Investigating the Role of Antibiotic in Acute Myeloid Leukemia

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In

Applied Biosciences

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DECLARATION

We certify that this research work titled *" To Invesligale ltte Role of Antibiotic (Gramicidin A) i i lt6, 9)(p23 ¡ q34) – High Aggi'essiie Acute Myeloid Leukemia"* is our own work. The worL has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Dedicated to

Our exceptional parents for all their efforts, encouragement, love, sacrifices and prayers throughout our educational voyage,

To our honored Supervisor for his prodigious guidance,support and inspiration,

To our friends and siblings who motivated and cheered us during these years ,

To all cancer patients,

And,

Lastly, we humbly honor ***Late Prof. Dr. Fauzia Maqsood****, whose unwavering dedication to education and passion for knowledge persisted even in the face of her courageous battle with lymphoma, leaving an enduring legacy for her family and her students.*

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LIST OF ACRONYMS

A

AML	Acute Myeloid Leukemia
AMLs	Acute Myeloid Leukemias
ASXL1	ASXL Transcriptional Regulator 1

C

CMP	Common Myeloid Progenitors
CLP	Common Lymphoid Progenitors
CML	Chronic Myelogenous Leukemia
CEBPA	CCAAT/Enhancer-Binding Protein Alpha
CDC25A	Cyclin-Dependent kinases by Cell division cycle 25A

D

DNMT3A	DNA Methyltransferase 3 Alpha
IDH2	Isocitrate Dehydrogenase 2
DEK-NUP214	DEK proto-oncogene- Nucleoporin 214kDa

E

ETO (MTG8)	Myeloid Translocation Gene on 8
ER	Endoplasmic Reticulum

F

FLT3	Fms-like Tyrosine kinase 3

G

G-CSF	Granulocyte Colony-Stimulating Factor

H

HC	Hematopoietic cell
HPCs	Hematopoietic Progenitor Cells
HSC	Hematopoietic Stem Cell
HSCs	Hematopoietic Stem Cells
J	
JAK	Janus Kinase
K	
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
L	
LT-HSC	Long Term- Hematopoietic Stem Cell
LSCs	Leukemic Stem Cells.
M	
MPP	Multipotent Progenitor Cell
MTT Assay bromide (MTT) assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium
miR	microRNA
mTOR	Mammalian Target of Rapamycin
N	
NK	Natural Killer
NPM1	Nucleophosmin gene
P	
P	Phosphorylation
PML-RAR gene	Promyelocytic Leukemia gene- Retinoic Acid Receptor alpha

PI3K	Phosphoinositide 3-Kinase
R	
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RBC	Red Blood Cell
RUNX1	Runt-Related Transcription Factor 1
RTr	Receptor Tyrosine residues
S	
ST-HSC	Short-Term Hematopoietic Stem Cell
STAT	Signal Transducer and Activator of Transcription
SOCS	Suppressors of Cytokine Signaling
SCs	Stem Cells
SYK	Spleen Tyrosine Kinase pathway
T	
t (8;21)	Translocation between chromosome 8 and 21
t (15;17)	Translocation between chromosome 15 and 17
t (6;9)	Translocation between chromosome 6 and 9
TET2	Tet methylcytosine dioxygenase 2
TYK	Tyrosine Kinase
W	
WHO	World Health Organization

SYMBOLS

µM	micro-Mol

ABSTRACT

Background: Acute Myeloid Leukemia (AML) is the abnormal proliferation of white blood cells that are still developing from myeloid stem cells. It is incredibly heterogeneous and has many subtypes based on the many translocations associated with it. One of its subtypes, the *t (6,9)* AML makes up only 1% of all AMLs. AML subtype with *t (6,9)* is rare, has early onset, is extremely aggressive and has poor prognosis. There is no specified targeted therapy for *t (6,9)* AML, which is particularly resistant to chemotherapy as well. Gramicidin A is an antibiotic and recently has been reported to inhibit tumorigenesis in solid cancers. Our preliminary studies also showed its toxic effect on cell lines for myeloid leukemia. However, the exact molecular mechanisms responsible for inhibiting cancer growth have not been explored to establish it as a therapeutic agent for both solid and liquid tumors.

Aim: Our aim is to investigate its effects on FKH-1 cell line specific to *t (6,9)* AML and U937 as control via in-silico and in vitro approaches.

Methodology: Molecular Docking of FKH1 and U937 cell line targets with ligand (Gramicidin A) was performed using PyRx to find targets for the drug. Proteins with the lowest binding energies were selected for further molecular analysis and modeling via Discovery Studio Visualizer. The Insilco data presented promising binding targets, particularly with the NUP214 domain of the oncofusion protein DEK-NUP214. The antiproliferative potential of Gramicidin A was observed through MTT assay and further validated through RT-PCR.

Conclusion: Interestingly, it was found that the drug Gramicidin A aggressively promotes growth in FKH-1 cell lines, while inhibiting cell growth in U-937, K-562, and Nb4 cell lines. The IC-50 value was achieved at drug concentration as low as 0.25 μM. Conclusively, these results suggest a favorable role of gramicidin A as a potent inhibitor in case of highly aggressive AML in U-937, K-562, and Nb4 cell lines while its encouraging role for FKH-1 growth calls for intricate studies to better understand tumorigenesis in *t (6,9)* AML.

Key words: AML (Acute Myeloid Leukemia), Gramicidin A, FKH-1, U-937, DEK-NUP214.

CHAPTER 1

1. INTRODUCTION

Leukemia is a pervasive global menace that continues to demand our unwavering attention due to its severity and progression level which is causing alarming situations worldwide. Leukemia is a heterogeneous clonal malignancy that produces abnormal white blood cells. According to the Fourth Edition of the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues, leukemia is broadly labeled into myeloid or lymphoid lineages. Overall, there are four types of leukemia acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphoblastic leukemia (CLL). Despite progress in hematopoietic malignancy treatment leukemia still accounts to be the 15^{th} and 11^{th} most frequent cause of cancer incidence worldwide with 474,519 incidentrate and 311,594 mortality rates (Du.M, 2022). According to WHO, leukemia is ranked fifthin cancer-related death in Pakistan and reckoned for 4.1% of all cancer cases (WHO, 2019). Ahmad, Yusuf, & Burney (2015) conducted a study in Pakistan enlightening that AML is the most common type of leukemia in Pakistan, after CML(Ahmad *et al.*, 2015). AML is blood and bone marrow cancer. It is the most common kind of acute leukemia in adults. This kind of cancer usually becomes fatal if it isn't treated properly. AML is likewise known as acute myelogenous leukemia and acute non-lymphocytic leukemia. In AML, the myeloid stem cells normally emerge as a sort of immature white blood cells referred to as myeloblasts (or myeloid blasts). The myeloblasts in AML are bizarre and produce abnormal progeny. They produce atypical white blood cells, red blood cells, or platelets and are referred to as leukemia cells or blasts. Leukemia cells can build up within the bone marrow and blood occupying the space of normal blood cells. When this happens, infection, anemia, or bleeding may also occur. The leukemia cells can spread outside the blood to different parts of the body, inclusive of the brain, spinal cord, skin, and gums. Sometimes leukemia cells form a solid tumor referred to as myeloid sarcoma. Myeloid sarcoma is likewise called an extramedullary myeloid tumor, granulocytic sarcoma, or chloroma. (Leukemia, 2022). With the latest molecular strategies, along with cost- and time-effective Next-generation sequencing (NGS) technology, a sizeable range of genetic mutations has been recognized. Six genes, including

FMS-like tyrosine kinase three (*FLT3*), nucleophosmin 1 (*NPM1*), CCAAT/enhancer binding protein alpha (*CEBPA*), Runt-associated transcription element 1 (*RUNX1)*, additional sex combs-like 1 (*ASXL1*), and tumor protein p53 (*TP53*), have already been included into the risk categories proposed through the European Leukemia Net (ELN) (Yu.J, 2020). AML is classified into several subtypes, one of which is highly aggressive, with a translocation between chromosomes 6 and 9, which accounts for only 1% of all AML cases. The *t (6,9)* translocation is a rare genetic anomaly that results in the formation of a chimeric oncofusion gene named *DEK-NUP214* (previously it was called CAN) (Huret *et al.*, 2013).

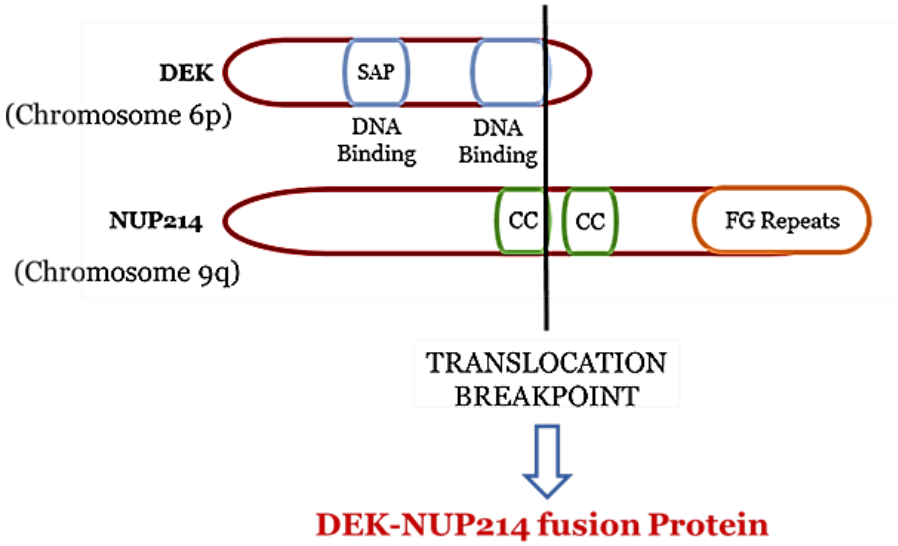

Figure 1 Schematic structures of the DEK and NUP214 proteins and Translocation breakpoint resulting in DEK/NUP214 translocation. Modified from (Huret *et al.*, 2013).

Conventional treatment for AML includes high-dose chemotherapy, radiation therapy gilteritinib, enasidenib, or ivosidenib targeted therapy, maintenance therapy with midostaurin, for AML with *FLT3* genetic mutation and bone marrow transplant. However, these treatments have not generated promising results making AML an alarming situation globally (Leukemia, 2022). Gramicidin A (GA) is a channel-forming ionophore that renders biological membranes permeable to precise cations which disrupt cell ionic homeostasis. It is a famous antibiotic, but its capacity as anticancerous agent has not been broadly evaluated yet (David *et al.*, 2015).

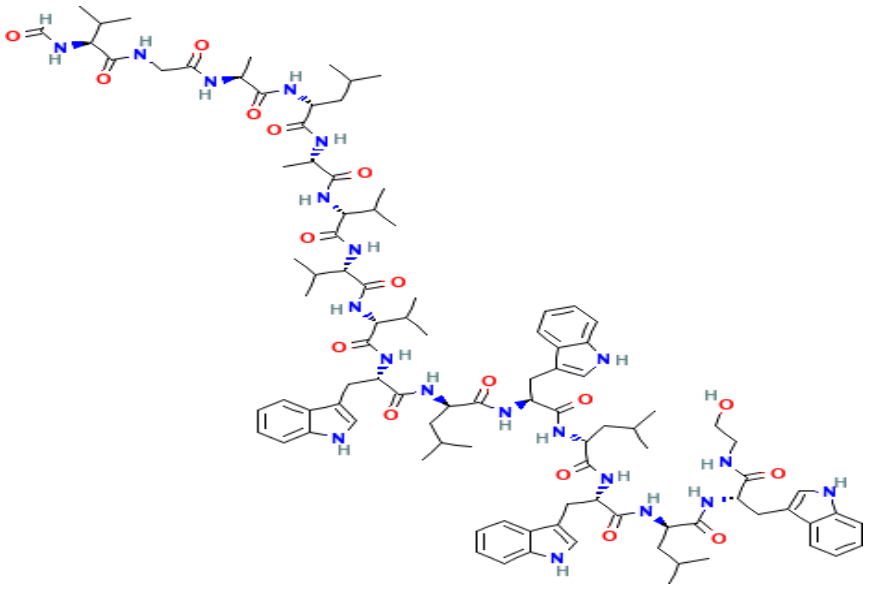

Figure 2 Structure Of Gramicidin A (Pub Chem**)**

Gramicidin is a heterogeneous mixture of six antibiotic peptides acquired from the soil bacterium Bacillus brevis. Gramicidin A is active against most Gram-positive bacteria and inactive against Gram-negative organisms (David *et al.*, 2015).

It is a cytotoxic drug as well as a targeted angiogenesis inhibitor that makes a significant contribution in compromising cancer growth *in vitro* and *in vivo* as it is an effective tumor suppressor (David *et al.*, 2015).

Gramicidin monomers make a β-helix conformation within membranes. Dynamic dimerization of two monomers forms a functional channel, which therefore induces local membrane deformation. Cells preserve a low concentration of intracellular Na+ and an excessive concentration of intracellular K+ relative to the extracellular surroundings. This dynamic dimerization of GA causes the perturbation of Na+ and K+ homeostasis that impairs cancer metabolism (David *et al.*, 2015).

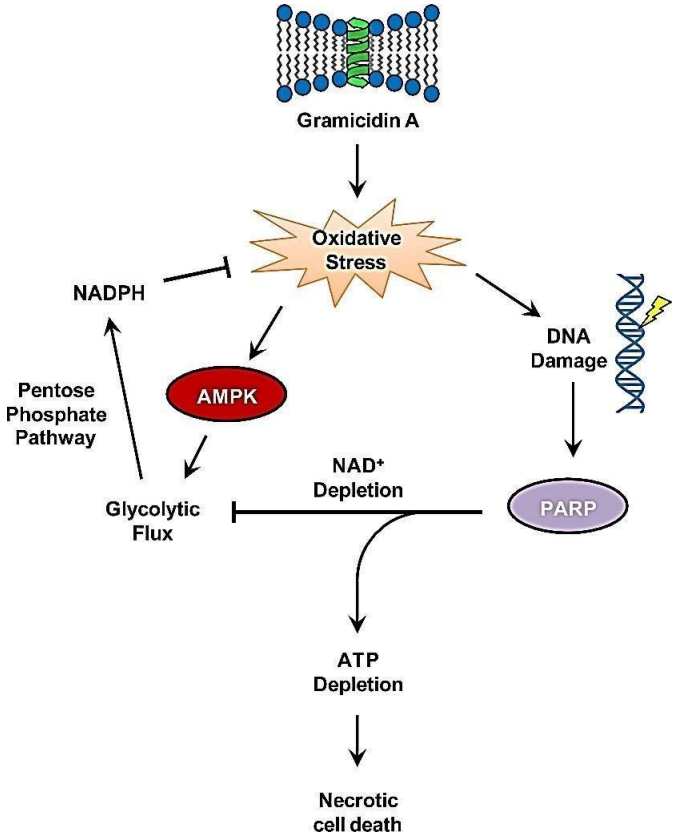

Figure 3 Gramicidin A mode of action depicting its anticancer potential (David et al., 2015).

1.1. Rationale

The antibiotic Gramicidin A, known for its efficacy against CML and Acute Promyelocytic Leukemia (APL) specific targets, is hypothesized to effectively bind to targets in *t (6,9)* AML, as all three leukemia types share similar targets, thus suggesting its potential as a targeted therapeutic approach for *t(6,9)* AML.

1.2. Research Objectives

The objectives of the research were:

- To find out potential targets for Gramicidin A in AML using *in silico* approaches.
- To check the antiproliferative potential of Gramicidin A in AML.
- To perform mechanistic studies of Gramicidin A in AML.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Hematopoiesis

Hematopoiesis is a process which involves the production of mature blood cells but in a controlled manner, all happening in hematopoietic system in our body. The system constitutes spleen, bone marrow and liver. This process initiates during early phases in the development of embryo and it implicates excessive cells input through division and differentiation from rejuvenating, pluripotent hematopoietic cells. (Kim et al., 2014). Hematopoietic stem cells (HSCs) can be differed into long term- hematopoietic stem cell (LT-HSC), short-term hematopoietic stem cell (ST-HSC), and multipotent progenitor cell (MPP) regarding the duration of proliferation. Under normal functional conditions, rare HSCs for example LT-HSC can propagate into all lineage of blood cells in the bone marrow. Whereas number of HSCs in the peripheral blood become higher under myelosuppression by granulocyte colony-stimulating factor (G-CSF) and drug stress conditions, assuming their rapid migration from the bone marrow. These progenitor HSCs in peripheral blood are ST-HSC, contributing directly to recovery of damaged cells and tissues. (Lee & Hong, 2020)

Process starts from HSC at the upper level which is multipotent, descending cell further differentiate into myeloid and lymphoid lineages of blood cells (Kim, Stachura, & Traver, 2014). Focusing on these lineages, leukopoiesis, the process of leukocyte production from HSC in the bone marrow and blood is further divided into myelopoiesis and lymphopoiesis. Myelopoiesis comprises the series of differentiation stages leading to the production of cells of innate immune system such as granulocytes, platelets and erythrocytes. However,

Lymphopoiesis generates the cells of adaptive immune system such as T and B lymphocytes (Mirza, 2020).

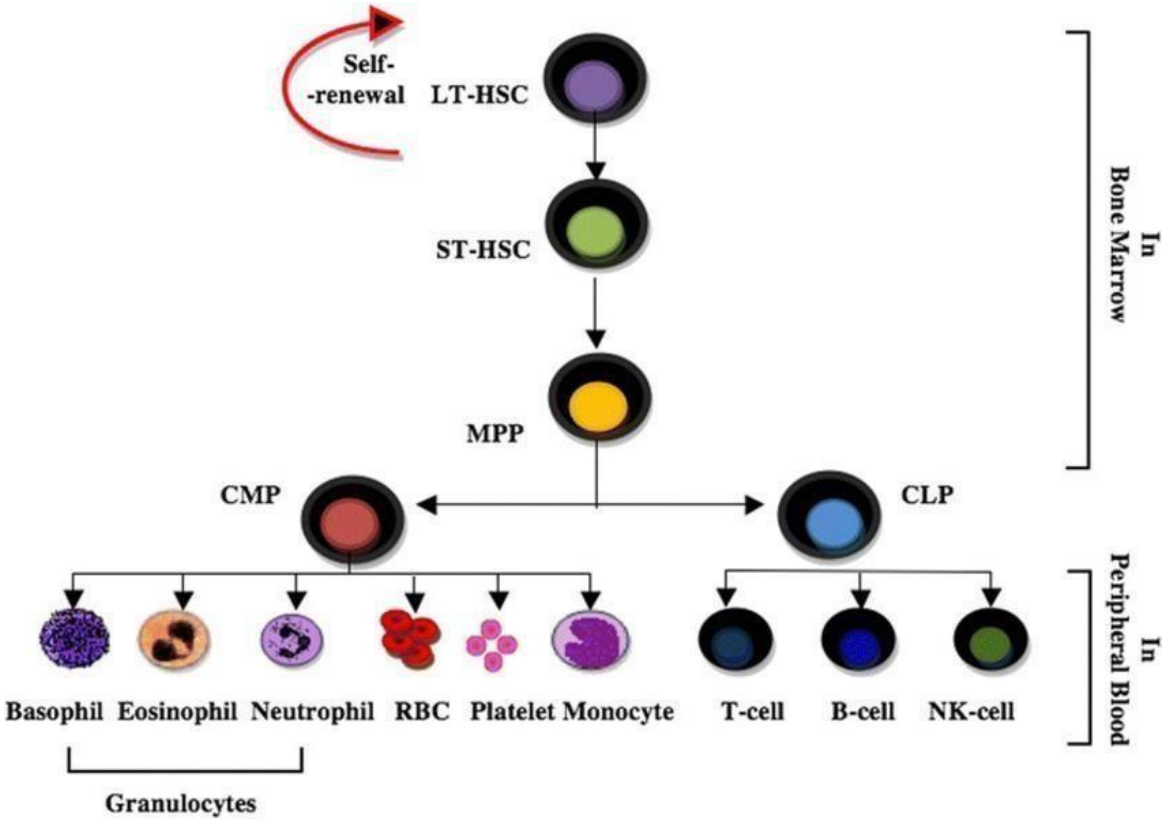

Figure 4 hierarchy of Hematopoiesis.
Hematopoietic stem cells (HSCs) developing into long-term (LT)-HSC and short-term (ST)-HSC forms in bone barrow. First, a LT-HSC having long-term self-renewal ability is transformed into a ST-HSC after which HSC generates a multipotent progenitor cell (MPP). This MPP committed in bone marrow to turn out to be either common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). These CLP and CMP generates mature blood cells upon differentiation such as granulocytes, erythrocytes or red blood cells (RBC), monocytes, platelets, natural killer (NK) cells, B cells, and T cells of innate and adaptive immunity. (Bakhuraysah, Siatskas & Petratos, 2016).

Leukemogenesis is the process that leads to development of leukemias. As they originated from discrete, single cells in the bone marrow, so are monoclonal diseases (Irons & Stillman, 1996). Leukemia is the production of abnormal leukocytes via primary or secondary process (Chennamadhavuni *et al.*, 2023). It arise from mutations or transformations of hematopoietic cells in the blood and bone marrow. One of the three major cancers of the hematopoietic system (Leukemia, lymphoma, and myeloma). Leukemias are recurrently manifested as liquid tumors because the abnormal cells from malignant progeny normally move out from the bone marrow and enter the blood stream. So, markedly amplified the myeloid, lymphoid or, in exceptional cases, erythroid lineage cells specifically abnormal cells in bone marrow

or blood. Whereas myelomas are emerged from plasma cells that are fully differentiated and can be found in bone marrow, tissue, or blood as dispersed clones or as solid masses. Plasma cells in myeloma continue to divide, far from normal plasma cells, which stop proliferating after differentiation. Lymphomas results from the malignant alteration of a single lymphocyte that is usually present in a lymph node or can be localized outside the bone marrow in another lymphoid tissue such as spleen or thymus. As a result, lymphomas are called solid cancer. (Hematopoietic Cancers, 2014).

2.1.1. Prevalence of Hematopoietic Cancers

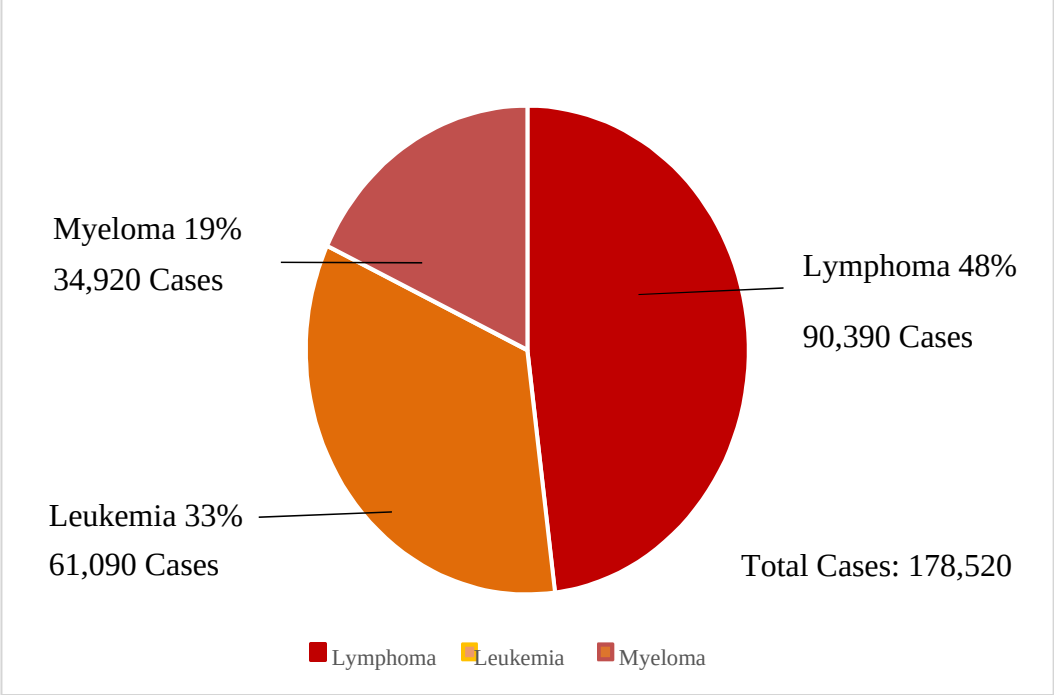

Figure 5 **Figure 6 Estimated percentage of new cases of leukemia in 2021** (Cancer Facts & Figures, 2021. American Cancer Society, 2021).

In United States, an approximation of 1,519,907 people (about the population of West Virginia) is having or in remission from leukemia, myeloma, lymphoma, or other hematopoietic diseases and total 186,400 people are anticipated to be diagnosed with leukemia, lymphoma, or myeloma in 2021. The total estimate of people in remission or having leukemia is 397,501, lymphoma and myeloma are 825,651 and 138,415 respectively in 2021. (Cancer Facts & Figures, 2021; American Cancer Society, 2021).

2.1.2. Prevalence of leukemia in Pakistan

In Pakistan, the increase in mortality with morbidity due to Leukemia has become a global health concern. Although it is affecting both genders, but males are being affected at higher rates (approximately 5.2%). According to WHO, leukemia is ranked fifth in cancer-related death in Pakistan and reckoned for 4.1% of all cancer cases (WHO, 2019). Ahmad, Yusuf, & Burney (2015) conducted a study in Pakistan enlightening AML is the most common typeof leukemia in Pakistan, after CML (Ahmad *et al.*, 2015).

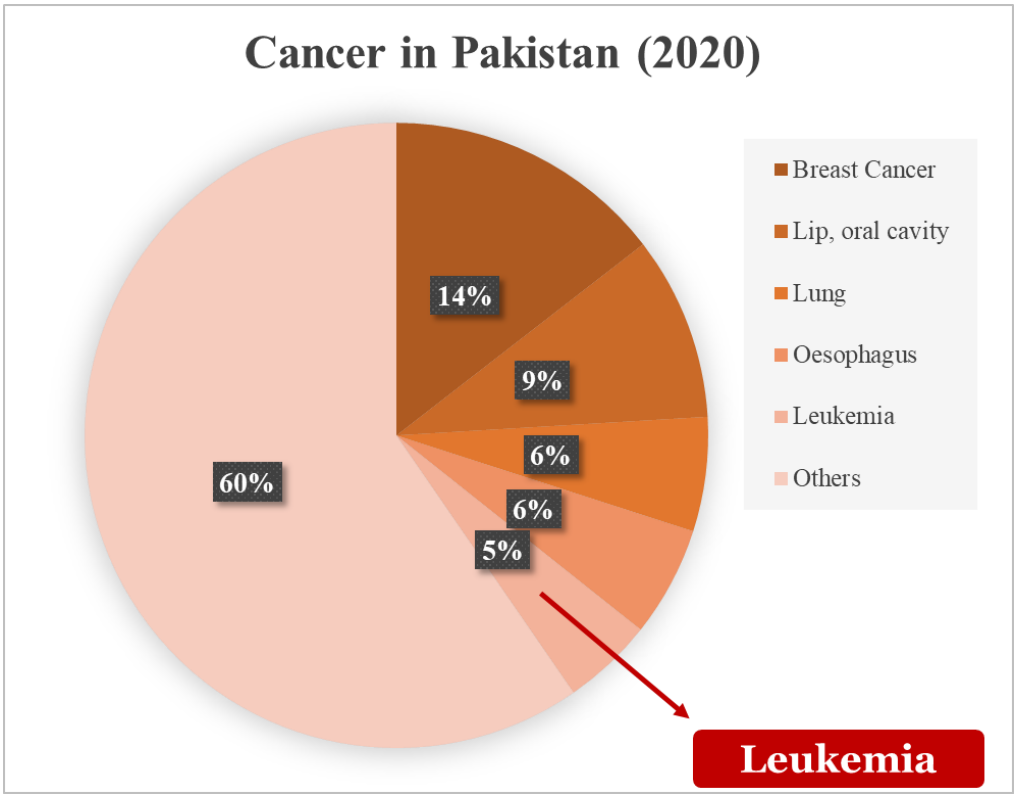

Figure 7 Leukemia prevalence in Pakistan (WHO, 2020)

2.2. Acute Myeloid Leukemia (AML)

Acute leukemia is a diversified set of hematological cancers categorized by clonal expansion of immature lymphoid or myeloid precursors (Memon *et al.,* 2017). Studies on etiology of acute leukemia have been emphasized for a long time, and Infection has been considered an etiology for many hematological cancers. (Ehsan *et al.*, 2015). As a result of several gene mutations and chromosomal rearrangements, hematopoietic precursor cells undergo clonal transformations leading to the development of AML, which is a heterogeneous group (Rubnitz *et al.*, 2010). AML is characterized by differentiation arrest & clonal expansion of progenitor cells of myeloid lineage. The cause of AML is heterogeneous. Most cases of AML persist without a clear cause, however in certain patients, previous exposure of pharmacological, industrial, or environmental DNA-damaging substances has been associated. (Shallis *et al.*, 2019). AML is bone marrow cancer, arising from the abnormal proliferation of white blood cells such as monocytes or granulocytes that are still developing from myeloid stem cells (Cancer Research UK, 2020).

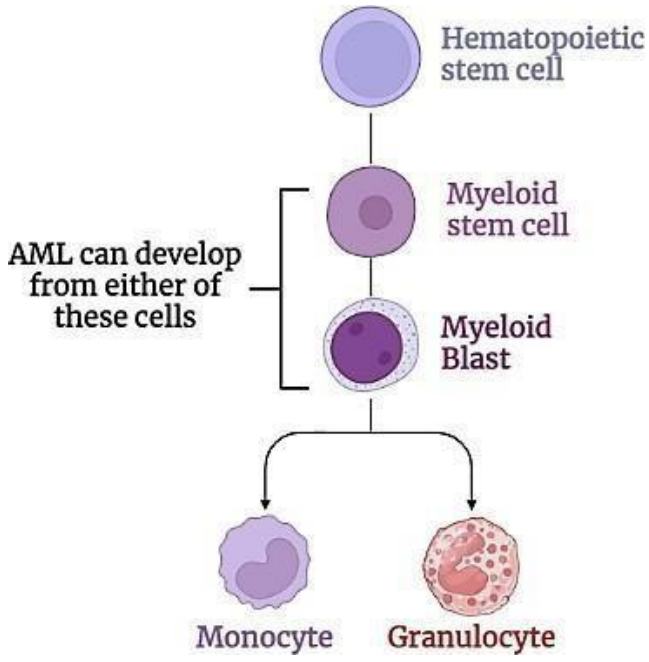

Figure 8 Origin of Acute Myeloid Leukemia.
AML can originate from abnormality either in myeloid stem cells or myeloid blast that give rise to abnormal monocytes or granulocytes. (Cancer Research UK, 2020)

2.2.1. Molecular pathogenesis of AML

AML is a clonal disease marked by several genetic abnormalities. At the time of diagnosis, most cases of AML can be defined by clonal heterogeneity, with an existence of an originating clone along with at minimum one sub-clone. (Enomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia, 2013). Cytogenetic studies revealed that recurrent variations in chromosomal structure are marked by acquired genetic defects (such as somatic mutations) which play critical roles in leukaemia pathogenesis (Mrózek, Heerema & Bloomfield, 2004). Several patterns of clonal expansion (occurring new genetic defects in leukemic blast cells) at the time of relapse are probable tolead to therapeutic resistance (Ding, Ley *et al.*, 2012).

Just recently, the finding of genetic and molecular changes has resulted in the refining of AML prognosis. Recurrent mutations in KIT, FLT3, NPM1, TET2 and CEBPA have been found using targeted DNA sequencing. (Acute Myeloid Leukemia: From Molecular Pathogenesis to Oral Targeted Therapies, 2019). Typical these genes play a role in the differentiation and regeneration of myeloid cells. These genes can lead to leukemogenesis upon mutations and alterations (Krönke, Bullinger, Teleanu, *et al.*, 2013). Genes linked to the epigenetic regulation in differentiation of myeloid cells which includes DNMT3A, IDH2, TET2 and ASXL1 are also found in preleukemic HSCs and appear early in the progression of AML. Preleukemic stem cells can differentiate into multiple lineages and resist chemotherapy, ultimately causing relapse. (Corces-Zimmerman, Hong, Weissman, *et al.*, 2014).

Molecular changes resulting in AML leukemogenesis are of two forms, for example, transcription control changes in HSCs leading to alterations in signal transduction molecules necessary for the growth receptors. Second is the mutations leading to the activation of signaling molecules causing changes in functions and expressions of transcription factors, impotent for usual differentiation of myeloid progenitor cells (Steffen *et al.*, 2005).

2.2.2. Deregulation of signaling pathways in AML

Several components of signaling pathways that drive hematopoietic cell proliferation and survival, such as RAS family members, receptor tyrosine kinases (RTKs), PI3K/AKT pathway and the RAF/MEK/ERK cascade, are disrupted in AML. The processes driving the dysregulation of signaling in AML involve either direct activation by gain-of-function mutations or increased expression, or indirect stimulation via mutation in upstream regulating molecules (Scholl, Gilliland, Fröhling *et al.*, 2008). Abnormal signal transduction promotes the survival and multiplication of hematopoietic progenitor cells in AML. Signal transduction may be activated by various kinds of genetic mutations affecting many signaling molecules, including RTKs such as KIT and FLT3 as well as members of the RAS family of guanine nucleotide-binding proteins. (Scholl *et al.*, 2008).

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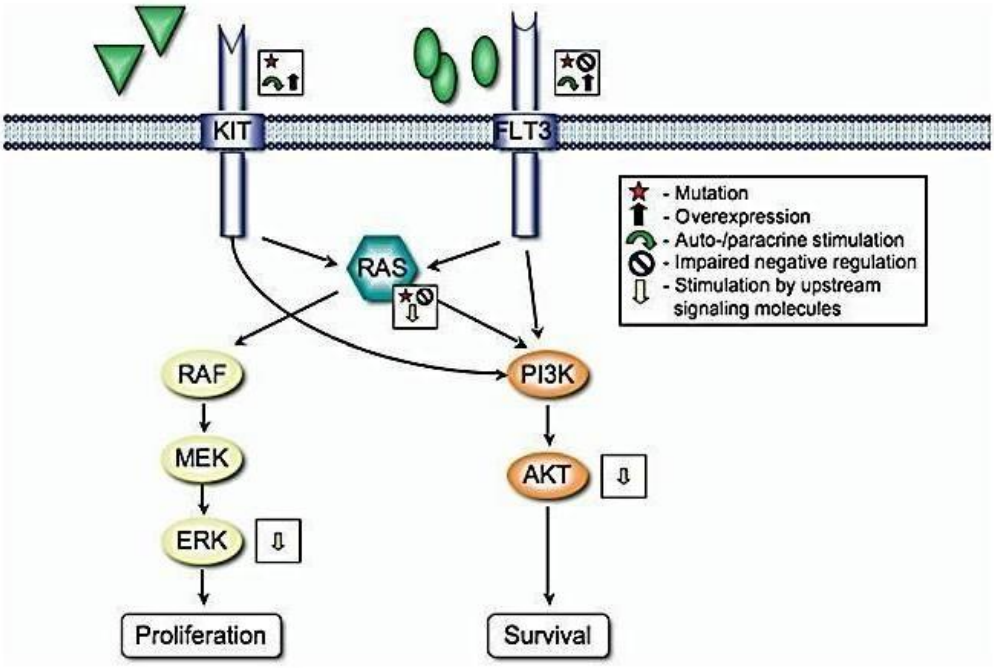

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Figure 9 Aberrations in signal transduction in AML
Activation of several upstream signaling molecules are often done by mutation, overexpression, autocrine/paracrine stimulation, or poor negative feedback. Abnormal activation of upstream signaling molecules induces continuous signaling via a small number of downstream effector cascades (for example PI3K/AKT and RAF/MEK/ERK), which results in increased survival & proliferation of HPCs (Scholl *et al.*, 2008).

2.2.2.1. JAK-STAT Pathway Activation

The JAK-STAT pathway is a signaling cascade that involves the activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) in response to extracellular signals such as cytokines, hormones, and growth factors. Once activated, JAKs phosphorylate STATs, which then dimerize, translocate to the nucleus, and bind to specific DNA sequences to regulate gene expression. This pathway plays an important role in immune response, hematopoiesis, and cell proliferation and differentiation (Moser, Edtmayer, Siepracka & Stoiber, 2021).

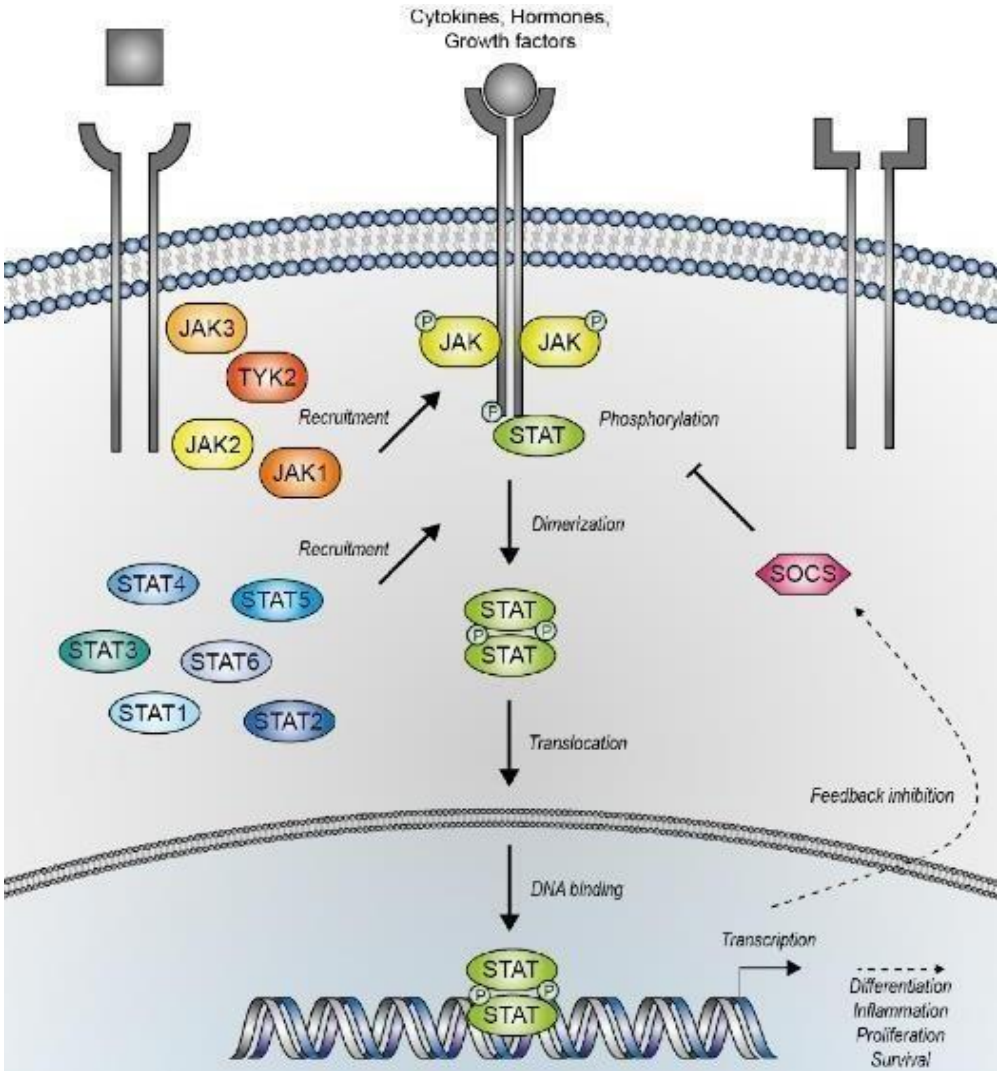

Figure 10 The JAK-STAT signaling pathway leading to differentiation, inflammation, proliferation and survival (Moser *et al*, 2021)

2.2.2.2. In Early Hematopoiesis

JAK-STAT pathway plays role in regulation of hematopoiesis, proliferation self-renewal and survival of HSCs, upon the phosphorylation of STAT5. However, its deregulation has been associated with many cancer types. Specifically, STAT5 has a central role in normal lymphoid and myeloid development (Wang & Bunting, 2013). Recent study revealed the role of JAK1 and JAK2 in HSCs. Conditionally, the deletion of JAK1 caused reduction in self-renewal capability and reformed differentiation in HSCs, whereas JAK2 knock-out was proved to be embryonically fatal because of inefficient erythropoiesis (Fasouli & Katsantoni, 2021). Furthermore, the deletion of JAK2 leads to the failure of BM and increased apoptosis in certain types of HSCs (Akada *et al*., 2014). Another study showed that JAK3 is vital for the development of innate lymphoid cells (Robinette *et al.*, 2018). TYK2 plays a critical role in regulation of B-cell tumors (Stoiber *et al.*, 2004).

A study confirmed that the activation of STAT5 is significant in the self-renewal of normal as well as leukemic SCs. (Kato *et al.*, 2005) and also helps to support the development and proliferation of blood cells. In mice that are deficient in STAT5A/5B, the hematopoietic potential to produce different blood cells is compromised (Snow *et al.*, 2002).

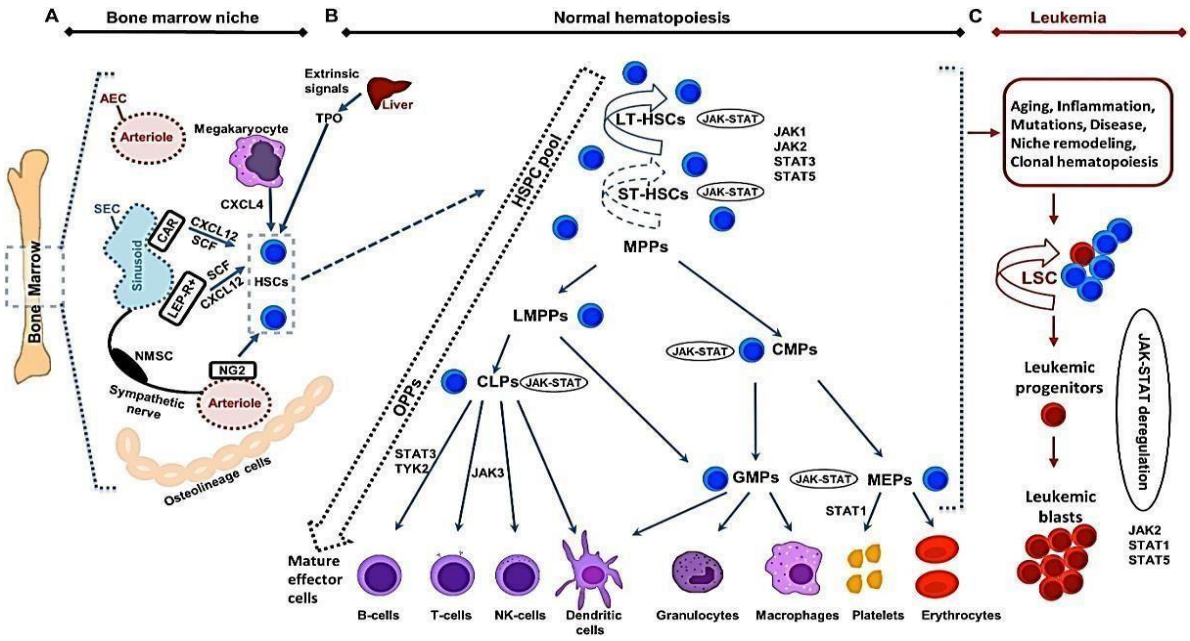

Figure 11 (A) Bone marrow niche. (B) Normal hematopoiesis and (C) Leukemic hematopoiesis- Aging, inflammation, mutations, disease, niche remodeling and clonal hematopoiesis leading to the formation of a LSCs (Fasouli & Katsantoni, 2021).

2.2.2.3. In Acute leukemias

Levels of CDC25A, a phosphatase important for proliferation and differentiation in AML expressing the FLT3-ITD mutation, are controlled by a complex STAT5/miR-16transcription and translation pathway, confirming that FLT3-ITD/STAT5/miR-16/CDC25Ainterplay is important for AML cell proliferation and differentiation (Sueur *et al.,* 2020). Furthermore, induced inflammatory response in the human AML niche leads to increased activity of the JAK/STAT pathway in AML blasts and BM stromal cells promoting leukemicproliferation (Habbel *et al.,* 2020).

Since the 1990s, STAT1, STAT3, and STAT5 have been consistently identified to be activated in all acute leukemic cells (Gouilleux-Gruart *et al.,* 1996, 1997). STAT1 has been presented to promote the growth of tumor in the leukemia development (Kovacic et al., 2006), whereas STAT5 has a vital role in the development of both myeloid and lymphoid lineage malignancies. AML development is a multi-step and- cause process (Gruszka *et al.*,2017), in which the most common gene to be mutated is FLT3 (Ley *et al.,* 2013). A study explained that mutated FLT3 specific to AML can cause the induction of STAT5 targeted genes (Mizuki *et al.,* 2003) as well as other mutation like FLT3-D835 (*Taketani et al.,* 2004).Another study showed that CDC25A levels, which is a phosphatase essential for differentiation and proliferation in AML with mutation of FLT3-ITD, are regulated by a complex pathway involving STAT5 and miR-16. This result suggested the interplay betweenFLT3, STAT5, miR-16 & CDC25A is fundamental for the of AML pathogenesis (Sueur et al., 2020; Fasouli & Katsantoni, 2021).

2.2.3. The PI3K-Akt-mTOR Signaling in AML

The PI3K-Akt- mTOR is an intracellular pathway which abnormally upregulated in AML and plays an important role in leukemogenesis, metabolism and in the bioenergetics of these cancer cells which may depend upon the downstream signaling (Nepstad *et al.,* 2020).

mTORC1 is activated in all cases of AML, whereas PI3K is activated only in 50% of the samples of leukemia. The activity of PI3K is related to the expression of p110δ, which is

an isoform of class IA PI3K in leukemic cells. However, in 70% of AML samples, autocrine signaling of IGF-1/IGF-1R results in PI3K activation, and still the targeted inhibition of this pathway fails to induce apoptosis. Moreover, In vitro, targeted blocking of mTORC1 or PI3K/AKT alone results in anti-leukemic effects that mainly work by inhibiting proliferation (Park *et al.*, 2010).

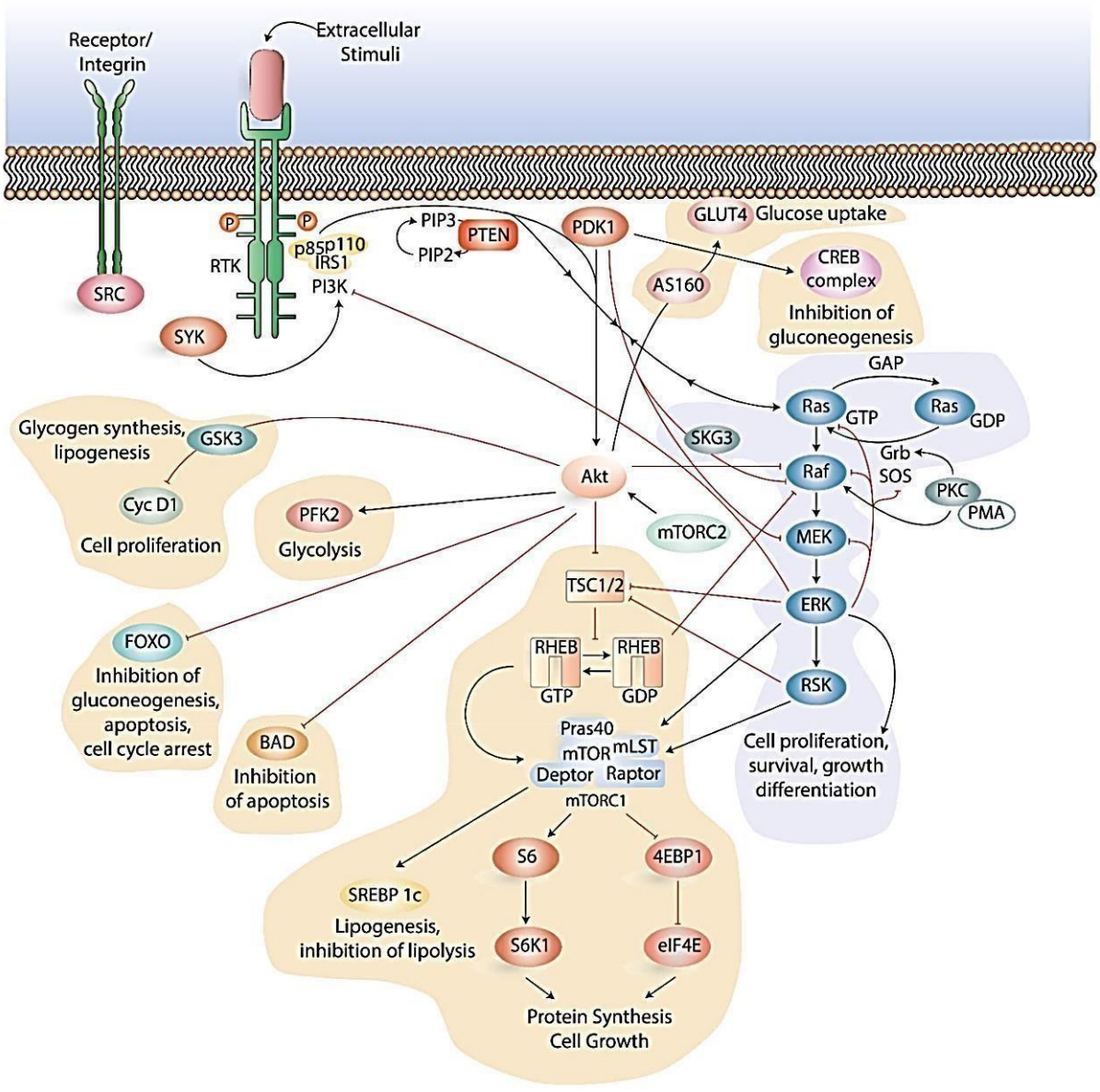

Figure 12 Crosstalk between the Ras-Raf-MEK-ERK pathway, the PI3K-Akt-mTOR pathway and the SYK pathway (Nepstad et al., 2020).

2.2.4. Wnt Signaling in AML

Wnt signaling pathway is necessary for leukemic stem cell maintenance. Studies showed the abnormal expression of components of the Wnt pathway such as WNT2b, LEF-1, and WNT1 (Gruszka et al., 2019). Moreover, the translocation products including AML1/ETO, PML/RARα, or PLZF1/RARα control the genes linked with Wnt signaling. (Müller-Tidow et al., 2004). Mutations in FLT3 constitute approximately 30% of all AML cases. In myeloid progenitor cells, the association between the Wnt signaling and FLT3-ITD mutation was examined. In cells with FLT3-ITD, a microarray study discovered higher mRNA expression of receptor FZD-4, further confirmed by Western blotting and RT-PCR (Tickenbrock *et al.*, 2005). Regardless of exposure to the ligand (Wnt3a), 32D/FLT3-ITD cells showed increased levels of β-catenin protein with respect to the control. Furthermore, wild-type FLT3 patients did not express large quantities of β-catenin levels, whereas 5/7 of the AML sampleswith these mutations expressed. TCF-dependent transcription, for example, the increase in Wnt target gene mRNAs, was induced by FLT3-ITD. In the presence of FLT3-ITD, Wnt3a slightly increased cell growth. AML occurs because of the mutations in HSCs or progenitorcells, further, up regulating the Wnt signaling by several mechanisms. Other mechanisms are inactivation of Wnt antagonist through methylation of promoter. The molecules modulating Wnt signaling are β-catenin, LEF-1, PSMD2, phosphorylated-GSK3β, PPARD, sFRP2, RUNX1, XPNPEP, PCDH17, AXIN2, CXXC5, PTK7 and LLGL1. (Gruszka et al., 2019).

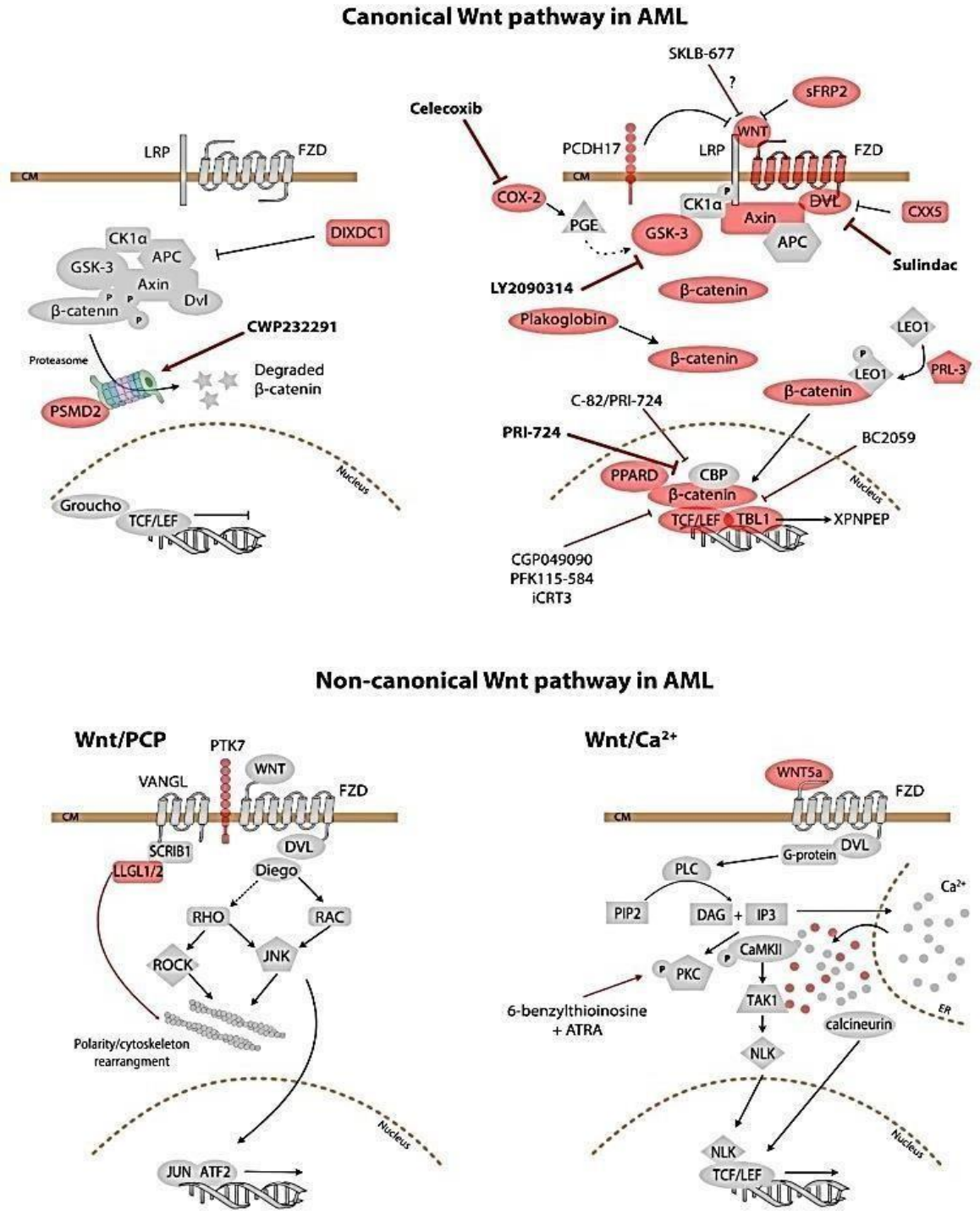

Figure 13 Wnt signaling in Acute Myeloid Leukemia (Gruszka et al., 2019).
Coral red-colored proteins have been involved in AML specifically. The thin arrows with red bar-head indicate the sites where inhibitors in investigation act, while thick arrows with bar-headed show the specific targets of agents that are used in clinical trials.

2.3. Acute Myeloid Leukemia with *t (6,9)*

AML is classified into several subtypes, one of which is highly aggressive, with translocation between chromosomes 6 and 9, which accounts for only 1% of all AML cases (Huret, 2013). The *t (6,9)* translocation is rare genetic anomaly that results in the formation of chimeric oncofusion gene named as DEK-NUP214 (previously it was called CAN) and is reported in less than 2% of the children or adult cases of AML as well as in less often in some cases of Ph-negative chronic myeloid leukemia and myelodysplastic syndrome (Moraleda, Alonzo, Gerbing, Raimondi, Hirsch *et al*, 2012). Studying disease-defining translocations in AML, such as t (8;21)-RUNX1-ETO, t (15;17)-PML-RAR, or t (6;9)-DEK-NUP214, has made it possible to better understand disease-causing molecular pathways and recognize the vital role that aberrant transcription regulators play in the development and progression of AML. (Alcalay, Orleth, Sebastiani *et al*, 2001). The WHO classification defines AML with the *t (6,9)*as a distinct disease due to its unique biology and clinical characteristics and unfulfilled treatment needs (Arber, Orazi, Hasserjian, Thiele, Borowitz *et al*, 2016). Studies reported *t (6,9)* AML patients are young adults mostly, with an average age of 23 to 40 years as compared to other AML subtypes having median age of about 66 years (Arber *et al*, 2016). The rate of complete remission is less than 50%, and the median survival following diagnosis is just around a year. (Tarlock, Alonzo, Moraleda *et al*, 2014). In the first Complete Remission, the only available curative therapy is hematopoietic stem cell transplantation (Kayser *et al*, 2020; Chiriches *et al*, 2022).

2.4. 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 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 inhibiting angiogenesis and HIF in RCC. In preliminary study (David JM. *et al.,* 2013), the GA turned out 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 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. Gramicidin A was more effective than Salinomycin in IC50 values against the cancer cells tested. GrA can induce apoptosis. 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 appeared. These microvilli-like protrusions were reduced through GrA treatment in pancreatic cell lines. In contrast, with salinomycin no discernible differences were observed in the 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 theprotrusions 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.

The chemical structure of gramicidin and its toxic effect on gastric cancer cells SGC-7901 and BGC-823 cells proliferation. a Chemical structure of gramicidin. The cell survival rate of b SGC7901 and c BGC-823 cells which were treated with 0, 0.3, 1, 3, 10 and 30 μM of gramicidin respectively in 96-well plate were quantitatively analyzed by CCK-8 assay. The

results are shown as the mean±SEM of three independent experiments (n=3, *P<0.05, **P<0.01 and ***P<0.001 vs. Control)

Gramicidin A induces apoptosis and cell proliferation of Human gastric cancer cells (Chen at el., 2019). G2/M cell cycle arrest is also caused by Gramicidin A in human gastric cancer cells. Gramicidin A effect on the human GSCs cell cycle was then investigated. Propidium iodide (PI) staining was used to determine cell cycle distribution, after that flow cytometry was used. After gramicidin treatment, SGC-7901 cells percentage was lower in the G1 phase as compared to control group G1 phase after 24 and 48 hours. The population of cells SGC-7901 were increased in the G2/M phase after Statistical analysis. The results showed that the cell cycle was stopped by gramicidin A at the G2/M phase.

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). For the identification of potential therapy targets in cholangiocarcinoma cells, total RNA isolated from RBE cells treated with PBS for 12 hours were used. The scientists identified differentially expressed genes (DEGs). 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).

CHAPTER 3

3. METHODOLOGY

3.1. Methodology-In Silico

In Silico approaches were used to assess the anti-cancer activity of the drugs against our targeted cell lines and the results were then used to compare the possible targets and add to the efficacy of the in vitro experiments. The molecular docking procedures can be employed to model the interaction among different ligands and our target proteins at the atomic level which allows us to study their mode of interaction at the binding site of the protein and contemplate the biochemical process they give rise to. (Meng et al., 2011)

In the docking procedures the binding site or pocket of the macromolecule is mostly known and if not, then the normally used approach in current docking resources employes a flexible ligand along with a rigid receptor however it's not the rule of thumb and a different approach may vary depending on the software being used. The docking process efficacy basically rests on two interlinked steps, first is scoring of the sampled conformations in which the ligand sits in the binding pocket of the target protein molecule and then ranking them accordingly based on their respective scoring functions (Kitchen et al., 2004b). Using the molecular docking procedures, we can get to know the possible targets and analyze their efficacy quantitatively which would yield our possible results, that would aid in the chain of experiments next in the line.

3.2 Molecular Docking

3.2.1 Molecular structure of ligand

1. Obtaining the protein SDF Structure

The molecular structure of Gramicidin A was obtained from PubChem and downloaded in SDF format to be further processed for its use in the molecular docking procedure. The SDF format of Gramicidin A as our drug was chosen as a ligand to interact with possible macromolecule targets down in the signaling line.

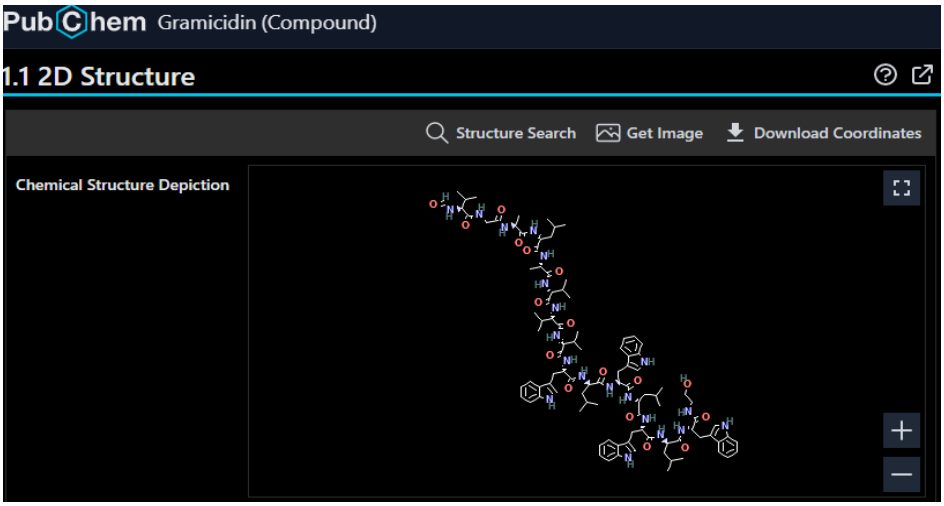

Figure 14: Obtaining Gramicidin A molecular structure from PubChem.

2. Open Bebel

The software Open Bebel was used to convert the saved SDF Gramicidin A structure to MOL2 format to be analyzed using the software, Chimera.

3. Chimera

Chimera is a 3D molecular visualization software used to analyze protein structure at molecular level down to their specific chains. The Gramicidin A ligand (protein) was uploaded, and the structure was cleaned for any unbound or residual chain of non-interacting atoms and then exported in PDBQT.

3.2.2 Molecular structure of target protein/macromolecule

3.2.2.1. UniProt

UniProt is a large resource of protein database, their sequences, along with their associated detailed annotations ("UniProt: The Universal Protein Knowledgebase," 2017). Uniprot was used to obtain the target protein sequences for both FKH-1 and U937 cell lines that were suspected to be involved in the downstream signaling of the cancer progression and relapse. 12 proteins for FKH-1 and 8 proteins for U937 were selected to serve as the target molecule, interacting with our ligand to see their mode of interaction and possible binding abilities with each other.

3.2.2.2. NCBI Protein BLAST (Basic Local Alignment Search Tool)

A protein BLAST was carried out by using NCBI protein BLAST function, for the selected U937 and FKH-1 proteins to get the already annotated and computed protein sequences similar to the one's we are targeting. The results were then analyzed on the basis of their percentage identity and similarity with other protein structures, choosing the one with the highest percentage identity and query cover.

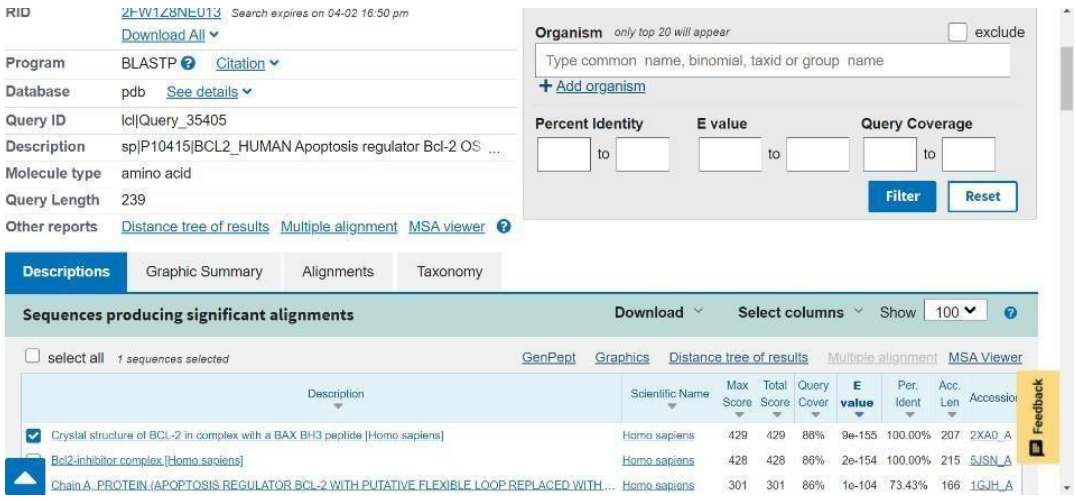

Figure 15 NCBI BLAST of selected proteins as macromolecule drug target.

The protein ID of the selected protein is then searched on PDB RCSB for its molecular structure at the chain and peptide level of the protein to look for the specific chain that is suspected to be interacting with Gramicidin A and the complete protein structure was then saved for its use as a macromolecule in the further steps of molecular docking.

3.2.2.3. Molecular Docking (Pyrx)

Molecular docking is performed by using PyRx software, it is a user-friendly tool that combines some more important tools like Autodock, Autodock Vina and Open Bebel which makes it a much better option than using autodock alone (Kondapuram et al., 2021).

The target protein/macromolecule and the ligand are uploaded in their designated PDBQT formats and the scoring sites are marked to look for interactions in those specific sequences for ligand and protein, as PyRx was used so there is no hard and fast rule to select the x, y and z grids to look for interaction, a general boundary was marked around the ligand and macromolecule to look for interactions in all possible sites and conformations of the ligand and macromolecule.

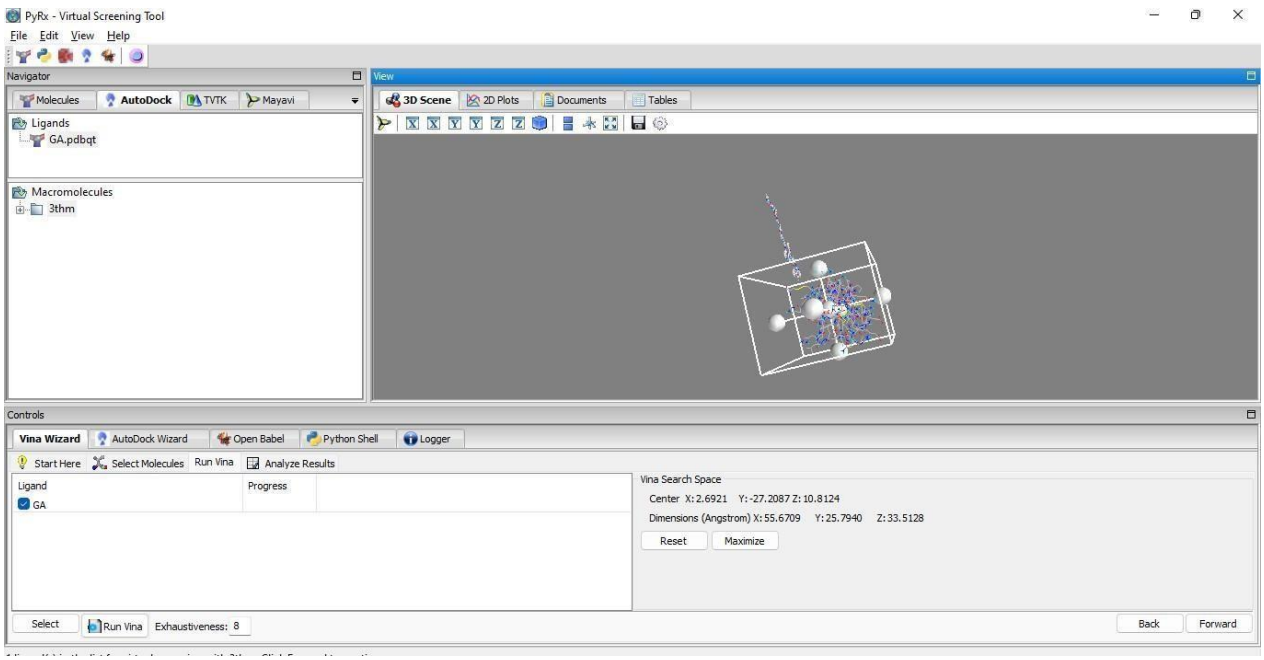

Figure 16: PyRx interface, showing the grid selection for the interaction among Gramicidin A and 3thm (An FKH-1 cell line target protein).

Docking is started, after some hours the results will be displayed in tabular form showing the B.E (Binding Energies) of the interactions among ligand and target molecule. As discussed earlier, the B.E refers to a specific interaction at a specific pose and that would be used as a reference in the visualization steps next in the line.

3.2.3 Visualization

This step comprises of ranking the scored interaction (in terms of B.E of interacting ligand and macromolecule while docking) and analyzing their best poses, also addressing the type of interaction they represent.

3.2.3.1. PyMol

PyMol software was used for visualization of the interactions, as it offers great 3D viewing functionalities and generate publication quality images of molecular structures along with other advanced rendering functions. (Seeliger & De Groot, 2010)

3.2.3.2. BIOVIA- Discovery Studio

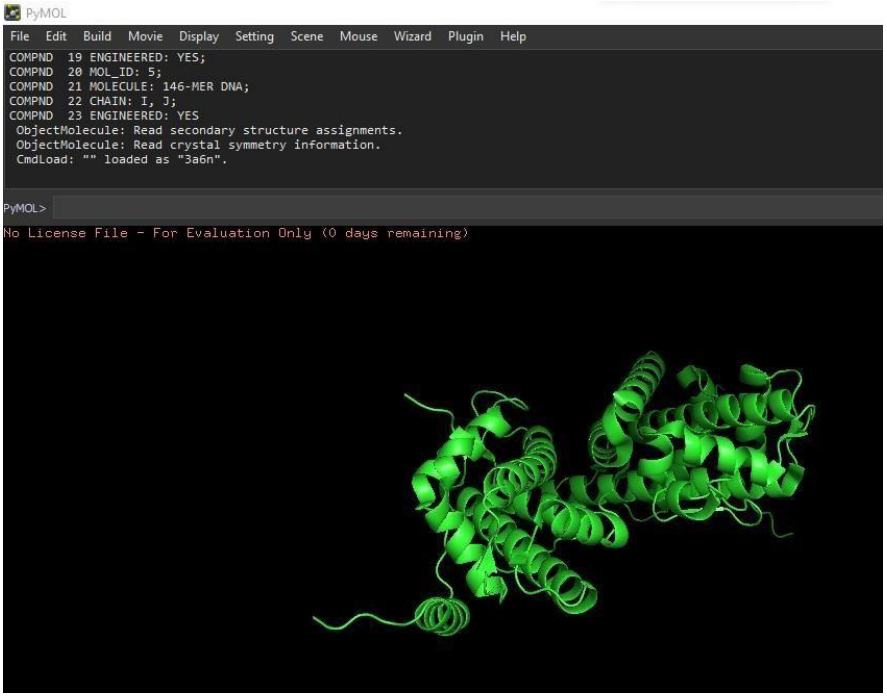

Figure 17 PyMol interface, showing 3a69 (An FKH-1 cell line GA target protein).

The next steps were performed on BIOVIA discovery studio visualizer software a protein modeling suite. It efficiently visualizes the superimposing poses and helps identify the type of interactions involved in the designated conformation (Nataliia, 2023). The ligand and the macromolecule were uploaded in the best pose with the lowest B.E, which was previously exported from Pymol.

The interactions between the target protein/macromolecule and Gramicidin A in different binding pockets were shown in the form of discrete bonds represented by lines and dots and following that, the interacting atoms of the ligand and macromolecule were labelled for the type of ionic or non-ionic bump interactions they were involved in giving a more precise idea of how our targeted protein would respond to Gramicidin A.

3.3. In Vitro Methodology

3.3.1. Cell lines in the study

In this investigation, the cell lines used for AML were FKH-1, U-937, NB4 and K562.

Table 1 Cell lines Used

FKH-1	Acute Myelocytic Cell line
U-937	Human Monocytic Leukemic Cell line
K-562	Chronic Myeloid Leukemic Cell line
Nb4	Acute Promyelocytic Leukemic Cell line

3.3.2. Culturing of cells

To prevent any contamination of the cells, both cell lines were maintained in sterileconditions using RPMI-1640 (Gibco Life Technologies) mix supplemented with 10% fetal bovine serum (Gibco Life Technologies) for all cell lines except FKH-1, for which 20% fetalbovine serum was used, 1% Penstrip (Biowest), and an antimycotic agent Amphotericin in concentration of 0.25 – 0.5 ug/ml was used. For the best growth of these cells, a water jacketed humidified incubator was used for maintenance at 5% CO2 and 37°C.

Approximately 1 x 10^6cells/ml for U-937, K-562, and Nb4 and 2 x 10^6 cells/ml were kept in culture to prevent any type of contact inhibition.

3.3.3. Freezing of cells

Two freezing solutions were created to freeze or cryopreserve these cells. Solution Icontained 80% RPMI 1640 and 20% DMSO, while solution II had 70% RPMI 1640 and 30%FBS.

Separate eppendorf tubes containing cells were used for centrifuging them at 1200 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. 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.

3.3.4. 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 generously with ethanol before being taken inside a laminar flow hood. Transferred to Eppendorf, the suspension was centrifuged at 1200 rpm. The pallet was resuspended and dissolved in media prepared beforehand which also contained FBS; 10% for all cell lines except FKH-1 which had 20% FBS. After that, the cells were grown in a 6 well plate in a C02 incubator. Media was replenished every 2 days for all cell lines except FKH-1 for which after every 3 days media was refreshed.

3.3.5. 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 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 centrifuge tubes, pelleted, and then resuspended in 1 ml of 10% culture medium for all cell lines except FKH-

1 for which 20% FBS was used. Reconstituted cells were vortexed before being put in 10ul portions onto a paraffin strip and then diluted with 40ul of trypan blue dye. By pipetting up and down, homogenized it. Finally, a hemocytometer was filled with 10ul of dilution. Due to a damaged plasma membrane, non-viable cells absorb the dye and appear 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:

No of cells in grid 1+ grid 2+ grid 3+ grid 4 X dilution factor X 10,000/ml

3.3.6. Drug Dilutions

The compound Gramicidin A was first dissolved in its solvent DMSO. The initial stock solution has a concentration of 2mM. From the stock solution, four drug concentrations were prepared in 10% FBS media (for U-937, K-562, and Nb4) and 20% FBS for FKH-1. 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:

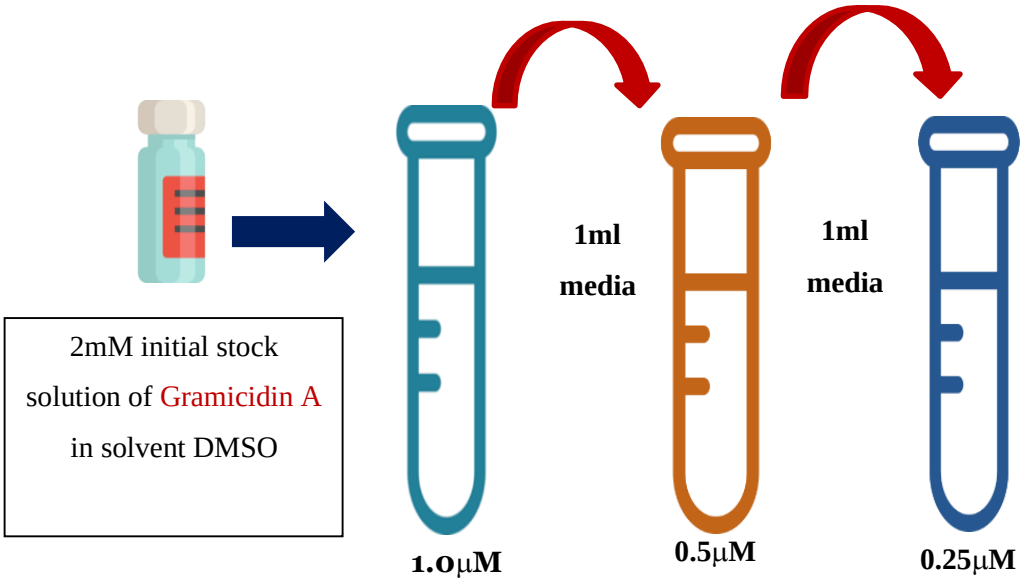

Figure 18 Serial Dilution of 2mM drug in 10% FBS media

3.3.7. MTT Assay

This is a colorimetric assay, and the live cells turn the tetrazolium dye into purple-colored, insoluble formazan crystals based on their me tabolic activity. In a 96-well plate, 15000 cells per 50ul were plated. In triplicate wells, 50ul of the inhibitor gramicidin A at various doses (1uM, 0.5uM, and 0.25uM and 0uM) were added. 50ul of cells were treated with 50ul drug with 0.01% DMS0 in the triplicate -ve control. Additionally, a duplicate plating of 100ul culture media (used to create drug dilutions) was performed. This was done to reduce, if any, the impact of the drug solvent and medium. The plate was then left in an incubator for 72 hours (the time it takes for the FKH-1, U-937, NB4 and K562 cell lines to double) 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 allow crystal formation after the MTT dye addition.

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.

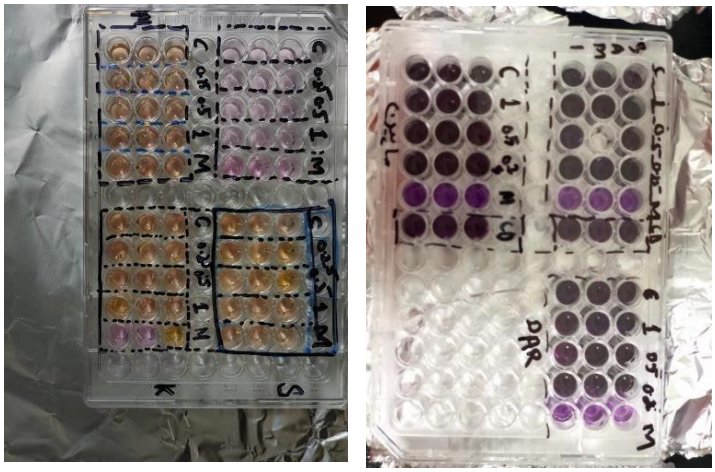

Figure 19 (a) MTT cytotoxic assay pouring for U937 cell line, (b) Day 2 of MTT assay after incubation period of 48 hours for U937

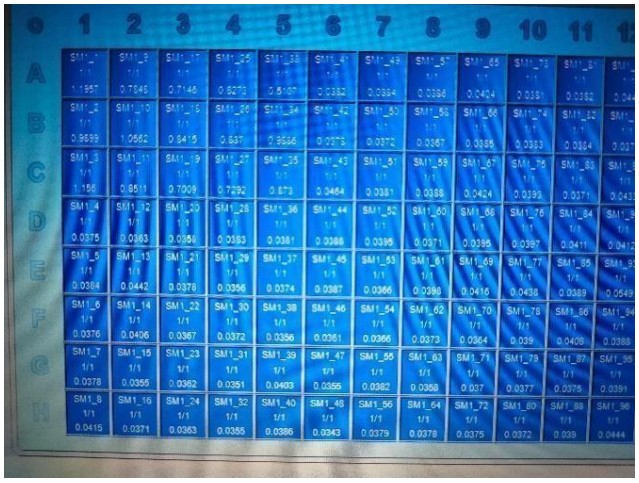

Figure 20: MTT analysis via spectrophotometery

3.3.7.1. Treatment of cells with gramicidin A

FKH-1, U-937, NB4 cells and K562 cells were grown in a 6-well plate at a density of 1.0 x 10^6 cells/ml and subjected to a 72-hour inhibitor treatment at a dose of 0.25 uM and 1uM for all cell lines. Following treatment, RNA was extracted from the treated cells, and cDNA was then synthesized for gene expression experiments.

3.3.8. RNA Extraction

A 2ml eppendorf was filled with 1-2 million treated cells, and the sample was spun for 5 minutes at 1200 rpm. The supernatant was discarded after which 500ul of chilled PBS was added and centrifuged once more at 1200 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 homogenize 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 12000xg and 4 °C. Three layers of the mixture were identified: an upper aqueous layer containingRNA, 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 Eppendorf. The remaining Eppendorf containing DNA and proteins was stored at -80°C for future use. Now inEppendorf in which RNA was added, 500ul of chilled isopropanol was added, and the tube was shaken well before being incubated on ice for 15 minutes. The tube was centrifuged for20 minutes at 12000 xg and 4°C. The resulting pellet was rinsed with 75% chilled ethanol and centrifuged once more at 7500 xg and 4°C for 5 minutes, discarding the supernatant in the process. Carefully removing 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.

3.3.8.1. RNA Quality and Quantity Check

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

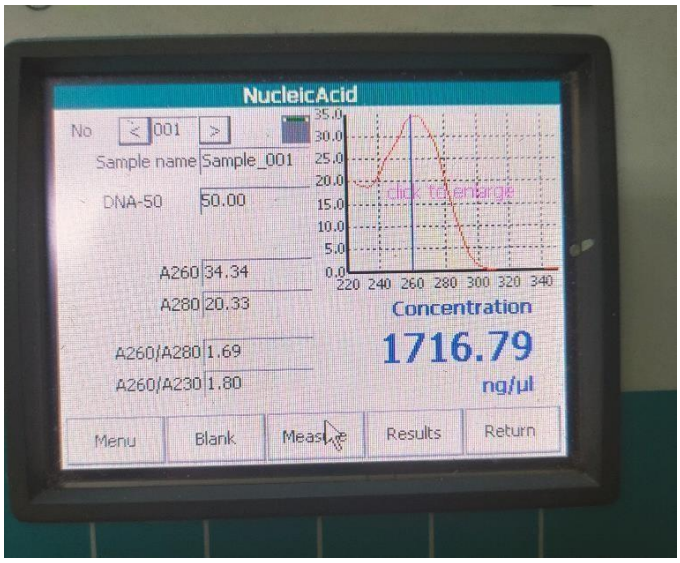

Figure 21 Nanodrop analysis for FKH1 cell line

3.3.9. Complementary DNA (cDNA) Synthesis

1000ng of RNA was used as the reverse transcription template to create the cDNA the very same day RNA was extracted. Thermofischer cDNA kit was used. 1uL (10uM) of oligo dT20 was taken 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.

3.3.9.1. 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. GAPDH is a housekeeping gene, and its presence ensures the presence of cDNA. 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.

3.3.9.2. 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.

3.3.10. 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. 3 primers were used which were c-myc, EYA3, and AXIN. 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.

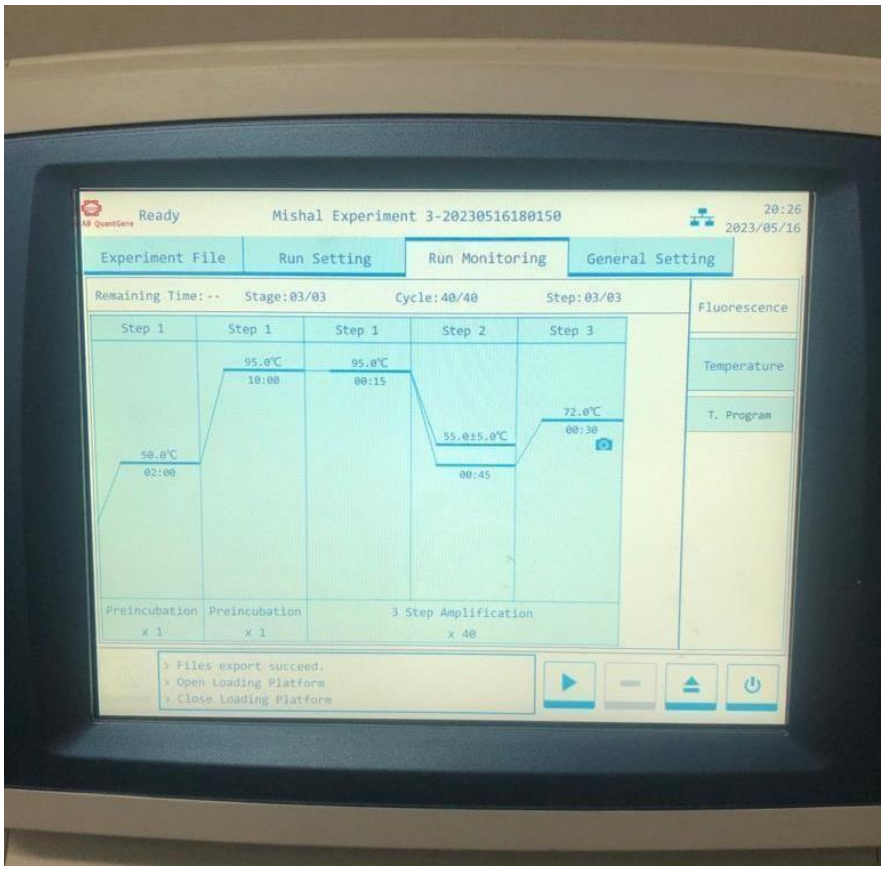

Figure 22 Optimization of RT-PCR for FKH 1 - cell line

3.3.11. Hemolysis Assay

To determine if Gramicidin A had a cytotoxic effect on healthy RBCs, a hemolysis assay was conducted in a 96-well microplate. Red blood cell death is referred to as hemolysis. Red blood cells and test materials were incubated at a pH range of 7.2-7.6 during this testing. Whole human blood was collected in an EDTA tube, centrifuged at 500 xg for five minutes at 4°C, 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 these 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 500 xg for 5 min at 4°C 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 = abs sample-abs negative control X100% abs positive control-abs negative control

CHAPTER 4

4. RESULTS

4.1. Insilico Results

The In-silico results yielded somewhat promising results that were pointing towards Gramicidin A being an effective drug against FKH-1 cell lines and U937 too. These results also supported the fact that our preliminary research also showed anti-cancer potential of Gramicidin A against the K562 and NB4 cell lines under the same drug concentrations.

The results were divided under the docking results and the visualization results of the previous data obtained. Docking results were in tabular form and contemplated the anti-cancer activity through different types of binding interactions in different poses.

4.1.1. Docking Results

The results of the specific cell lines who target proteins were selected and potentially targeted with our drug Gramicidin A. Docking results from PyRx were obtained in tabular form and then the best pose showing the most favorable interaction was selected for further analyzation of the results in the in-silico part.

Table 2 Some best target proteins that had the lowest B.E in interaction with u937 indicating good binding.

PDB ID	Cell Line	Protein name	Chain	Binding Energy
6JN2	U937	DOT1 in complex with AF10	A, B	-7.2
3THM	U937	TNFR (superfamily 6)	C (F)	-6.6
2ODB	U937	CDC42	A	-5.9

4.1.1.1. U937

A total of 8 protein targets were selected in case of U937 to be targeted by Gramicidin A. As we can see from Table 2, 6jN2 protein of the U937 cell line is having -7.2 B.E, which is the lowest among all the B.E of the target proteins which were selected for the U937 cell lines. This points towards the fact that these proteins will in turn be inhibited by the drug action of our selected drug. The docking results of U937 yielded promising results further validating our preliminary data of gramicidin A showing its anti-cancer potential and inhibiting the proliferation of the cancer cells as indicated by the B.E of the best interactions among the selected potential target proteins and gramicidin.

4.1.1.2. FKH-1

The family of selected proteins for the FKH-1 cell line were selected on the criteria of finding the similar target proteins against the gramicidin A so that there are favorable interactions among the protein and our targeting drug that would ultimately point towards its role in inhibiting the proliferation of the selected cell line.

In case of FKH1, 12 proteins were selected as the target proteins and were made to dock with Gramicidin A whose docking results were obtained in tabular form and then ranked in the order of their increasing B.E indicating the lesser favorable interaction trend.

Table 3 Results of FKH1 target proteins, the one's with the lowest of the B.E pointing towards the fact that they are good potential targets for Gramicidin A.

PDB ID	Cell line	Protein name	Chain	B.E
3FMO	FKH-1	Nup214	A	-8.8
1Q1V	FKH-1	DEK	A	-5.6
4KFZ	FKH-1	LMO & anti-LMO VH complex	A, C	-6.2

4.1.2. Importance Of Fkh1 Results

The results of FKH1 B.E were promising and important in a sense that the target protein which has the lowest B.E among all the 12 selected proteins in the cell line turned out to be Nup214. Nup214 is the nucleopore protein which was involved in the development and the manifestation of *t (6,9)* mutation which led to development of AML symptoms and its early onset.

The mechanism involved binding of the Nup214 protein with the DEK protein leading to the formation of the DEK/CAN complex which is the actual onco-fusion protein which was sought to be targeted as indicated in the objectives of our experiments.

4.1.3. Visualization Results

The visualization results followed the docking results. On the data available we used the B.E of the interactions in the best poses to visualize them on Pymol and look for the favorable and the best pose, that led, we were able to get the visualized results of each of their favorable interactions, in case of both FKH-1 and U937 respectively. After that using the exported poses to label and visualize the type of bonds were involved in the interactions and the type of bump interactions were leading to the changes in the mode of interaction of drug and the target protein.

4.1.3.1. PyMol

Pymol was used to visualize the best poses of our interacting protein and gramicidin A in the respective cell lines. The results were then exported to BIOVIA Discovery Studio for labelling the type of bonds and bond lengths.

4.1.3.2. BIOVIA Discovery Studio

BIOVIA Discovery Studio gave us a better publication quality image of what type of bonds and interactions were present at the molecular level among our protein and ligand.

Our motive was to find the potential target in the U937 and FKH1 cell lines, to eliminate them in the in vitro experiments and look for the effect of that on the proliferation of the cells of the respective cell lines. The Pyrx results already indicated Nup214 to be the protein with the lowest of the B.E making it the best among all the targeted proteins.

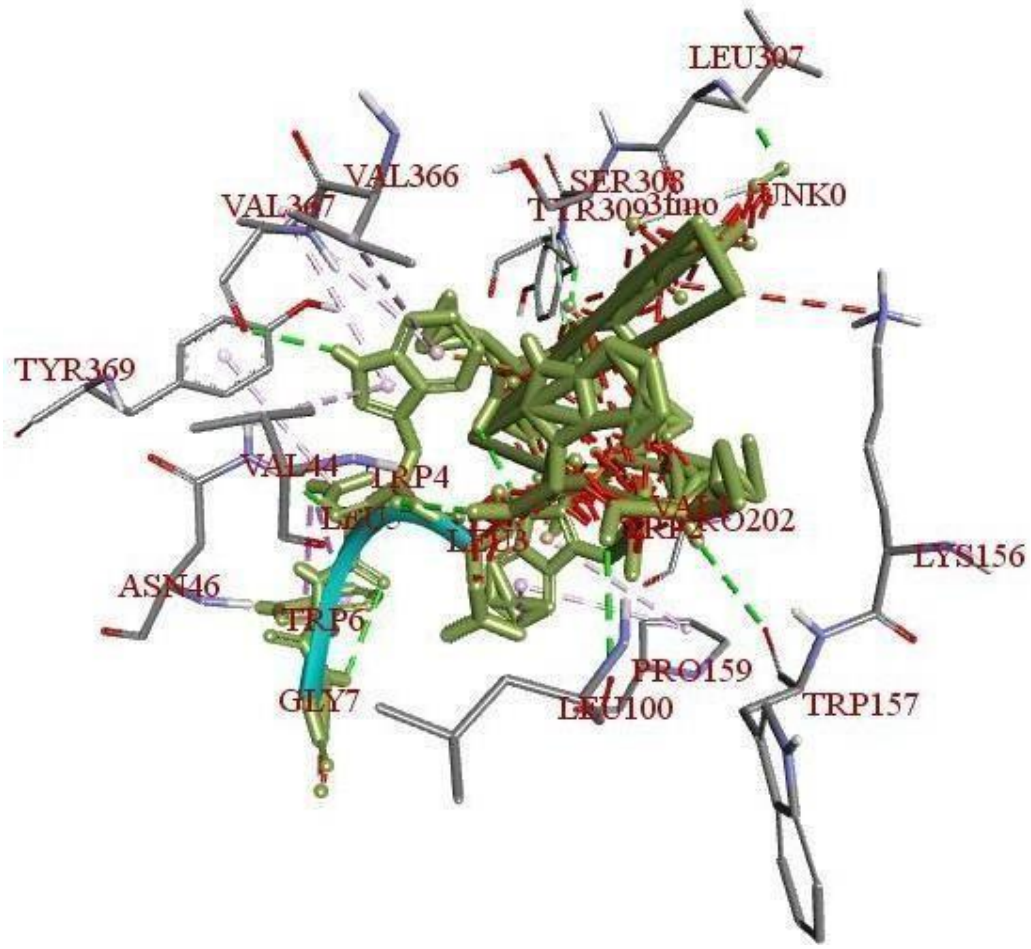

Figure 23 The type of bond interactions among Nup214 and gramicidin A along with their detailed bond lengths and type of residues involved in the bond.

Table 4 Shows the types of bonds involved among the 3FMO protein and Gramicidin A along with the residues involved in the bonds.

All these results point to Nup214 being the best target candidate for Gramicidin A in FKH1

Protein	Drug (Ligand)	Binding energy	Bonds/Interactions	Bond Length	Interacting residues
3FMO	GA	-8.8 KCAL/MOL	Conventional hydrogen bond	2.39 Å	VAL367
			Conventional hydrogen bond	2.70	GLY7
			Conventional hydrogen bond	3.22	LEU100
			Conventional hydrogen bond	3.06	TRP157
			Conventional hydrogen bond	2.27	TRP2
			Conventional hydrogen bond	2.73 Å	TYR309
			Pi-Cation Interaction	3.87 Å	TRP4
			Carbon hydrogen bond interaction	3.71	PRO202
			Pi-Alkyl Interaction	5.22	PRO159

cell line and the results from in vitro experiments were still required to validate these in silico results.

4.2. Invitro Results

4.2.1. Gramicidin A interferes with the proliferation potential of U937 cell line

In this study, the action potential of gramicidin A was assessed against U937 cell line, Cells were treated with different concentrations of gramicidin A and an MTT assay was performed after 48 hours to assess the proliferation.

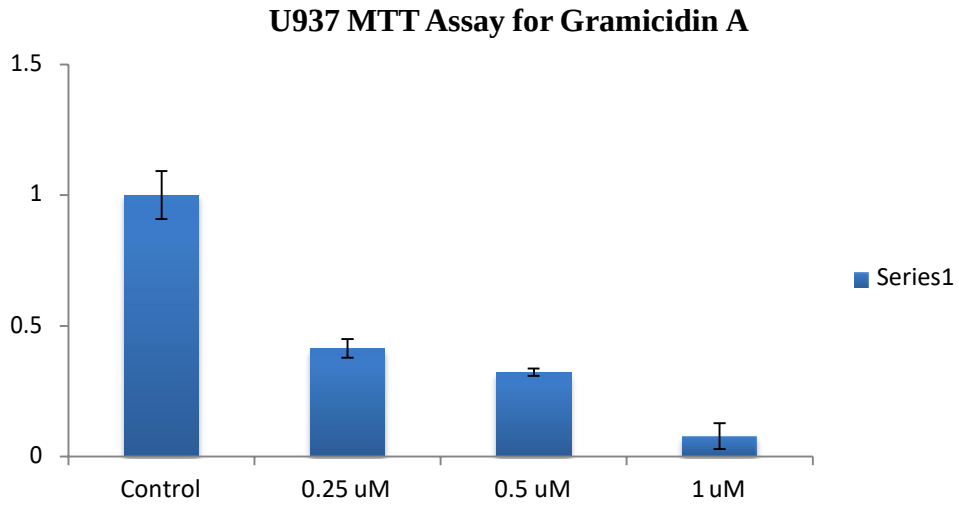

Figure 24 MTT assay results of Gramicidin A in U937 cell line

Cells were cultured in liquid medium (RPMI + 10% FBS+1% L-Glutamate and 1% Penicillin and Streptomycin) to determine the proliferation ability of U937 cells in the presence of 0.01% DMSO and different concentrations of Gramicidin A. Table shows that the antibiotic gramicidin A interferes with the proliferation potential of U937 cell lines.

4.2.2. Gramicidin A does not interfere with the proliferation potential of FKH cell line:

In this study, the action potential of gramicidin A was assessed against FKH cell line, Cells were treated with different concentrations of gramicidin A and an MTT assay was performed after 48 hours to assess the proliferation.

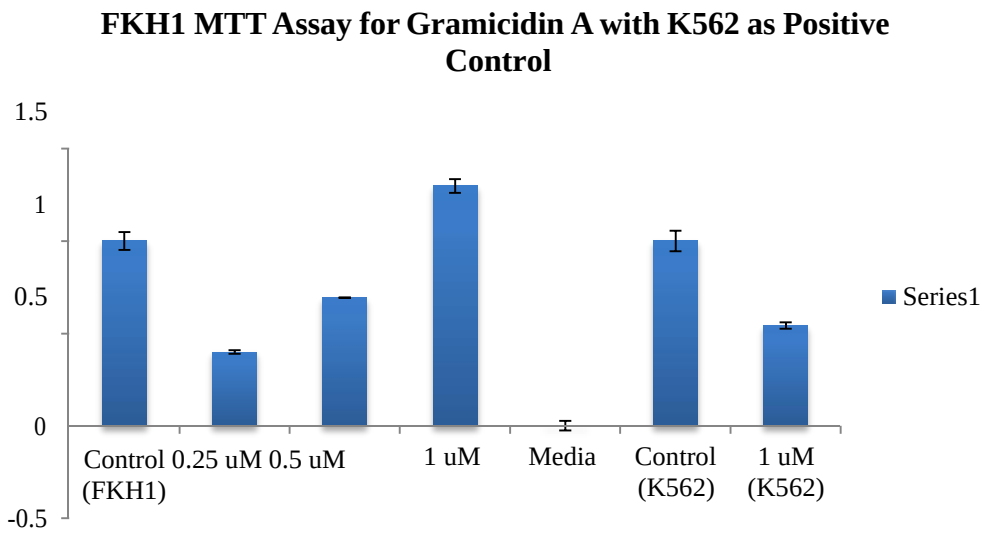

Figure 25 FKH1 MTT assay for Gramicidin A with K562 as positive control

Cells were cultured in liquid medium (RPMI + 20% FBS+1% L-Glutamate and 1% Penicillin and Streptomycin) to determine the proliferation ability of FKH cells in the presence of 0.01% DMSO and different concentrations of Gramicidin A. K562 was taken as positive control. Table shows that the antibiotic gramicidin A does not interferes with the proliferation potential of FKH cell lines and they became more aggressive after the treatment with the Gramicidin A .

4.2.3. Hemolysis Assay

To determine whether Gramicidin A has hemolytic effect or not, a 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:

$$\text{Hemolysis Percentage}=\frac{Sample\ Absorbance-Negative\ Control\ Absorbance}{Positive\ Control\ Absorbance-Negative\ Control\ Absorbance}\times 100$$

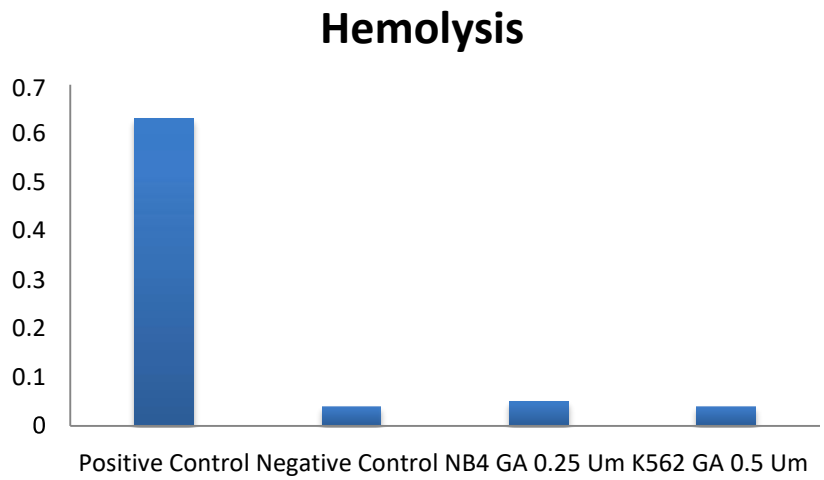

Figure 26 Hemolysis assay results of Gramicidin A in NB4 and K562 cell lines

In our study we found 0 % hemolysis in both of the cell lines. Hence proved that gramicidin A is nontoxic.

4.2.4. Effect on downstream signaling cascade in NB4 and K562 cell lines:

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 NB4 and K562 cell. Expression of *AXIN 2* gene, EYA 3, *c-Myc* genes were analyzed with different drug concentrations. In NB4, we observed reduction of gene expression by *AXIN 2* and *EYA 3* while *c-Myc* showed progression in tumorigeneses at 0.25μM gramicidin A concentration. However, in K562 at 0.5μM gramicidin Aconcentration *AXIN 2*, *c-Myc* and *Axl* enhanced the proliferation while *EYA 3* significantly caused the reduction in the gene expression.

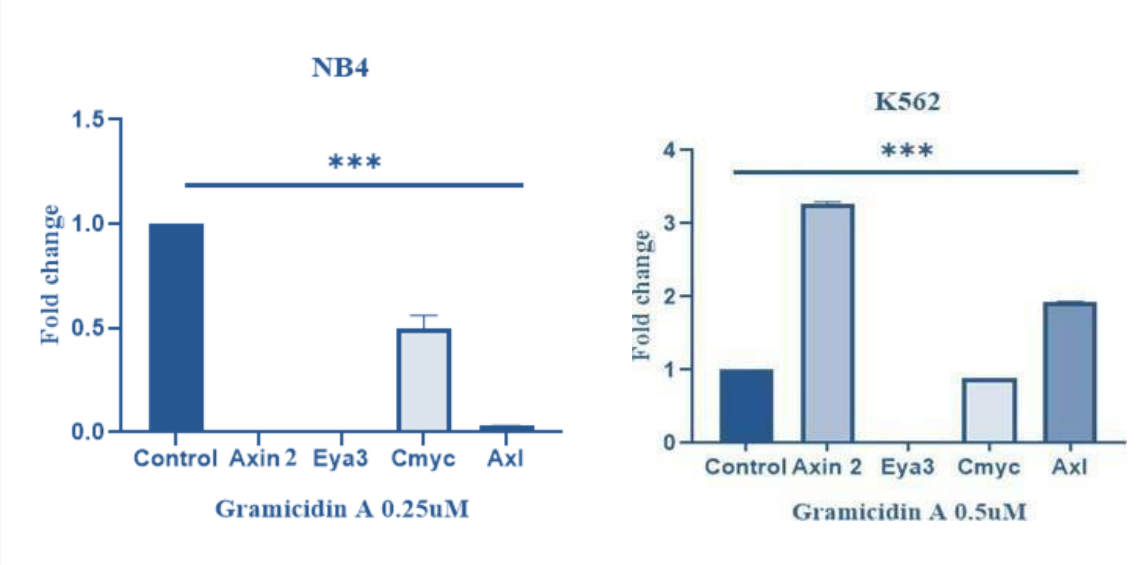

Figure 27 RT- PCR results of Gramicidin A in Nb4 and K562 cell lines

4.2.5. Gene Expression Analysis of Gramicidin A on FKH 1 cell line using RT-PCR:

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 FKH cell line. Expression of *EYA 3* and *c-Myc* genes were analyzed with different drug concentrations.

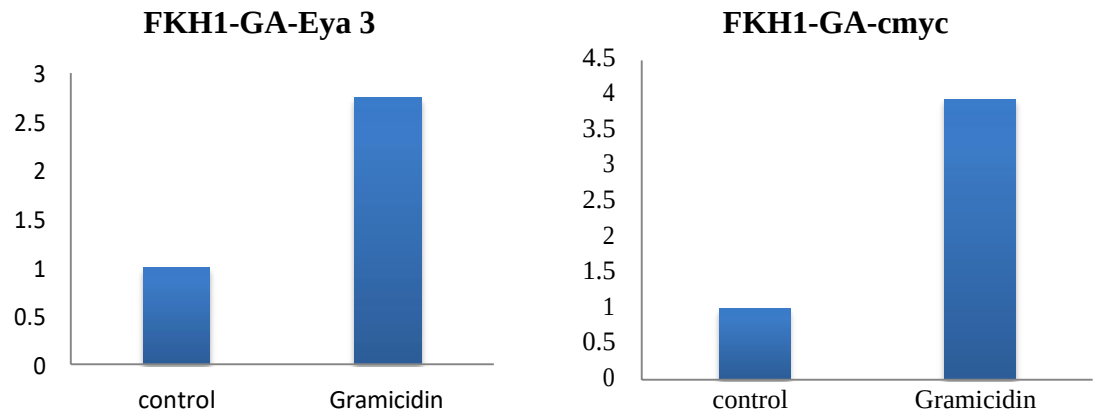

Figure 28 RT- PCR results of Gramicidin A in FKH-1 cell lines

CHAPTER 5

5. DISCUSSION

Gramicidn A is a natural linear peptide product of 15 –mer. Double dimerization is formed due to alternating D and L chirality that forms β-helix incorporated in a lipid bilayer. This specific structure works as a transmembrane channel for monovalent cations (Na^+, H^+, and K^+). (Xue.Y.W, 2022). Studies have revealed that it worked as a cytotoxic drug as well as a targeted angiogenesis inhibitor that makes a significant contribution in compromising cancer growth *in vitro* and *in vivo* as it is an effective tumor suppressor. (David.J.M, 2015)

Gramicidin A has shown anti cytotoxicity effect against many tumors. GA, the channel-forming ionophore, has cytotoxic and antiangiogenic activities in RCC (Renal cell carcinoma). The cytotoxic activity is because of ATP depletion, and the anti-angiogenic impact is because of the inhibition of HIF (hypoxia-inducible factor) through the induction of endogenously expressed VHL (von Hippel-Lindau tumor suppressor protein) (Xue.Y.W, 2022). Gramicidin (0.3 mg/mL) in combination with different concentrations of BisBAL NPs has shown promising synergistic antitumor effect towards HeLa cells, achieving an < 86% tumor growth inhibition. (Romero.C, 2021)

Our preliminary data suggested that Gramicidin A strongly interferes with leukemogenesis in CML and APL and showed no hemolysis. Gramicidin A upon testing in vitro interferes with the proliferation potential of PML/RARα-positive APL cells and BCR-ABL positive CML cells (Maham *et al.*, Manuscript in preparation).

For understanding its anti-cancerous potential, we hyphothized that the antibiotic Gramicidin A, known for its efficacy against CML and APL specific targets, can effectively bind to targets in *t (6,9) AML*, as all three leukemia types share similar targets, thus suggesting its potential as a targeted therapeutic approach for *t (6,9)* AML.

In context to *in-silico* study, Gramicidin A was docked with all the possible protein targets from AML cell lines, finalizing the potentially best targets based on lowest ligand-protein binding energy. The In-silico results yielded somewhat promising results that were pointing towards Gramicidin A being an effective drug against FKH-1 cell lines and U937 too. These

results also supported the fact that our preliminary researches also showed anti-cancer potential of Gramicidin A against the K562 and NB4 cell lines under the same drug concentrations. Regarding FKH 1 cell line *in silico* results, we found that Gramicidin A can target the NUP 214 region in the DEK- NUP 214 *t (6,9)* translocation. Through computational techniques, we were able to discover that Gramicidin A specifically targets the Nup214 gene in the DEK-NUP214 (previously CAN) translocation of AML.

After *in-silico* results, *in vivo* experimentations were performed suggesting that Gramicidin A has anti cancerous potential that can target AML cell lines of NB4, U937 and K562. These results after MTT cytotoxicty assay were also confirmed by gene expression analysis using RT-PCR technique. Expression of *AXIN 2* gene, EYA 3, *c-Myc* genes were analyzed with different drug concentrations. In NB4, we observed reduction of gene expression by *AXIN 2* and *EYA 3* while *c-Myc* showed progression in tumorigenesis at 0.25μM gramicidin A concentration. However, in K562 at 0.5μM gramicidin A concentration *AXIN 2*, *c-Myc* and *Axl* enhanced the proliferation while *EYA 3* significantly caused the reduction in the gene expression.

It was found that the genes were downregulated as Gramicidn A may have interfered with the signaling pathways of these AML cell lines. 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. (Maham *et al.*, Manuscript in preparation).

Recent findings have proved that Gramicidin A is effective against solid tumors but there is no authentic data that proves its efficacy against liquid tumors like AML. Our study is a novel approach that proved its efficacy against leukemic cell lines. In context to the FKH- cell line, we found that Gramicidin A was not able to inhibit the proliferation in FKH1 cell line. FKH 1 genes are poorly annoted and little is known about them. The inability of Gramicidin A drug at different concentration against FKH1 cell line, and instead leading to an aggressive

proliferation at even increased drug concentrations, shows a peculiar behavior, hence, pointing out significant gaps in literature corresponding to the lack of understanding of this cancer subtype. It also concludes that even the elimination of *t (6,9)* and reduced expression of DEK- NUP 214 complex was unable to contain the proliferation of this specific AML subtype pointing towards the fact there are some other underlying signaling pathways involved in these cell lines' proliferation that have not been studied or analyzed yet.

CHAPTER 6

6. CONCLUSION

Gramicidin A, an ionophore antibiotic, depicts tremendous cytotoxic and antiproliferative characteristics, making it a powerful tumor suppressor. Its centralized angiogenesis inhibition diminishes most cancer growth both in vitro and in vivo. The anticancerous abilities of Gramicidin A have been confirmed through MTT cytotoxicity assays carried out on U937, Nb4, and K562 cellular lines, further supported by the RT-PCR results. The promising anti-proliferative results of Gramicidin A that are targeting the leukemic cell lines is a novel therapeutic approach. Notably, the drug attains IC-50 value, with powerful inhibition even at low drug attention of 0.25 μM. These findings indicate the favorable position of Gramicidin A as a strong inhibitor in acute myeloid leukemia, specifically in U-937, K-562, and Nb4 cell lines. Additionally, Gramicidin A aggressively promoted proliferation in the FKH 1 cell line which highlights the need for comprehensive studies for a better understanding of *t (6:9)* High Aggressive AML.

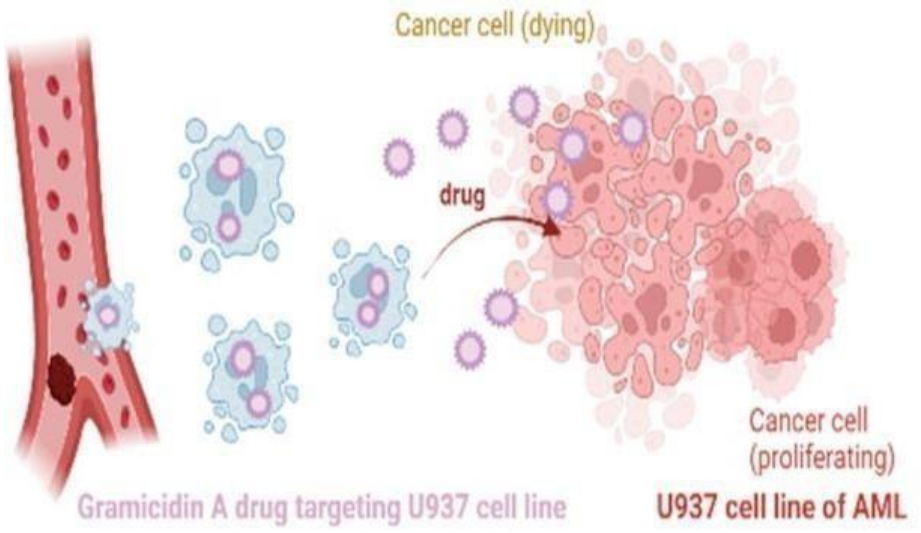

Figure 29 the anti-cancer potential of Gramicidin A has been proved through cytotoxicity assay in U937, Nb4 and K562 cell lines Except FKH1 cell line

CHAPTER 7

7. FUTURE PROSPECTS

- The anti-cancer potential of Gramicidin A, as has already been pointed out in various research is an avenue for targeted cancer therapy.
- It also points out significant gaps in literature corresponding to the lack of understanding of this cancer subtype.
- Our research serves to open avenues and provide opportunity to further investigate:

  - The role of genes involved in leukemogenesis.
  - The pathways pertaining to leukemogenesis.
- Further investigations may potentially validate the use of Gramicidin A as a targeted anti-cancer treatment for AML and its subtypes (CML, APL)

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