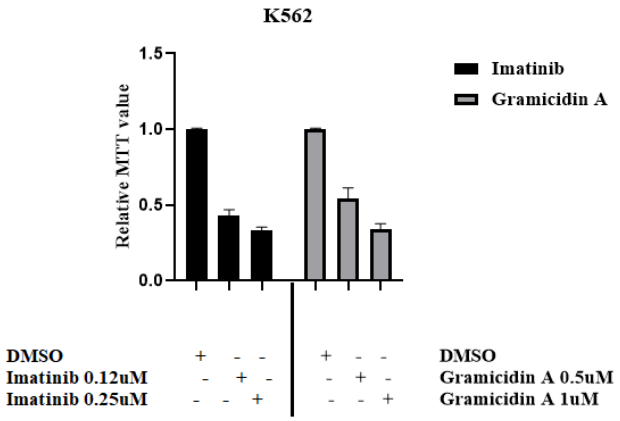


Figure 4.1.8: Comparison between Gramicidin A and imatinib

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

4.1.9 Combinatorial effect of Gramicidin A and ATRA on PML/RARα-positive NB4 Cells

To increase the overall effectiveness of the treatment, two drugs are frequently combined.

Although there are many cancer treatments available, their efficacy is constrained by their toxicity to normal cells. In addition, over time, cancer cells can become resistant to a particular therapy that was once a successful treatment. Therefore, it makes sense to utilize a variety of medications in combination to treat this problem.

Due to its promising outcomes, ATRA is known to be the preferred treatment for PML/RARα- positive APL patients. However, as was mentioned above, ATRA is known to cause differentiation syndrome in APL patients, which is accompanied with a massive rise in leukocyte numbers. As a result, regression is observed in individuals who exclusively get ATRA as APL therapy after achieving CR. Therefore, a combination therapy is required to reduce the concentration of ATRA while still achieving good outcomes. Due to the significant decrease in the ability of NB4 cells to proliferate following treatment with Gramicidin A as compared to ATRA, we investigated the impact of combined treatment to determine if there is any additive effect on the proliferation of NB4 cells as compared to the single treatment as well as if combined treatment at different concentrations in uM can reduce the concentration of ATRA, in order to reduce the differentiation induction caused by a reaction involving ATRA. After 72 hours, the proliferation was evaluated using the MTT test.

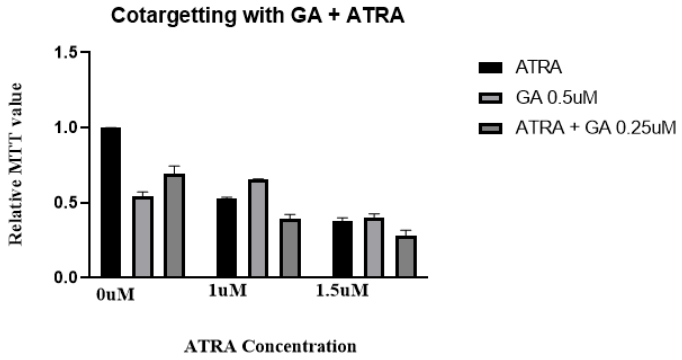


Figure 4.1.9: Effect of the combination treatment on the proliferation potential of PML/RARα-positive NB4 cells.

The results showed additive effect in case of ATRA and Gramicidin A.

4.1.10 Combinatorial effect of Gramicidin A and imatinib on BCR ABL-positive CML Cells

Due to the promising outcomes, imatinib is known to be the preferred treatment for BCR ABL positive CML patients. However, as mentioned above, imatinib is known to cause resistance to treatment in CML patients, which is accompanied with a massive

rise in leukocyte numbers. As a result, regression is observed in individuals who exclusively get imatinib as CML therapy. Therefore, a combination therapy is required to reduce the concentration of imatinib while still achieving good outcomes. Due to the significant decrease in the ability of K562 cells to proliferate following treatment with Gramicidin A as compared to imatinib, we investigated the impact of combined treatment to determine if there is any additive effect on the proliferation of K562 cells as compared to the single treatment as well as if combined treatment at different concentrations in uM can reduce the concentration of imatinib, in order to reduce the resistance caused by imatinib. After 72 hours, the proliferation was evaluated using the MTT test.

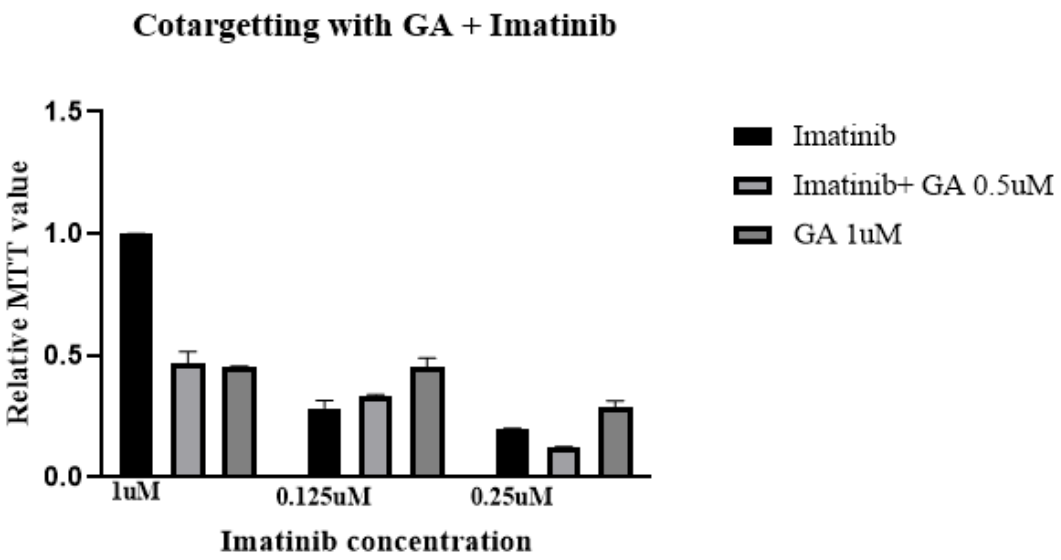


Figure 4.1.10: Effect of the combination treatment on the proliferation potential of BCR-ABL positive cells.

The results showed additive effect in case of imatinib and Gramicidin A.

4.1.11 Effect on downstream signaling cascade in PML/RARα-positive NB4 cells

We also studied the impact of targeting the genes as described in our in-silico studies on downstream signaling pathways as the above analysis shows a reduction in the proliferative potential of the APL cell line. Axin 2 expression was first analysed as a

necessary step towards the downstream signaling. As can be shown, gramicidin A 0.25 uM treatment significantly reduces the expression of Axin 2. The wnt-beta catenin pathway is activated by the Axin 2 gene, which is expressed more strongly in the APL cell line. But in this case, its expression is suppressed. Like this, PML/RARα-positive NB4 cells showed downregulated AXL-RTK expression (Figure). As previously mentioned, AXL-RTK plays a significant role in leukemogenesis. The stabilization of beta-catenin, which results in the activation or up-regulation of Wnt-target genes like c-Myc, Axin2, etc., is mediated by the fusion protein PML/RARα.

As shown in Fig. In APL, AXL-RTK role in Wnt/beta-catenin signaling can be indicated by the fact that downregulation in AXL-RTK expression suppresses the proliferation of NB4 cells, which results in downregulation of Wnt/beta-catenin target genes including c-Myc and Axin 2 as PML/RARα leukemogenesis is Wnt/beta-catenin dependent. We can see the effect of beta-catenin target genes This route is the primary factor controlling how the CSCs renew themselves (Fatima et al., 2021).

Eya3 controls PP2A, a significant cellular Ser/Thr phosphatase, and participates in maintaining the stability of the important oncogene c-Myc. Figure shows that after treatment of NB4 cells with Gramicidin A 0.25uM concentration, the expression of Eya3 is reduced. This is because Eya3 combines with PP2A to generate c-Myc stability and tumor progression.

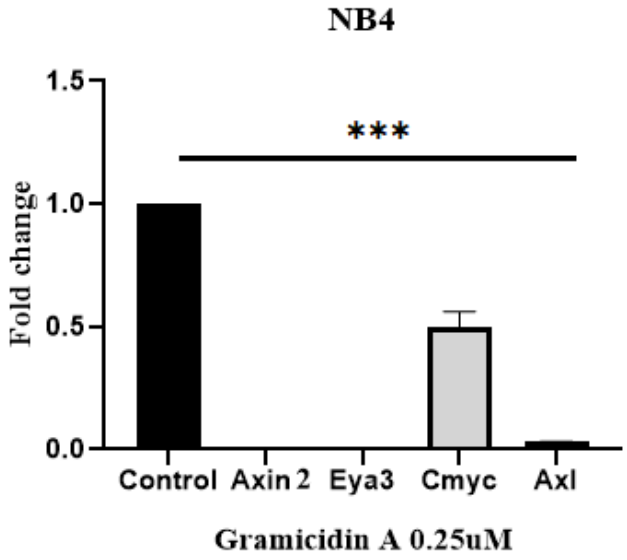


Figure 4.1.11: Expression of Axin 2, Eya3, c-Myc and Axl in PML/RARα-positive NB4 cells after treatment with Gramicidin A.

4.1.12 Effect of Combinatorial treatment with gramicidin A + ATRA on downstream signaling cascade

As shown by the results above, Gramicidin A+ATRA when combined has some additive effect on the proliferation of NB4 cells that are PML/RARα- positive. Following the treatment of the cells with 0.25 uM gramicidin A and 1 uM ATRA, we looked at the expression of Axin 2, AXL, c-Myc, and EYA3. Axin 2, AXL, cMyc, and EYa3 were significantly downregulated, according to the findings.

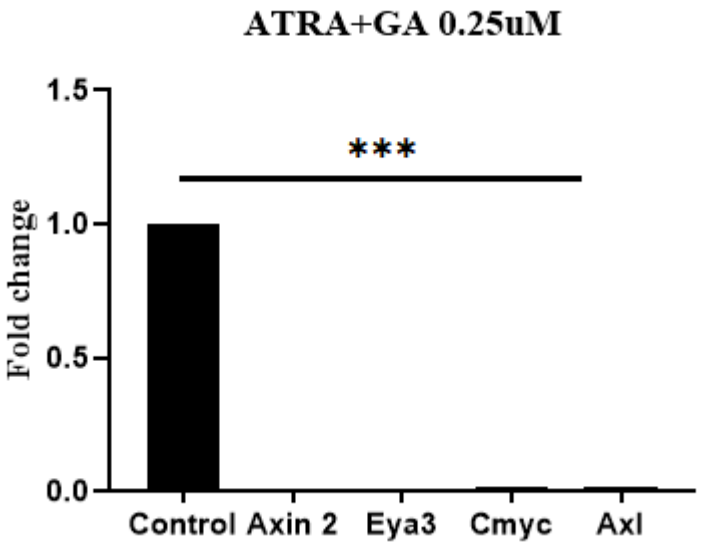


Figure 4.1.12: Expression of Axin 2, Eya3, c-Myc and Axl in PML/RARα-positive NB4 cells after treatment with Gramicidin A and ATRA.

4.1.13 Effect on downstream signaling cascade in BCR-ABL positive CML

To check the effect of gramicidin A on the downstream signaling cascade in BCR-ABL positive, cells were treated with 0.5uM to check the expression of different genes. As it can be seen in figure (4.1.13)that c-myc and Eya3 are downregulated in case of BCR ABL positive CML while Axin 2 and Axl are upregulated.

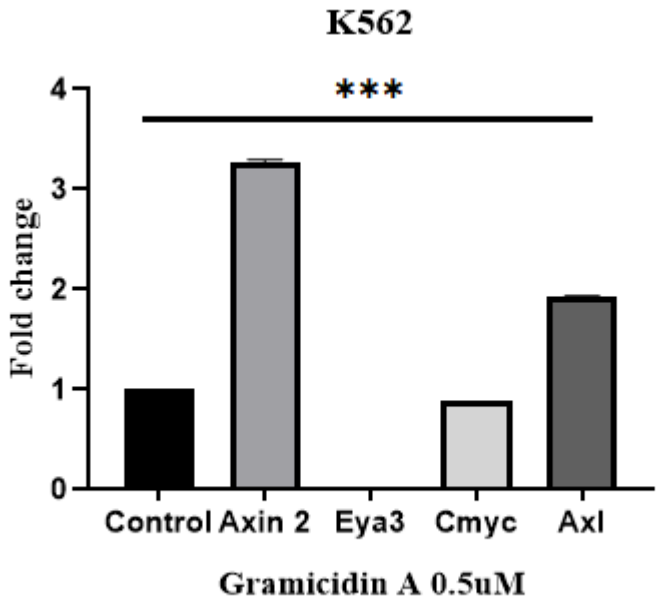


Figure 4.1.13: Expression of Axin 2, Eya3, c-Myc and Axl in BCR ABL-positive CML cells after treatment with Gramicidin A.

4.1.14 Effect of Combinatorial treatment with gramicidin A +Imatinib on downstream signaling cascade

As shown by the results above, Gramicidin A+imatinib when combined has some additive effect on the proliferation of CML cells that are BCR-ABL positive. Following the treatment of the cells with 0.5 uM gramicidin A and0.25uM imatinib, we looked at the expression of Axin 2, AXL, c-Myc, and EYA3. Axin 2, AXL, cMyc, and EYa3 were significantly downregulated, according to the findings.

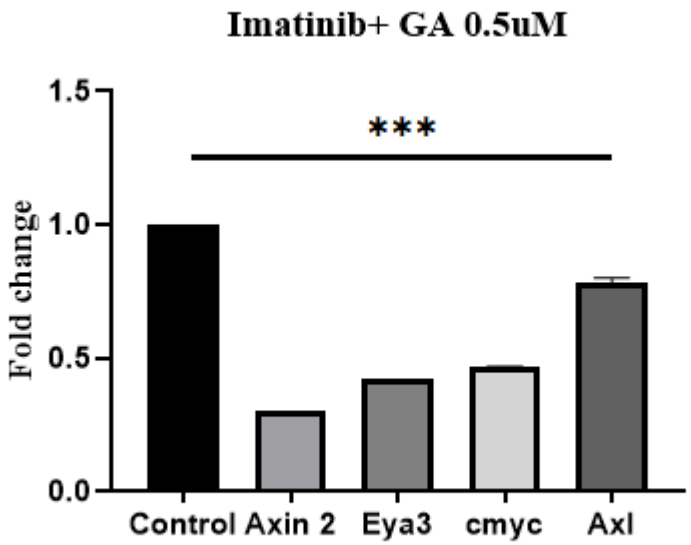


Figure 4.1.14: Expression of Axin 2, Eya3, c-Myc and Axl in BCR ABL-positive CML cells after treatment with Gramicidin A + imatinib.

4.1.15 Apoptosis Check in PML/RARα-positive NB4 cells after targeting with Gramicidin A

Cells were treated with 0.25µM concentration of Gramicidin A. DNA was extracted after 48-72 hours of incubation. Extracted DNA was then run on 1.5% agarose gel through electrophoresis.

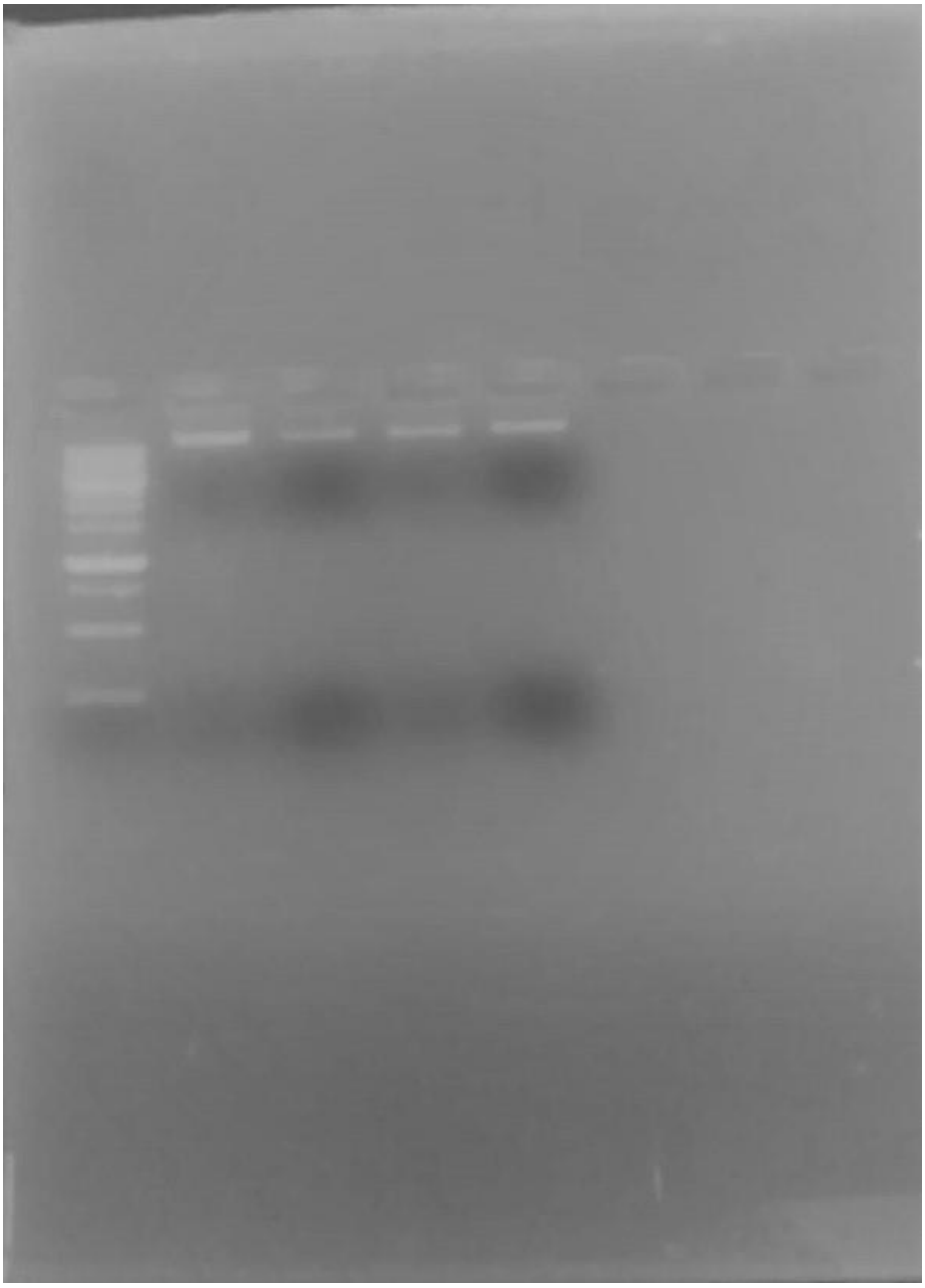


Figure 4.1.15: Gel image for DNA fragmentation assay for NB4 cells -targeted with gramicidin A

Thus, after performing apoptosis assay, it was observed that no apoptosis is induced by Gramicidin A in NB4 cells.

4.1.16 Apoptosis Check in BCR ABL positive CML cells after targeting with Gramicidin A

Cells were treated with 0.5µM concentration of Gramicidin A. DNA was extracted after 48-72 hours of incubation. Extracted DNA was then run on 1.5% agarose gel through electrophoresis.

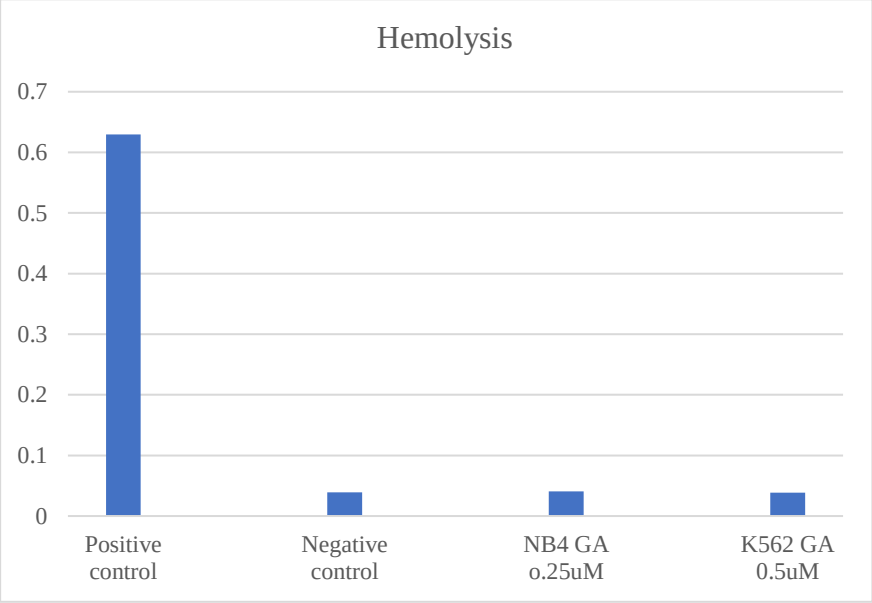
Figure 4.1.16: Gel image for DNA fragmentation assay for K562 cells -targeted with gramicidin A

Thus, after performing apoptosis assay, it was observed that no apoptosis is induced by Gramicidin A in K562 cells.

4.1.17 Hemolysis assay

To determine whether Gramicidin A has hemolytic effect or not, hemolysis assay was performed. For NB4 cells, drug concentration was 0.25uM and for K562 the concentration was 0.5uM. Hemolysis was measured by using the following formula:

$$\%\text{Hemolysis} = \frac{\text{Abs sample- Abs negative control}}{\text{Abs positive control- Abs negative control}} \times 100$$



In both k562 and NB4, 0% hemolysis was observed.

5) DISCUSSION

Gramicidin A, a hydrophobic linear pentadecapeptide derived from microorganism, belongs to the ionophore antibiotics that some of them have been proven to target cancer stem-like cells and used as chemosensitizers. (Kaushik V, Yakisich JS, Kumar A, Azad N, Iyer AKV. Ionophores: potential use as anticancer drugs and chemosensitizers). Recent findings revealed that gramicidin A possesses anticancer properties in renal cell carcinoma, which indicate the therapeutic potential for the treatment of other solid tumors.

Gramicidin A upon testing in vitro interferes with the proliferation potential of PML/RARα-positive APL cells and BCR-ABL positive CML cells. In context to in-silico study, Gramicidin A was docked with all the possible protein targets from APL cells and CML cells, finalizing the potentially best targets based on lowest ligand-protein binding energy.

The current study evaluated the cell cytotoxicity of gramicidin A and its anti proliferative activity on NB4 and K562 cell lines. Due to chromosome translocations, APL, a distinct subtype of AML, expresses an oncogenic fusion protein involving the RARA gene. In hematopoietic precursor cell lines, expression of PML-RARA prevents differentiation and promotes survival (L.-Z. He et al., 1997; Puccetti & Ruthardt, 2004; Vitoux, Nasr, & de The, 2007). We found that Gramicidin A was able to interfere with the proliferation potential of APL cells.

Our study has found for the first time that Gramicidin A is highly active against APL and CML. Thus, its the novelty of Gramicidin A in case of liquid cancer i-e leukemia. Compared in terms of IC50 values, the efficacy of Gramicidin A was stronger than that of Salinomycin, the well-known CSC-active compound. GrA displayed potent cytotoxicity to leukemic cancer cells at the levels which were much lower than the borderline concentration that could induce hemolysis. Generalized toxicity is a significant challenge to the development of ionophores as therapies for human cancer. GA causes hemolysis and is toxic to the liver, kidney, meninges, and olfactory apparatus.(Wang F, Qin L, Pace CJ, Wong P, Malonis R, Gao J. Solubilized gramicidin A as potential systemic antibiotics) but in our study in case of NB4 and k562 by performing hemolysis assay, it has shown 0% hemolysis in both the cases. Thus, indicating that it is nontoxic to normal red blood cells.

Our findings demonstrated that Gramicidin A was preventing the growth of APL and CML cells. Next, we looked at how targeting the fusion genes affected PML/RARα NB4 cells' downstream signaling. The receptor tyrosine kinase AXL, which was found to be expressed lesser following Gramicidin A treatment of NB4 cells, is crucial for the development and transformation of CML (O'bryan et al., 1991). It promotes increased cell survival and proliferation by mediating intracellular signaling through the PI3K/Akt and Erk pathways (Graham, DeRyckere, Davies, & Earp, 2014; Schmidt, Ben-Batalla, Schultze, & Loges, 2012). In PML/RAR positive APL, AXL-receptor tyrosine kinase is implicated in leukemogenesis and may be a potential therapeutic target (Fatima et al., 2021).

According to Lemaitre, Buckle, and Méchali (1996) and Uribesalgo, Benitah, and Di Croce (2012), up-regulation of the transcriptional activation c-Myc is linked with treatment resistance in AML and CML because it promotes cell growth while also regulating apoptosis and differentiation. If Wnt is not controlled, beta-catenin may increase the expression of c-Myc, which is thought to contribute to treatment resistance (Cardona-Echeverry & Prada-Arismendy, 2020). Unspecific Wnt/beta-catenin target gene c-Myc promotes cell division as well as other biological functions (Dang, 2012). As a "master regulator," the c-Myc gene controls cellular metabolism and proliferation.

Because it is triggered by a large number of oncogenic pathways, which in turn causes a large number of metabolic alterations that can result in malignant transformation. According to numerous studies (Coluccia et al., 2007; Miller, Thomas, Islam, Muench, & Sedoris, 2012; Müller-Tidow et al., 2004; K. Wang et al., 2010), the activation of Wnt-target genes such as c-Myc and Axin2 is dependent on mitogenic stimulation. Two well-known Wnt/b-catenin target genes are AXIN2 and MYC (T.-C. He et al., 1998; Yochum, Cleland, & Goodman, 2008). MYC is a transcription factor responsible for promoting the expression of genes whose products promote cellular proliferation (Dang et al., 2006).

The Wnt/-catenin signaling cascade is a pro-survival signaling system that interacts closely with signaling pathways like the phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT). More recently, Wang et al. (Dunne et al2014) showed that, in MCF7 breast cancer cells, AXL knockdown led to a decrease in nuclear B-catenin whereas AXL stimulation by Gas6 dramatically raised B-catenin levels and triggered its nuclear translocation. demonstrating AXL's involvement in both the development of cancer and the stabilization of B-catenin.

According to research by Corno et al. (2016), AXL-RTK regulates Wnt signalling by stabilizing B-catenin and activating signaling pathways like PI3K/AKT/mTOR, Ras/Raf/MAPK/ERK. The downregulation of AXL-RTK expression can also affect the stabilization of B-catenin and the downregulation of its target genes, as shown by our findings. Gramicidin A decreased the proliferation of APL cells, which led to the downregulation of c-myc, a key regulator of cell proliferation (Miller et al., 2012).

It is evident from the MTT experiment that gramicidin A prevents the growth of NB4 cells. The PCR results showed that Gramicidin A treatment of NB4 and K562 cells downregulates AXL-RTK, which in turn reduces the expression of beta-catenin dependent genes, c-Myc, and Axin 2 in NB4 APL cell line that is PML-RARA positive.

It was also found that the combined treatment (gramicidin A + ATRA) and (Gramicidin A+ imatinib) had a additive effect on the proliferation of NB4 cells and CML cells. Due to the differentiation induction by ATRA and differentiation syndrome (Asou, 2017), and resistance caused by imatinib in CML positive patients (Alves R et al, 2021), combinatorial therapy (Gramicidin A+ ATRA) and (Gramicidin A+ imatinib) might be a better targeted therapy than the current therapy ATRA and tyrosine kinase inhibitor imatinib.

Crosstalk between several cell-to-cell signal cascades, which promotes the survival and maintenance of cancer cells, is fundamental to the progression of cancer. Therefore, it is necessary to develop a therapy that would focus on the primary causes of leukaemia. Gramicidin A, an antibiotic, could potentially be used as a targeted therapy for leukaemia in the future if the above findings are confirmed and further in-vitro and in-vivo research is conducted.

6) CONCLUSION

Gramicidin A, an antibiotic from the ionophore class, prevents the growth of the NB4 and K562 cell lines. The downregulation in the expression of AXL-RTK, which stabilizes and regulates beta-catenin, leads to downregulated expression of beta-catenin target genes, and B-catenin is the main player in regulating the self-renewal of CSCs, was observed because our in silico results showed that PML and BCR are the main targets of Gramicidin A and that these target proteins are the main key players of PML/RARα-positive APL leukemia and BCR-ABL positive CML. Additionally, the Axin 2 gene, which activates the wnt-beta catenin pathway, is expressed more highly in the APL cell line. However, in our investigation, Gramicidin A was able to decrease this expression. Gramicidin A, being non-hemolytic, can be used in combination to mitigate the side effects of ATRA and imatinib when given in a dose-dependent manner together with these drugs. This combination might be a viable substitute for the topical leukemia treatment. In vivo models should be used going forward, nevertheless, to increase efficacy and effectiveness.

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