Development of High Throughput Flowcytometric Assay for Immunophenotypical Quantification of Leukemic Stem Cells and Phenotype Based Drug Sensitivity Approach in Acute Myeloid Leukemia

Debashish Deb

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Tiivistelmä – Referat – Abstract

There is significant reduction in number of approved drugs for acute myeloid leukemia in recent years. Partially it may be due to the failure of discovery and validation approach to new drugs as well as the complexity of the disease. *Ex vivo* functional drug testing is a promising approach to identify novel treatment strategies for acute myeloid leukemia (AML). In ideal condition, an effective drug should eradicate the immature AML blasts, but spare non-malignant hematopoietic cells. However, current strategies like conventional cell viability assay fail to measure cell population-specific drug responses. Hence, development of more advanced approaches is needed. Using multiparameter, high-content flow cytometry (FC), we simultaneously evaluated the *ex vivo* sensitivity of different cell populations in multiple (10) primary AML samples to 7 FDA/EMA-approved drugs and 8 drug combinations. Amongst the 7 tested drugs, venetoclax, cytarabine and dasatinib were very cytotoxic with venetoclax had the highest blast-specific toxicity, and combining cytarabine with JAK inhibitor ruxolitinib effectively targeted all leukemic blasts but spared non-malignant hematopoietic cells. Taken together, we show that the *ex vivo* efficacy of targeted agents for specific AML cell population can be assessed with a cell phenotype, FC-based approach. Furthermore, we put an effort to analyze the potential of this assay and biomarkers to predict the clinical outcome of individual patients and future perspectives.

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Table of contents

Abbreviations 5

List of Figures 6

List of Tables 7

Chapter One: Introduction 8

1.1 Acute myeloid leukemia 8

1.2 Taxonomy of AML 8

1.3 Genomic landscape of AML 9

1.4 Prognosis 10

1.5 Current therapies 11

1.6 Anti-cancer drugs/agents 13

1.7 Cancer Research and Limitations 15

1.8 High Throughput Flow cytometry in hematology 16

Chapter Two: Aim of the thesis 19

Chapter Three: Materials and methods 20

3.1 Workflow 20

3.2 Sample processing and handling 21

3.3 Culture media, reagents and antibodies 21

3.4 Assay development: scale up and optimization 21

3.5 Cell Viability Assay 22

3.6 Staining protocol/FACs analysis of AML 22

3.7 Data acquisition and analysis (HTFC) 23

3.8 Moab combinations used to characterize AML cells 24

3.9 Statistical analysis 24

Chapter Four: Results 25

4.1 Assay development: scale up and optimization 25

4.2 Immunophenotyping of leukemic cells 29

4.3 Assay sensitivity and reproducibility 36

4.4 Blast-specific drug sensitivity profiling in heterogeneous AML patient samples 37

Chapter Five: Discussion & Concluding remarks 45

Acknowledgements 48

References 49

Abbreviations

- AML - Acute Myeloid Leukemia
- WHO - World Health Organization.
- FMAT - fluorometric microvolume assay technology
- HTFC - High throughput flow cytometry
- LSC – Leukemic stem cells
- HSC – Hemapoietic stem cells
- CTG assay - CellTiter-Glo® Luminescent Cell Viability Assay
- 7-AAD - 7-Aminoactinomycin D
- PS - Phosphatidylserine
- CD marker – marker for ´´cluster of differentiation”
- FC - flow cytometric
- NPM1 - Nucleophosmin 1 gene
- FSC-H - Forward scatter height
- FSC-A - Forward scatter area

List of Figures

§ **Figure 1.1:** Acute myeloid leukemia.

§ **Figure 1.2:** Different types of mutated genes commonly found in acute myeloid leukemia.

§ **Figure 1.3:** Schematic presentation of linear and branching evolution.

§ **Figure 1.4:** Basic working principle of a flow cytometer.

§ **Figure 2.1**: Workflow with high throughput screening system.

§ **Figure 3.1:** Comparison between expression patterns for different experiment condition in AML sample.

§ **Figure 3.2:** Comparison between expression patterns for different incubation time in AML sample.

§ **Figure 3.3**: Comparison of cell growth in different cell culture plate.

§ **Figure 3.4:** Comparison of cell counts for different plate types in flow-cytometry assay.

§ **Figure 3.5** Removal of doublets using FSC-A (log) vs. FSC-H (linear) dot plot.

§ **Figure 3.6** Removal of debris using FSC-A (linear) vs. SSC-H (linear) dot plot.

§ **Figure 3.7** Removal of debris using double negative population gating strategy (Annexin V vs. 7AAD).

§ **Figure 3.8:** Identification of stem cells population within AML primary sample.

§ **Figure 3.9:** Identification of AML Blast cells in primary sample of Acute Myeloid Leukemia.

§ **Fig. 4.0:** Identification of LSCs in 'CD34+CD38-' AML population.

§ **Figure 4.1:** Demonstration of flow cytometric (FC) analysis of an acute myeloid leukemia based on CD45/SSCA gating.

§ **Figure 4.2:** Statistical presentation of immunophenotypical tally of a patient blood sample based on the designed assay.

§ **Figure 4.3**: Drug sensitivity of an AML diagnosed patient sample for venetoclax.

§ **Figure 4.4**: Drug sensitivity of an AML diagnosed patient sample for dexamethasone.

§ **Figure 4.5**: Drug sensitivity of an AML diagnosed patient sample for cytarabine.

§ **Figure 4.6**: Drug sensitivity of an AML diagnosed patient sample for GSK2126458.

§ **Figure 4.7**: Drug sensitivity of an AML diagnosed patient sample for Ruxolitinib.

§ **Figure 4.8**: Drug sensitivity of an AML diagnosed patient sample for temsirolimus.

§ **Figure 4.9**: Drug sensitivity of an AML diagnosed patient sample for Dasatinib.

§ **Figure 5.0**: Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for combinatorial drug.

List of Tables

§ **Table 1:** Types of anticancer drugs**.**

§ **Table 2:** List of FDA approved drugs for acute myeloid leukemia.

§ **Table 3:** List of antibody panel.

§ **Table 4:** Immunophenotypical expression data of 10 acute myeloid leukemia patients.

§ **Table 4.2** Customized anti-cancer drug panel.

Chapter One: Introduction

1.1 **Acute myeloid leukemia (AML)** is clonal hemapoietic stem cell disorder which is known to be the most aggressive among all adult leukemia. According to National Comprehensive Cancer Network (NCCN) review report 2014, an estimated 18,860 were diagnosed with AML only in USA, 60% of them are most likely to die from the disease. For patients aged ≤ 60 years, the curability is up to 35-40% whereas for older patients the outcomes are further dissatisfactory. The survival rate among older patients is only about 5-10 months (Adult Acute Myeloid Leukemia Treatment–Patient Version (PDQ®), 2015; Döhner, 2015) .

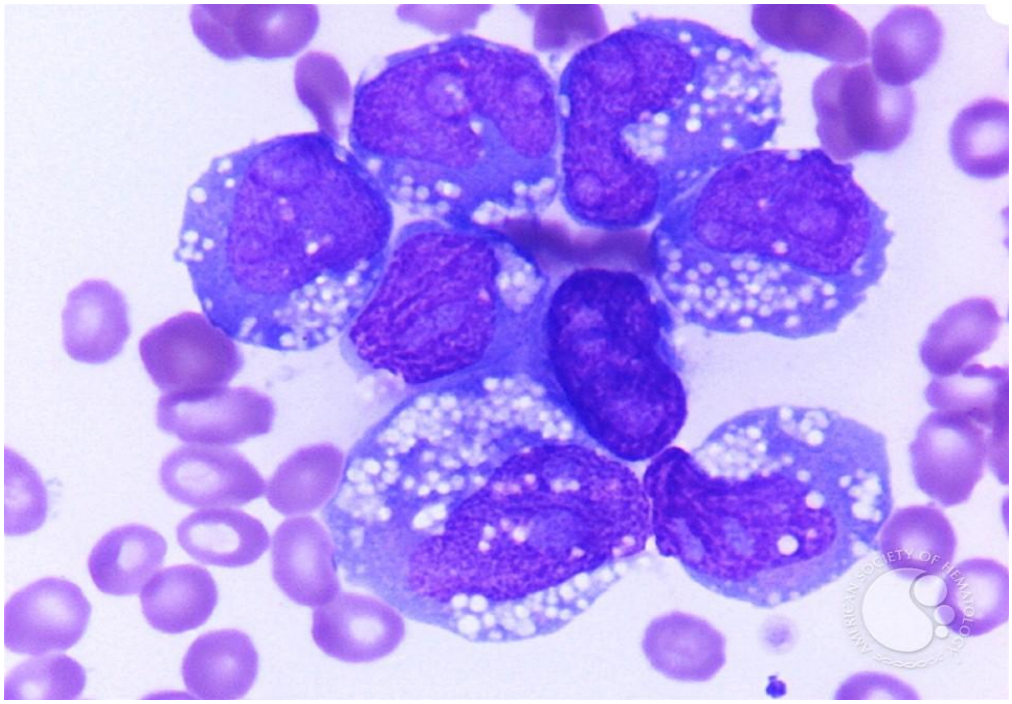


Figure 1.1: Acute myeloid leukemia; Source: Hashim A, & Sabeeh N,

1.2 Taxonomy of AML

AML have been categorized by two systems: French-American-British classification system, and World Health organization (WHO) classification system (recent). Based on the available genetic and clinical features WHO characterized AML into four major groups-

- Acute myeloid leukemia with recurrent genetic abnormalities
  AML with t (8; 21) (q22; q22), RUNX!-RUNX1T1
  AML with inv (16) (p13.1q22) or t (16; 16) (p13.1q22), CBFβ-MYH11
- Acute myeloid leukemia with myelodysplasia-related changes
- Therapy related neoplasms
- Acute myeloid leukemia, not otherwise specified
  - Myeloid sarcoma
  - Myeloid proliferations related to Down syndrome
  - Blastic plasmacytoid dendritic cell neoplasm

1.3 Genomic landscape of AML

The cytogenetic is very well-known scenario. In 2012 Cancer genome Atlas research network revealed one of their genomic studies on 200 AML patients anticipating to explicate the complex interplay of genetic lesions in individual AML patients. The experiment lacked enough data to provide such genomic insight but was enough to identify and categorize the AML-specific mutated genes. They categorized those mutated genes in eight functional classes, like mutation in - signaling genes, myeloid transcription factors, transcription factor fusions, nucleophosmin gene, spliceosome-complex genes, cohesion-complex genes, genes involved in epigenetic homeostasis, and tumor-suppressor genes (The Cancer Genome Atlas Research Network and co-authors, 2013; Döhner, 2015).

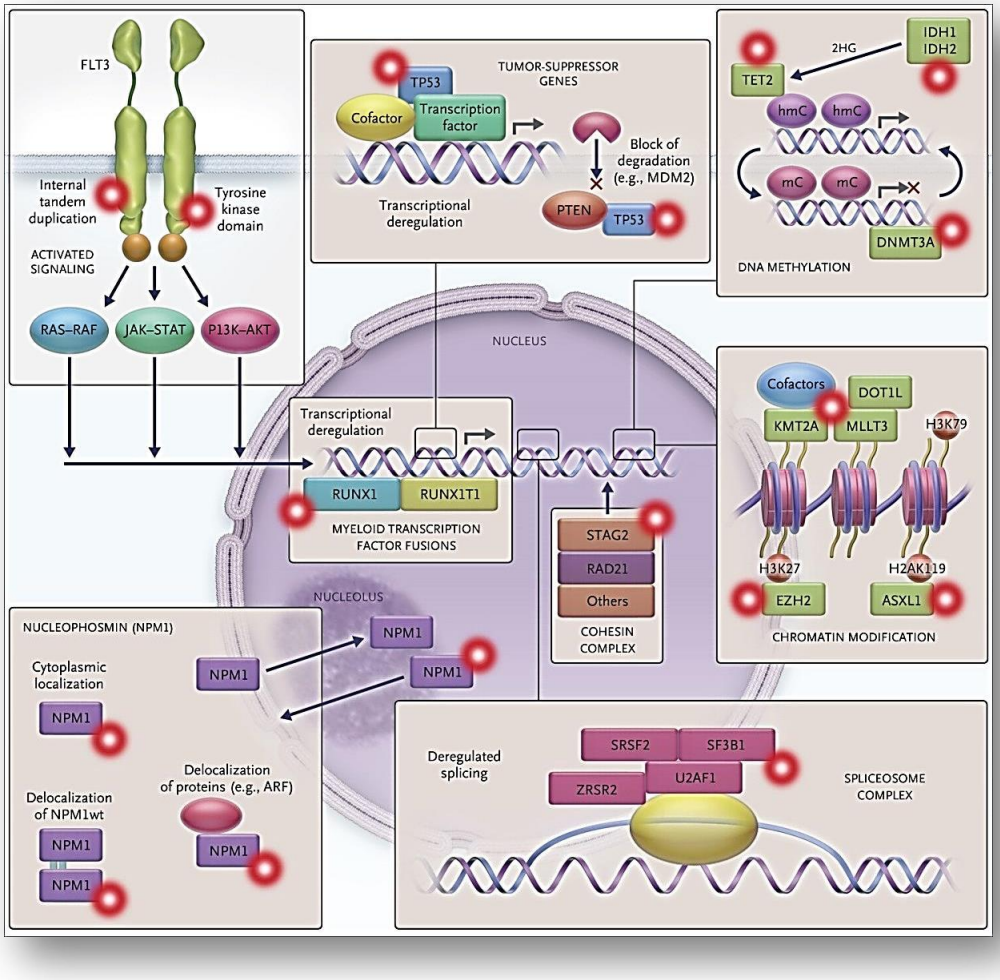


Figure 1.2: Different types of mutated genes commonly found in acute myeloid leukemia (Source: Döhner, 2015)

Studies also found that two diver mutations might be enough for AML initiation but in most cases, patients found to carry more. The study suggests that AML can be characterized by clonal heterogeneity during diagnosis and at relapse it might contribute to disease's resistance against different therapies (Döhner, 2015; Tallman 2005; Welch 2012; Grove, 2014). High variability in progression is very characteristic to cancer.

Cancer evolution is a complex and unpredictable process. Thus far there have been two proposed concepts of AML evolution, namely linear evolution and branching evolution.

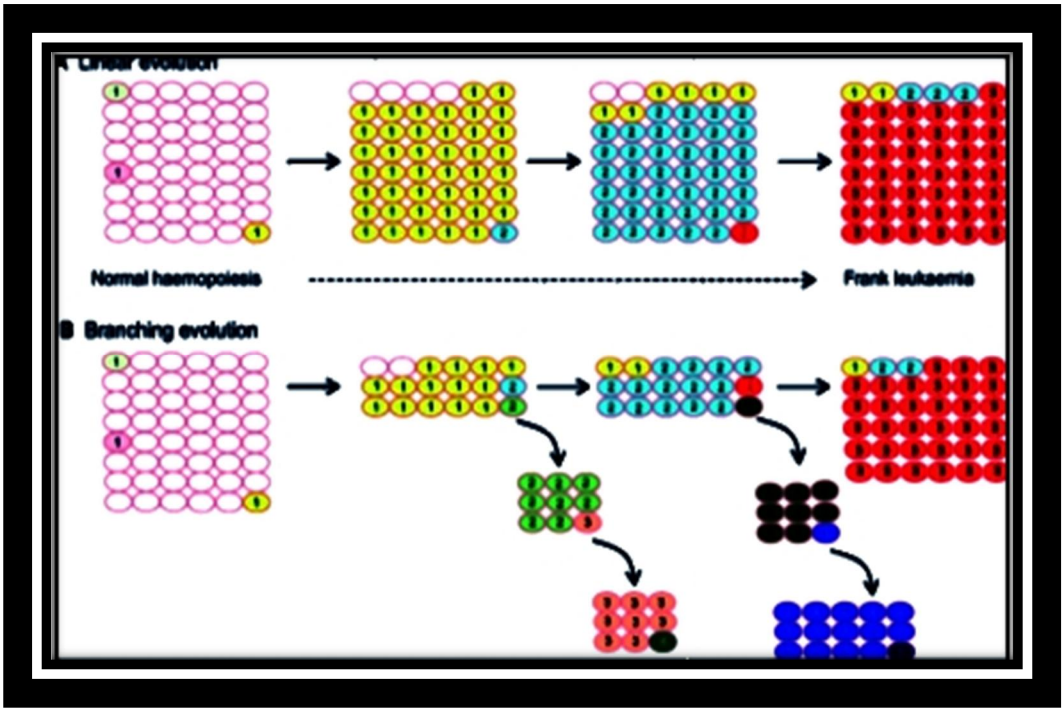


Figure 1.3: Schematic presentation of linear and branching evolution. Source: Grove, 2014

Linear evolution can be defined as stepwise accretion of driver mutations resulting in sequential dominant clones. The final tumor dominates/overpowers earlier clones-which contains only few mutations compared to the final tumor that carries all mutations occurred throughout its history. But several studies reported AML as an expressive and mutational complex disease as well as they overthrow the idea of final tumor being over dominant (by numbers) to the earlier subclones. They rather agreed on variation in relative dominance among subclones throughout the disease [(Welch 2012; Grove, 2014, Anderson 2013)]. The continuous acquisition of mutations and associated expansions in population sizes intensely increase genetic and clonal heterogeneity in cancer, and therefore it is expected that most cancers evolve with a complex branching architecture.

1.4 Prognosis:

Poor prognostic value of AML has been the major drawback in treating patients. Researchers are always in quest for established marker of AML for better predictability but so far, the outcome has not been satisfactory. Several factors have been identified till date; those can be divided into two major groups-

- Patient related like age, coexisting condition, poor performance status, and,
- Disease related like white cell count, prior myelodysplastic syndrome, and leukemic-cell genetic changes.

Knowing the biology of AML is very important for leukemogenesis and can be of great clinical significance. For example, autocrine proliferation in AML seem to adversely affect prognosis. Angioregulatory molecules like angiopoietin-2 positively affect prognosis whereas antiangiogenic agent's effect is adverse. Furthermore, Intracellular signaling also affect prognosis. Intracellular phosphoresponses specific to exogenous cytokines are related to prognosis (Bruserud 2001; Irish 2004, Del Principe 2005). In addition, molecular genetic lesions are considered as active prognostic marker for AML like NPM1 and CEBPA mutations and FLT3 internal tandem duplications. These are already in use as prognostic marker in clinical practice while few others (RUNX1, ASXL1, TP53) waiting for the recommendation (Dohner, 2010). Disease outcome can also be predicted monitoring leukemia associated aberrant phenotypes which can be identified using high throughput flow cytometer.

1.5 Current therapies

Till to date AML is considered as a treatable disease. The general strategy for treating AML patients is long term process. The aim is to acquire complete remission and maintain it, although patient first must need to go through an assessment period to determine whether s/he is eligible to undergo such long-term process or not. Among current strategies consolidation therapy with conventional chemotherapy and autologous stem cell transplantation has relatively lower mortality count but for allotransplantation treatment related mortality rate is quite high. At present cytogenetic profile of individual patient is used to measure prognostic feature for determining the patient's category, like favorable, intermediate and poor risk groups leaning on the outcomes by chromosomal abnormalities in several large series of patients (Lin, 2010). The whole conventional treatment process can be divided in to two main steps- first achieving complete remission via induction therapy and secondly post remission that includes consolidation and maintenance.

1.5.1 Induction therapy: The target for induction therapy is to lower the number of malignant cells in bone marrow as well as resuming normal blood cell production. Till date continuous infusion cytarabine with anthracycline is the established strategy for induction therapy. Normally patients aged ≤60 YRS. receives Daunorubicin 45 to 60 mg/m2; Idarubicin 10–12 mg/m2; Mitoxantrone 10–12 mg/m2) and 7 days of continuous-infusion cytarabine (100–200 mg/m2). The success rate to complete response is about 60-85% for younger (≤ 60 yrs.) patients but for older patients the response is way inferior. Patients with partial remission generally receives a second cycle of induction. Patients older than 60 yrs. with favorable-risk and intermediate-risk cytogenetic findings and no coexisting conditions, induction therapy is similar as to that in younger patients (Dohner et al., 2015; Burnett et al., 2005; Derolf et al., 2008). During treatment older patients often accompanied with some other conflicting situations like cytogenetic abnormalities or clinically co-existing situation or both which most likely adversely affect the patient's situation. Therefore, older patients are mostly not suitable candidates for intensive induction therapy. Few other options are also emerging besides cytarabine, like daunorubicin (Dohner et al., 2015; Tallman et al., 2005, Haferlach, 2008).

1.5.2 Post remission therapy: Once complete remission achieved, appropriate post remission therapy is needed to preserve complete remission. Post remission therapies include conventional chemotherapy as well as hemapoietic cell transplantation. Post-remission therapy can be described in two steps (Dohner et al., 2015; Tallman et al., 2005, Haferlach, 2008; Dohner et al., 2010). -

Ä Consolidation therapy: to achieve prolonged remission by preventing relapse as well as further reduction in the remaining leukemic cells.

Consolidation with conventional chemotherapy: Patients with favorable ELN genetic risk profile should receive 2–4 cycles of intermediate-dose cytarabine (1000–1500 mg/m2 intravenously, usually every 12 hrs. over 3 days, or 1000–1500 mg/m2 intravenously on days 1–6). This treatment along with chemotherapy might bring better results for patients with favorable genetic profile as well as for adverse-risk AML patients. Patients older than 60 yrs. normally receive intermediate does of cytarabine (500–1000 mg/m2 intravenous, every 12 hrs. (Days 1–3), or 500–1000 mg/m2 intravenous, (days 1–6).

Consolidation with allogenic hematopoietic stem cell transplantation (alloSCT): For patients who fail to reach the extended complete remission with conventional approaches. AlloSCT is more considered as investigational therapy for patients. The eligibility of patients is esteemed based on his/her medical co-exiting conditions, recent history, and performance status as well as patient's agreement to participate in this experiment. Patients receive low dose of cytarabine 20 mg/12 hr. intravenously (days 1–6 for 4 weeks) (Dohner et al., 2015) It is hitherto the best supportive care systems for patients who are unable to receive antileukemic therapy, but it doesn't guarantee positive outcome or complete remission. The outcome of treatment is affected by few factors like- patient's age: health issue might cause older age patients (older than 60 yrs.) unfit for such process. High risk genetic factors, Graft source, and graft-versus-leukemia effect can severely affect the whole transplantation process. The chance of relapse increases with persisting minimal residual diseases. Another important factor is the performance index of the patient, it also highly effects the outcome of the treatment.

Ä Maintenance therapy: It is less myelosuppressive than induction or consolidation therapy. The aim of this therapy is to prevent relapse by further reducing the number of residual leukemic cells. But so far no validation has been made that patients who has received intensive therapies are benefiting from maintenance therapy (Robak et al., 2009; Buchner et al., 2007).

1.5.0 Treatment of relapsed and refractory patients: Till to date, with standard chemotherapy 50-70% of AML patients who achieve CR are expected to relapse over 3 years and 20-40% of AML patients do not even achieve remission (Robak et al., 2002; Mato et al., 2008). Patients with relapsed or refractory AML has poorer response and overall survival to treatments than patients who achieve CR lasting ≥6 months. Many strategies have been considered but none emerge to be the optimal. AlloSCT proved to be curative for a minority of patients but it's not the solution for the majorities and largely depends on the availability of the donor. In most cases additional chemotherapy is recommended in the hope of achieving remission. Salvage therapies mostly include high (2-3g/m2) or intermediate (1-1.5 g/m2) dose of Cytarabine (Ara-C) in combination with other agents. With salvage therapy 40-60% remission is achieved for patients who has first CR lasting ≥ 1 years but for patients CR lasted ≤ 1 years the success rate goes down to 10-15%. Till to date no single approach or treatment can be considered as the standard for the relapsed patients (Tallman et al., 2005; Estey et al., 2000, Robak et al., 2009).

1.6 Anti-cancer drugs/agents:

The history of anti-cancer drugs starts back in 1940s. Mechlorethamine, an alkylating agent was the first clinically used anti-cancer drug. Ever since hundreds of anticancer drugs have been developed. The successful use of anti-cancer drugs depends on several factors, like type and location of cancer, side effects of drugs, disease status, treatment strategy etc. but above all specificity of anticancer drugs as it plays important role in reducing the severity of side effects associated with the drugs' use.

1.6.1 Classes of anticancer drugs

1. Regardless of the type of cancer, anti-cancer drugs can be listed into several categories (See the table below). (Goodman, The Pharmacological Basis of Therapeutics. 11th editon; Tripathi, Essentials of Medical Pharmacology. 5th Edition; Snyder, 2014)

Types	Example
Alkylating agents	Nitrogen mustards: Cyclophosphamide, Ethyleneimines, Nitrosoureas, Methyl Hydrazines, Alkylsulfonates, Platinum Coordination complexes.
Anti- Metabolites	Folate Antagonists: Methotrexate, Purine antagonists, Pyrimidine antagonists: 5-Fluorouracil.
Anti-Tumor Antibiotics	Fludarabine, Raltitrexed
Plant Alkaloids/ Microtubule Inhibitors	Etoposide, Irinotecan
DNA Linking Agents	Oxaliplatin, Carboplatin
Biological agents	Bevacizumab, Ipilimumab
Bisphosphonates	Pamidronate, Ibandronic acid
Hormones/other	Anastrozole, Thalidomide

Table 1: Types of anticancer drugs

Not necessarily all approved anti-cancer drugs can be used for every types of cancer. So far Food and Drug Administration (FDA), US has approved the following list of drugs for treating AML. Apart from the names mentioned in Table -2, one drug combination (ADE) has also been approved by FDA to treat AML patients. The combination referred as ADE and is mainly used to treat acute myeloid leukemia in children. The drug combo includes the following drugs (Drugs Approved for Different Types of Cancer, 2016).

Drugs in ADE combination:

A = Cytarabine (Ara-C)

D = Daunorubicin Hydrochloride

E = Etoposide

FDA APPROVED DRUGS FOR ACUTE MYELOID LEUKEMIA	
¨ Arsenic Trioxide	¨ Daunorubicin Hydrochloride
¨ Cerubidine	¨ Doxorubicin Hydrochloride
¨ Clafen (Cyclophosphamide)	¨ Idamycin (Idarubicin Hydrochloride)
¨ Cyclophosphamide	¨ Idarubicin Hydrochloride
¨ Cytarabine	¨ Mitoxantrone Hydrochloride
¨ Cytosar-U (Cytarabine)	¨ Neosar (Cyclophosphamide)
¨ Cytoxan (Cyclophosphamide)	¨ Rubidomycin
¨ Trisenox (Arsenic Trioxide)	¨ Tabloid (Thioguanine)
¨ Vincasar PFS (Vincristine Sulfate)	¨ Tarabine PFS (Cytarabine)
¨ Vincristine Sulfate	¨ Thioguanine

Table 2: List of FDA approved drugs for acute myeloid leukemia (Drugs Approved for Different Types of Cancer, 2016).

1.6.2 Novel and emerging drugs for AML:

Multiple studies reported that AML patients are unlikely to be benefited clinically from further dose intensifications in post remission therapy. Optimization of AML therapy is very much needed, and the process might include new anticancer drug/agent or can be even entirely novel therapeutic strategy. Some of the potentially emerging anticancer agents include monoclonal antibodies, immunotoxins, hypomethylating agents, alkylating agents, FMS-like tyrosine kinase (FLT3) inhibitors, multidrug-resistant modulators, and farnesyltransferase inhibitors. Then again study suggests that such agents might not effective in clinical trial when given as monotherapy. Therefore, compounds that have showed great potentiality in early investigational phases, should be subject to further trial in combinations with approved standard drug/s or another new agent/s (Kern et al., 2006; Robak et al., 2009).

1.7 Cancer Research and limitations

1.7.1 Incompetence of current anticancer drugs/agents:

Despite all the efforts dug discovery in AML has met only modest amount of success. Discovering new and effective drug for AML is not straightforward. Heterogeneity of AML makes it further difficult. Drug development would have been more effective if preclinical models could estimate clinical outcome more accurately. Cell line derived from specific tumor or primary cell cultured in an animal model generally lacks the disease complexity. Cells in culture or in animal model grows in normal condition and may descend from a selected subpopulation of cells, therefore might retain/represent the disease complexity inefficiently. Moreover, the genetic landscape of AML also reveals another major reason for failure of many anticancer drugs as single therapeutic agent. Generally, AML has more than one driver mutation, on average 13 and typically on average 5 of them occurring in genes that are recurrently mutated and consistent with a pathogenic role in AML (Estey et al., 2015; Klco et al., 2014). Most mutations are stochastically acquired events in normal hematopoietic stem/progenitor cells, within which hematopoietic stem/progenitor cell retains relatively small number of "driver" mutations. Many of the anticancer agents might be successful reversing the mutational effect but those mostly ignores the abnormalities/mutations acquired during AML evolution. These new critical abnormalities may further act as active drivers in disease progression. (Duque-Afonso et al., 2014, Krönke et al., 2013, Klco et al., 2014). These newly acquired mutations may bypass the founding mutation(s) by using a parallel cellular pathway or might act as the downstream of the original mutation(s). Therefore, it is evident that, cells from the establishing clone repeatedly give rise to a variety of subclones which are potentially resistant to many therapies or drugs and can predominate at relapse making AML a progressive disease. (Duque-Afonso et al., 2014, Klco et al., 2014). The development and growth of AML highly depends in multiple distinct pathways; therefore, it can be argued that each of these coactive pathways need to be targeted therapeutically for better success.

1.7.2 Limitations of Cell line as cancer study model:

AML is a major cause of human death worldwide and its well-known molecular heterogeneity makes it further challenging to understand its biology. Many different experiment models are available to study cancer or to investigate for novel and effective anticancer drugs. The advancement in cancer pathobiology owes greatly to these experiment models like cancer cell line model, primary cell culture, in vivo animal model etc. Use of cell lines in cancer study as an experimental model has been very popular and valuable. Even after being used successfully for decades in cancer study still many scientists doubt the significance of cell line model in cancer. Nelson-Rees and colleagues in 1970s first questioned the clinical significance of cell lines in cancer. They further concluded that cell lines undergo numerous passages leading to extensive modifications in its characteristics which in turn make the cell lines clinically insignificant (Gillet et al., 2013; Nelson-Rees, 1974, 77, 78). Some other issues include- i) cross contamination, a major problem in cell line model. ii) Genotypic and phenotypic drift is also common in cell line. Cell lines are easily stored but studies reported if stored for long time, cell lines lean towards some genotypic as well as phenotypic changes. This is frequently caused by the rise of subpopulation within a culture followed by changes over a period of time by the selection of specific, more rapidly growing clones. Even though the cells appear identical morphologically, differences in cell growth rate, clonogenicity, hormone receptor content, and Karyotype have been observed. Genomic instability also causes genomic differences between the original tumor and the cell line derived from it (Ferreira et al., 2013, Burdall et al., 2003). Cell line losses the natural heterogeneity of the tumor. Therefore, cell line culture

environment doesn't reflect the original tumor microenvironment which might explain the failure of anticancer drugs in clinical trial even though they show high potency in laboratory test (Van Staveren et al., 2009, Ferreira et al., 2013, Burdall et al., 2003; Gazdar et al., 2010, Bahia et al., 2002; Vargo-Gogola et al., 2007).

1.7.3 Limitations of animal modelling

Animal modelling have been the front-line strategies for drug discovery research. However, several limitations in mouse modeling, such as species-specific differences, limited recapitulation of de novo human tumor development, and differences in drug response as compared to humans (Cheon et al., 2011; Testa et al., 2011) makes it a unsuitable perspective. For instance, mice tend to be more resistant to many anti-cancer compounds than patients (Teicher et al., 2009). The lack of functional immune system in these models may also interfere with the outcome, as immune surveillance is known to contribute to several aspects of tumorigenesis. For instance, certain populations of lymphocytes and leukocytes play a tumor-supporting role in cancer (Grivennikov et al., 2010; Ustun et al., 2011). Consequently, immunocompromised mouse models often fail to recapitulate the human microenvironment properly, which might contribute to the fact that the results obtained in xenograft models do not often correlate with clinical outcomes. Furthermore, high-throughput screening in mice is challenging. Therefore, combinatorial approaches that use several model systems in a more closely resembled environment are required to further understand the complexity of human cancers (Cheon et al., 2011) and further to find appropriate treatment strategy.

1.7.4 Primary cell culture as an alternative?

The ability to detect sensitivity towards therapy in the laboratory before clinical treatment in similar environment, has long been an aspiration in AML. With the concomitant discovery of specific markers as well as technologies, primary culture can be the long-awaited model system to study drug sensitivity for AML. Primary cultures provide detailed pathology compare to cell line. This detailed pathological knowledge can then be to be compared with those of the original cancer environment which will help us in better understanding the disease. Primary cell culture mimics the disease microenvironment closer than cell line.

1.8 High Throughput Flow cytometry in hematology

Flow cytometry is a widely used method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population. It provides multiple parameter analysis of single cells flowing in single file in a stream of fluid (Brown, 2000). Döhner et al., described multi-parameter flow cytometry as a powerful tool in drug discovery for identifying leukemia associated aberrant phenotype (Döhner, 2015).

1.8.1 General principle of Flow cytometry: Flow cytometry measures optical and fluorescence characteristics of single cells or any other particle for example, latex beads etc. passes through the detector channel. Forward angle light scatter characterizes physical properties, for example size, and

right-angle scatter characterizes internal complexity. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Antibodies conjugated to fluorescent dyes can bind to specific proteins on cell membranes or inside cells. So, when labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. While returning to their resting states these fluorescent molecules emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously (Figure 1.4).

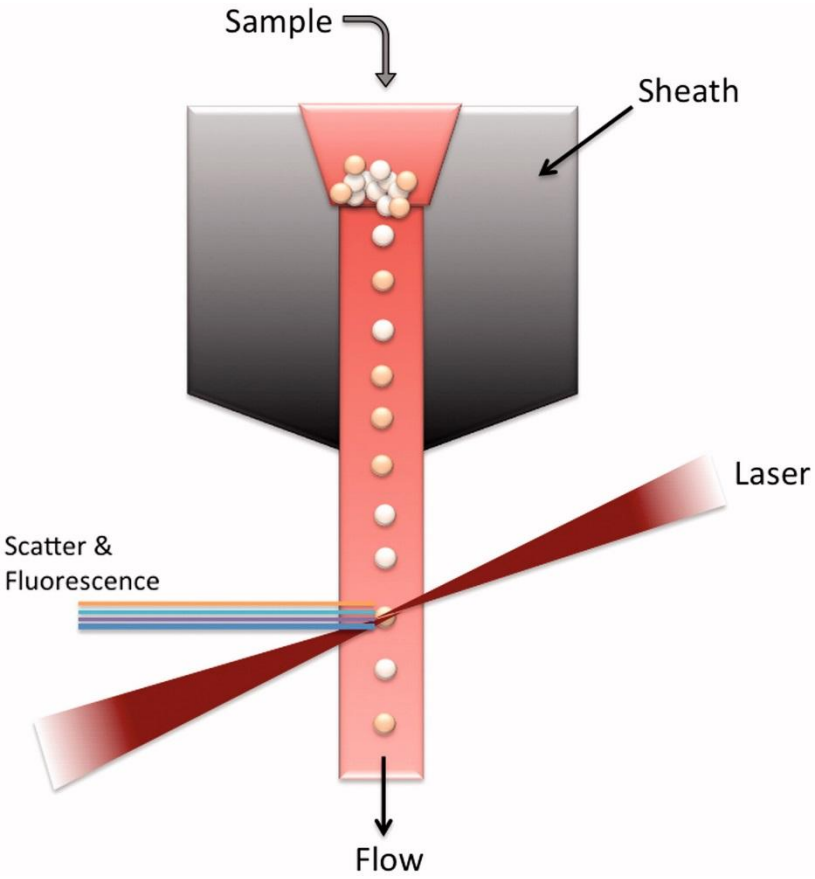


Figure 1.4: Basic working principle of a flow cytometer. Source: Aysun, 2017

1.8.2 High throughput flow cytometry (IntelliCyt iQue screener hd and plus): HTFC outweighs traditional flow cytometry by many features like plate level annotation, analytics to result visualization that are unlikely available with traditional flow cytometer. It has continuous, air gap-delimited stream which ensures accurate tracking and minimize cross-contamination. Its laser trimming technology that enables accurate counting and analysis of a wider range of particle sizes in the same sample (Comley, 2015).

1.8.3 High throughput flow cytometry: Advantages

There are many other benefits of using HTFC in developing drug sensitivity assay. Many technologies like, ELISA, Flow cytometry (traditional), and fluorometric microvolume assay technology (FMAT) are in use for drug discovery but most suffers due to some limitations. For example, ELISA includes numerous wash steps that introduce variability, which is unwelcoming, and this extra washing might

destroy important epitopes during antigen immobilization which might fail the whole experiment. Most importantly ELISA lacks multiplexing capabilities which is one of the major demands of today's science. FMAT has multiplexing capabilities but with limited capacity. Moreover, this technique lacks sensitivity for low binding. Flow Cytometry might be better option when comparing to the above-mentioned techniques, but it is comparatively slow, complex to set up and use than HTFC. Another major concern for traditional flow cytometry is the requirement of large sample volumes. The advantages of using HTFC over other applications can listed as bellow-

- HTFC screener can analyze multiple cell populations within each microplate well.
- HTFC can work with suspension cells/blood cells.
- Multiplexing is another true feature of HTFC.
- It provides opportunity for novel approach to phenotypic screening.
- It has higher sensitivity and wider dynamic range than traditional FC or other related applications.
- It is fast and highly accurate.
- Resolution of free and bound signals for cellular and molecular targets.

1.8.4 High throughput flow cytometry: Immunophenotyping in drug discovery

Immunophenotyping is the technique that allows to identify various populations of interest and even their portions within a heterogeneous cell population. The principle behind the use of antibodies to identify such population is their specificity towards antigens expressed by these cells. These antigens are known as markers. These markers are usually functional membrane proteins involved in cell communication, adhesion, or metabolism. However, these markers will often be expressed on more than one cell type. In this circumstance HTFC comes in use as it allows immunophenotyping cells with two or more antibodies concurrently. By evaluating the unique repertoire of cell markers using several antibodies together, each coupled with a different fluorochromes, a given cell population can be identified and quantified.

Chapter Two: Aim of the thesis

The aim of the study is to use cancer markers (cluster differentiation (CD) markers that are widely used in cancer diagnosis and treatment) in primary AML sample to study different but specific populations using HTFC.

My objective is to develop a primary AML sample based High throughput flow-cytometry assay that is able to successfully measure ex-vivo drug response of specific cell populations.

With further improvement this platform may provide a stronghold for individualized systems medicine strategy to tailor treatments for patients with acute myeloid leukemia.

Chapter Three Materials and methods

3.1 Workflow:

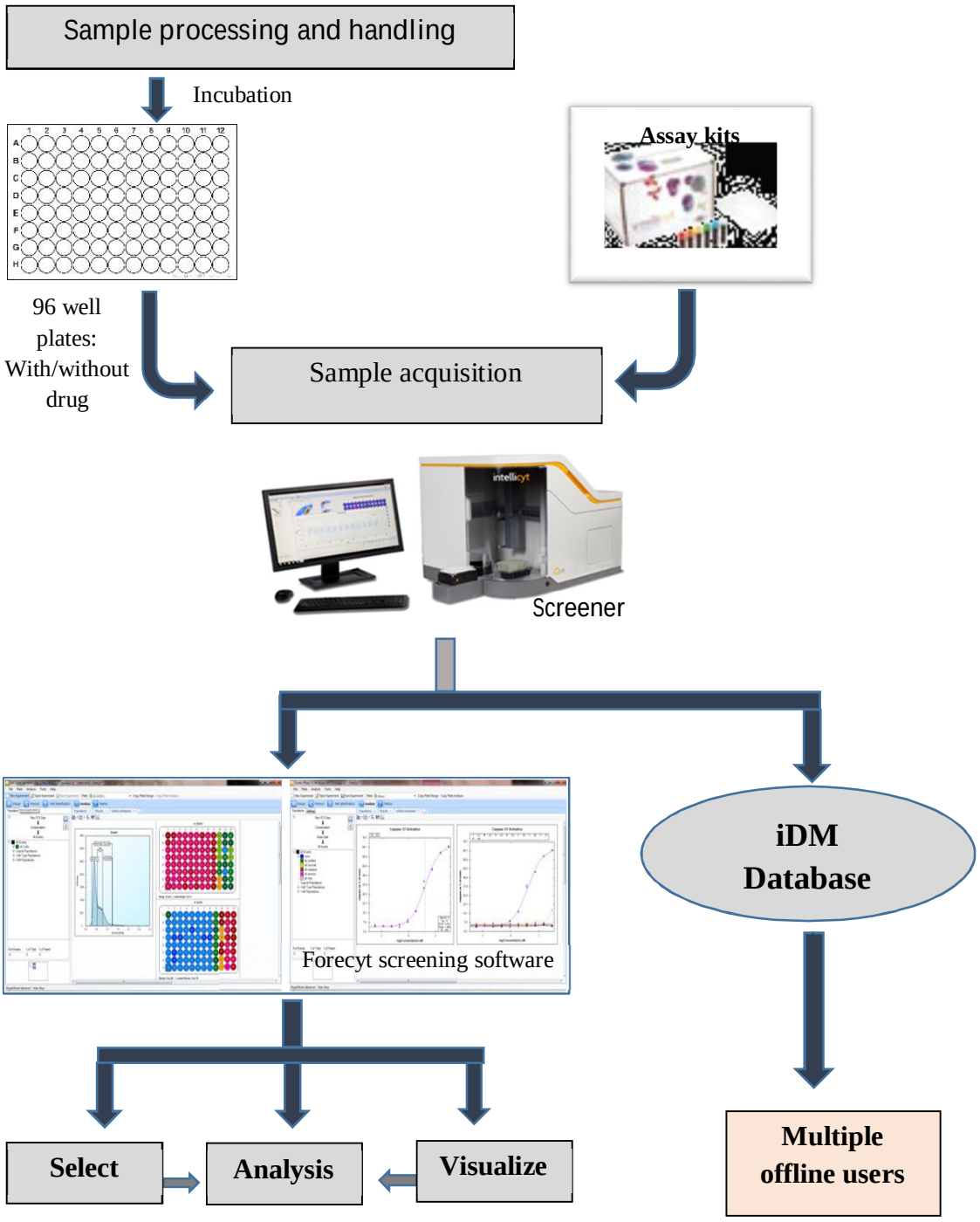


Figure 2.1: Workflow with high throughput screening system

3.2 Sample processing and handling

AML patient sample studied in this project were primarily collected with written informed consent and ethical approval of the Institutional Review Board of the Hospital in accordance to Finnish legislation. Upon receipt, Bone marrow or peripheral blood aspirates from leukemia patients were subjected to Ficoll centrifugation (Ficoll-Paque PREMIUM; GE Healthcare) in order to isolate the mononuclear cells by density gradient separation and immediately used for analysis or stored as pellets at -70 or bio-banked in liquid nitrogen in FBS and 10% DMSO for future use.

3.3 Culture media, reagents and antibodies

Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium from Medicago Ltd., Uppsala, Sweden. Fetal calf serum (FCS), RPMI-1640 medium was obtained from Lonza Group Ltd. (Verviers, Belgium); Annexin V FITC apoptosis kit and CD123 were ordered from Miltenyi biotec ltd. (Lund, Sweden); other antibodies (CD96, CD34, CD38, CD45) are from BDBiosciences (San Jose, CA, USA), and CellTiter-Glo® reagent was ordered from Promega Ltd., USA. L-glutamine, streptomycin, and Penicillin were lab made. Cell culture plates (96 well) were ordered from Thermo Scientific, Denmark.

<u>12.5% conditioned media Composition:</u>

- 12.5% HS-5 medium
- 87.5% complete cell culture medium

Roswell Park Memorial Institute medium (RPMI) 1640,

10% fetal calf serum,

1% L-glutamine, and

1% penicillin-streptomycin.

3.4 Factors in assay development

On the process of developing the drug sensitivity test assay we took all the prospects in count that might have substantial effect over the growth and phenotypic expression of cells and /or any sub-populations of cells, as well as their overall sensitivity towards drugs. Therefore, in every occasion cell were treated almost equally, keeping some of the comparable factors different to encounter every possibilities. Cells were plated at a concentration 200,000/well in 100 µl 12.5% conditioned media. The parameters observed are

Factors	Condition
Washing or no wash	Wash group is subject to extra washing (than no wash group) afterwards staining step to minimize background intensity.
Plate type (v- bottom, u- bottom, flat bottom)	Cells were cultured in every plate at same culturing environment to examine whether well bottom type has any significance for our assay.
Incubation period (day 0,1…3)	Cells were incubated for different time period, like 24 Hrs. and 72 Hrs.
Antibody concentration	Antibody titration was done to learn the optimum concentration (criteria considered- cost effectiveness, duration, background noise minimization and better separation of stained populations)

3.5 Cell Viability Assay (ATP based)

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. Here, cell viability is directly correlated with the amount of ATP in cells. The Assay relies on the properties of a proprietary thermostable luciferase, which produces a stable "glow-type" luminescent signal. Detection is based on using the luciferase reaction to measure the amount of ATP from viable cells. There is a linear relationship (r2 = 0.99) between the luminescent signal and the number of cells from 0 to 50,000 cells per well. Values represent the mean ± S.D. of four replicates for each cell number. Among the homogeneous viability assays, the ATP assay is the fastest to perform compare to any other ATP based methods that require multiple steps to extract ATP and measure luminescence and can detect the smallest number of cells, making it useful for 384- and 1,536-well formats. The CellTiter-Glo® reagent principally works by lysing cell membranes to release ATP; it also inhibits endogenous ATPases and provides luciferin and luciferase necessary to measure ATP using a bioluminescent reaction [(Riss, 2005)].

3.5 Staining protocol/FACs analysis of AML (iQue Plus)

§ Centrifuge the cells (300gX6min) in a 15 ml falcon tube, then remove supernatant.
§ Re-suspend the cells in 0,5ml staining buffer (0.5% BSA in PBS). If the cells start to clump treat with DNase (frozen sample only).
§ Dilute the cells with staining buffer to the right concentration 2 million cells/ml.
§ Add 50ul of cells to 96-well V-bottom plate (100 000cells/well).
§ Staining: Add the following antibodies to the wells. The study panel included the following monoclonal antibodies: CD33, CD34, CD38, CD45, CD96, and CD123.
§ Incubate at RT for 15min and cover plate with folia.
§ Add 100ul PBS on top of each well and mix properly.
§ Centrifuge the plate, 500g for 6min. Remove supernatant by turning the plate upside down.
§ Vortex the plate shortly to remove cells from the bottom.

§ Dilute the cells to 25ul staining buffer.
§ Add 2ul of 7AAD to one well and one well with all antibodies.
§ Read the plate immediately with iQue plus.

3.7 Data acquisition and analysis (HTFC)

The experiment was always carried on 96 well (v bottom) plate. The following setting was used during the acquisition, intending to achieve ≥40,000 events per well.

§ Preplate priming: 60 seconds, prior to sampling a plate.
§ Pre-plate Shake: 15 seconds at 2000 rpm
§ Sample Order: By Column
§ Sip Time: 18 seconds
§ Additional Up Time: 2 Seconds
§ Sample Height: 0.50
§ Pump: 29 rpm
§ Plate Model: V bottom 96 well
§ Interwell Shaking: 4 Seconds at 2000 rpm after every other well.
§ Cytometer speed: Medium
§ Threshold: FSC-H < 150000
§ Flush duration: 30 secs;
§ Post-plate Clean: Decon: 30 secs; Clean: 30 secs; Water: 60 secs.

IntelliCyt Corporation provides powerful data acquiring software ForeCyt for rapid analysis of all experimental data. After the acquisition period it generates analysis on a template file. To minimize the fluorescence, overlap between detection channels compensation adjustment (provided by IntelliCyt Corp.) is also added to the template file. ForeCyt software comes with electronic gating feature that create hierarchical population tree in that template file. From the scatter profile (SSC vs. FSC), different cell population percentages were determined by SS/CD marker gating (manually drawn) excluding dead cells and debris. These gating are uniform for all wells for specific fluorescence. Gating to define “Positive” and “negative” cell population (fluorescence signal) was drawn based on fluorescence intensity of the positive and vehicle-only control samples, and/or as appropriate to the endpoint.

3.8 Moab combinations used to characterize AML cells

The following drugs are used in this experiment.

Antibody	Fluorophore	Specificity
CD 3	BL4/FL4	
CD 8	BL3/FL3	
CD38 FITC	BL1	Marker of cell activation
CD34 PE-Cy7	BL5	Hematopoietic stem cells marker
CD96 APC	RL1	Leukemic stem cells marker
CD123 APC-V770	RL2	Myeloid differentiation marker
CD45 BV605	VL4	Leukocyte activation marker

Table 3: List of antibody panel

3.9 Statistical analysis

Statistical analysis was conducted with Graph Prism version 7.0 (GraphPad Software). Differences between drug responses were analyzed by Mann-Whitney U test, and for multiple t-tests p-values were adjusted using the Benjamin-Hochberg method ($q<0.10$ used to determine significance).

3.9.1 Calculation of the drug sensitivity and drug combination scores

Here drug sensitivity score (DSS) method (20) is used to measure ex-vivo drug sensitivity of AML and healthy BM against tested drugs. DSS is a measure of drug response based on the area under the curve (AUC) calculation with further modifications. It captures both the potency and the efficacy of the drug by integrating all four curves fitting 140 parameters: inhibitory concentration (IC50), slope and minimum and maximum asymptotes. Drug combination efficacies were calculated as the difference between observed and expected values. The expected value is computed using the Bliss independence model20 as reference, which assumes that two drugs exhibit their effect independently21. If Ya is percent inhibition of drug A at dose a and Yb is percent inhibition of drug B at dose b, then Bliss independence model is written as YBliss = Ya + Yb – YaYb.

Chapter Four: Results

Disease microenvironment plays important role in stability and efficacy of an anticancer drug candidate. While developing an assay it is important to keep it error free as much possible while taking care of the basic requirements. But studies have shown that in research for many diseases meeting standards might not be enough like, in AML. So far, no assay has been developed in AML research that can provide us with actual complex disease environment.

It is therefore essential to develop a screening platform that can assess the drug candidates' therapeutic significance within appropriate or similar disease microenvironment. The primary sample of patients negates all other available option in this respect. In this study, we aim to develope a 96-well plate high throughput, multiplexed flow cytometric (FC) assay for immunophenotypic quantification of AML via specific identification of CD34+CD38− LSCs which can be used for population specific drug studies.

4.1 Assay development: scale up and optimization:

In this study we tried to build a screening platform that mimics (partially) the disease complexity while scaling up the assay set up to come up with best possible option for AML study. The following factors were investigated to optimize the consistent cell culture growth as well as their analysis, like washing condition, incubation period, types of culturing plates, and antibody concentration.

4.1.1 Treatment condition

Background noise is a major concern in immunophenotyping based drug sensitivity assay. We believed an added washing step will further reduce background noise. Therefore, we considered extra washing step while preparing the sample for FACS analysis. Same sample was prepared separately with and without extra washing step. Afterwards the results were compared (Fig. 3.1). Sample with extra washing procedure appeared to have less background noise which is beneficial for further analysis, for example separating different population or finding the expression of a small amount of any subpopulation. But only disadvantage is the requirement of high number of cells compare to no-wash procedure. Multiple samples (10) were run to confirm the result.

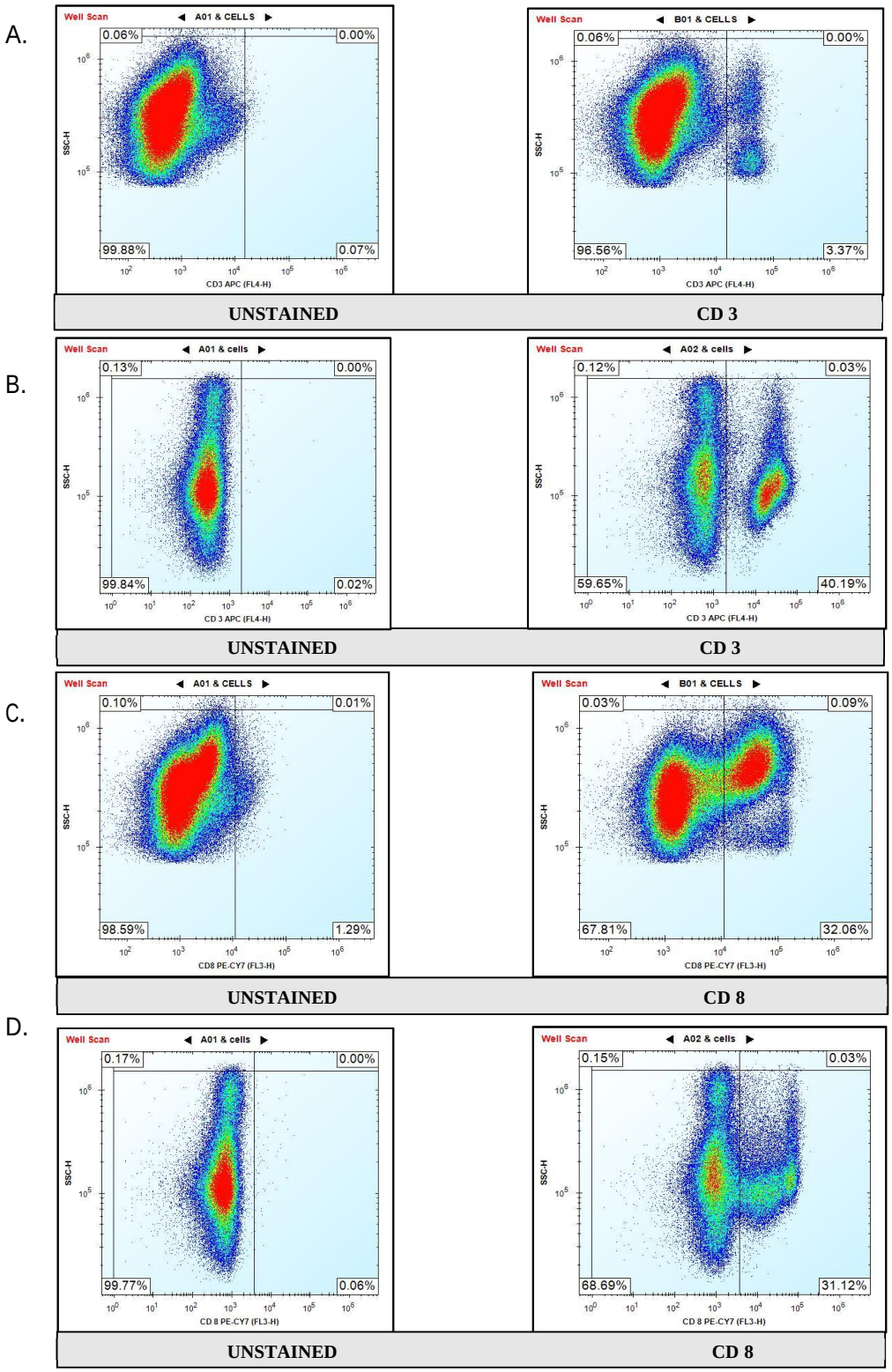


Figure 3.1: Comparison between expression patterns for different experiment condition in AML sample. Same sample has been labeled with UCHT1 monoclonal antibodies CD3, and CD8, analyzed by flow cytometry. The CD3 and CD8 positive populations were gated (right panel). Fig. 3(A) & Fig. 3(B) represents CD3 expression for 'wash' condition and 'no wash' condition respectively. Fig. 3(C) & 3(D) represents CD8 expression for 'wash' condition and 'no wash' condition respectively. The experiment was done on same sample for different incubation times.

4.1.2 Incubation period:

To find optimum incubation period for cell culture growth, same sample was incubated for different period of time viz. '0 Hr.' and '72 Hr.' (Fig. 3.2). Both samples were processed and treated similarly for FACS analysis subsequently. We haven't noticed any significant difference between two study groups (Fig. 3.2). Result was confirmed after testing multiple samples (10). It can be concluded that incubation period has little or no effect on cell culture growth as well as their surface marker expression.

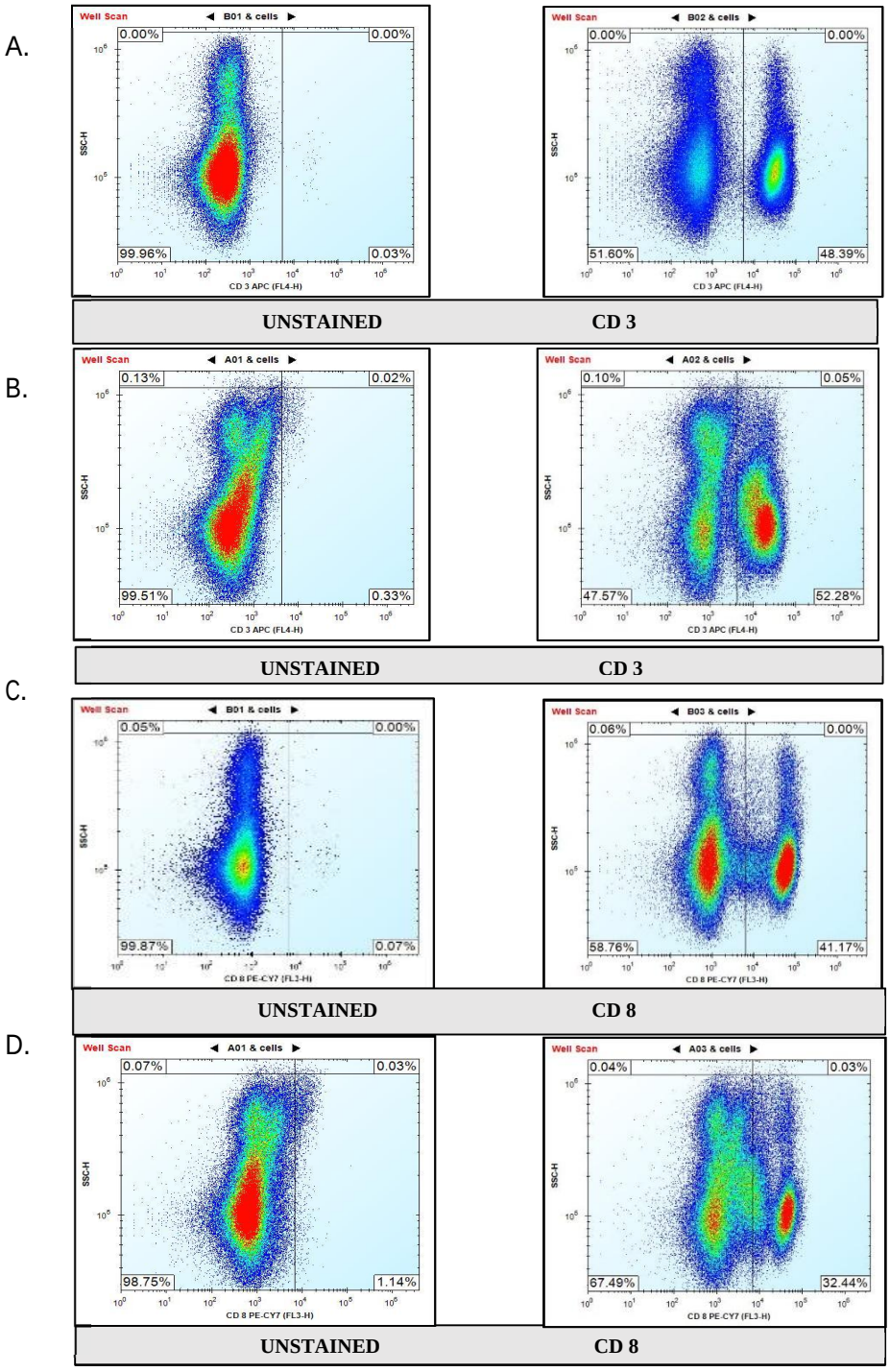


Figure 3.2: Comparison between expression patterns for different incubation time in AML sample. Same sample has been labeled with UCHT1 monoclonal antibodies CD3, and CD8, analyzed by flow cytometry. The CD3 and CD8 positive populations were gated (right panel). Fig. A & Fig. B represents CD3 expression

for ‘0-day incubation’ and ‘3-day incubation’ respectively. Fig. C & D represents CD8 expression for ‘0-day incubation’ and ‘3-day incubation’ respectively. The experiment was done on same sample for different incubation times.

4.1.3 Comparison between different plate types

Commercially many types of culturing plate are available that can be used for FC study. Depending on the requirement of experiment, certain types will likely to have edge over others. Count of cells has significant role in current study. Here we have used 96-well plate of three different well bottom types, namely 96 well- V bottom, 96 well- U bottoms, 96 well- FLAT bottoms. We checked the plates both with flow cytometry and cell viability assay (CTG) to assess the feasibility (based on cell count) of different types. For ‘FLAT bottom’ FC count was done for two given situations, like sampling from middle of bottom or sampling from corner of well to check if it had any added effect to cell number. FC result shows no significant difference as they all seem to be similarly effective (Fig. 3.4). For CTG assay ‘V bottom’ found to be the most consistent among those types (Fig. 3.3).

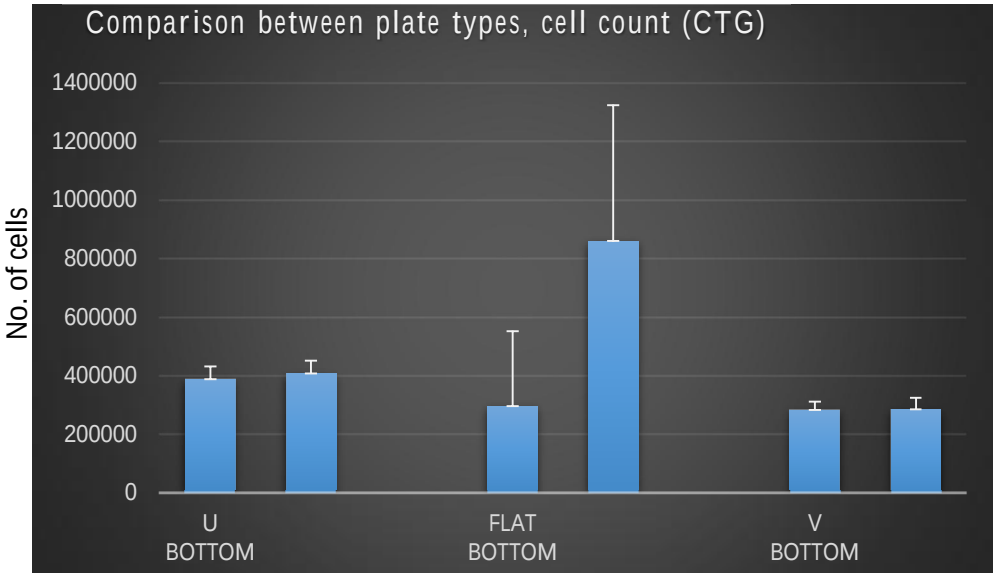


Figure 3.3: Comparison of cell growth in different cell culture plate. CTG assay was used to count the number of cells in different plates. U bottom, V bottom, and FLAT bottom are used. Y-axis represents the number of cells.

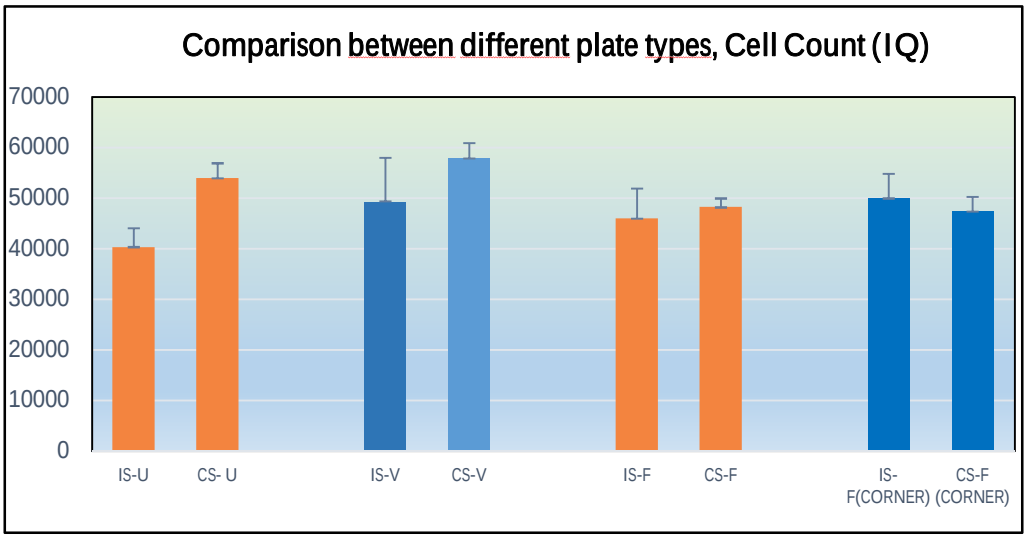


Figure 3.4: Comparison of cell counts for different plate types in flow-cytometry assay. IS-U = Interwell shaking, U bottom, IS-V = Interwell shaking, V bottom, CS-U= Continuous shaking, U bottom, CS-V= Continuous shaking, V bottom, IS-F= Interwell shaking, FLAT bottom, CS-F= Continuous shaking, FLAT bottom. Y-axis represents the number of cells.

4.1.4 Sample/antibody ratio

Primary sample/antibody ratio was also checked to further reduce the background noise as well as cost. Five different sample/antibody ratio was used, like 200:1, 100:1, 40:1, 20:1, 10:1 and all samples were incubated for two different periods (30 mins and 60 mins). We found that 20:1 patient sample/antibody ratio works best for most of the antibodies while for CD38 FITC 40:1 ratio worked better. Different incubation period with antibody didn't affect cells expression pattern, therefore we chose 30 mins incubation time to reduce the experiment time.

4.2 Immunophenotyping of AML cells

In the present work, we established high throughput 96-well flow cytometry workflow for detecting different population subsets within heterogeneous AML culture. Either whole bone marrow or peripheral blood cells were used. HTFC screening systems, like from IntelliCyt, has the ability to analyze samples in small volumes as well as simultaneously assess different cellular parameters from a single well. This allows us to adjust the HTFC platform (IntelliCyt iQue) and its automated sampling system to accurately process samples directly from multi-well plates and investigate different types of markers (both extracellular and intracellular markers) (Fereshteh, 2016). Flow cytometry allows user to analyze multiple populations within each well of a plate. User can create a hierarchical tree of population based on fluorescence and scattered properties of each cell type in the data set. Electronic gates are drawn around the subpopulations of interest and each population is identified, named, and color coded separately. The whole identification process can be described stepwise as bellow-

Commercially available antibodies like CD34, CD38, CD45, CD96, and CD123 are used in this study. Antibody selection was made based on their specificity (see Table 3) with the aim to separate leukemic

blast cells from other normal cells. 7-AAD, and Annexin V are used here as a viability probe for methods of nonviable cell exclusion.

4.2.1 Removal of Doublets

During analysis when aggregate cells/nuclei pass through the lasers, they take longer time than single cells. This in turn, affects the area of the signal. Therefore, it is essential to resolve singlets from aggregates. There are different ways of discriminating doublets. A pulse geometry gate (such as FSC-H x FSC-A), can be very efficient in discriminating doublets. In this method the fundamentals of doublet discrimination are to identify disproportions between cell sizes vs. cell signal. Many of the modern digital instruments (here IQue Plus) have a function that allows one to correlate 'Area' and 'Height' in dot plot for further presenting the same variation when PMT voltages are changed, so that they can be correlated one to the other. To start with all events are presented using an FSC-A vs. FSC-H dot plot and SC Area is used as scaling factor as if all events came from a same cell population (Fig: 3.5).

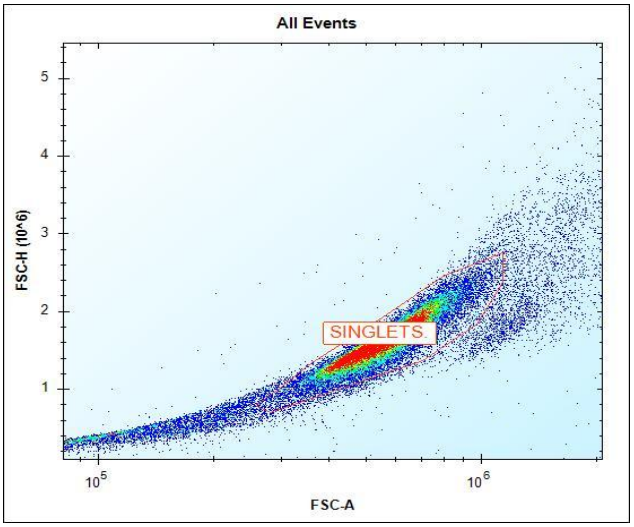


Figure 3.5 Removal of doublets using FSC-A (log) vs. FSC-H (linear) dot plot

4.2.2 Removal of Debris

In current study, only live and viable cells are included in the analysis. Therefore, one of the earliest steps is to eliminate any debris, dead cells and clumps or doublets. Following the step of doublet discrimination, we managed to remove further debris with the negative selection of these unwanted events. The selection was based on size, which is estimated by forward scatter. Cellular debris is generally appearing in the lower section of FSC. And dead cells often have lower forward scatter and higher side scatter than live cells. Based on gating principle that excludes 'low FSC and high SSC' events as debris, we manage to exclude most of debris and dead cells from further analysis (Figure 3.6).

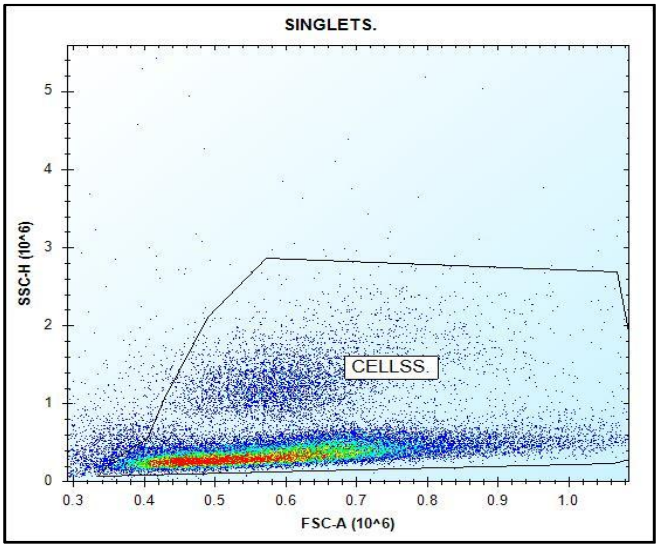


Figure 3.6 Removal of debris using FSC-A (linear) vs. SSC-H (linear) dot plot

4.2.3 Exclusion of dead cells

Dead cells can generate impractical data as a result of nonspecific antibody binding or unwanted uptake of fluorescent probes. In current study, cell viability measurement is very important to evaluate the physiological state of cells. It provides data like response to cytotoxic drugs and environmental factors, or disease status. We here used '7-Aminoactinomycin D (7-AAD)' and 'Annexin V' to detect dead cells in a cell suspension to exclude them from analysis. Annexin V is used here as a non-quantitative probe to detect apoptotic cells that have expressed phosphatidylserine (PS) on the cell surface. PS is an important phospholipid membrane component, generally located on the cytoplasmic surface of the cell membrane but during apoptosis, PS is translocated from the inner to the outer leaflet of the membrane, exposing itself to the external cellular environment where is detected using highly fluorescent Annexin V conjugates. On the other hand, 7AAD is high affinity DNA binding dyes that are efficiently excluded from live cells. As live cells miss both criteria to be stained, they are gated as double negative population. Live cells appeared and gated as negative population for both Annexin V and 7AAD staining (Fig. 3.7)

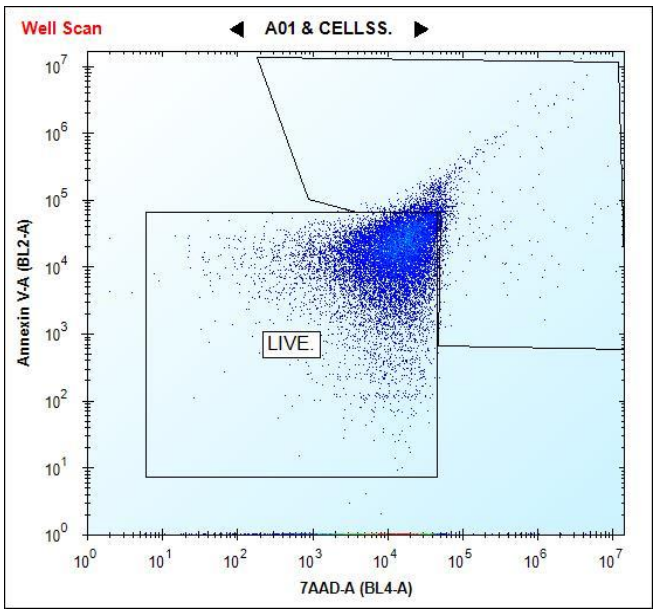


Figure 3.7 Removal of debris using double negative population gating strategy (Annexin V vs. 7AAD). Here the double negative population (gated live) is the live population/population of interest.

4.2.4 Identification of Stem cells

AML is widely considered as a stem cell disease and LSCs are well-known culprit for treatment failure and relapse in AML. LSCs are thought to be insensitive to chemotherapy. Therefore, LSCs identification and analysis is very critical to AML study. The most recognized strategy for LSCs identification is to start with stem cells (CD34+/CD38-). Here, unstained patient sample is considered as negative control for all analysis. Studies have already established the few stem cell markers in AML. Rhenen and his colleagues showed in their study that for CD34-positive AML, CD38 is generally absent in leukemic stem cell (van Rhenen, 2007). CD34+CD38- (stem cell) population is usually a minor population and has been described before. Then the CD34+CD38- compartment was defined within this CD34+ population. The required minimal significant number of CD34+CD38- events was set at 30%. Same sample without stain was used as a negative control while comparing (Fig. 3.8). 11 samples were tested and CD34 expression was best in 81% (9/11) cases whereas CD38 expression was good in every case. CD34 was highly expressed in 8 cases, and low on others.

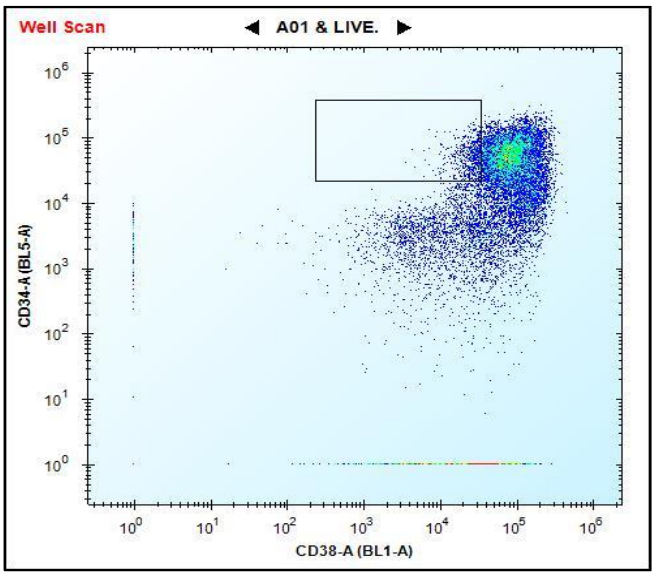


Figure 3.8: Identification of stem cells population within AML primary sample. Lin CD34+CD38- gating is used to select stem cell population.

4.2.5 Identification of Leukemic Stem cells

Though cells with CD34+CD38- surface antigen can be regarded as important feature of LSCs in AML, reports have suggested that normal HSC also shared the same surface markers of CD34+CD38–. Therefore, the specific identification of LSCs from normal HSCs is essential for scientific research as well as clinical investigation (Ding, 2017). With the help of additional LSC markers, leukemic cells can be distinguished from normal hematopoietic stem cells who generally lacks those markers. Some of the validated leukemic stem cell (LSC) markers for AML are CD33, CD44, CD47, CD90, CD96, CD123, CD32 (chemotherapy resistant AML only) etc (Zeijlemaker, 2016). In this study we preferred CD45, CD96, and **CD123** as LSC marker as these markers are highly expressed in LSCs but mostly absent in nHSCs.

4.2.5.1 Expression of CD123 on leukemic population

CD123 is one of most investigated LSC marker used in AML biology. In this experiment, we used CD123 expression as the primary target to identify LSC population. 'Lin CD34+CD38-CD123+' gating is used to select AML blast cells as suggested by several earlier studies and rest of the population ('Lin CD34+CD38-CD123-', 'Lin CD34-CD38-CD123+' and 'Lin CD34-CD38-CD123-') is identified as normal BM cells (Zeijlemaker, 2016). Blast population is color coded red while normal BM cells are coded green (See Figure 3.9). CD123 was expressed in 90% cases (highly expressed in 55% (6/11) cases, while 36% had low expression level and in 9% (1/11) cases the marker was absent.

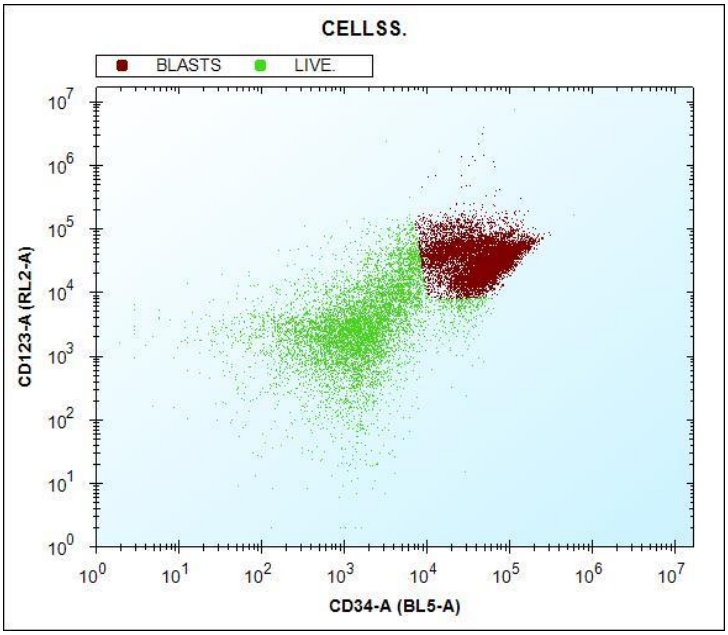


Figure 3.9: Identification of AML Blast cells in primary sample of Acute Myeloid Leukemia. Lin CD34+CD38-CD123+ gating is used to select AML Blast cells population.

4.2.5.2 Expression of CD96 on leukemic stem cells

CD96, a trans-membrane glycoprotein that belongs to immunoglobulin superfamily. It only expresses on T and NK cells. Ding et al., reported that CD96 expression can be used to distinguish AML LSCs from normal HSC (Ding, 2017). CD96 is a LSC-specific marker in human AML and excellent candidate target for targeting LSCs. Furthermore, CD96+ LSCs have been reported to have high ability for engraftment in mice making it suitable to use for LSC-targeted antibody therapy. This provides with the opportunity to therapeutically target LSC while leaving nHSC unharmed. Here, LSC (CD34+ CD38- CD96+) selection is color coded red and nHSC (CD34+ CD38- CD96-) selection is coded in blue color green. CD96 has been found to be expressed in all (11) cases but the expression was best in 91% (10/11) cases (Figure 4.0).

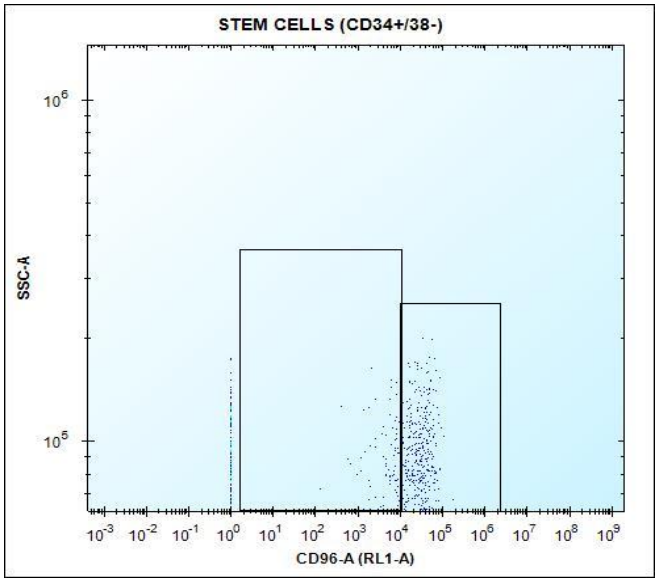


Figure 4.0: Identification of LSCs in 'CD34+CD38-' AML population. Lin CD34+CD38-CD96+ population denotes LSCs and Lin CD34+CD38-CD96- population denotes nHSCs.

4.2.6 Identification of other cells

The identified CD34+CD38- blasts in a population were further characterized by faint expression of CD45 and low SSC. During investigation identification of granulocytes and basophils are very important as improper identification of these may lead blasts to be incorrectly seen as nonconcurrently positive for mature granulocyte antigens (e.g., CD11b, CD15) or under normal expression of blast antigens (e.g., HLA - DR, CD34, etc.) (Harrington, 2012). Specific markers of blast cells like CD34 can be used for definite identification of different Blast cells (Al-Fatlawi, 2016). CD45 is common and strongly expressed marker among AML patients (Osman et al., 2015). Current experiment found all tested samples (10 samples) positive for CD45 marker expression. Amongst those 90% cases have more than 80% positivity for CD45 expression (Fig. 4.1). Unstained CD45 positivity allowed us to discriminate leukemic blast cells from residual normal cells. From the CD45/SSCA gating we are able to see the actual number as well as the ratio (population ratio) for lymphocytes and granulocytes. Analysis was performed on non-leukemic population with intend to observe the fate of normal cells like lymphocytes and granulocytes (See Figure 4.1).

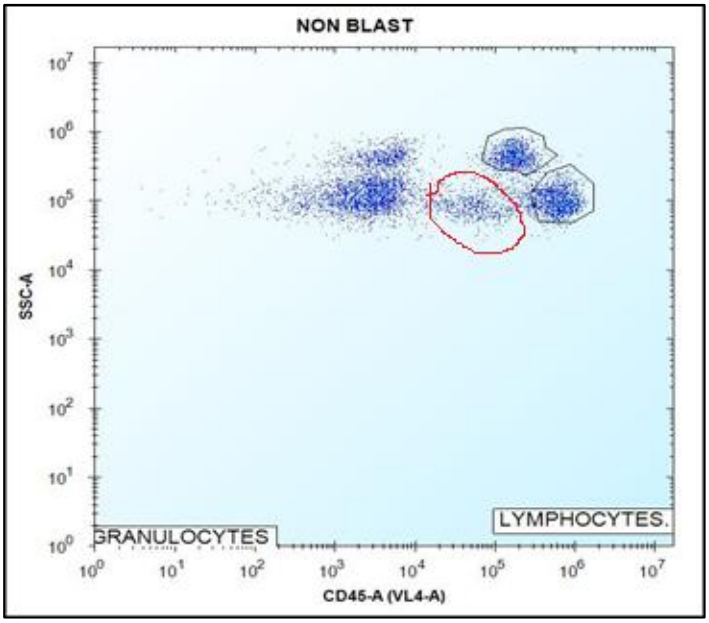


Figure 4.1: Demonstration of flow cytometric (FC) analysis of an acute myeloid leukemia based on CD45/SSCA gating. It includes lymphocytes (right-bottom), monocytes (left-bottom, circled red), granulocytes (right-top) and other cells.

4.7 Assay sensitivity and reproducibility

To immunologists and hematologists, high throughput flow cytometry is a well-known established technology for cell sorting and analysis to drug discovery applications. To define the specificity and performance of the assay in a clinical setting, primary samples from 10 diagnosed AML patients were assayed (Table 4). Figure (4.2) shows the detail immunophenotypical expression of an AML patient sample (BM) in statistical arrangement (constructed using assay markers). The assay was able to detect minute population like nHSC population which 0.5% of total live population (Figure 4.2).

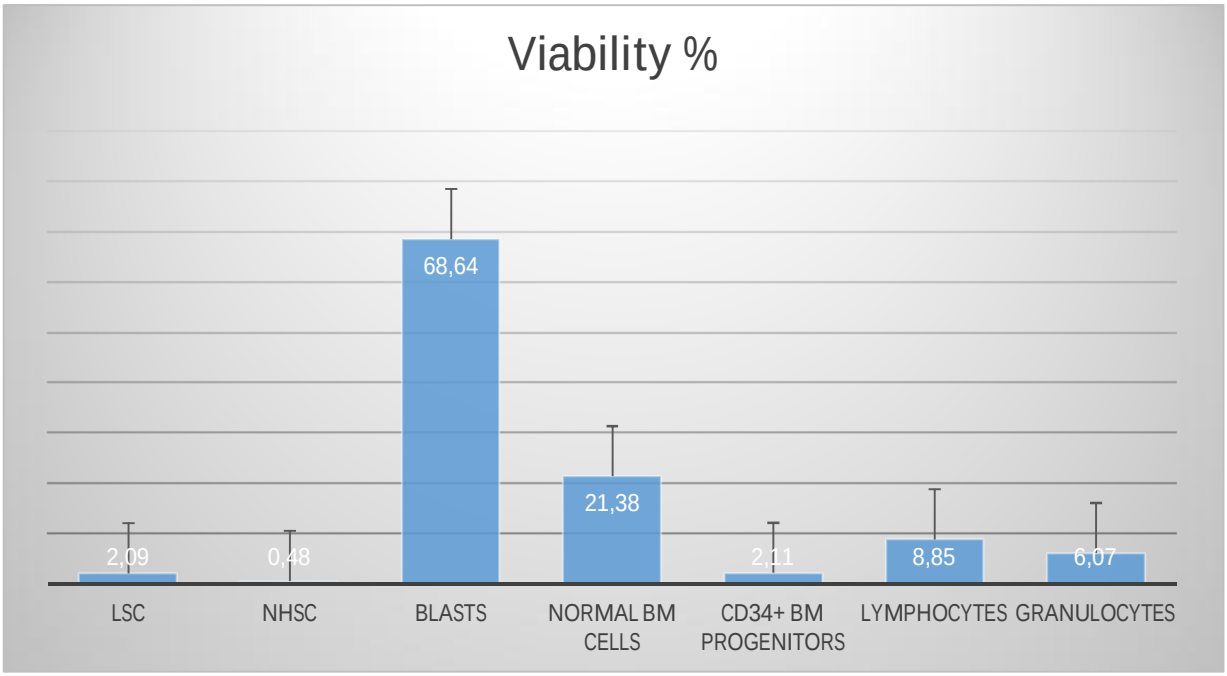


Figure 4.2: Statistical presentation of immunophenotypical tally of a patient blood sample based on the designed assay. Markers includes in this assay are CD34, CD38, CD45, CD96, and CD123.

Sample	Source	CD34	CD38	CD45	CD96	CD123
1	BM	90		99	97	87
2	BM	84		95		77
3	BM	69	87	97	84	38
4	BM	Low	91	97	90	50
5	BM	Low		89	78	Low
6	BM	72	5	4,5	30	Low
7	BM	13		80	19	Low
8	BM	13	91	96	82	87
9	BM	38		96	87	Low
10	BM	81		93	Low	Low

Table 4: Immunophenotypical expression data of 10 acute myeloid leukemia patients. Each patient sample were treated and analyzed in the same way.

4.8 Blast-specific drug sensitivity profiling in heterogeneous AML patient samples

To investigate the efficacy on different anticancer drugs we developed a 96-well custom-made drug plate which contains standard anticancer drugs as well as some novel combinations of some drugs. BzCl

containing unstained patient sample is considered as positive control and DMSO containing unstained patient sample as negative controls. DMSO was added to negative control sample in minute amount to minimize the effect of DMSO as most anticancer drugs were dissolved in DMSO (Table 5).

		Venetoclax	Ruxolitinib	Temsirolimus	Dasatinib	Dexamethasane	Cytarabine	GSK2126458				
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0,01	0,01	0,001	0,01	0,01	0,01	0,001	KOMB1	KOMB5	DMSO	
C		0,1	0,1	0,01	0,1	0,1	0,1	0,01	KOMB2	KOMB6	DMSO	
D		1	1	0,1	1	1	1	0,1	KOMB3	KOMB7	BzCl	
E		10	10	1	10	10	10	1	KOMB4	KOMB8	BzCl	
F												
G												
H												

Table 5: Customized anti-cancer drug panel. KOMB1= Venetoclax 0,1uM+ Dasatinib 0,1uM, KOMB2= Venetoclax 0,1uM+ Ruxolitinib 1uM, KOMB3= Venetoclax 0,1ηM+ Temsirolimus 0,01uM, KOMB4= Venetoclax 0,1uM+ GSK2126458 0,01uM, KOMB5= Dexamethasane 1uM+ Dasatinib 0,1uM, KOMB6 = Dexamethasane 1uM+ GSK2126458 0,01uM, KOMB7 = Cytarabine 0,1uM+ Dasatinib 0,1uM, KOMB8= Cytarabine 0,1uM+ Ruxolitinib 1nM+ Temsirolimus 0,01uM.

The result shows that few drugs effectively kill the leukemic cells whereas other fail to show any response. To show the result more efficiently we conducted comparative study between individual drug responses data of FC assay and cell viability assay. The following figures show while treating the whole live population. Venetoclax, cytarabine and dasatinib all effective killed all cells. Ruxolitinib, and dexamethasone was moderately effective, whereas temsirolimus and GSK2126458 was rather ineffective as a sole treatment. In many cancer patient's cases the cytotoxic effect of anticancer agents has been a major concern which causes lot of suffering for the patient. The cytotoxic effect of anticancer drugs sometimes outweighs their benefit, therefore here the aim was to develop an assay that can observe the effect of anticancer drugs on all cell populations, from blast population to normal HSCs. Therefore, in this study we tried to analyze the effect of different drugs in specific cell population as well.

Venetoclax is selective as well as a potent BCL2 inhibitor. Our study (Fig. 4.3) showed that venetoclax is effective against Blast and LSC population which reaffirms the previous findings. Our study found venetoclax to be effective against leukemic cells at intermediate dose, but it was toxic to lymphocytes at the same time. At highest dose venetoclax kills or attenuate all cells including normal BM cells.

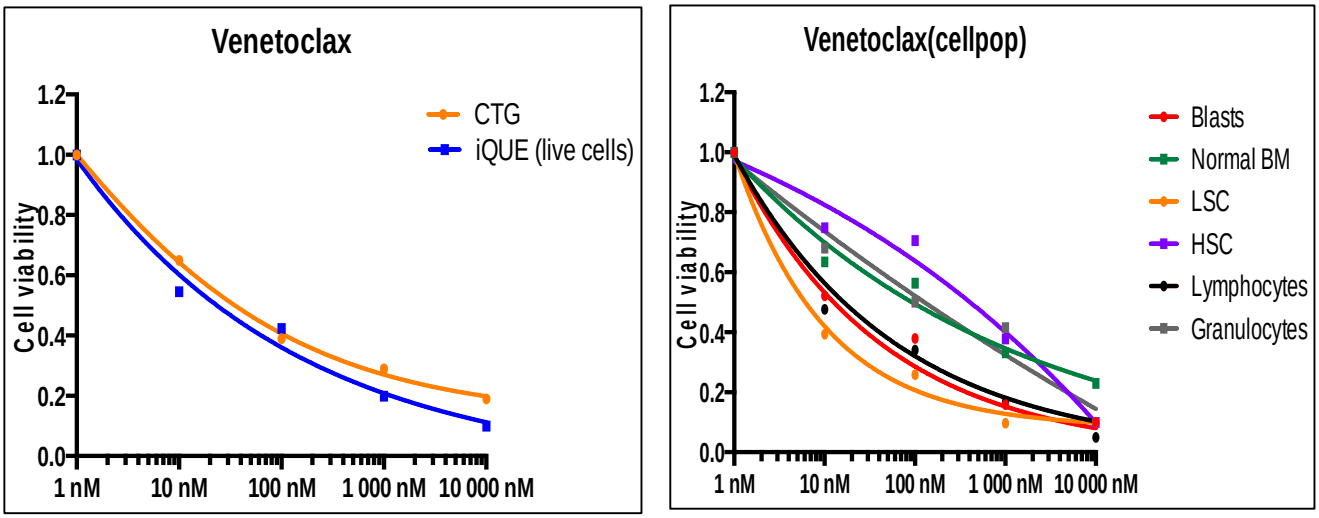


Figure 4.3: Drug sensitivity of an AML diagnosed patient sample for venetoclax. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for venetoclax. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

Dexamethasone is an anti-inflammatory drug widely used in lymphoid malignancies but for myeloid disorders their use is much limited. In current experiment, dexamethasone was found ineffective against blast or mature LSC, but non-blast population was affected. Both lymphocytes and granulocytes were either killed or attenuated by dexamethasone. Even HSC numbers were depleted very quickly after treating with dexamethasone (Fig. 4.4).

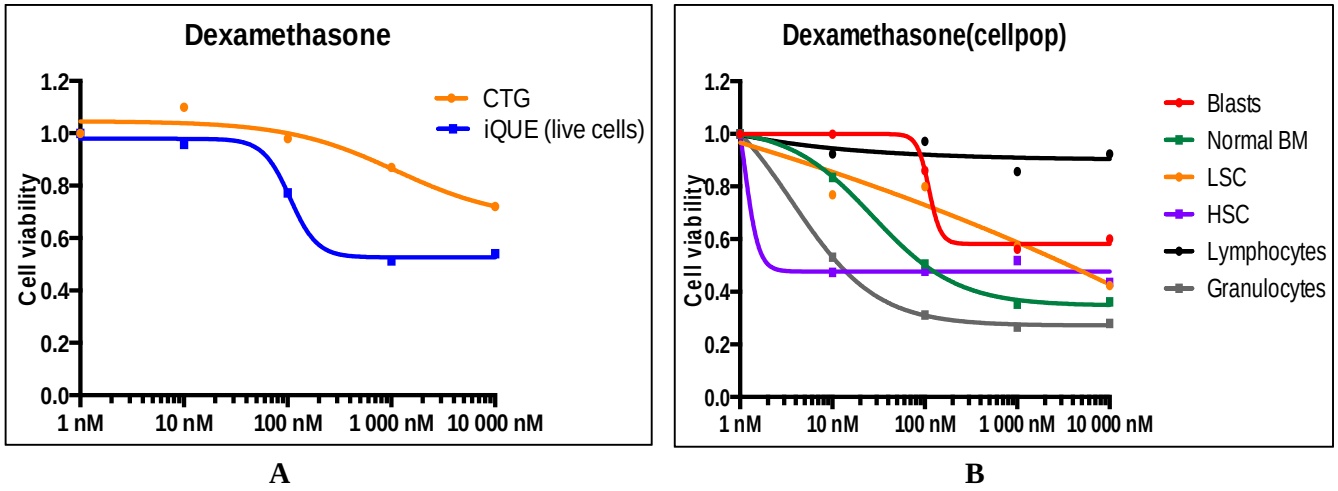


Figure 4.4: Drug sensitivity of an AML diagnosed patient sample for dexamethasone. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for dexamethasone. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

Cytarabine is one of most commonly used drugs in the treatment of acute myeloid leukemia. Earlier reports suggested that high dose of cytarabine might result in higher rate of relapse free survival than conventional dose but may have severe toxic effect. Our study found that cytarabine effectively kills leukemic cells at intermediate level dose (100 nM/well) but at highest dose (10,000 nM/well) it kills almost all live cells. In this experiment, our aim is to find drug that eradicates all leukemic cells while leaving normal cells. But at the highest dose cytarabine not only kills blasts and LSC but also kills normal BM, granulocytes, and HSCs (Fig. 4.5).

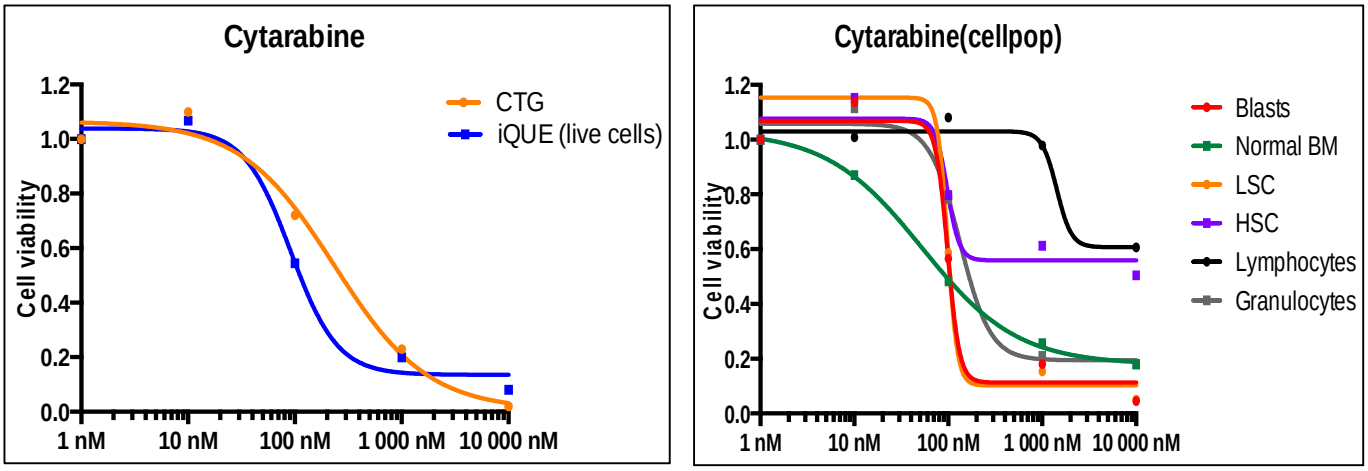


Figure 4.5: Drug sensitivity of an AML diagnosed patient sample for cytarabine. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for cytarabine. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

GSK2126458 aka Omapalisib is a kinase inhibitor, to be specific inhibitor of phosphatidylinositol 3-kinase (PI3K). In this experiment we found that as a sole treatment it was hardly effective in low to mid-range concentration. At highest concentration (1000 nM) the drug managed to lower the number of blasts or leukemic cells, but it adversely affects the non-malignant hematopoietic cells as well (figure 4.6).

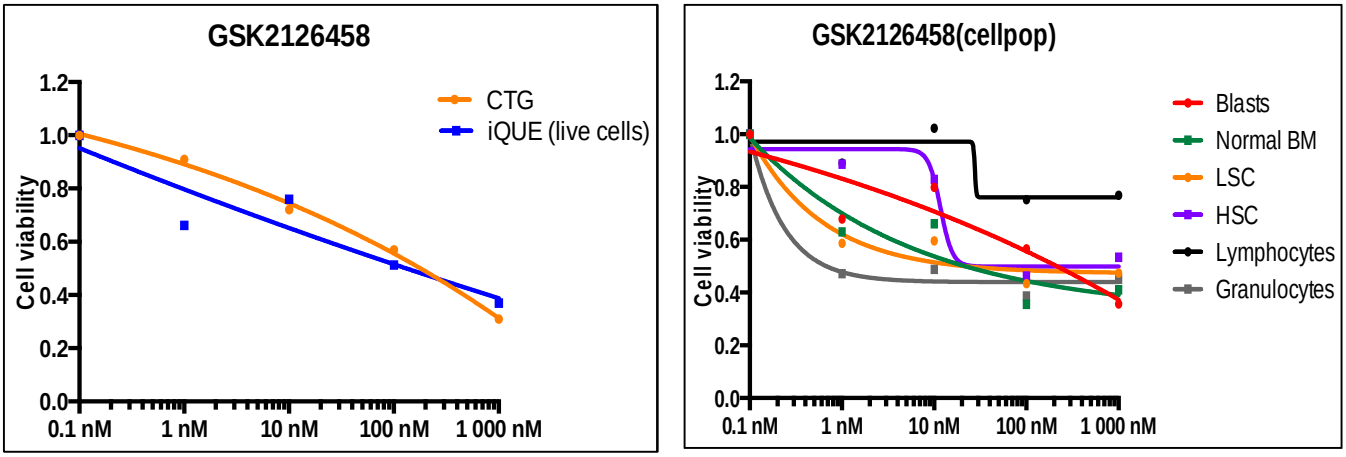


Figure 4.6: Drug sensitivity of an AML diagnosed patient sample for GSK2126458. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for GSK2126458. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

Ruxolitinib (JAK inhibitor) also fail to show significant efficacy against leukemic cells as sole treatment. Surprisingly it killed most mature LSCs at lower concentration (10nM), but blast population was no not affected at all. At highest concentration (10000 nM) it managed to kill most blast population, but its cytotoxic effect might have killed non-leukemic cells as well (figure 4.7).

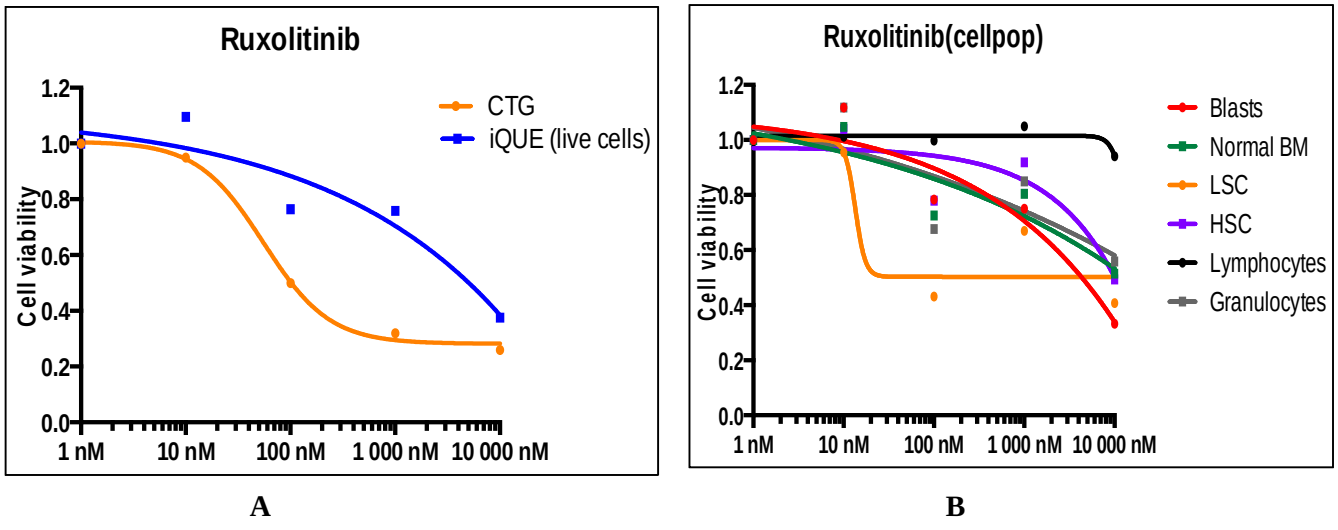


Figure 4.7: Drug sensitivity of an AML diagnosed patient sample for Ruxolitinib. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for ruxolitinib. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

Temsirolimus is an inhibitor of mammalian target of rapamycin (mTOR). Unfortunately, it also showed no significant effect against blast population. Even in highest concentration it had negligible effect on blast population (figure 4.8).

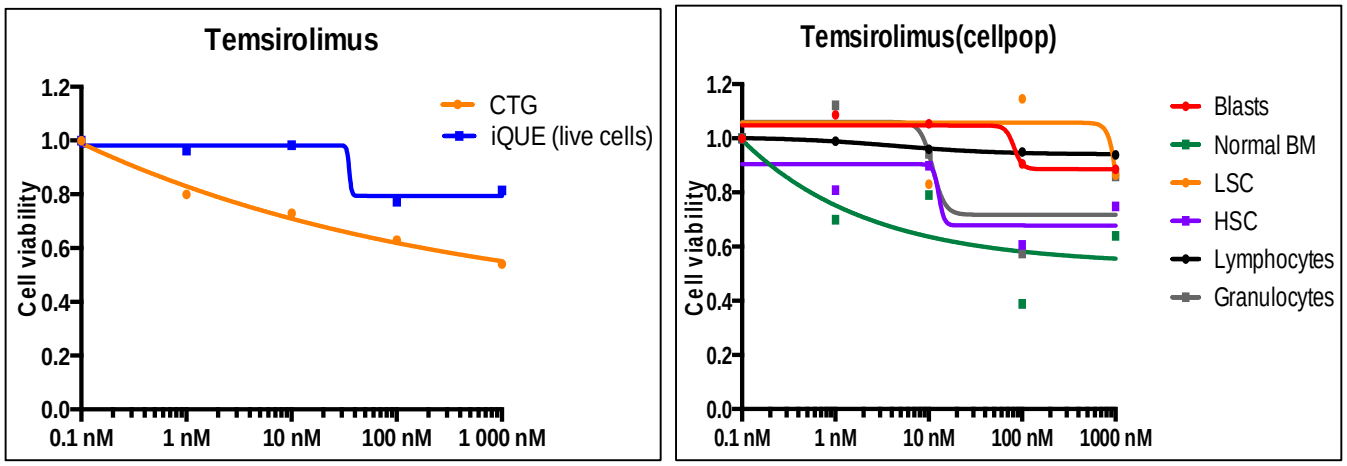


Figure 4.8: Drug sensitivity of an AML diagnosed patient sample for temsirolimus. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response (FC data) on specific AML cell populations for temsirolimus. Data were plotted and graphed using GraphPad Prism (GraphPad Software). Y-axis represents viability of cells and X-axis presents concentration of anticancer compound.

Dasatinib has been reported to be used successfully along with chemotherapy in AML treatment especially for AML patients carrying $KITD^{816V}$ mutation (Ustun, 2009). Our current experiment found dasatinib to be significantly effective against leukemic cells at intermediate to higher dose (Fig 4.9). Though at higher dose normal cells were also affected significantly.

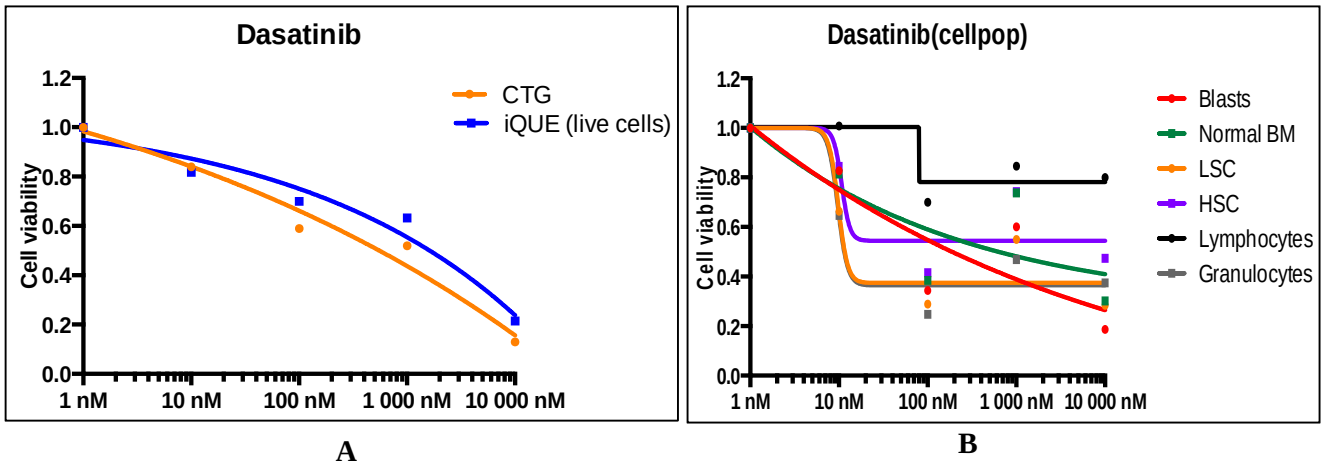
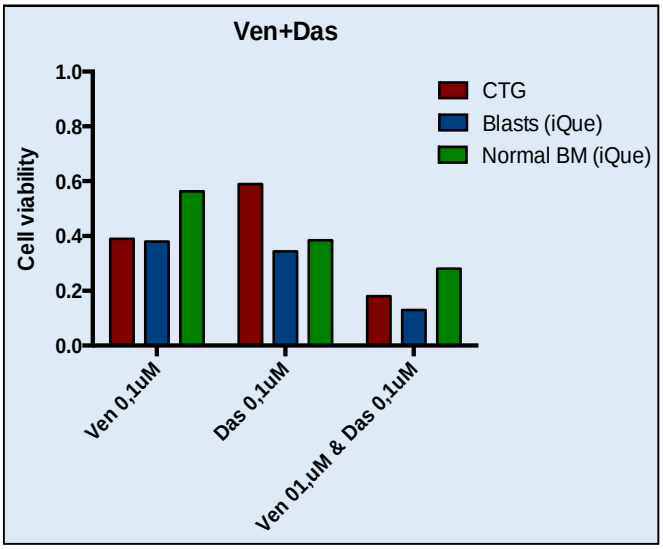


Figure 4.9: Drug sensitivity of an AML diagnosed patient sample for Dasatinib. (A) Representation of drug mediated response based on live cell count. iQUE (in blue colored line) represents FC data and CTG represents data from cell viability assay (orange colored). (B) Representation of drug mediated response on specific AML cell populations for dasatinib. Data were plotted and graphed using GraphPad Prism (GraphPad Software).

In current study we have examined few combinations of anticancer agents as well based on previous studies and suggestions. While analyzing drug efficacy our main aim was whether we can observe ex-vivo drug sensitivity for individual cell population or not. In current experiment, we found that all combinations with venetoclax (venetoclax+dasatinib/GSK2126458/temsirolimus) successfully eradicate most blast cells but unfortunately it kills or attenuates most normal BM cells also. Only cytarabine and ruxolitinib combination at highest concentration (1 µM) showed expected anti-leukemic efficacy. It effectively eradicated most blast cells while not affecting normal BM cells. Dexamethasone combined with dasatinib/GSK2126458 didn't show any significant anti-leukemic activity (Figure 5.0).



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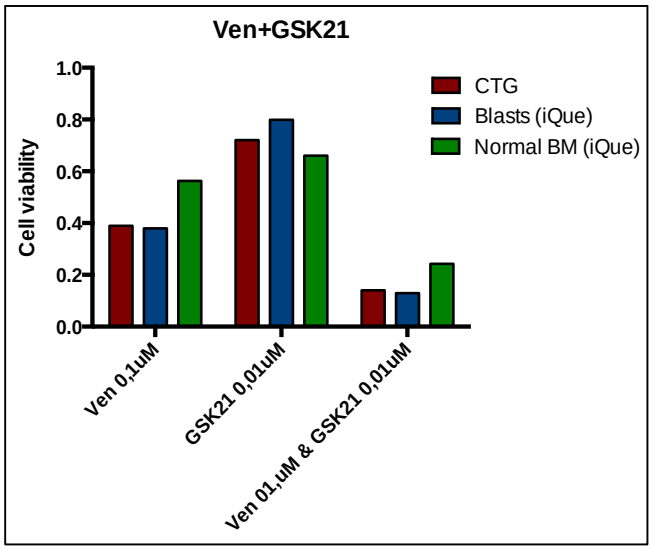

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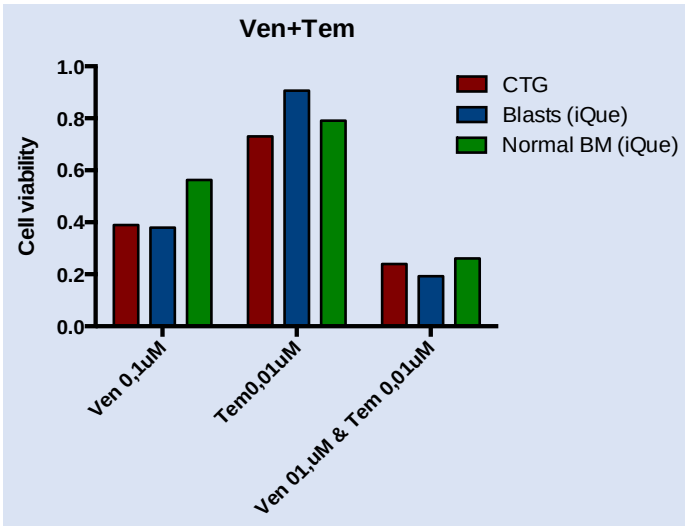
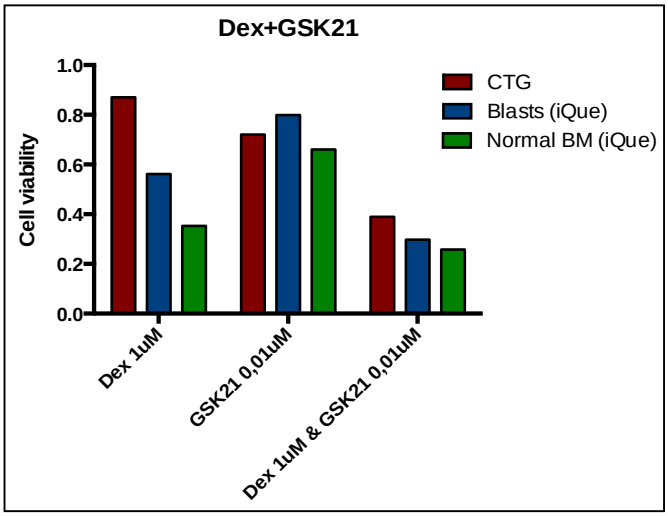


C

Figure 5.0 (continued): Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for combinatorial drug. (A). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for venetoclax and dasatinib combination. (B). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for venetoclax and GSK2126458 combination. (C). Ex-vivo response of blast (leukemic cells) and other (non-

leukemic) cells for venetoclax and temsirolimus combination. The assay was conducted on same patient sample at the same time. Data were plotted and graphed using GraphPad Prism (GraphPad Software).



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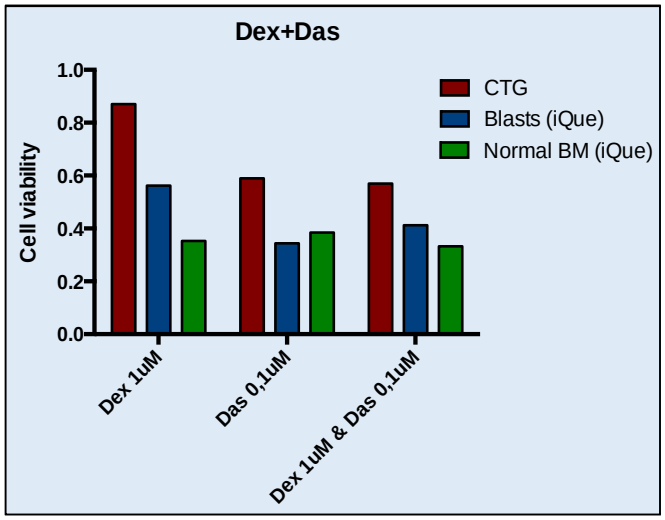

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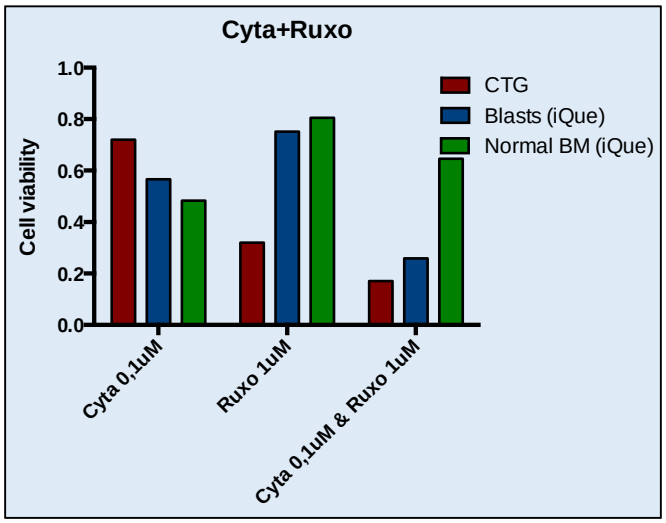

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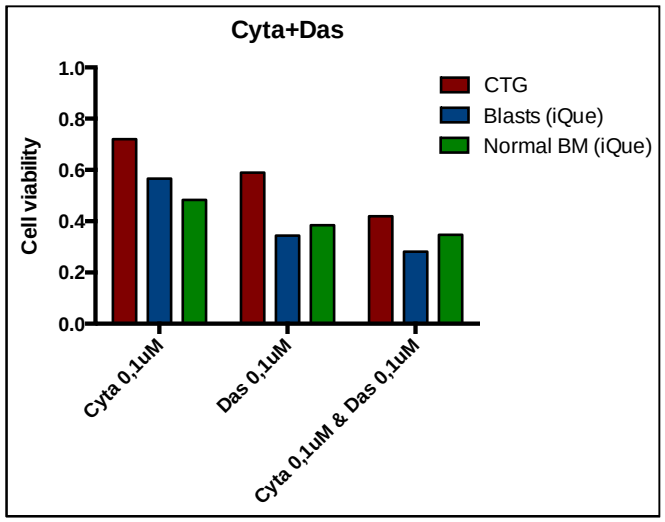

G

Figure 5.0: Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for combinatorial drug. (D). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for dexamethasone and GSK2126458 combination. (E). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for dexamethasone and dasatinib combination. (F). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for cytarabine and ruxolitinib combination. (G). Ex-vivo response of blast (leukemic cells) and other (non-leukemic) cells for cytarabine and dasatinib combination. The assay was conducted on same patient sample at the same time. Data were plotted and graphed using GraphPad Prism (GraphPad Software).

Chapter Five: Discussion & Concluding remarks

Microenvironment plays key role in disease progression by supporting the proliferation and survival of cancer cells. It also interferes with drug efficacy and resistance [(Zhou et al., 2005; Sison et al., 2011)]. In many cases failure of anticancer drugs might be directly linked to the difference between experiment condition and actual patient condition. Ex vivo models using primary patient sample can be used as a screening platform which can partially recapitulate the species-specific disease microenvironment.

Successfully predicting outcomes is very important from a clinical perspective, but the ability to predict also has major implications on preclinical cancer research as well, per se while developing assays where the focus is to bridge the translational gap. So far animal models and cell line cultures have been the front-line strategies in predicting outcomes for drug discovery research. The predictive values of these strategies are always debatable as both models are limited to address the true disease microenvironment which can significantly affect the drug efficacy in vivo [(Testa et al., 2011; Cheon et al., 2011)]. Transgenic animal models may recapitulate a specific cancer pathway but fail to capture the heterogeneity of a complex disease like AML. Furthermore, successful drug development requires large-scale compound screening platforms that allows identification and optimization of lead molecule in physiologically relevant cells [(Fereshteh et al., 2016)]. High throughput flow cytometry can be considered as an ideal platform for phenotypic screening and drug discovery studies for its ability to perform high-speed multiplex quantitative analysis of suspended cells and other particles [(Comley, 2015)].

In current experiment we introduced a 96-well plate high throughput, multiplexed flow cytometric (FC) assay for immunophenotypic quantification of AML with the aim to observe ex-vivo sensitivity of specific cell populations for different anti-cancer drugs/agents. Previous studies suggested that, dividing cells is an inefficient target while treating AML as the disease regains its ability from minimal residual disease (MRD) cells [(Ishikawa et al., 2007; Costello et al., 2000)]. MRD is a significant prognostic factor for relapse and adverse outcomes in AML. MRD are likely to contain LSCs, which is the possible true cause of disease relapse [(Kern et al., 2004, Feller et al., 2004; Venditti et al., 2000; Loken et al., 2012; San Miguel et al., 2001; Zhu et al., 2013)]. Therefore, targeting dividing cells only like conventional chemotherapeutic strategy might be fruitless [(Pollyea et al., 2014)]. Parallelly our objective was also to be able to find if or any anti-cancer drugs solely affect leukemic or blast cells or not as cytoxicity related disorders are very common in cancer treatment.

Leukemia initiating cells or leukemia stem cells has been reported to contribute not only in leukemic initiation, progression but also in chemotherapeutic drugs resistance, and disease relapse [(Venton etal., 2016; Crews et al., 2016)]. Therefore, targeting specific surface markers of LSCs (like CD34, CD96, and CD123) is considered to be a great potential strategy for eliminating LSCs selectively. The prognostic importance of CD34+CD38− LSC frequency both during diagnosis and post-treatment has been established by

numerous reports (Terwijn.et al., 2014. Van Rhenen et al., 2005). Bonnet and Dick in 1997 first reported CD34+CD38− cells with the ability to reconstitute human AML in NOD/SCID mice (Bonnet and Dick, 1997. Ding, 2017).

With CD123 we found the significant expression in 90% cases (highly expressed in 55% (6/11) cases, while 36% had low expression level and in 9% (1/11) cases the marker was absent) which reaffirms the earlier reports (Al-Fatlawi, 2017). CD123 aka interleukin-3 receptor alpha (IL-3Rα) heterodimeric cytokine receptor consist of the alpha and beta units. In normal hematopoiesis, IL-3Rα may bind to high-affinity IL-3 receptor heterodimer stimulating the development and survival of multi-lineage colonies from normal BM, while in leukemic condition, stimulation induced by IL-3 cause's proliferation of AML blasts. (Al-Mawali et al., 2017). The ability of CD123 to discriminate between normal cells and LSCs establishes it as a unique but highly specific marker for the identification and targeting of LSCs. CD123 have been found to aberrantly overexpressed on CD34+CD38– LSCs but not expressed on normal CD34+CD38– hematopoietic cells (Jin et al., 2009; Han et al., 2017; Jordan et al, 2000). Larsen et al., has reported strong correlation between the level of expression of CD123 and relapse in AML (Larsen et al., 2012). CD96 also expresses frequently on $CD34^{+}CD38^{-}$ AML-LSC population but not on normal HSC enriched population (Hosen et al., 2007). In current experiment we found in 91% cases there was significant expression for CD96 in $CD34^{+}CD38^{-}$ AML population which establishes the previous report by Hosen et al., 2007.

CD45 is a myeloid cell marker also known as transmembrane tyrosine phosphatase. CD45 is universally expressed by white blood cells (WBCs). CD 45 expresses high on lymphocytes and monocytes, but for granulocytes, precursor granulocytic cells, precursor B-cells, and proerythroblasts the expression level of CD45 is rather low. Furthermore, mature erythroid cells are CD45 negative. Therefore, CD45 can be used in flowcytometric plot against SSC scatter to distinguish the major leukocyte (sub) populations (Al-Fatlawi, 2016). Saksena et al., reported the use of SSC scatter plot versus CD45 (in flow cytometry) in detecting different AML subtypes (Saksena, 2016). BLAST cells are known to be located in the CD45 weak positive region. Blast gate by CD45/SSC contains various cell types in addition to myeloid Blast. Granulocytes and basophils are the largest contributor in blast gating. Therefore, failure to identify these contaminating populations may give rise to overestimation of blast percentages.

With combined use of all these markers we successfully managed to differentiate leukemic populations as blast or mature LSCs and non-leukemic population like lymphocytes, monocytes, granulocytes etc. This allowed us to effectively witness the ex-vivo drug related response for different cell populations. We have examined few FDA approved drugs in current experiment. While few of them showed significant efficacy against leukemic cells, others failed. Venetoclax was found significantly potent against leukemic population. The single□agent activity of venetoclax in relapsed and refractory AML has been confirmed by Konopleva, 2014. Dexamethasone was rather ineffective against leukemic cells but it adversely affected granulocytes and normal BM cells. Dexamethasone is mostly used in order to prevent or treat severe inflammatory status (Inaba et al., 2010; Kelaidi et al., 2009; Sanz et al., 2014). Reports has suggested

that dexamethasone improves patient's condition (treated with chemotherapy) reducing relapse rates (Récher et al., 2016). Cytarabine is an anti-metabolite that kill cancer cells by stopping them making and repairing DNA needed for growth and multiplication. In our experiment cytarabine was found to kill or attenuate all live cells at highest dose which supports the previous claim (Löwenberg et al., 2011).

While trying different drug combination we come across one surprising result with cytarabine and ruxolitinib combination treatment. Surprisingly we noticed that the combo drug effectively eradicated all leukemic population but not non-leukemic populations (Figure 5.0). There are previous reports suggests the success of combination therapy with ruxolitinib plus low dose cytarabine in patients with blast□phase myeloproliferative neoplasms (Mwirigi et al., 2014). Reports has suggested JAK inhibitors like ruxolitinib, target not only disease□related spleen enlargement and other symptoms but also encourage epigenetic and immunoregulatory modification (Mwirigi et al., 2014). Whereas, cytarabine acts via direct DNA damage and incorporation into DNA. Cytarabine is cytotoxic to proliferating mammalian cells. Their heterogenous yet co-operative mechanisms made the combination approach more effective than a single agent approach.

If the selection of therapies for individual patients should ideally be based on the AML phenotype, functional ex-vivo analysis may be the ultimate method for personalized cancer therapy. In this experiment we have showed that it is feasible to accurately detect LSC using markers with specific properties in the different fluorescence channels, which is broadly applicable in a multi-parametric setting. Although biomarker-based strategy has emerged as commanding concept in personalized cancer therapy, only biomarker may not bring expected result clinical efficacy due to heterogeneity of cancers. Genetic and epigenetic discrepancies within clonal populations could critically regulate outcome of a particular drug combination for specific patient. Reports have suggested that microenvironment and heterogeneity is crucial to predictability of current biomarker-based strategies for chemotherapy (Majumder et al., 2015). With the detailed molecular profiling of certain disease, this assay might prove very beneficial for personalized treatment strategy. Additionally, for specific purposes, the panel can be further extended with additional antibodies.

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