Finding the effects of Novel Organometallic Compound Phenanthroline in High Risk Acute Myeloid Leukemia

By

Maidah Shafique (Registration no.00000152386)

Navira Aslam (Registration no. 00000200630)

Sabahat Habib (Registration no. 00000151187)

Zoya Orangzeb Abbasi (Registration no. 00000150187)

Thesis Supervisor: Dr. Dilawar Khan

Atta-Ur- Rahman School of Applied Biosciences (ASAB) National University of Science and Technology (NUST) Islamabad, Pakistan.

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Thesis Supervisor: Dr. Dilawar Khan

Atta-Ur- Rahman School of Applied Biosciences (ASAB) National University of Science and Technology (NUST) Islamabad, Pakistan.

(2020)

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of BS FYP Thesis written by **Ms. Maidah Shafique (Registration No. 152386), Ms. Navira Aslam (Registration No. 200630), Ms. Sabahat Habib (Registration No. 151187)** and **Ms. Zoya Orangzeb Abbasi, (Registration No. 150187)**, of School **Atta-Ur-Rahman School Of Applied Biosciences (ASAB)** has been vetted by undersigned, found complete in all respects as per NUST Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of BS degree. It is further certified that necessary amendments as pointed out, during final presentation of the scholar, have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor: Dr. Dilawar Khan

Date: 10/8/2020

Signature (HOD): _____

Date: _____

Signature (Principal);

Date: _____

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Name of students:

Maidah Shafique

Navira Aslam

Sabahat Habib

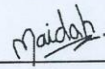Zoya Orangzeb Abbasi

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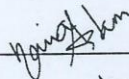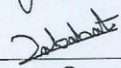
Team Members **Signature**

Maidah Shafique

Navira Aslam

Sabahat Habib

Zoya Orangzeb Abbasi

Date: 06-08-2020

Supervisor **Signature and Stamp**

Dr. Dilawar Khan

Date: 10/8/2020

Dedicated to our beloved parents

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1. ABSTRACT

Acute myeloid leukemia (AML) is one of the common forms of acute leukemia, is a malignant disorder that is identified due to the aberrant growth and differentiation of hematopoietic stem cells (HSCs), leading to the accumulation of immature myeloblasts (precursor cells) in peripheral blood and the bone marrow. Being a heterogeneous disorder. AML is consisting of various combinations of genetic aberrations, one of them is t(6;9) DEK/CAN that results in the formation of chimeric gene encoding leukemia associated fusion proteins (LAFP). DEK/CAN is associated with subtype high risk AML that has poor prognosis. Currently no therapy other than standard chemotherapy is available for DEK/CAN positive AML. So, there is a need for effective targeted therapy for DEK/CAN-positive AML. The purpose of this research study was to find out the antiproliferative activity and candidate targets of novel organometallic compound in high risk AML by using MTT assay and in silico approaches respectively. As an AML model we used DEK/CAN positive FKH-1 cell line. Our novel organometallic compound strongly interfered with the proliferation potential of FKH-1 cell line and added additive antiproliferative effects with imatinib. In addition, in silico methodology was established to carry out computer-based modeling and know about the active sites of different genes which can be targeted by different compounds/analogues. Further studies are required to find some novel candidate targets for therapeutic intervention

2. INTRODUCTION

The word leukemia corresponds to the cancer of white blood cells that begins in the blood forming cells known as hematopoietic stem cells in the bone marrow (Adamietz, Bremer, Hoelzer, & Strumberg, 2009). Under the normal circumstances, mature blood cells i.e. RBCs, WBCs and platelets are formed due to differentiation and maturation of blast cells. In case of leukemia, there is aggregation of malignant blasts in the bone marrow due to differentiation blockage as a result of some mutation. In leukemia, abnormal leukocytes are produced due to abnormal functioning of bone marrow (Stein & Tallman, 2016).

As reported in several studies, the incidence of leukemia is progressively increasing in Pakistan. Leukemias are the second most common cancer in northern part of Pakistan reportedly ("PATTERN OF BASIC HEMATOLOGICAL PARAMETERS IN ACUTE AND CHRONIC LEUKEMIAS," 2019)

However, AML is a complex disease biologically and is molecularly and clinically heterogeneous, hematopoietic fatal clonal disease distinguished by unlimited division of abnormal, immature blast cells, and abnormal synthesis of cells (Tamamyan et al., 2017).

It is caused by multiple somatic mutations which influence genes of different functional groups. Large translocations in chromosomes and mutations in the genes involving multiplication and differentiation of hematopoietic stem cells cause aggregation of abnormally differentiated myeloid cells. (Kouchkovsky & Abdul-Hay, 2016)

Mutated genes that encode epigenetic modifiers, like DNMT 3A (DNA Methyltransferase 3 Alpha), TET 2 (tet methylcytosine dioxygenase 2), IDH 1 ((Isocitrate Dehydrogenase (NADP (+)) 1) and IDH 2 are first obtained and expressed in the base clone. Mutations involved in NPM 1 or signaling molecules (e.g., FMS Like Tyrosine Kinase 3 (FLT 3), family of RAS genes) are generally secondary mutations which happen late in the course of leukemogenesis (Michael Medinger, 2017).

AML can occur in patients having existing blood disease or due to prior therapy (e.g., topoisomerase II exposure, radiation or alkylating agents); although, in most cases it appears to be a new malignancy in people who are healthy (Kouchkovsky & Abdul-Hay, 2016).

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AML is commonly present in adults and the frequency of AML is 3–4 individuals per 1 lac. Average age of AML diagnosis is about 70 years. Mostly cases develop in the male population. AML is common in adults, while acute lymphoblastic leukemia (ALL) mostly occur in children. In Pakistan the median age of ALL patients is 6 years ("PATTERN OF BASIC HEMATOLOGICAL PARAMETERS IN ACUTE AND CHRONIC LEUKEMIAS," 2019).

AML accounts for around 25 per cent of all Western world adult leukemia. Worldwide, AML has the highest incident rate in the United States, Australia and Western Europe. AML in adults predominates marginally among men in most countries ("Acute myeloid leukemia," 2017).

The prevalence of AML is seen to be increased with age at the rate of ~1.3 per 1 lac to 12.2 cases per 1 lac population. Even with existing therapies, as many as 70% patients (age 65 years) will die from their illness within 1 year of disease (Kouchkovsky & Abdul-Hay, 2016). However, onset of DEK/CAN positive leukemia occurs at the age of 23 to 40 years

AML patients express symptoms due to bone marrow failure, symptoms caused by leukemic cell infiltration in different the organs, or both. Symptoms related to failure of the bone marrow are associated with loss of RBCs, reduced neutrophils and thrombocytopenia. Patients commonly experience symptoms of dyspnea, dizziness, fatigue, nausea and bleeding. Spleen, liver, gums, and skin are the most frequent areas of infiltration. Infiltration is the most common occurrence in patients with AML monocytic subtypes. ("Acute myeloid leukemia," 2017)

With a few exceptions, AML diagnosis is based primarily on identification of peripheral blood or bone marrow for 20% or higher amount of myeloblasts (Tamamyan et al., 2017).

The frontline treatment used for AML is "7 + 3" induction therapy. In this treatment, there is intravenous infusion of cytarabine for straight 7 days and daily doses of anthracycline for 3 days (Saygin & Carraway, 2017).

Since then, most centers still consider the standard of care to be the 7 + 3 regimen. Latest advances in leukemia research has shown benefit with the administration of cytarabine (Ara-C) and high dose nucleoside analog carrying regimens over standard 7+3.

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Nowadays, in treating young adults, 7 + 3 or Ara-C (HiDAC) in high dose in addition with a nucleoside analog is used. The main objective of this therapy is to reduce the cancer cells to an unnoticeable quantity I.e. less than 109 cells. Experiments have revealed that the value of residual leukemia cells that disappear during treatment, induce relapse afterwards, and are called as minimal residual disease (MRD) (Tamamyan et al., 2017).

After induction therapy the post-remission therapy is used to eradicate leftover cells of leukemia. This therapy consists of three main procedures: consolidation chemotherapy, extended maintenance therapy, conditioning chemotherapy accompanied by allogeneic or autologous hematopoietic stem cell transplant (HSCT). Cellular therapies are another inspiring area for research in future that have presented favorable outcomes in many clinical trials (Tamamyan et al., 2017).

Treatment of older AML patients is very difficult, and the older age is correlated with lower results independently. This is mainly because of low performing ability and concurrent state, which can higher the mortality rate associated with the treatment related mortality (TRM). In addition, age may be related to worst cytogenetics, which may result from secondary AML or treatment-related AML and may be linked with resistance to medication. Given all these features, however, studies suggest the "treatment attempt" approach for older adult patients is valid by the "just assistive care" strategy. For patients under 75 years of age with better performing ability, 7 + 3-based induction may result in a 50 percent complete remission (CR) rate, but for the unfavorable risk group, the remission rate is below 30 percent and overall survival <5 percent. Dose reductions can be introduced for patients with comorbid conditions, and clinical trials for the unfavorable risk community or chemotherapy with lower dose (mild cytoreductive induction) could be an option. (Tamamyan et al., 2017)

In older adults unhealthy for standard induction chemotherapy, lower-potency treatment outcomes (low-dose azacitidine, cytarabine or decitabine) are often < 1 year of overall curative and median survival (MS). (Saygin & Carraway, 2017)

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INTRODUCTION

Most individuals with AML react to starting chemotherapy and attain a complete remission. Only few experiences long-term survival because in a high number of patients, relapse occur due to therapy-resistant disease. Therefore, relapse is a main challenge in the AML treatment.

Toxicity of the current cancer therapeutics as well as their side effects, both long and short term, are restricting the cancer treatment options in aged patients too. Tyrosine kinase inhibitors (TKIs), has not only lowered the issue of toxicity due to chemotherapy but has also increased the survival of many leukemia patients. However, the issue of therapy resistance is a major one and is still a challenge.

Keeping in view the scenario of issues at hand, new targets as well as targeted therapies for AML needs to be explored to increase the treatment options for AML patients. Such therapies might be able to reduce the dosage as well as toxicity of the current therapies with the achievement of promising and effective results and reduced aftereffects. In this respect, Organometallics are complexes of metals which contain at least one, covalent metal−carbon bond. These are recently reported as promising anticancer drug candidates.

Though organometallics have been explored massively as reagents to fight cancer, compound cisplatin continued to be the most extensively used anticancer drug and is still being used to treat almost 70% of all cancer patients since its discovery in 1965(Rosenberg, Van Camp, & Krigas, 1965). Several new ionic titanocene compounds have been outlaid and distinguished. They have a significant cytotoxicity activity against different human tumor cell lines (Allen, Croll, Gott, Knox, & McGowan, 2004). Metal association complexes offer a flexible stand for the production of new anticancer compounds.

Cisplatin and other platinum-based anticancer drugs (e.g. carboplatin, oxaliplatin) have shown that metal complexes may play a significant role in the clinic's treatment of anticancer therapies. Moreover, administration of such targeted therapies along with the use of Organometallic compounds in combination with the standard therapies may have the ability to lower the resistance developed from single targeting.

1.1   Hypothesis

The effective therapy for high risk AML is need of the time, the current problem which we are facing is the availability of effective therapy which can overcome the adaptive resistance to current therapeutics and destroy leukemic cells. Different signaling pathways like JAK/STAT, RAS/RAF, Akt/PI3K are activated by DEK/CAN fusion gene which are involved in AML leukemogenesis. More research is still needed for better understanding. Organometallics were thoroughly investigated as cancer-fighting reagents; compounds such as cisplatin remain the most commonly used anticancer drug. Hence by using the anti-cancerous potential of organometallic compounds, high-risk AML can be deduced. It can set a new hallmark in world of cancer therapeutics.

1.2   Aims and Objectives

- Evaluation of antiproliferative activity of organometallic compound Phenanthroline in FKH-1 cell line through in-vitro analysis

- To find out gene expression profile of FKH-1 cell line and potential anti-leukemic drug targets of phenanthroline using *in silico* approaches.

2 LITERATURE REVIEW

2.1 Leukemia

Leukemia is a cancer of white blood cells caused by somatic mutations. .

2.1.1 TYPES:

Leukemia is of two types as per the number of progenitor cells: Leukemia raised from lymphoid origin is known as Lymphoid leukemia whereas that of myeloid origin (myeloid lineage) is known as Myeloid leukemia. This myelogenous leukemia is further split into two subtypes: Slow-growing myeloid leukemia is called Chronic Myeloid Leukemia (CML) while fast-growing leukemia that progresses quickly is called AML. AML is a clonal differentiation arrest of myeloblasts in peripheral blood and Saultz Garzon, 2016).

2.1.2 ETIOLOGY:

Myeloid leukemias may occur due to more than one reason. Sometimes they are genetically inherited, otherwise they may occur due to exposure to DNA-mutation causing agents. Most of the time, etiology remains unknown. (direct, 2019, July). Myeloid leukemia may also occur due to some previously present bone marrow disorder (hematologic disease) or a person exposed to a genotoxic agent. In the beginning, disorder affects multipotential hematopoietic stem cells, ultimately converting into a disastrous disease. (Löwenberg & Rowe, 2016)

2.1.3 PATHOGENESIS:

Considering AML, it may appear for the first time in previously healthy individuals. The pathogenesis includes peculiar growth and proliferation of myeloid cells. Some patients have symptoms such as fatigue, leukocytosis and symptoms of anemia. If left untreated, death typically occurs within months. It is common in adults and of ~80 per cent of cases are confirmed in this group. Advances in care have resulted in substantial changes in results for

youngsters but the prognosis in older patients remains low (De Kouchkovsky & Abdul- Hay, 2016).

A research to study the occurrence of different leukemias in Khyber Pakhtunkhwa population Pakistan, was conducted in January2015. Tests showed a comparatively greater incidence of acute leukemia (80 percent) as compared to chronic leukemia (20 percent). Among different types of leukemia, AML 31.25% (n=125) was more common as compared to CML 10% (n=40). It was also discovered that male patients were more prone to leukemia i.e. 64.5% (n=258) as comparedto female patients i.e. 35.5% (n=142) and mostly patients were teenagers.

2.2 CLASSIFICATION:

2.2.1 French American-British classification:

Eight main subtypes of AML were classified by French American-British classification system in 1970s. This system was based on morphology, cytochemical criteria and immunophenotyping of the cells. These eight subtypes were FAB M0 to M7 (Some researchers do not include M0 in the classification and consider M1 to M7 as FAB classification types). Based on morphology, this FAB classification is divided on identification of granulocytic (types M1 to M3), granulocytic-monocytic (type M4), only monocytic (types M5a and M5b), erythroid (type M6) and megakaryocytic (type M7) cell types. This classification is highly reproducible and is widely accepted in cancer research. Usually acute myeloid leukemias are morphologically identified using Romanowsky's stain, but in order to diagnose FAB types, cytochemical staining procedures should be used. The types M1 and M5a require a cytochemical evidence whereas type M7 can be identified by using monoclonal antibodies and electron microscopy (Hassan *et al., 1993).*

Prognostic differences have not been found amongst these leukemia FAB types, but some evidences show poor prognosis in monocytic variants as well as longer rate of survival in M3 type cases. It was also seen that clinical status and age of patient are important factors to predict the therapy response by patient.

2.2.2   WHO Classification:

Then the World Health Organization (WHO) modified this AML classification. In this system, seven AML subtypes are included which are listed below: (Kouchkovsky & Abdul-Hay, 2016)

Table 1 WHO classification of AML and related neoplasms

WHO Classification Types	Subtypes	
AML with recurring cytogenic abnormalities	With t(8;21)(q22;q22), (AML1/ETO)	Independent of Numbers of Blasts in PB or BM PB: Peripheral Blood BM: Bone Marrow
	With inv (16)(p13.1;q22) or t(16;16) (p13.1;q22), (CBFβ/MYH11)	
	Acute promyelocytic leukemia: with t(15;17)(q22;q12), (PML/RARα) and variants	
	AML with t(9;11)(q22;q23), MLLT3/MLL or other 11q23 (MLL) abnormalities	≥ 20% Blast cells in PB or BM
	AML with t(6;9)(p23;q34), DEK/NUP214	

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	AML with inv (3)(q21;q26.2), RPN1/EVI1	
	AML (megakaryoblastic) with t(1;22)(p13;q13), RBM15/MKL1	
	Temporary entities: AML with mutated NPM1 and AML with mutated CEBPα	---------
AML with myelodysplasia related changes	---------	---------
Therapy related myeloid Neoplasm	- - - - - -	---------
Myeloid sarcoma	---------	---------
Myeloid proliferation by Down Syndrome	- - - - - -	---------
AML, not otherwise classified	M0 AML with minimal maturation	---------
	M1 AML without maturation	
	M2 AML with maturation	
	M4 Acute myelomonocytic leukemia	

	M5 Acute monocytic leukemia	
	M6 Acute erythroid leukemia	
	M7 Acute megakaryoblastic leukemia	
	Acute Basophilic leukemia	
	Acute panmyelose and Myelofibrose	

2.3 DIAGNOSIS:

There are various protocols for the diagnosis of AML as given below:

2.3.1 Morphology

For suspected AML patient, aspiration of bone marrow is performed as a part of the usual diagnostic practice. Blood and marrow smears are structurally analyzed by treating it with stain from Wright-Giemsa or May-Gru nwald-Giemsa. A blood or marrow blast count of greater or equal to 20% is demand for AML diagnosis, excluding some AML cases such as erythroleukemia cases. It is suggested that at least 500 nucleated cells should be determined on marrow smears which contain spicules and 200 leukocytes on blood smears. In myelomonocytic or monocytic differentiation AML subtype, promonocytes and monoblasts are calculated as equal to blast but abnormal monocytes are not considered. (Döhner et al., 2010)

2.3.2 Immunophenotyping

Immunophenotyping defines and distinguishes cell subgroups, on the basis of appearance of the specific kinds of antigens or markers on nucleus or cytoplasm cell surface. This technique utilizes antibodies labelled with fluorochromes which detects certain antigens. (Wang & Liberec, 2017)

Immunophenotyping through multiparameter flow cytometry (MFC) gives appropriate data for monitoring, diagnosis and classification of AML. MFC permits lineage assessment, quantification, and identification of aberrant blast cells and classification of disease in compliance with the maturation stage of cell. Due to genetic diversity, AML presents highly heterogeneous immunophenotypic profiles. (Costa et al., 2017).

To identify positive marker for AML, there is no accordance on the cutoff point. For most markers the commonly used criteria are, expression of marker by 20% or more leukemic cells, while for selected markers like, CD117, CD34 and CD3, a lower cutoff value of 10% is applicable. (Döhner et al., 2010)

2.3.3 Cytogenetics

A patient with suspected AML, conventional cytogenetics analysis is one of the required elements in the diagnostic evaluation of this disease. In adults, about 55% of AML patients are recognized with chromosomal rearrangements. For initiating the diagnosis of a standard karyotype and defining an aberrant karyotype, a minimal of 20 metaphase cells must be examined from bone marrow. From blood smears, abnormal karyotypes can be diagnosed. (Döhner et al., 2010)

2.3.4 Molecular cytogenetics

For molecular diagnostics a blood or marrow sample should be taken regularly. The DNA and RNA must be separated for molecular screening. RNA isolation should be a prime concern if

the cell number is limited since RNA is well matched for molecular screening for leukemia-associated mutations and fusion genes. If the morphology of chromosome is of not good quality, then RT-PCR is an alternate to the analyze rearrangements. (Döhner et al., 2010)

2.3.5 Genome-wide studies

New developments in genetic engineering have led to the recognition of unique genetic aberrations and the feasible development of systematized and structured cancer genome construction. For example, microRNA-expression and gene-expression characterization has shown to be valued for the locating new leukemia subgroups (Döhner et al., 2010).

2.3.6 Biobanking

It is recommended that leukemic blood and marrow pretreatment of patients be deposited inside a biobank. The prior condition for biobanking is patient informed acknowledgement which allows for a broad range of related laboratory studies. (Döhner et al., 2010)

2.3.7 Other tests

Other tests for AML diagnosis are aspiration of bone marrow, bone marrow biopsy and additional tests are also done at the time of diagnosis which are biochemistry, analysis of comorbidities, urine analysis coagulation tests, lumbar puncture, serum pregnancy test, bio banking, detailed family history and demographics and medical history. (Döhner et al., 2017)

2.4 PROGNOSIS:

The accurate evaluation of prognostic factors by the physician is important for deciding between treatments or therapies like consolidation therapy, hematopoietic stem cell transplant. (Kouchkovsky & Abdul-Hay, 2016) These factors related to prognosis can be divided into:

- Patient related factors that includes general health condition and patient characteristics, it predicts the treatment related mortality.
- AML related factors, it predicts resistance to therapies. (Döhner et al., 2010)

2.4.1 Elements of a prognostic study

Prognostic studies should start at a specific point of time in the disease course, patients follow up should be done for proper required time and all relevant outcomes should be measured properly. Other aspects included are:

To obtain sample without any biasness, the population under study should be a defined one including all those suffering from the disease, for example all those on a disease register.

To make sure that prognostic study is accurate all the patient's follow up should start from same specific point in the disease course.

Patients follow up should be carried out long enough so that the important outcomes have occurred in that time period.

All aspects of the disease should be included in a prognosis study and not just death and recovery.

Cohort study is claimed to be the best design for a prognostic study. Normally, it's not possible and it's not considered ethical to haphazardly classify patients to different prognostic factors. This particular study involves individuals who are suffering from the disease but yet no symptoms have appeared. The number of outcome events should be carefully monitored. (Taskesen et al., 2011)

At the moment, management of patients suffering from AML, is determined by many factors including age, performance status and cytogenetics.(Grimwade & Hills, 2009)

Performing control case study of prognosis on AML patients may also be possible, those who have already suffered from the outcomes are compared to those who have not suffered from any adverse effects also called a control group. The purpose being to analyze the number of individuals exhibiting any kind of prognostic factors in any group. However, it should be mentioned here that this type of study is bias prone and information about absolute risk is not provided.

Advanced patient care, stem-cell transplantation (SCT) along with Cytarabine and anthracycline based chemotherapy have improved the outcome of AML patients, but still fatality cause is relapse in these patients. in these patients is **relapse. The chance of relapse is related to the occurrence of risk factors** such as age, cytogenetics, performance status and number of chemotherapy cycles needed to achieve the first full hematological response (CR).

Table 4. Simplified Prognostic Score (0-14 points) for Acute Myeloid Leukemia at First Relapse

Prognostic Factor	Coefficient	Points
RFI, relapse-free interval from first complete remission, months		
> 18	0	0
7-18	0.69	3
≤ 6	1.28	5
CYT, cytogenetics at diagnosis		
t(16;16) or inv(16)*	0	0
t(8;21)*	0.68	3
Other†	1.19	5
AGE, age at first relapse, years		
≤ 35	0	0
36-45	0.21	1
> 45	0.47	2
SCT, stem-cell transplantation before first relapse		
No SCT	0	0
Previous SCT (autologous or allogeneic)	0.49	2

NOTE. Simplified prognostic score (range, 0-14 points) = RFI + CYT + AGE + SCT. The prognostic score is simplified by refitting the Cox regression model in which the continuous factors age at relapse and RFI are replaced by indicator variables for different ranges. The subdivision of age at relapse and RFI in this simplified score is based on an isotonic regression analysis. Coefficients are estimated from the simplified score. Division of the coefficients by 0.25 and rounding to the nearest integer yields the score points.
*With or without additional cytogenetic abnormalities.
†Normal, intermediate, unfavorable, and unknown cytogenetics.

Figure 1 Simplified Prognosis Score.

. First-relapse AML therapy is related to fairly low response times. The median duration of the second relapse-free interval (RFI) is usually shorter than that of the first RFI when the second CR is reached. As only a small number of patients suffering from relapse will benefit from current reinduction therapy for long-term durability. This knowledge could then assist doctors in making therapeutic decisions for patients at this stage of disease and could also help them in designing specific and investigational strategies for treatment. The contributing factors in predicting outcome for AML patients are reported and include RFI, cytogenetics, performance status and age.

After occurrence of first relapse, about 29% patients survived for about 12 months and the survival rate of 11% patients was 5 years. The chances of OS have been studied with respect to FAB classification, White Blood Cells count and diagnostic cytogenetics, number of induction

cycles of chemotherapy required to achieve first CR, RFI after first CR, age at recurrence, and whether SCT was taken on before first recurrence. These three different groups were combined to make one single group of another cytogenetics. Variable quantity analysis showed that favorable cytogenetics FAB classification, non-M5, less number of induction cycles toward first CR, longer RFI after first CR, no previous SCT and younger age predicted for improved OS. (Breems et al., 2005)

2.4.2   Patient-related factors

One of the patient related factor is the age, older patients have a poor outcome of therapy than younger patients. So, one of the deleterious prognostic factors is increasing age. Another more important factor than age is the comorbidities. Patients with other medical condition along with AML have the worse outcomes. (Döhner et al., 2010)

Analyses of multivariate model indicate that other variables like serum creatinine or albumin, platelet count are involved in the high risk of treatment related mortality (TRM) in elderly patients. (Kouchkovsky & Abdul-Hay, 2016)

2.4.3 AML-related factors

AML-related factors consist of WBC count, prior cytotoxic treatment for another disease, previously existing myelodysplastic syndrome (MDS) and molecular genetic or cytogenetic alterations in the leukemic cells at diagnosis. (Döhner, 2010)

2.4.4 Cytogenetics

The one strongest prognostic factor in patients with AML is the changes in cytogenetics. Different cases of AML build on their cytogenetic profiles is classified into favorable, intermediary or unfavorable prognostic risk groups. The favorable prognosis includes the chromosomal rearrangements such as t (15;17), inv (16)or t (8;21) . In contrast, high chance of treatment failure and death is related with cytogenetic changes like complex karyotype. Complicated karyotype includes three or more than three chromosomal rearrangements e.g. monosomy 7 or 5, inv (3), t (6;9) are included in adverse prognostic risk groups. Patients with normal cytogenetics (CN-AML) constitutes intermediary prognostic risk group. (Kouchkovsky & Abdul-Hay, 2016)

2.4.5 Molecular genetics

Molecular genetics constitutes irregular gene expression and mutations that interprets the genetic differences within established cytogenetic groups, specifically the heterogeneous and large group of patients with CN-AML. (Döhner et al., 2010)

It helps on the further refining of risk stratification. The gene mutations in patient lower prognosis and increase the risk of treatment failure as compared to patient with intermediate-risk AML. For example, the existence of a c-KIT mutation in patient having t(8;21), remarkably decreases overall survival and elevates the risk of relapse of disease. (Kouchkovsky & Abdul-Hay, 2016)

2.4.6 Minimal residual disease monitoring

Minimal residual disease (MRD) monitoring helps in early evaluation of response of patient

towards therapy which provides guidance in performing preemptive therapies. The tracking of MRD is done through RT-PCR which detects particular point of leukemia such as mutations, fusions or overexpression of genes. It is also done through multi parameter flowcytometry which identifies leukemia-associated aberrant phenotypes. (Döhner et al., 2010)

2.5   TREATMENTS OF AML:

2.5.1   CHEMOTHERAPY

Chemotherapy is the therapy of cancer in which drugs are utilized that block or slow down uncontrolled cell division. That's why Chemotherapy is regularly used with other cancer treatments, which include radiation or surgical operation. For instance, a patient can be given chemotherapy to reduce a tumor before surgical operation to help kill any cancer cells that are left which can cause cancer relapse. (Deborah Tolmach Sugerman, 2013)

Chemotherapy is the fundamental treatment for about all individuals with AML and is frequently given in stages. The medication session of AML includes:

Remission induction or induction therapy*:* The target of this portion is to rapidly kill as many leukemia cells inside the marrow and blood. This usually places leukemia into the state of remission which means that the patient doesn't have signs or manifestations of the malignancy. The standard induction treatment for AML is 7 + 3 regimen, which comprises of constant administration of cytarabine for 7 days along with a small portion of anthracycline given on days 1 through 3. But lately, the progression of cytarabine or daunorubicin portion have showed more advantage in the induction treatment. (Wang, 2018)

Consolidation therapy*:* Also known as intensification therapy, its objective is to kill any left-out leukemia cells and keeping the patient in remission. A substitute method to intensify standard induction therapy is by introducing fludarabine or cladribine, which is a purine analog. Clofarabine has also proven efficacy in AML patients which is a second-generation purine analog. (Wang, 2018)

2.5.2   STEM CELL TRANSPLANTATION

After the treatment of chemotherapy, infusion of healthy stem cells is given to the patient for replacing the stem cells that were destroyed during the intensive therapy. These new infused stem cells reestablish healthy stem cells inside the bone marrow that can shape new WBCs, platelets and RBCs. The two fundamental sorts of stem cell transplantation are:

- Allogenic bone marrow transplantation: In this transplant, stem cells of a healthy donor are introduced into patient. This transplantation is possible for patients on a small-scale i.e. those having an HLA-matched donor and those having young age. But it has complications like Graft Versus Host Disease (GVHD), if the patient is immunocompromised, its immune system is taken over by donor's immune system. (Ivana Trajkovska, 2017)

- Autologous bone marrow transplantation (ABMT): In this transplant, own stem cells of patient are introduced. In contrast, ABMT is accessible to all patients in remission less than the age of 55 years. The chief drawback of using ABMT in leukemia is that the graft of patient is infected with leukemia. Efforts are being done to remove this remaining leukemia through medications like cyclophosphamide derivatives. (A. H. GOLDSTONE, 1986)

2.5.3   IMMUNOTHERAPY

Immunotherapy works by boosting the body's immune system so that it can efficiently recognize and destroy cancer cells. This therapy activates the adaptive immune system of the body by inducing its memory function against cancer. (Schirrmacher, 2018) Immunotherapeutic approaches for treating cancer are mainly divided into two groups:

- Cellular i.e. HSCT, DLI (Donor Lymphocyte Infusion), and adoptive cellular immunotherapy.
- Non-cellular i.e. tumor antibodies, immune modulators like cytokines and vaccines.

Due to immune tolerance mechanisms, the side effects of immunotherapy are low.(M, 2007). Another name for this limitation of non-cellular and cellular immunotherapy is the anti-self-immune responses. The major example for this response is GVHD which causes remarkable mortality and morbidity. (Molldrem, 2010)

2.5.3.1   *Monoclonal antibodies:*

These are man-made form of proteins by the immune system (antibodies) that are made to bind with certain receptors on the surface of cancer cells. These antibodies act as a homing device, delivering poison right away to cancer cells and killing them. For the treatment of AML, the most reassuring targets are the cell surface receptors i.e.CD33 and CD123, both exists on normal hematopoietic cells and leukemic cells. An investigation proved that GO (anti-drug conjugate) can be more productive in patients with increased CD33 expression. Other drugs like SL-401 and CSL362 specific to CD123, are currently under investigation in various studies and showing encouraging results. (Molldrem, 2010)

2.5.3.2   *Immune checkpoint inhibitors:*

Normally, immune checkpoints behave as a defense mechanism in opposition to autoimmunity, but the tumor cells use their chance to provoke immune response and fight off the immune system. Thus, these inhibitors work by turning on immune responses. Programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) are the two key checkpoint receptors. It was proposed that administering nivolumab i.e. inhibitor of PD1 could be an efficient salvage therapy for the patients having relapsed AML after allo-HSCT. Phase II trials are ongoing, utilizing nivolumab alone or along with CTLA4 antibodies after chemotherapies and allo-HSCT. (Molldrem, 2010)

2.5.3.3   CAR T therapy

In CAR T therapy, novel receptors are genetically engineered which are known as Chimeric Antigen Receptors (CARs). These receptors can both provide proliferation, memory function and T cell activation and identify cancer-associated antigens (Michael M. Boyiadzis, 2018). In mouse model studies, it is proved that targeting with CAR T-cells against the receptors CD33 and CD123 had some anti-AML activity, but the side effect was severe myeloablation (myelosuppression) which was unavoidable. So, it is to be referred that the continued use of CAR T cells is linked with extended myeloablation and anti-cancer efficiency. This proposed that it is crucial to do a following rescue HSCT. (Molldrem, 2010)

2.5.4   RADIOTHERAPY

Radiotherapy uses radiations like x-rays that are used to demolish cancer cells. Intense radiation breaks down DNA present in cancer cells which blocks their capacity to proliferate and divide further. The purpose of this treatment is to minimize its radiations towards normal cells which are in the path of radiation and maximize its exposure to abnormal mutated cells. Generally, cancer cells are not that efficient in comparison to normal cells in mending the destruction caused by radiotherapy which results in distinctive killing of cancer cells (Rajamanickam Baskar, 2012). Radiation is usually included in conventional chemotherapy regimens that provides better grades of complete remission. Its observed that radiation therapy is recommended to those AML patients that have extramedullary disease e.g. myeloid sarcoma (Faye M. Feller, 2014). In a study, results indicated that a modest radio therapy (RT) dose of 20-30 Gy (Gray: unit of absorbed dose) achieves good local control of Myeloid Sarcoma (MS). Radio therapy response is affected depending upon the patient's age, previous bone marrow transplant (BMT), and underlying hematologic disease. (Wan-Yu Chen, 2013)

2.5.5   TARGETED THERAPY

In targeted therapy, drugs are used that recognize and attack cancer cells by blocking the activity of certain enzymes or molecules or proteins that are involved in spreading cancer. In some targeted therapies, drugs are directly delivered to cancer cells and kill them, or monoclonal antibodies are delivered that assist the immune system to destroy the cancer cells.

Targeted therapy has lesser side effects than other therapies of cancer (Eytan M. Stein 2016). Some of the drugs that are in their clinical trials for targeted therapy are:

2.5.5.1 FLT3 inhibitors

FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor of type 3. It is present on immature leukemic cells and hematopoietic stem cells. When phosphorylated, it activates multiple pathways involved in the apoptosis, proliferation and differentiation of myeloid stem cells present in bone marrow. So, mutation in this gene causes the production of too many abnormal leukemic cells. Flt3 inhibitors are required for blocking the function of mutated FLT3 gene. Few of the inhibitors that are in clinical trials are gilteritinib, crenolanib, midostaurin and quizartinib. (Eytan M. Stein 2016)

2.5.5.2 Kinase inhibitors

In targeted therapy of CML, the well accepted strategy is the use of kinase inhibitors for the inhibition of BCR-ABL. It is appeared that kinase inhibitors are much efficient in treating this disease. But, leukemic stem cells (LSCs) cannot be efficiently killed by these inhibitors which can cause cancer relapse. It depicts that this curative treatment does not lead towards the complete treatment of the disease. For the development of curative therapies for CML, it is required to recognize the genes or pathways that are concerned in the maintenance of leukemic stem cells.

2.5.6 Emerging therapies

The treatment of AML (intensive induction of 7 days of cytarabine plus 3 days of anthracycline (7 + 3) with consolidation chemotherapy or hematopoietic cell transplantation (HCT) has remained effective over the past 40 years. Only 40% of AML patients < 60 years of age stay on traditional intensive chemotherapy regimens for more than 5 years, and only patients with leukemia having favorable-risk core-binding factor have a death rate of 56% at 10 years. Instead of scarcity of novel drug approvals for treatment with AML, the identification of possible driver mutations through next-generation sequencing has revealed the biological

complexity of AML and led to new research drugs. With a greater understanding of pathogenesis of AML, there is a clear momentum towards various actionable goals to produce novel agents. Some of these agents have expressed impressive single-agent activity, such as IDH inhibitors, but logical consolidation can offer the highest advantage in terms of the vast genetic landscapeCPX-351 can be used in the regular first-line induction therapy for older adults with secondary AMLs, depending on the cost / benefit ratio, for a decade now. Additionally, novel agents such as STAT inhibitors, IDH inhibitors, FLT3 inhibitors (Sorafenib, Midostaurin, Quizartinib, Crenolanib), monoclonal antibodies, and vadastuximab (SGN-CD33A) may routinely be combined in select patients with conventional induction and consolidation therapy (Kouchkovsky & Abdul-Hay, 2016).

Improvements in results can be obtained in AML patients by combining traditional rescue regimens with vosaroxin, guadecitabine, venetoclax, and possibly even tosedostat. In addition, attempts to improve the host immune system for increased tumor surveillance and killing by adding checkpoint inhibitors (ipilimumab, nivolumab, and others) may change therapy delivery at multiple phases, including intensive chemotherapy, and at relapse, and post-HCT. Timely use of targeted therapies (e.g., FLT3 inhibitors) in post-transplant maintenance setting may also drive improvements for patients with leukemias (Saygin & Carraway, 2017).

Drug production for AML is a challenging process with a long history of numerous failures and limited progress.

2.6 Organic compound phenanthroline

A unique copper (II) complex of the antibacterial fluoroquinolone product N-propyl-norfloxacin (Hpr-norf) in the existence of 1,10-phenanthroline (Phen) has demonstrated biological characteristics as an antibacterial, antitumor and antimicrobial agent. Human acute myeloid leukemia cell line HL-60, MTT assay, and Trypan blue assay were performed to evaluate the antileukemia, cell proliferation, or viability, and structural integrity cell membrane and (chloro)(Phen)(N-propyl-norfloxacinato) copper (II) (complex 1), respectively. They establish that HL-60 cells decreased their proliferation rate and viability after complex 1 treatment, leads to apoptosis (programmed cell death) in a time-dependent manner. (Katsarou, Efthimiadou, Psomas, Karaliota, & Vourloumis, 2008).

In the number of 25 bis(cyclopentadienyl)vanadium (IV) and 14 oxovanadium (IV) compounds manufactured and investigated for antitumor activity, bis (4,7-dimethyl-1,10-phenanthroline)sulfatooxovanadium(IV)(metvan) has been determined as the major satisfying multi-targeted, apoptosis-inducing anticancer vanadium complex. In human leukemia cells, metvan cause apoptosis at low micromolar and nanomolar concentrations. Metvan is much more effective than the typical chemotherapeutic agents: vincristine and dexamethasone   in inducing programmed cell death of primary leukemia cells in patients with acute lymphoblastic leukemia, acute myeloid leukemia or chronic acute myeloid leukemia. Metvan-induced programmed cell death is associated with a reduction in capacity for mitochondrial transmembrane, the development of reactive oxygen species, and glutathione diminution. Treatment of leukemia cells from acute lymphoblastic leukemia, acute myeloid leukemia and chronic acute myeloid leukemia prevents both the metalloproteinase-9 and -2 matrix's constitutive expression and gelatinolytic operation. (D'Cruz & Uckun, 2002).

2.7   Cell Cycle

The cycle of eukaryotic mammalian cells is broken down into four separate stages: G1, S, G2, and M. G1 is the gap phase in which the cells are preparing themselves for DNA replication. Phase G1 is a very crucial phase as it is this phase in which cells combine mitogenic and growth-inhibitory signals and decide whether the cell will move, pause or exit the cell cycle further. There is an important checkpoint, found in both yeast and mammalian cell cycle. In mammalian cell cycle, it is referred to as restriction point and in yeast, it is called start point. This the point at which cell confirms to proceed with DNA replication and completion of cell cycle. In S phase DNA synthesis occurs. G2 is defined as second gap phase and is characterized by cell preparation for division process. M indicates Mitosis in M phase and is characterized by separation of replicated chromosomes into separate nuclei, and two daughter cells formation following cytokinesis. For cells that exit the cell cycle, and become dormant, the term G0 is used. (Johnson & Walker, 1999)

Every type of life, on earth, need to stand DNA damaging agents in environment such as Sun's radiations. Nature has provided cells with cell cycle checkpoints pathway and Highly conserved DNA repair system to fight against both endogenous and exogenous DNA damaging agents. The amount of these agents being exposed to individuals, and the time duration of exposure, are very important factors in determining whether that individual will have cancer or

not. These factors are also very crucial in determining cellular response towards treatment and in determining toxicities.

In mammalian cells, DNA is continuously facing attacks from agents that could damage either one of many nitrogenous bases found in DNA or phosphate back bone on which these bases are found. For example, free oxygen radicals that can cause either base damage or backbone damage, are product of natural metabolism or can be produced by exposure to any ionizing radiation present in surrounding environment. Life has managed to create mechanisms such as DNA repair systems to avoid any damage to DNA by metabolic or external ionizing agents. (Kastan & Bartek, 2004)

In cancer biology, one of the most important aspects, is the cellular response to DNA damage. Firstly, cancer is caused by damage to cellular DNA. Epidemiological studies proved this theory in animal models and the observations that many human-cancer-susceptibility syndromes occur due to mutations in genes, involved in DNA damage response system. Secondly, the cure for cancer comes from DNA damage. Most of the therapeutic strategies used to cure cancer, focus on repairing or reversing DNA damage, such as many radiotherapy and chemotherapeutic agents. Thirdly, most of the side effects of cancer are because of DNA damage. Bone marrow suppression, hair loss and gastrointestinal toxicities, all occur due to DNA-damage-induced cell death of dividing progenitor cells in these tissues. So, from cancer point of view, DNA damage is root cause of cancer. It is used to cure cancer and all the side effects are due to DNA damage. (Elledge, 1996).

The elegant but not perfect process called DNA repair system is the defense system of a cell against DNA attacking agents. A variety of different repair mechanism exist because there are a variety of DNA lesions that can occur. In addition to DNA repairing system, cell also

responds to DNA damage by stopping the cell cycle process or by committing suicide that is the programmed cell death called apoptosis. Although a little is known about how apoptosis and cell cycle arrest are working in accordance with DNA repair system, but such coordination must occur to reduce the outcome for the cell. Apart from damage to the DNA, cells also must cope up other stresses such as exposure to abnormally low levels of nutrients as well as oxygen. Although different cells use different aspects of DNA repair system but there are some factors that are common in different cells while fighting DNA damaging agents. (Martin, Hamilton, & Schilder, 2008)

Cell cycle checkpoint refers to the halting of any progress in cell cycle until it is confirmed that DNA replication and chromosome segregation has been completed without any error. In the image below some of the pathways are shown that are responsible for DNA repair system and if cells manage to escape these pathways it results in onset of cancer.
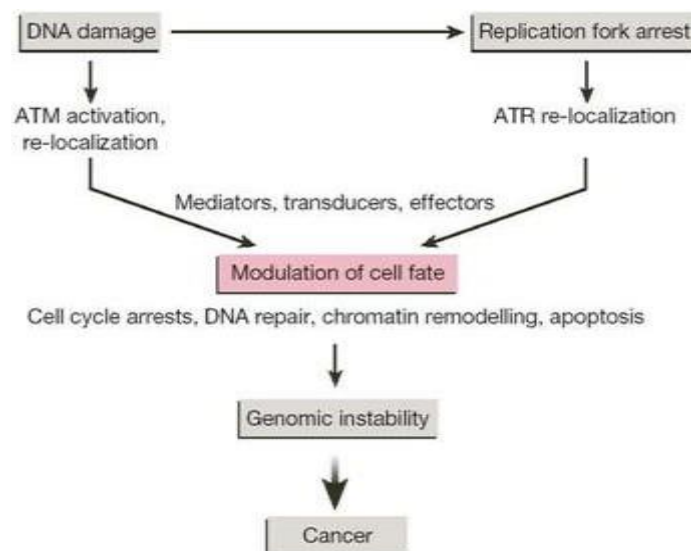

Figure 2 DNA Repair mechanisms

(Elledge, 1996)

2.8   Cell Lines

Cell lines are an important tool to study biological processes without causing any damage to any life. But cell lines must not be mistaken with primary cells as cell line results are subjected to human error and environmental limiting conditions. Cell lines can't be used in place of primary cells because of its limitations. To make experiments conducted on cell lines error free precautions must be taken and experiments should be carried out in a controlled environment.(Thomson et al., 1998)

2.8.1   FKH1

Translocation t(6:9) (p23; q34), resulting in DEK / CAN fusion, is a recurring chromosomal abnormality associated predominantly with different AML and MDS subtypes. Patients suffering from this particular translocation appear to be young and have poor prognosis. The role of fusion protein produced by dek-can chimeric transcript on the leukemogenesis of t (6;9) AML or MDS is not known yet. It identified the first permanent cell line (FKH-1) with t(6;9), taken from a patient's peripheral blood with t(6:9) AML transformed from Philadelphia chromosome (Ph1)-negative CML. Dek / can transcripts and myelomonocytic markers were expressed by the FKH-1 If there is a recombinant human granulocyte (G-CSF) factor of 10 ng / ml, the cells divided every 54 h and displayed differentiation, resulting in heterogeneous morphologies. This proves that this cell line can be used to check the effects of any targeted therapy on malignancy carrying dek/can fusion. (Hamaguchi et al., 1998)

2.9   Relapse and resistance

In response to initial chemotherapy, most individuals achieve full remission, and only a small number of patients experience long-term survival because majority suffer from relapse owing to drug resistance. Hence, Relapse is the main obstacle in achieving 100% recovery following AML treatment. But still nothing to very little is known about the drug resistance mechanisms in AML. (Zahreddine & Borden, 2013)

Cancer relapse is one of main reasons because of which cancer treatments are failing. It can occur due to incomplete eradication of tumor cells using radiotherapy and chemotherapy. Recent advances in model developments and research have provided a deeper knowledge to scientists about the mechanisms by which tumor relapse occur. The main contributing factor to

drug resistance is the survival of cancer stem cells (CSCs) in malignant cancers. CSCs have a relatively quiescent metabolism and show resistance to therapeutic agents using a variety of mechanisms (Hackl, 2017).

 A P-glycoprotein (P-gp), a 170-kDa protein encoded by the MDR1 gene, is one of many MDR multidrug resistant gene products. This is made up of two transmembrane and two nucleotide-binding domains. It is a type of efflux pump that can pump out amino acids, organic ions and many other things. For both newly diagnosed and relapsed AML, over-expression of (P-gp) results in poor prognosis in AML. Patients with elevated P-gp rates have had higher counts of white blood cells, worse chromosomal anomalies and shorter overall survival (OS). In the multivariate review, MDR1 expression was reported in a retrospective study of 331 adult AML patients as an independent prognostic factor of induction therapy and of OS (Shaffer et al., 2012).

The main reason for treatment failure in cancers is drug resistance. Several forms of gene mutations, irregular expression of drug-resistance-related miRNAs, upregulated PI3K / AKT and autophagy signal pathways, over-expression of certain kinds of drug-resistance-related enzyme may lead to relapse and drug resistance in patients suffering from AML. MDR work has been comprehensive and a variety of inhibitors have been identified targeting certain drug-resistant mechanisms (Mechetner & Roninson, 1992).

 To reverse resistance, overcoming these crucial factors is very important. When a patient is diagnosed with AML, it should be noted that whether he or she carries high-risk factors for drug resistance or not. Therefore, the risk of drug resistance can be forecasted through identifying gene mutations by Next Generation Sequencing (NGS) and the expressions of proteins and enzymes. More resistance mechanisms are expected to be discovered. But still confirmation on clinical bases on many cases is needed to effectively use the above-mentioned mechanisms in overcoming drug resistance in AML and to improve prognosis and overall survival ratee. (Jing Zhang, 2019)

3 MATERIALS AND METHODS

3.1 Preparing cell lines

Whole experiment was conducted on suspension cell line FKH-1:

FKH-1 is a study model for high risk AML that carries translocation (6;9) (p23; q34) leading to DEK-NUP214 (DEK-CAN) fusion gene.

The cells were first cultured in a 6-well tissue culture plates in pre-warmed RPMI 1640 (Gibco by Life Technologies). 20% fetal bovine serum and 1% of Pen Strep (Gibco by Life Technologies) were also added to cell culture medium to prevent contamination of micro-organisms.

Culture plates were placed in a humidified water-jacketed incubator at 37˚C temperature and 5% CO_2 to allow for cell lines to grow. The cells were always maintained at a density of less than 1.0 x 106 /ml to avoid contact inhibition.

3.1.1 Freezing and thawing cells

For freezing/cryopreservation of cells, two solutions were made i.e. freezing solution I and freezing solution II.

Solution I contained RPMI 1640 70% (V/V) and FBS 30% (V/V) while solution II contained RPMI 1640 80% (V/V) and DMSO 20% (V/V).

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

3.1.2 Thawing

When the cells were required, cryopreserved cells from the liquid nitrogen freezer were taken out and were thawed in incubator at 37˚C and were resuspended in culture medium. Then they were washed once with PBS to get rid of Dimethyl Sulfoxide (DMSO) and pellet was again resuspended in culture medium and plated in 6-wells culture plates and were put in CO_2 incubator at 37˚C.

3.2 Trypan Blue Exclusion Assay

Trypan blue exclusion assay was done in order to determine the number of cells, present in the medium per ml of the medium, this was done to seed the constant number of cells viable for MTT assay, to obtain accurate results.

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

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

4

3.3 MTT Assay

MTT assay is used to evaluate the cells effectiveness and their division by the conversion of tetrazolium salt, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] to Formazan crystals. This reduction is catalyzed by dehydrogenase enzymes present in mitochondria of the living cells and is therefore an indicator of cell viability (Riss TL, 2013).

Cells were plated at a density of 1.0 x 10^4 cells/50 μl into 96-well plate followed by the addition of 50μl/well of different concentrations of Phenanthroline (10mM) (Med Bio Express), in μM in triplicates. Drug was allowed to produce its effect on the cells for about 48-72 hours. After completion of the required time duration, cell viability was assessed by the addition of 15μl of

filter sterilized MTT (5 mg/ml in PBS) to the microtiter wells containing cells and Phenanthroline concentrations and plates were again kept in CO_2 incubator for about 3-4 hours to allow crystals formation. 50µl of media was then removed carefully without disturbing the crystals after 3-4 hours. 150µl of DMSO was put to dissolve the crystals. After complete dissolution of crystals, the absorbance at 550 nm was measured with a spectrophotometric plate reader.

Similar protocol was followed to conduct co-targeting experiment of Phenanthroline with Imatinib. Only difference was addition of both drugs combined in each well 25µl/well.

3.4 *In-silico* Methodology

In silico means "performed via computer simulation" in reference to biological experiments. The term was used in 1987 with other Latin phrases in vivo, in vitro, and in situ widely used in biology.
Drugs have traditionally been found by evaluating compounds produced against a battery of biological in vivo screens in time-consuming, multi-step processes. Viable compounds were then further studied in growth, where they examined their pharmacokinetic properties, metabolism and possible toxicity. There have been numerous adverse studies at this point (Kennedy, 1997)

This concept was reconfigured in a variety of ways today. Drug metabolism, pharmacokinetics, and toxicity testing is performed much earlier today; that is, before a clinic decision to assess a compound is made. However, the pace at which biological testing data are collected has increased significantly, and (ultra)high-throughput screening (HTS) facilities are now popular in major pharmaceutical companies and advanced biotech(van de Waterbeemd, 2002). *In silico* in medicine research, the ability to increase the rate of prognosis while eliminating the need for expensive lab work and trials is considered. One approach to attain this is through the more effective production and screening of drug candidates. To find out Potential Drug Targets *In silico* following steps were taken:

- Using the online chemical structure library, the structure of organometallic compound Phenanthroline was extracted and studied.

MATERIALS AND METHODS

- As the organometallic compound is to be tested on FKH-1 cell line so the candidate gene list for the respective cell line was formulated by the help of gene databases that are NCBI; National Centre for Biotechnology Information and Gene Card; The Human Genes Database. Literature review was done for finalizing the rest of important genes.

- Afterwards to find out the genes related to our candidate genes, UNIPROT Database i-e specific for protein sequence and functional information was used. The FASTA sequence after selecting a functional domain of the each of the gene was taken and Blast using PSI Blast (BLAST is a software used to compare primary biological sequences.) resulted in the PDB ID of the related proteins.

- Considering the Percent Identity of the related proteins, only the proteins with percent identity greater than 60% were selected. All the other protein below this criterion were excluded.

- The selected proteins were further analyzed in a way that protein with percent identity below 90% were modelled using SWISS-MODEL (software used for homology modeling of 3D protein structures) and duplicate chains were deleted by entering the modelled protein PDB ID in UCSF Chimera, a software used for visualizing and analysis of molecular structures.. However, proteins with percent identity greater than 90 were directly analyzed in UCSF Chimera for deletion of duplicate chains.

- For the protein structure analysis to find out the best binding site for the drug, a server called DoGSiteScorer (an automated pocket detection and analysis tool) was used. The binding cavity i-e a pocket was selected based on drug score, usually the drug score nearer to 1 is considered good and then amino acid residues of that pocket were noted.

- The next and the most important step was Docking, using *PyRx* software.

4   RESULTS

4.1   Phenanthroline and Imatinib interferes with proliferation potential of FKH-1 cell line

Pharmacological targeting of Phenanthroline and Imatinib was performed on FKH1 cell line at increasing concentrations in μM and proliferation was assessed through MTT assay after 96 hours. Our results indicated a reduction in the proliferation potential of FKH-1 cells in a dose dependent manner as shown in figures 1 and 2 respectively. Phenanthroline was noted to be effective against tumor cells between concentrations of 5 and 10μM as shown in figure 1. Lower concentrations did not show much reduction in tumor cells i.e. 0.625 and 1.25μM. Similarly, Imatinib showed reduction in cell number between concentrations of 1-2μM in dose dependent manner as shown in figure 2. Taken together our results showed that both phenanthroline and imatinib interferes with proliferation potential of FKH-1 cell line.
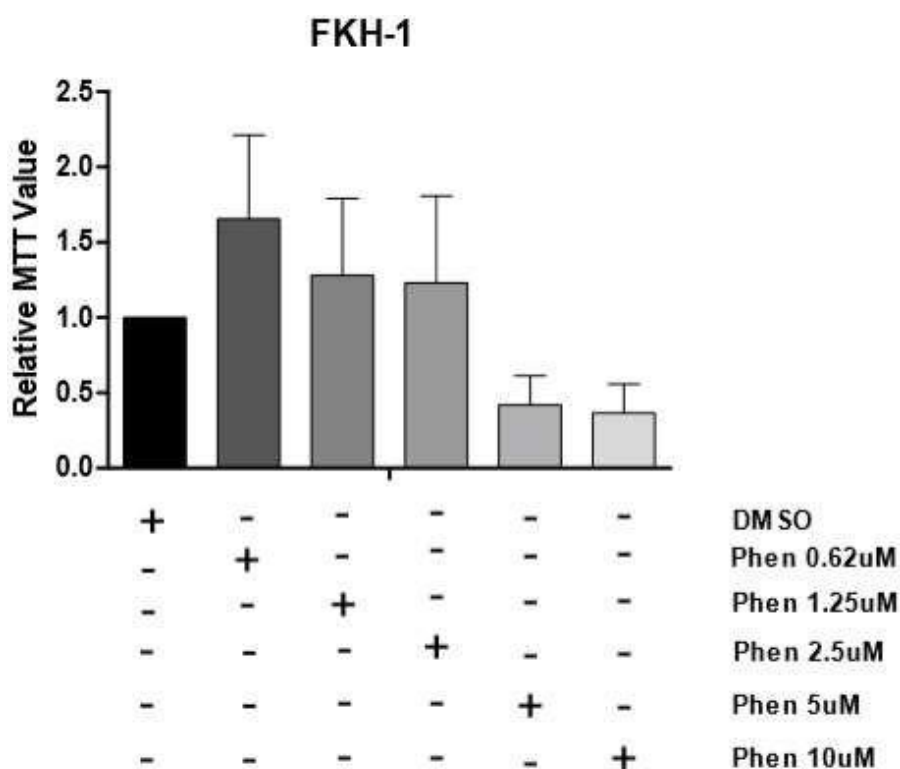

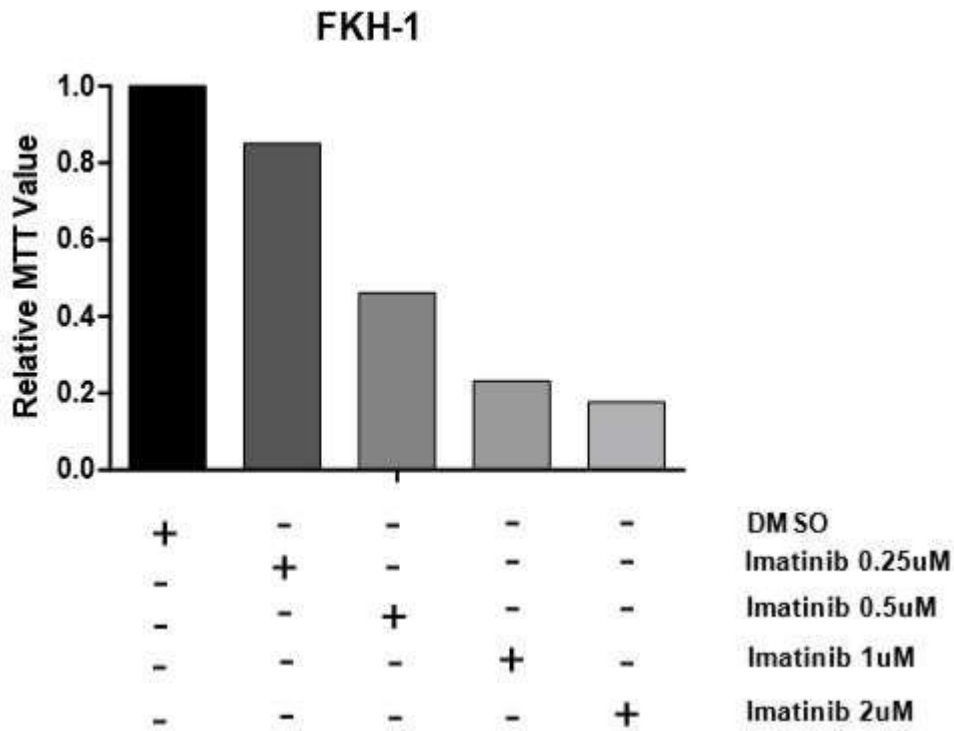*Figure 3 Antiproliferative effect of phenanthroline on FKH-1 cell line*

Figure 4 Antiproliferative effect of imatinib on FKH-1 cell line

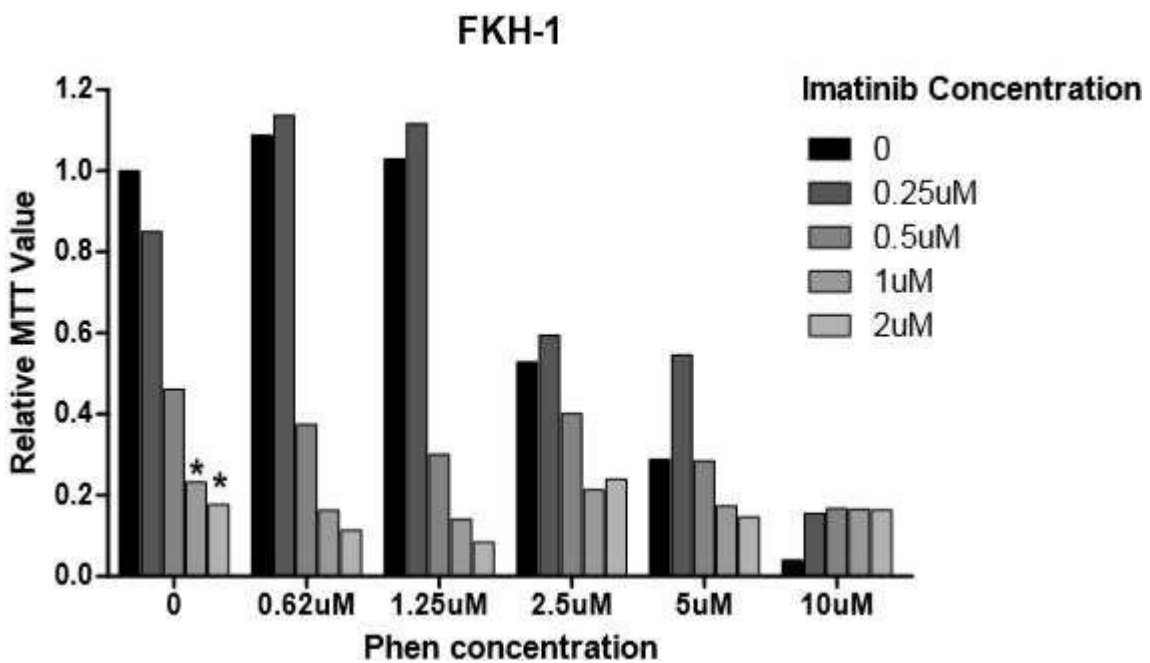

Figure 5 Co-targeting: combinatorial anti-proliferative effect of phenanthroline and imatinib

4.2 *In silico* results:

After extracting all genes (from NCBI GenBank) and their FASTA formats, their domains were selected on basis of high amino acid number and functionality. Both FASTA sequences retrieval and domains selection was done using UNIPROT. FASTA formats of each domain was blasted on NCBI Blast to obtain accession ID of respective chain. RCSB PDB software was used in order to extract PDB files of our concerned proteins or domains using the respective accession IDs. Then for pockets and residue details of each protein domain, using Dogsitescorer, amino acids detail for each protein domain was extracted.

Table 2 List of genes of fkh1 cell line with their pockets having high drug score

GENES	Description	Pockets selected	Drug score
FKBP1A	FKBP prolyl isomerase 1A [*Homo sapiens* (human)]	**P-2**	**0.77**
FOXK1	forkhead box K1 [*Homo sapiens* (human)]	**P-0**	**0.76**
FOXK2	forkhead box K2 [*Homo sapiens* (human)]	**P-4**	**0.48**

FOXP2	forkhead box P2 [*Homo sapiens* (human)]	**P-0**	**0.87**
RPS6KB1	ribosomal protein S6 kinase B1 [*Homo sapiens* (human)]	**P-0**	**0.82**
FHL1	four and a half LIM domains 1 [*Homo sapiens* (human)]	**P-0**	**0.65**

5 DISCUSSION

The aims of this study were to study the effects and candidate targets of organic metallic compound phenanthroline in high risk AML using invitro and Insilco approaches. The findings demonstrate that Phenanthroline showed effectiveness and blocked the further proliferation of FKH-1 at a higher concentration. It also showed additive antiproliferative effect with imatinib, an ABL kinase inhibitor in FKH-1 cell line.

It is to be noted that in a previous study on a novel ruthenium based organometallic compound targeting AML cell line published by Daniel Azar (2015), experiments were performed on phenanthroline ligand and its corresponding metal complex (Ru II). The activity against AML cell lines was to be detected by phenanthroline bound to its metal complex and by ligand free metal compound. The analysis confirmed that the metal alone didn't showed any cytotoxic activity but when bound with the ligand phenanthroline, it showed cytotoxic activity against AML. Also, it depicted that the ligand phenanthroline showed significant activity by intercalating DNA. By adding phenyl groups in this ligand, it could become more active DNA intercalator therefore more significant activity could be observed against AML. (Azar, 2015)

While the previous research has focused on the activity of phenanthroline bound with metal complex and its effectiveness with different metal compounds, this study provides the co relation between dosage of phenanthroline and reduction potential of proliferation of AML cells. With high dosage of Phenanthroline, more reduction in the proliferation of FKH1 was observed.

FKH-1 cell line was targeted with Imatinib and Phenanthroline. An additive effect was observed at a lower concentration of both Imatinib but at higher concentration (uM-uM). In single targeting, the effects of phenanthroline were not observed at lower concentration but were observable in co-targeting. It can be possible that targeting FKH-1 cell line with imatinib may activate certain genes which may be involved in proliferation and are targeted by Phenanthroline in co-targeting. Thus, it adds additive antiproliferative effect at lower concentration.

From the in-silico results, pocket list of protein domains was selected based on their high drug score. These pockets were obtained for finding out the key candidate target for phenanthroline compound by performing the step of docking which has to be done in future.

DISCUSSION

Further research is required on this compound to understand its mechanism of action and study at molecular level is needed to reveal the signaling pathway involved in cancer cell proliferation targeted by phenanthroline.

6 CONCLUSION

AML is a biologically a heterogeneous disease. Although progress in supportive care and prognosis have improved the therapies, but still long- term survival of patients remains poor. The available treatments for AML are not much effective in eliminating leukemic stem cells, therefore, relapse occur and some of the therapies may also develop resistance. This research aimed to identify effective drug to eliminate leukemia stem cells from the bone marrow to avoid relapse of AML after the treatment. Based on the experiments done, it can be said that the organometallic compound phenanthroline is effective in killing leukemia cells at higher concentrations. Also, its activity was checked along with imatinib in co targeting assay. Some promising results have been achieved which need further investigations to provide effective treatment for AML

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7 Appendix

Heterogenous disease A disease caused by different genes.

Antiproliferative Inhibit cell growth

Thrombocytopenia Low blood platelets count

Dyspnea Shortness of breath

Additive effect Sum of effect of two drugs taken independently

Leukemogenesis Production of leukemia

Acute lymphoblastic leukemia Accumulation of large numbers of lymphocytes

Comorbid state Presence of two or more conditions in a patient

Megakaryocytes Platelets involved in blood clotting

Monoclonal antibodies Made by single clone of immune cell

Monoblast Cells that give rise to monocytes

Promonocytes Cell developing into monocytes

Electrocardiogram Test to check function of heart

Radiograph Image produced by radiations

Myeloablation Decreased bone marrow activity

MDS Myelodysplastic syndrome, immature blood cells in bone marrow do not mature

Philadelphia chromosome Abnormal chromosome formed by translocation between chr 9 and chr 22

Antineoplastic Inhibit development of neoplasm (tumor)

Antiapoptotic Inhibit programed cell death (apoptosis)