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The Effects of Frondoside A in Acute Leukemia

Fatma Hussain Sajwani

UAEU

جامعة الإمارات العربية المتحدة
United Arab Emirates University

United Arab Emirates University

College of Medicine and Health Sciences

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Fatma Hussain Sajwani

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Under the Supervision of Professor Thomas E. Adrian

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Declaration of Original Work

I, Fatma Hussain Sajwani, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*The Effects of Frondoside A in Acute Leukemia*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Professor Thomas E. Adrian, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

Student's Signature: _____ Date: 20/08/2017

Advisory Committee

1) Advisor: Thomas E. Adrian

Title: Professor

Department of Physiology

College of Medicine and Health Sciences

2) Co-advisor: Basil Ramadi

Title: Professor

Department of Microbiology

College of Medicine and Health Sciences

3) Member: Milos Lijubisavijevic

Title: Associate Professor

Department of Physiology

College of Medicine and Health Sciences

4) Member: Sherif Karam

Title: Professor

Department of Anatomy

College of Medicine and Health Sciences

5) Member: Srdjen Denic

Title: Associate Professor

Department of Internal Medicine

College of Medicine and Health Sciences

Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Thomas E. Adrian

Title: Professor

Department of Physiology

College of Medicine and Health Sciences

Signature _____ Date 20/08/2017

2) Member: Milos Lijubisavijevic

Title: Professor

Department of Physiology

College of Medicine and Health Sciences

Signature _____ Date 20.08.2017

3) Member: Srdjen Denic

Title: Associate Professor

Department of Internal Medicin

College of Medicine and Health Sciences

Signature _____ Date 20/8/2017

4) Member (External Examiner): Kourosh Lotfi

Title: Professor

Department of Hematology

Institution: Linkoping University (Sweden)

Signature _____ Date 2017-AUG-20

This Doctorate Dissertation is accepted by:

Dean of the College of Medicine and Health Sciences: Professor Ruth Langer

Signature _____ Date 11-08-2017

for Dean of the College of Graduate Studies: Professor Nagi T. Wakim

Signature _____ Date 8-11-2017

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Abstract

Among hematological malignancies, acute leukemia is the major cause of mortality. Despite improvement of survival with current chemotherapies, some patients still die from the disease or the treatment side effects. Thus, new therapeutic agents are needed. Anti-cancer drugs derived from natural products are of interest. Frondoside A is a triterpenoid glycoside form the sea cucumber, *Cucumaria frondosa* that has shown potent antitumor effects in various cancers. Previous studies in acute leukemia are limited. The current study investigated the effects of frondoside A in acute leukemia cell lines alone and in combination with drugs currently used for this malignancy. This study is the first to attempt comparing the efficacy of this compound to available conventional drugs.

The acute leukemia cell lines used were CCRF-CEM, HL-60 and THP-1. Cells were cultured and treated with different concentrations of frondoside A, vincristine sulphate, asparaginase and prednisolone each compound alone and in combination with frondoside A. Experiments were conducted with treatment incubation periods of 24, 48 and 72h. The inhibitory concentration 50 (IC_{50}) for each compound at each time point was determined for the three cell lines using the CellTiter-Glo luminescence assay. Induction of apoptosis was examined using Annexin V test and expression of apoptosis-related genes (low-density expression array) was investigated in two acute leukemia cell lines. The expression of protein products of selected genes was also investigated. The effect of frondoside A combined with NFκB pathway inhibitor, andrographolide in acute leukemia cell lines was also examined.

CCRF-CEM cells were very sensitive to frondoside A treatment while HL-60 and THP-1 were less sensitive. Frondoside A markedly enhanced the anticancer effects of all of the conventional drugs in all cell lines. Synergistic effects were seen in some of the combination concentrations. Induction of apoptosis was confirmed morphologically and by Annexin V in CCRF-CEM and THP-1 treated cells. Analysis of the effect of frondoside A on expression of apoptosis-related genes showed marked changes in multiple pro- and anti-apoptotic genes. Expression of some genes coding for both pro-apoptosis and anti-apoptosis proteins were increased, suggesting that a

survival pathway was also activated in the frondoside A-treated cells. Frondoside A treatment increased the gene expression of multiple members of both the intrinsic and extrinsic as well as the executioner pathways. The compound also up-regulated the genes encoding multiple death receptors and death effector domains. In THP-1 cells, frondoside A treatment resulted in the increased expression of the tumor suppressor protein p21 and it decreased the expression of the mutated p53 protein in CCRF-CEM cells. Frondoside A treatment also markedly up-regulated multiple genes in the NFκB pathway with changes being more marked in the THP-1 cell line, which is more resistant to the effects of frondoside A. Combining andrographolide IC_{50} concentration with frondoside A in the treatment of acute leukemia cell line resulted in marked enhancement of frondoside A anti-leukemia effect.

Frondoside A has marked anti-leukemia effects. It decreased the viability of acute leukemia blasts and induces apoptosis. The apoptosis appeared to be due to the activation of both extrinsic and intrinsic pathways. Resistance to frondoside A can be due to the activation of the NFκB survival pathway in the treated cells and combining the treatment with NFκB pathway inhibitors results in dramatic enhancement of the anti-leukemic effect of frondoside A. Frondoside A affected different genes and pathways in leukemia blast cells and inhibiting malignant cells by targeting multiple pathways might be more beneficial in the treatment strategy. It potentiates the anti-cancer effects of all three drugs currently used to treat acute leukemias and it may be a valuable addition to the therapeutic options in these deadly diseases especially in high-risk patients by sparing the side effects of high dose therapy and bone marrow transplantation.

Keywords: Frondoside A, acute leukemia, pro-apoptosis, anti-cancer, chemotherapy, sea cucumber, NFκB pathway, Andrographolide.

Title and Abstract (in Arabic)

تأثير مادة الفروندوسايد أ في سرطان الدم الحاد

الملخص

اللوكيميا الحادة (سرطان خلايا الدم البيضاء الحاد) سبب رئيسي للوفيات. بالرغم من تحسن معدل النجاة، لا يزال هناك مرضى يموتون بسبب هذا المرض أو الأعراض الجانبية للعلاج. الأدوية التي تعالج الأمراض السرطانية والمشتقة من مواد طبيعية تستجلب الانتباه لقلة نسبة الأعراض الجانبية المصاحبة لها. Frondoside A مادة تحتوي على 30 ذرة كربون جليكوسايدية مشتقة من خيار البحر (*Cucumaria frondosa*) و الذي يظهر خواص ضد الأورام في السرطانات الصلبة. في هذا البحث قمنا بدراسة تأثير هذا المركب في اللوكيميا الحادة. هذا البحث هو الأول من نوعه في مقارنة فعالية هذا المركب للعلاج المتوفر حالياً لهذا المرض.

خطوط خلايا سرطان الدم الحاد التي استخدمت شملت CCRF-CEM, THP-1 وHL-60. عولجت الخلايا بتركيزات مختلفة من frondoside A, asparaginase, vincristine و prednisolone كل دواء على حدة و أيضا" مزيج من كل دواء مع frondoside A. تم تقييم بقاء الخلايا بعد العلاج و تم اختبار الحث لموت الخلايا المبرمج. كما تمت دراسة التعبير الجيني والبروتيني للجينات المتصلة بموت الخلايا المبرمج. تم دراسة أثر علاج الخلايا بمزيج من frondoside A مع مثبط مسار NFκB، andrographolide.

أوضحت التجارب أن إضافة frondoside A يحسن عمل الأدوية التقليدية التي تستخدم في علاج سرطان الدم الحاد. مادة frondoside A حثت الموت المبرمج في الخلايا السرطانية كما زادت التعبير الجيني للجينات المؤثرة في مسار الموت المبرمج الداخلي والخارجي والجلاد. في خلايا THP-1 قام frondoside A بزيادة البروتين p21 والذي يعمل على دورة الانقسام الخلوي. كما أظهر تأثيره على زيادة التعبير الجيني للجينات الخاصة بمسار NFκB. مزيج frondoside A وAndrographolide أظهر قدرته على زيادة فاعلية frondoside A ضد الخلايا السرطانية.

يظهر frondoside A تأثير كبير ضد اللوكيميا الحادة ومزيج من العلاج به مع مثبط مسار NFκB يزيد من فاعليته. كما يقوم المركب بتعزيز عمل الأدوية المستخدمة حاليا" وقد يكون إضافة فاعله لخيارات العلاج لمرضى سرطان الدم الحاد.

مفاهيم البحث الرئيسية: فروندوسايد أ، مساعد الموت المبرمج، مضاد للسرطان، علاج كيميائي، خيار البحر، مسار NFκB، أندروجرافولايد

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Special thanks go to my mother, sisters and nieces who supported me along the way.

Dedication

To my beloved family

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP-1	Activated protein-1
Asp	Asparaginase
CAD	Caspase activating DNase
Cdk (CDK)	Cyclin dependent kinase Cyclin dependent kinase
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
FrondA	Frondoside A
IAP	Inhibitor of apoptosis proteins
ICAD	Inhibitor of caspase activating DNase
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cell
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI3K/Akt	Phosphatidylinositol-3-kinase/ Protein kinase B
Pred	Prednisolone
PTM	Post translational modification
Raf	Rapidly accelerated fibrosarcoma kinase
ROS	Reactive oxygen species
7-AAD	7- Aminoactinomycin D
TNF	Tumor necrosis factor
Vin	Vincristine

Chapter 1: Introduction

1.1 Overview

Acute lymphoblastic leukemia (ALL) is one of the successes of the chemotherapy era and has led to over 75% of children being cured. However, there remain 25% of children who will still die of their disease despite chemotherapy. Acute myeloid leukemia (AML) is less common in pediatrics age group but has lower cure rates. Survival rate in AML is about 55% in spite of intensive chemotherapy and hematopoietic stem cell transplantation. Clearly there is a need to find better anticancer agents/compounds that specifically target cancer cells and have lower side effects.

Anti-cancer effects of compounds derived from seafood sources have not been fully studied on such patients' cells. One compound identified is frondoside A, a triterpenoid glycoside from the Atlantic sea cucumber *Cucumaria frondosa* which has a potent anti-cancer effects in solid tumors but its effect on hematopoietic malignancies has not been fully elucidated.

1.2 Statement of the Problem

Acute leukemia is a major health concern worldwide. It is the main hematological malignancy diagnosed both in pediatrics and adult age groups. With currently available chemotherapeutic, cure is possible in many of the cases, but not without side effects. Some of the synthetic drugs are also associated with long term side effects that compromise the cure and add a considerable burden on the health system.

The cure rate is less achievable by these drugs in high risk patients and intensive chemotherapy followed by bone marrow transplantation is the only option to achieve cure.

The search for new compounds with potential anti-leukemia effects is essential if we aim for curing this fatal disease. Much research is currently exploring the anti-cancer effect of naturally available compounds; hoping for the discovery of agents with potent effect on cancer cells but with minimal systemic side effects.

1.3 Relevant Literature

1.3.1 Cancers and Acute Leukemia

Cancer is a pathological condition that develops in response to multiple changes in normal tissue. It can be induced, promoted and progressed by different interacting factors. Genetic and epigenetic alterations have been proven to play a major role in the natural history of cancer but recent studies also demonstrated the importance of the interaction between cancer microenvironment and the immune system of the host (Uzan et al., 2014).

1.3.1.1 Characteristics of Cancer

For normal cells to transform into malignant phenotype, certain characteristics should be gained to achieve that. These characteristics are highlighted in general in this section with the factors contributing to leukemogenesis described in more detail later.

1.3.1.1.1 Continuous proliferating signals

Normal cells proliferate when stimulated transiently by a growth factor and stop when the growth factor is withdrawn. In cancer cells, stimulation of proliferation can be sustained by the ability of these cells to produce signaling molecules that stimulate proliferation receptors. Cancer can stimulate tumor-associated stromal cells (tumor microenvironment) to secret required signals (Goldar, Khaniani, Derakhshan, & Baradaran, 2015) or up-regulate receptors expressed on the cell membrane. Mutations in the receptors may render them active without external stimuli. Mutations downstream proliferation pathways may activate cell proliferation independent of receptors and ligands. Loss of negative feedback is another mechanism (Hanahan & Weinberg, 2011).

1.3.1.1.2 Avoiding suppressors of growth

Cell proliferation is controlled by tumor suppressor genes that inhibit cell cycle progression if growth conditions are not optimal. Cancer cells usually lose the function of tumor suppressor genes, which allow them to proliferate regardless of wellbeing of the cell and environment (Hanahan & Weinberg, 2011).

1.3.1.1.3 Resisting apoptosis

Programmed cell death is highly controlled by a balance between pro-apoptotic and anti-apoptotic proteins. This balance is altered in cancer favoring anti-apoptotic proteins (for detail, refer to section 1.3.3.3).

1.3.1.1.4 Manifesting unlimited replication ability

The number of times a cell can replicate depends on telomere length. This is a protective mechanism to maintain integrity of the genetic material. With each cell

division, the telomere shortens till a critical limit is reached, normal cells then inter senescence and apoptosis mediated by p53 (refer to section 1.3.3). If cells continue to divide beyond this limit, chromosomal end-to-end fusion occurs. In many cancer cells, the function of such tumor suppressor gene is lost. Cancer cells also express telomerase that activates the expression of this enzyme with the ability to maintain the telomere length (Hanahan & Weinberg, 2011). This is probably due to the fact that cancers arise in stem cells (blast cells in the case of leukemias) that already express telomerase.

1.3.1.1.5 Inducing angiogenesis

Angiogenesis is the process of producing new blood vessels from existing ones. This process is achieved by a balance between pro-angiogenic and anti-angiogenic factors. The angiogenic drive in cancer comes from stimulation of oncogenes to the up-regulation of factors or their respective receptors. An example for that is the vascular endothelial growth factor A (VEGF-A) (Hanahan & Weinberg, 2011).

Although acute leukemia is a blood disorder where the role of angiogenesis is conflicting, recent studies have shown increase vascularization of the bone marrow in ALL and AML. The leukemic blast cells were found to secret VEGF and angiopoietin that act as an autocine stimulus for the leukemic cell growth. Some studies have even shown that VEGF levels do correlate with the higher risk of the disease (Ayala, Dewar, Kieran, & Kalluri, 2009; Buga Corbu, Glűck, & Arion, 2014).

1.3.1.1.6 Invading locally and distant metastasis

Cancer cells are able to change shape, gain or lose adhesion molecules to enable them to invade and metastasize. In solid tumors, this process is known as epithelial-mesenchymal transition (EMT). There are multiple factors that help cancer

cells to exert migratory phenotype including interaction between the tumor cells and the microenvironment that provide the appropriate signals and proteins (Hanahan & Weinberg, 2011).

For cancer cells to gain these characteristics they require enabling properties to be present. Studies highlighted the function of two properties; genomic instability and tumor-promoting inflammation.

Genomic instability: Mutations in cancer cells and tumor phenotype gained through epigenetic alteration serve as survival advantage for these cells allowing them to further divide and accumulate more mutations. Some mutations can aid the escape of cells from apoptosis that is triggered in response to DNA damage.

Tumor-promoting inflammation: Immune cells in cancer microenvironment have contradicting function. They serve to eradicate cancer, but doing so (in the presence of the immune evading property of cancer cells) they provide the tumor cells with essential growth signals. Immune cells interaction with other tissues lead to the production of oxygen radicals which has a further mutagenic effect on cells.

Cancer cells use inflammatory factors and pathways to maintain their survival signals. One of the main factors is nuclear factor kappa β (NFκB) which is a major transcription factor involved in the inflammatory response. It is secreted by many cells in the body and has 5 subunits; RelA (p65), RelB, c-Rel (Rel), NFκB1 (p105) and NFκB2 (p100). Activation of specific subunits is stimulus-type dependent. The NFκB1 and NFκB2 are cleaved to produce the active forms p50 and p52 respectively. Normally NFκB is retained in the cytoplasm in an inactive form bound to its inhibitor IKβ (Gilmore & Herscovitch, 2006; Sethi, Shanmugam, Ramachandran, Kumar, &

Tergaonkar, 2012). NFκB is activated by a classical (canonical) pathway, triggered by pathogen liposaccharide, inflammatory secreted tumor necrosis factor (TNF) and interleukin-1 (IL-1) and involves RelA/p50. The non-canonical alternative pathway is activated through LTBR (lymphotoxin beta receptor), CD40, tumor necrosis factor receptor-2 (TNFR2) and B-cell activation factor (involves RelB/p52). Both pathways are IKβ/ inhibitor of NFκB kinase (IKK) dependent.

Atypical activation triggered by hypoxia and reactive oxygen species (ROS) is a third way of activation and is IKβ independent (Aivaliotis et al., 2012). Upon activation, NFκB dissociate from its inhibitor and translocate into the nucleus where it binds specific Igκ light chain enhancer region on DNA. This leads to the activation of B-lymphocytes as well as activation of other genes involved in cell survival and proliferation. Once the acute inflammation is controlled, the activity of the mediators subside and NFκB is inactivated through up-regulation of the inhibitor IKβ by NF-κB feedback inhibition (Hoesel & Schmid, 2013).

In case of cancer, there is a continuous secretion of mediators and NFκB is persistently activated. High levels of NFκB are associated with activation of many genes involved in cell signaling. Studies showed NFκB to activate anti-apoptotic genes which helps cancer cells to avoid cell death. It also activates neutrophils to produce ROS leading to DNA damage and tumorigenesis. On the other hand, high levels of ROS can reciprocally activate NFκB through an atypical pathway, as mentioned above, producing a vicious cycle (Hoesel & Schmid, 2013).

Continuously active NFκB in cancer contributes to the inactivation of tumor suppressor gene TP53 via RelA subunit. Many cancer cells are resistance to apoptosis mediated via cell receptors because of the over-expression of c-FLIP, a negative

regulator of death receptors. One of the target genes for activated NFκB is c-FLIP and NFκB can also up-regulate c-FLIP through the activation of MAPK and PI3K/Akt pathways, both leading to the inhibition of death signals through Fas, tumor necrosis factor receptor-1 (TNFR1) and other death receptors (Hoesel & Schmid, 2013). NFκB also induces genes from the inhibitor of apoptosis protein (IAP) family including XIAP, BIRC1, 2, 3, 5, 6, 7 and 8. These proteins hide the protein-protein interaction site on caspase substrates, hence, inhibiting apoptosis (Goldar et al., 2015). The function of NF-κB in cancer is summarized in Figure 1.

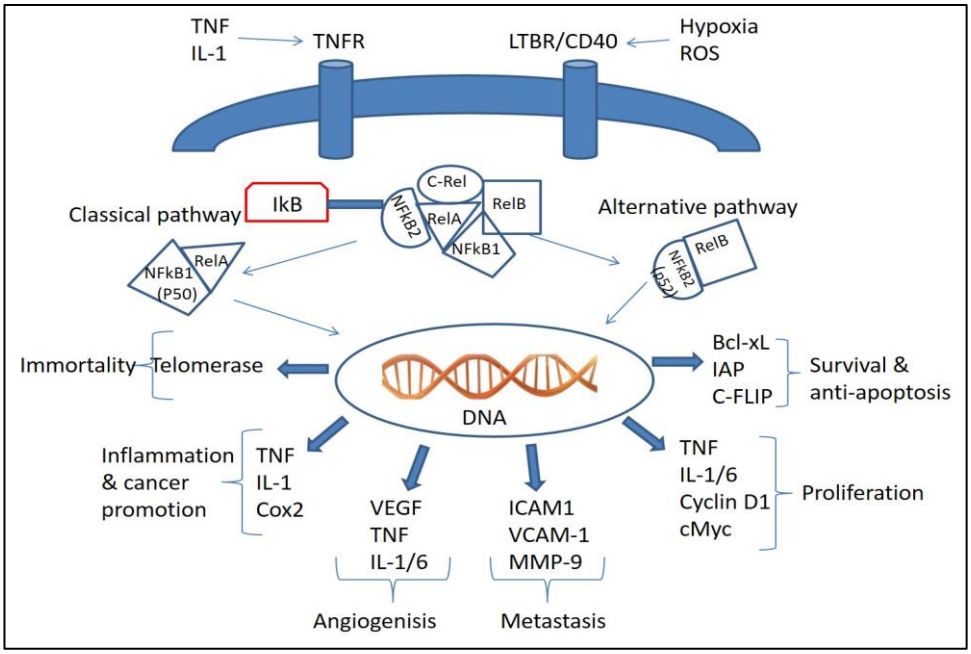

Figure 1: Pro-tumor function of NFκB

1.3.1.2 Acute Leukemia

Acute leukemia is a blood cancer and is regarded as a group of disorders characterized by a clonal expansion of a hematopoietic cell type leading to disruption of normal proliferation and differentiation process of blood cell precursors.

Normal hematopoiesis involves a complex interaction between hematopoietic stem cells (HSC), the surrounding microenvironment, different growth factors and growth inhibitory molecules.

1.3.1.2.1 Pathogenesis

Hematopoietic SC (also known as pluripotent SC) are undifferentiated cells with special properties allowing them to self-renew and indefinitely replenish stem cell pool in addition of being able to differentiate to any of the more lineage committed cells. The majority of these cells are kept quiescent in G0 phase of the cell cycle by the action of transforming growth factor- β (TGF-β) which acts through the tumor suppressor protein p53 and cyclin-dependent kinase (CDK) inhibitor p21. The TGF-β is usually secreted by the surrounding stromal cells (Buga Corbu et al., 2014; Hoffbrand , Catovesky, & Tuddenham, 2006). There is a tight balance between the stimuli that controls SC renewal or differentiation.

Under different influences of body requirements, SC can be stimulated to shift the balance towards differentiation to progenitor cells that have less self-renewal capacity and more differentiation potentials to multipotent SC. Further differentiation leads to a lineage commitment towards erythroid, megakaryocytic, eosinophilic and granulocytic/monocytic lineages.

After final commitment, cells undergo maturation. Abnormal cells are detected and eliminated by the immune system through the process of apoptosis (see section 1.3.3).

As in any cancer, acute leukemia arises as a result of accumulation of multiple genetic abnormalities involving key cellular processes such as cell cycle,

differentiation, maturation, regulation of transcription, cell death (apoptosis) and intracellular signal transduction (Buga Corbu et al., 2014; Rubnitz & Inaba, 2012).

Recent studies investigating the process of leukemogenesis revealed a complex interaction of different pathways as well as the bone marrow compartments. Although genetic alterations/mutations are considered a major factor for developing leukemia, they are not the only factors. Interaction with cellular epigenetic changes as well as the vital supportive contribution of the surrounding microenvironment are important in the process of transformation of a normal cell into a malignant one.

The genetic alterations in acute leukemia are found to affect genes involved in normal leukocyte development (O'Brien, Morin, Ouellette, & Robichaud, 2011). Activation of an oncogene, silencing a tumor suppressor gene or production of a fusion gene that encodes a protein with a transcription factor or kinase receptor activation property (Mullighan, 2009).

Broadly, the genetic alterations associated with acute leukemia can be classified based on the number and structure abnormalities of chromosomes, as listed in Table 1.

Examples of oncogene activated mutations are:

Translocation (12,21)

This translocation t(12,21)(p13;q22) involves TEL gene on chromosome 12 and the AML1 gene on chromosome 21 (Burg et al., 2004; Mullighan, 2012). TEL gene encodes a sequence-specific-DNA binding transcriptional regulator (from the ETS family) while AML1 is a transcriptional factor that binds specific DNA sequences inducing hematopoiesis development. The fusion resulting from the translocation

alters AML1 to a transcriptional suppressor causing disruption in normal lymphocyte development. The fusion gene also has the capacity to activate downstream JAK-STAT signaling pathway leading to the promotion of self-renewal of B-lymphocyte progenitors.

Classification	**Subtype**	**Frequency**
Aneuploidy	Hyperdiploidy	20-30%
	Hypodiploidy	1-2%
Chromosomal Translocations	t (12,21)	15-25%
	t (4,11)	1-6%
	t (1,19)	2-6%
	t (9,22)	2-5%
	t (17,19)	1%
	t (8,14)	1-2%
Submicroscopic alterations	PAX5	2% (31% in B-cell ALL)
	IKZF1	15% of B-cell ALL
	CRLFZ	2-5%
	JAK1/2	5-7% of B-cell ALL
	CREBBP	1-7%

Table 1: Classification of common genetic mutations in ALL

Translocation (4,11)

MLL rearrangements: these include translocations involving the mixed lineage leukemia gene on chromosome 11 and multiple other genes on different chromosomes such as t(4,11), t(9,11) and t(10,11) as well as t(11,19) that is seen in T-cell ALL and carry a poor prognosis (Campos-Sanchez et al., 2011; Heerema et al., 2005). MLL rearrangement is also seen in AML. Since t(4,11)(q21;q23) accounts for 50% of all MLL rearrangements; it will be discussed in more details. MLL gene is important for hematopoeisis regulation through maintaining normal gene expression. It acts partially through HOX gene family. The MLL-AF4 translocation places MLL gene under the

activation of AF4 gene promoter which encodes a transcriptional factor leading to up-regulation of HOX family proteins promoting a stem-cell like state of maturation. The HOX family proteins are involve in cell transformation and some of those proteins act by delaying cell differentiation. Other MLL rearrangements results in fusion proteins that affect the regulation of transcription leading to transcription deregulation (Bach et al., 2010).

Translocation (9,22)

Translocation (9,22)(q34;q11.2): is only seen in 3-5% of pre-B-cell ALL cases (Mullighan, 2012) but is significant due to its association with poor prognosis and the development of targeted therapy. It results in the production of Philadelphia chromosome with BCR region on chromosome 22 being translocated to chromosome 9. The BCR-ABL fusion gene encodes a novel protein with a tyrosine kinase (TK) activity. There are three fusion proteins produced from such translocation, depending on the breakpoint site within BCR region. A 190 kDa protein, mainly associated with ALL cases. Another two proteins of 210 kDa and 230 kDa seen in cases of chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) respectively (Hunger, 2011). This fusion protein activates multiple signaling pathways (such as Ras/Raf/MEK/ERK, JAK/STAT and SRC family of TK) (Brown, Seif, Reid, Teachey, & Grupp, 2008) leading to progression of cell through cell cycle and hence, the promotion of cell proliferation and adhesion.

Sub-microscopic alterations: as mentioned before and from earlier studies, genetic abnormalities are detected in 75% of ALL cases. With the advancement in technology the remaining cases were found to harbor submicroscopic abnormalities that might play a role in the leukemogenesis of this subtype of ALL. Some of the

microscopic abnormalities include deletion of lymphoid transcriptional factors (such as PAX5, IKZF1, EBF1 and LEF1), tumor suppressor genes (NF1, PTEN, RB1 and ATM), abnormal regulation of apoptosis (BTG1), signaling molecules and DNA circulating nucleic acid (Mullighan, 2009).

PAX5

The PAX5 oncogene from the Paired Box (PAX) family encodes an important transcription factor that is involved in B-cell differentiation (Thomas-Tikhonenko & Cozma, 2008).

Studies have shown PAX5 expression to be low in early B-cells (pro-B and earlier stages) and the expression increased in pre-B cell stage, after which it is down-regulated in plasma cells (terminally differentiated B cells). From the expression pattern, it is clear that the PAX5 gene has a role in B-cell lineage commitment, differentiation and development (Campos-Sanchez et al., 2011). In normal B-cells, transcription from PAX5 is controlled by multiple factors including an upstream regulator (early B cell factor 1, EBF1) that mediates histone remodeling allowing access for PAX5 transcription (O'Brien et al., 2011).

Epigenetic modifications are defined as changes that affect gene expression without a direct change or alteration to the DNA/gene sequence. These include DNA methylation and histone modifications such as acetylation and methylation.

DNA methylation occurs on the pyrimidine nucleotide cytosine. The methylation at the promoter of the genes is usually associated with the repression of the gene and majority of gene are kept quiescent by this mechanism. In contrast, the methylation of the coding part of the gene leads to the activation of the transcription

(Gutierrez & Romero-Oliva, 2013). The methylations is facilitated by the enzymes DNA methyl transferase 1, 3a and 3b.

DNA hypomethylation due either to defects in those enzymes or other factors have been shown to cause chromosomal instability and activation of oncogenes. Hypermethylation of the promoter regions of tumor suppressor genes was recorded to be associated with inactivation of these genes. A study to identify differences in DNA methylation in AML patients compared to normal hematopoietic cells showed a unique difference (Gutierrez & Romero-Oliva, 2013).

As a general rule acetylation of histone is associated with gene activation while methylation causes gene repression. Histone acetyltransferase (HAT) and histone methyltransferase (HMT) are responsible of these changes. Mutations involving these enzymes have been seen in many acute leukemias as in case of t(8;16). The MLL mutations that are known to be associated with aggressive ALL as well as AML involves a mutation in the mixed lineage leukemia protein which is a HMT (Gutierrez & Romero-Oliva, 2013).

Studies attempting to classify patients into risk groups and tailor treatment based on the epigenetic signature of the disease are ongoing and many epigenetic compounds are under investigation for possible use as a treatment option in acute leukemia (Gutierrez & Romero-Oliva, 2013).

The concept of leukemia (cancer) stem cell and tumor reprograming in response to the close interaction with bone marrow cellular compartments/ microenvironment has been emerging recently highlighting the importance of this in the process of leukemogenesis (Gojo & Karp, 2014). It is well known that the behavior

of the hematopoietic stem cells is determined by its interaction with the surrounding cells as well as the different proteins in the extra cellular matrix (ECM), including cytokines, growth factors and adhesion molecules.

The bone marrow consists of cellular and vascular parts. The cellular niche consists of the hematopoietic cells and the mesenchymal cells that supports the hematological components including osteoblasts, endothelia cells and neurons (Ayala et al., 2009). These are in contact with the ECM consisting of collagen, fibronectin and laminin. The vascular part functions as a boundary separating mature from immature cells. To demonstrate the importance of the microenvironment in the development of acute leukemia we will take the cytokine CXCL12 produced by the bone marrow reticular cells and vascular endothelial growth factor (VEGF) as examples.

The chemokine CXCL12 and its receptor CXCR4 have shown to be crucial for cellular homing, it controls the migration of normal hematopoietic cells in the bone marrow(Ayala et al., 2009). It has been shown that this function is also demonstrated in both acute lymphoid leukemia and acute myeloid leukemia blast cells. The expression levels of CXCR4 on leukemia blast are usually increased in hypoxic environment (such as the bone marrow) and CXCL12 is activated through hypoxia inducing factor-1 and promotes blast cell survival through NFκB and JNK/AP-1 pathways (Rashidi & Uy, 2015).

As mentioned before, VEGF secreted from blast cells promotes angiogenesis and this enhances the recruitment of other growth factors and molecules into the surrounding microenvironment leading to the support of leukemia cell survival (Ayala et al., 2009).

Cross-talk between leukemic blast cells and the marrow endothelial cells also found to promote blast cell survival through changing the cellular expression of the proteins involved the apoptosis pathways (Ayala et al., 2009).

1.3.1.2.2 Classification

Broadly, acute leukemias are classified based on the stage of differentiation/ maturation arrest and the type of SC involved. Hence, acute leukemias are classified as acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML). These major groups are further sub-grouped based on details of morphology, immunophenotyping and cytogenetics.

Previously, French-American-British (FAB) classification system was used. It is a purely morphological/cytochemical staining based system. For the diagnosis of acute leukemia it requires the presence of at least 30% of blast cells. If the blast cells are of lymphoid origin then they are further sub-classified to L1, L2 and L3 subtypes (Hoffbrand et al., 2006). The morphological details are shown in Table 2 and Figure 2. If the blast cells are of myeloid origin then the subtypes include M0 to M7 based on the degree of maturation (Buga Corbu et al., 2014; Hoffbrand et al., 2006). Table 3 demonstrates the morphological description of each subclass.

Classification	**Morphology findings**
L1	Blast cells small, uniform, high nuclear to cytoplasm ratio
L2	Blast cells larger, heterogeneous, lower nuclear to cytoplasm ratio
L3	Vacuolated blast cells, basophilic cytoplasm (usually B-ALL)

Table 2: French-American-British (FAB) classification of ALL

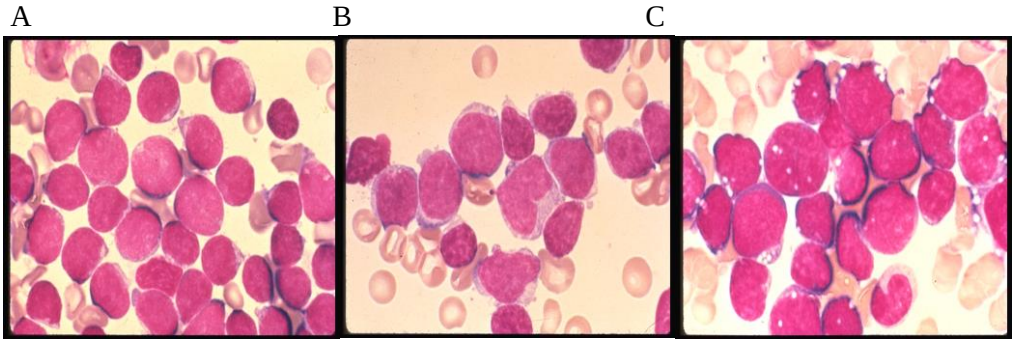

Figure 2: FAB classification of ALL. Based on morphology, A) L1, showing small blast cells with high N:C ratio. B) L2, the cells are larger with lesser N:C ratio. C) L3, shows basophilic cytoplasm with vacuoles. http://www.thecrookstoncollection.com

Currently, the World Health Organization (WHO) classification is in-use and it lowered the blast percentage for the diagnosis to 20% and extended the former system to involve surface/cytoplasmic markers using immunophenotyping techniques and also molecular cytogenetic analysis of detected abnormalities. A more complex and detailed sub-classification of acute leukemia has been produced which was found to better correlate with prognosis (Khaled, Al Malki, & Marcucci, 2016). Table 4 represents the recent revised version of the WHO classification system.

FAB subclass	**Morphological description**
M0	MPO/SBB positive, blast cells<30%
M1	MPO/SBB positive, blast cells≥30%, Mature cells≤10%
M2	Blast cells 30-89% with granulocyte maturation in>10%
M3	Promyelocyte maturation with strongly positive MPO/SBB and typical Auer rods.
M4	Blast cells≥30% with granulocyte maturation≥20% and monocyte components≥20%
M5	M5a) Blast cells≥30% with monocyte components≥80% and monoblast≥80% M5b) Blast cells≥30% with monoblasts<80%
M6	Erythroid lineage cells≥50% of the bone marrow
M7	Blast cells showing mainly megakaryoblasts features

Table 3: FAB morphological classification of acute myeloid leukemia. (MPO: myeloperoxidase, SBB: Sudan black B)

WHO category	Subclasses
AML with recurrent genetic abnormalities	AML with t(8;21) AML with inv(16) AML with t(15;17) AML with t(6;9) AML with inv(3) AML witht(1;22)
AML with myelodeysplasia	
Therapy related myeloid neoplasms	
AML not otherwise classified	AML minimally differentiated AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Acute Erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferation related to Down's syndrome	Transient abnormal myelopoiesis Myeloid leukemia associated with Down's syndrome
Blastic plasmacytoid dendritic cell neoplasm	

Table 4: WHO classification of acute myeloid leukemia

1.3.1.2.3 Incidence

Acute lymphoblastic leukemia accounts for two-third of acute leukemias in children and young adults. Data from the national cancer institute showed acute leukemia to account for 30% of childhood malignancies (Moriyama, Relling, & Yang, 2015) with ALL being the diagnosis in more than 60% of all hematological malignancies while AML accounts for 18% of the cases and is associated with more complex pathogenesis. In USA, there are 4000 cases diagnosed per year (Pui & Evans, 2006).

Eighty percent of pediatrics ALL are of pre-B cell ALL while T-cell ALL are around 10-15% of the cases (Mullighan, 2012; Pui & Evans, 2006). ALL in adults is

less common and the main diagnosis is AML in patients aged between 65-70 years (Buga Corbu et al., 2014; Nazha & Ravandi, 2014; Pui & Evans, 2006).

In the UAE, cancer is the 3rd cause of death after cardiovascular diseases and trauma (mostly due to road traffic accidents) (Badrinath, Ghazal-Aswad, Osman, Deemas, & McIlvenny, 2004; Tadmouri & Al-Sharhan, 2008). Data from the ministry of health showed leukemia to be the 3rd most frequent cancer accounting for 8% of all cases but it is the most common among children accounting for 39.4% of childhood malignancies. ALL accounts for 23% of all hematological malignancies, while AML is diagnosed in 14% of cases and it is more common in females with male: female ratio of 1:1.23 in the years 1998-2001 (Tadmouri & Al-Sharhan, 2008).

1.3.1.2.4 Diagnosis

The diagnosis of acute leukemia depends on the clinical presentation suggestive of the disease as well as the confirming investigations. In the following section, a brief diagnostic plan and findings will be described.

Symptoms at presentation include those of bone marrow failure such as fatigue and irritability due to anemia (Choi & Pai, 2003; Ek, Mellander, & Abrahamsson, 2005), easy bruising due to thrombocytopenia and infections due to a relative neutropenia. Central nervous system (CNS) involvement can also occur mainly in ALL, causing headache, vomiting and cranial nerve palsies. Lymphadenopathy, hepatosplenomegaly and osteolytic bone lesions may be found on examination (Hoffbrand et al., 2006; Hoffbrand & Pettit, 1994).

The diagnosis is usually confirmed by bone marrow examination showing replacement of normal hematopoiesis by blast cells of the malignant lineage.

Radiological studies are important to assess the extent of the disease, any complications, and to have baseline information about the patient's physical condition before starting any treatment. Chest x-rays might show a mediastinal mass due to infiltration of malignant cells. Testicular ultrasound can be used to look for testicular involvement (in ALL). Echocardiogram and Electro-Cardiogram (ECG) are important to perform before the start of treatment with anthracyclines due to their potential cardiotoxicity.

Bone marrow aspirate examination is mandatory for the diagnosis and classification. The microscopic examination of the aspirate will show a blast count of ≥20% (typically 50% or more).

Cytochemical staining of the BM aspirate cells is used to diagnose and broadly classify acute leukemia (Hoffbrand & Pettit, 1994). The diagnosis is further refined by immunological phenotyping (Bain, Clark, & Wilkins, 2010).

Incorporating cytogenetics analysis in the diagnosis of acute leukemia has emerged due to its correlation with the prognosis (Hoffbrand et al., 2006). Specific chromosomal abnormalities are associated with specific acute leukemia subclasses and these genetic abnormalities found to contribute largely to the disease behavior in terms of severity, response to treatment and risk of relapse (Jaffe, Harris, Stein, & Vardiman, 2001).

1.3.1.2.5 Prognosis

It is vital in acute leukemia patients as in patients with other types of malignancies to be assigned into risk groups based on prognosis. This determines the possible cure rate and risk of relapse and help in better tailoring the treatment options.

Recent treatment protocols have taken into accounts the prognosis of patients in order to design the best treatment schedule in regards to drug types, doses and duration of treatment.

The determination of the prognosis depends on many factors including; both clinical and laboratory findings. It was found that risk group stratification of patients with childhood ALL correlates well with the cure rate while it is less predictive of the outcome in AML (Liersch, Müller-Tidow, Berdel, & Krug, 2014).

The diagnosis of ALL at 1-9 years of age with B-cell types is considered a good prognostic factor with better cure rates than those diagnosed before age of 1 year or at 10 years and older. The white blood cell (WBC) count at diagnosis is another powerful factor with counts more than 50,000 cells/ml^3 being associated with less cure chances. Females were observed to do better than males and hence, gender is also included in the prognostic list. ALL subtypes of good prognosis are Pre-B-Cell ALL, common-B-Cell ALL and early Pre-B-Cell ALL achieving better cure rates than mature-B-cell ALL. Previously, diagnosis with T-cell ALL subtypes was considered a poor prognostic factor but with recent advances in treatment, its prognosis is as good as B-cell ALL. The involvement of extramedullary organs such as central nervous system (CNS) and the testicles in boys is a high risk factor. Cytogenetic abnormalities with chromosomal aneuploidy and translocations are recently been found associated with the prognosis of the disease (Cooper & Brown, 2015; Hoffbrand et al., 2006; Pui & Evans, 2006). Early response to treatment measured by minimal residual disease (MRD) detection techniques is also a powerful prognostic indicator, with some investigators considering it with the cytogenetic markers to be the two major factors affecting the disease outcome (Rubnitz & Inaba, 2012).

Using these multiple factors, pediatric ALL risk groups are divided into low, standard, high and very high risk groups while adults with ALL are grouped into either standard or high risk groups. AML risk stratification uses similar prognostic factors (Hoffbrand et al., 2006).

1.3.1.2.5 Treatment

Childhood ALL is one of the successes of the chemotherapy era and has led to over 75-90% of children being cured of this once uniformly fatal malignancy (Kreis, Louwen, & Yuan, 2015; Mathisen, Jabbour, & Kantarjian, 2012; Silverman & Deitcher, 2013). However, there remains a 10-25% of children who will still die of their disease or the treatment side effects. There is a need to investigate other compounds with more potent effect for these high risk/treatment failing cases if we are to improve treatment in the future. Acute myeloid leukemia is less common in pediatrics age group but has lower cure rates. Survival rate in AML is about 55% in spite of intensive chemotherapy and hematopoietic stem cell transplantation.

In adults, ALL is usually of poor prognosis with cure rates up to 40% due to the association with high risk cytogenetic alterations (Boissel & Sender, 2015; Mathisen et al., 2012; Pui, Mullighan, Evans, & Relling, 2012).

The treatment options for ALL depend on the prognostic factors, as well as the involvement of extramedullary sites such as CNS. The treatment protocols consist of four main stages (2): induction therapy, CNS prophylaxis, intensification therapy and maintenance treatment.

Current induction therapy consists of three to four main drugs including vincristine (to block mitosis), prednisolone (to block activation of AP-1 and NFkB

responsive elements), L-asparaginase (to starve leukemia cells of asparagine that, unlike other cells, they cannot synthesize) with or without anthracyclines (to inhibit nucleic acid synthesis and topoisomerase activity). It usually lasts 4-6 weeks and remission can be achieved in 95% of patients.

Subsequently, CNS prophylaxis is used to prevent leukemia cells reaching the nervous system. That is a specific treatment aimed to cross the blood-brain barrier preventing leukemia cells seeding and eliminating any CNS disease. The CNS prophylaxis consists of cranial irradiation and intrathecal methotrexate. Alternative protocols include 3 intrathecal drugs (usually methotrexate inhibits dihydrofolate reductase), hydrocortisone and cytarabine (inhibits DNA synthesis) and no irradiation, to minimize toxicity and the risk of developing secondary brain tumors. The decision of the protocol depends on the clinical and prognostic status of the patient.

Intensification therapy is used with the aim of eradicating any residual leukemia cells left soon after the induction of remission. The benefit of this therapy is seen in high risk patients. Different combinations of chemotherapy drugs are given including high dose methotrexate, cyclophosphamide, L-asparaginase, 6-mercaptopurine and cytarabine (Ara-C) (Pui & Evans, 2006).

Maintenance therapy is continued for 2-2.5 years after induction treatment with the aim of ensuring that all leukemia cells are eradicated and minimize the risk of relapse (the return of the disease after treatment, i.e. failure to “cure”).

High risk patients may be considered for allogenic bone marrow transplantation in their first remission.

Acute myeloid leukemia is more heterogeneous. Current treatment protocols rely on the molecular diagnosis. Treatment also include an induction cycle, intensification and maintenance cycles. With the addition of anthracyclines and cytarabine in most of the recent treatment protocol has improved the survival rate to reach to 70% of the cases (Nazha & Ravandi, 2014).

The ideal anti-cancer and more specifically anti-leukemia drugs should be targeting specific features of the tumor cells that are different from normal cells. In this way, normal cells are spared from being affected by the drugs and their side effects.

One of the main tumor characteristics as discussed before is the high rate of proliferation which requires large amounts of amino acids to produce proteins for cell survival. Asparagine is an amino acid that is produced by the cells using the enzyme asparagine synthase. Small quantities of asparagine are sufficient for normal cells but not for tumor cells. Lymphoid cells showed to have limited capacity to produce asparagine and their high requirement of it makes it an essential component for cancer cell survival (Boissel & Sender, 2015).

The introduction of L-asparaginase in the treatment of acute lymphoblastic leukemia deprives the blast cells from this amino acid. L-asparaginase hydrolyses asparagine into aspartic acid and ammonia. Initially it was extracted and purified from biological sources such as bacteria and plants, recently recombinant L-asparaginase is available.

The enzyme has been modified to decrease its immunogenicity and prolong its half-life; pegylated asparaginase (PEG-Asp) is one of the successful modifications

(Moriyama et al., 2015). Studies have shown L-asparaginase to cause cell cycle arrest in leukemia cells at G1 phase and eventually induce apoptosis. Some AML cells were shown to over express mRNA of asparagine synthase and treatment with l-asparaginase decreased its effectiveness. Asparaginase is also associated with decrease in plasminogen and anti-thrombin levels. Although high proliferation rate and asparagine dependence are features of leukemia blast cells, asparaginase is not specific and other normally highly proliferating cells are affected by the treatment. Side effects of asparaginase include allergic reactions, vomiting, hyperlipidemia, pancreatitis, immune suppression consisting of neutropenia but the most serious and major side effect is hypercoagulability and thrombosis. Thrombosis can affect different organs and lead to cerebral thrombosis, myocardial ischemia, renal dysfunction and acute liver injury (Verma, Kumar, Kaur, & Anand, 2007).

Vincristine is another chemotherapeutic drug that is used for the treatment of ALL, AML and many solid tumors. It is a vinca alkaloid with anti-mitotic effects. It binds the β subunit of tubulin in the spindle apparatus during cell division in M-phase leading to cell cycle arrest and interference with chromosomal segregation (Mora, Smith, Donohoe, & Hertz, 2016; Silverman & Deitcher, 2013). As part of its anti-cancer mechanism it binds other cellular microtubules and interferes with cellular transportation of molecules. Studies have shown vincristine to inhibit capillary formation and the secretion of VEGF *in vitro*. Vincristine action is not specific to cancer cells and it affects mitosis in normal cells as well.

Vincristine has to be given intravenously, since it is not active if given orally and can be lethal if given intrathecally. It is metabolized in the liver using the normal cytochrome P450 pathway. This can affect its plasma concentration and dosing

schedule since it can interact with other drugs/compounds using the same metabolic pathways. It has a large plasma distribution volume and a long half-life. It distributes in normal tissues which makes its effect in tumor cells limited.

The side effect of vincristine include hair loss, constipation and hyponatremia. It is teratogenic and the most serious side-effect is peripheral neuropathy (Moriyama et al., 2015). Vincristine binds to neuronal fibers leading to sensory, motor and autonomic neuropathies. As a consequence, patients can present with peripheral sensory loss, foot drop, decrease muscle power of upper as well as lower limbs and orthostatic hypotension. These neurological symptoms can be severe necessitating dose reduction and skipping of doses that can affect the treatment outcome. The neurological symptoms can start within a week of treatment and last for many years after stopping the drug. This side-effect can affect the quality of life as well as the effectiveness of the treatment (Mora et al., 2016). There is no effective treatment to protect or reverse vincristine induced peripheral neuropathy.

Glucocorticoids such as prednisolone/hydrocortisone are incorporated in the treatment regimen of acute leukemia. Prednisolone binds glucocorticoid receptors and translocate into the nucleus binding to steroid response element and activates/suppresses different genes through the interaction with activated protein-1 (AP-1) and NFκB. It can lead to cell cycle arrest and apoptosis. It's known anti-inflammatory effects also add to its anti-leukemia properties. It crosses the blood brain barrier which makes it suitable for CNS prophylaxis/treatment.

The side-effects of prednisolone include infections, osteonecrosis, osteopenia and fractures as a consequence. It also causes steroid induced psychosis,

hypogammaglobulinemia and proximal myopathies. The side-effects are dose and duration dependent (Inaba & Pui, 2010).

Treatment being non-specific for leukemia blast cells and affect other normally highly proliferating cells such as the gastrointestinal tract, the respiratory system and the skin. Drugs used in the treatment regimens of acute leukemia are mostly toxic causing immediate as well as longer term complications. These unwanted effects may either resolve with discontinuation of the drug or manifest themselves as long-term effects with more permanent end organ damage, affecting the patient's quality of life.

Naturally available compounds with medicinal properties such as anti-cancer/ anti-leukemia properties might be an alternative options for the treatment of such diseases with reduced side effects, lower toxicities and perhaps lower costs of treatment.

1.3.2 Frondoside A

Sea cucumbers, scientifically known as 'Holothuroidea' are echinoderms, living deep on the floors of the oceans (Menchinskaya, Pislyagin, et al., 2013; Wijesinghe, Jeon, Ramasamy, Wahid, & Vairappan, 2013). Their shape ranges from spherical to elongated cucumber like, hence, the name. *Cucumaria frondosa* is one of the species from the *Cucumaria* genus, cucumariidae family in the dendrochirotida order of the holothuroidea class. It is one of the largest sea cucumbers (Figure 3) and the most common type in New England, it is also abundant in the North Atlantic Ocean and Russia's Barents Sea. *Cucumaria frondosa* is harvested, dried and processed using segmented chromatography to extract multiple biologically active components (Aminin et al., 2008; Bordbar, Anwar, & Saari, 2011). Many of which have been

investigated for different possible pharmacological functions including anti-inflammatory, anti-bacterial and immune-modulatory functions (Aminin et al., 2008; Janakiram, Mohammed, & Rao, 2015) as well as its anticancer effect in different types of cancer.

Figure 3: *Cucumaria frondosa* from the deep Atlantic Ocean

Frondoside A is a soluble triterpenoid glycoside, extracted from the skin of *Cucumaria frondosa*. Different natural products extracted from sea cucumbers have been used as dietary supplements (Al Marzouqi et al., 2011; Janakiram et al., 2015) and as traditional remedy in old Chinese medicine (Li , Himaya, & Kim, 2013). Yet there have been 14 biologically active glycosides extracted from *C. frondosa*. Other forms of triterpenoid glycosides are available from other types of sea cucumbers and have some structural differences compared to frondoside A, these forms include holothuria A_2-2, holothuria A_4-2 and holothuria A_7-1 (Park , Bae, Kim, Stonik, & Kwak, 2014).

Structurally, frondoside A consists of a pentaoside with one sulfate group (Menchinskaya, Pislyagin, et al., 2013). It has an aglycone steroid back bone with a xylose attached as a 3rd monosaccharide residue, 3-*O*- methylglucose as a terminal

monosaccharide and an acetoxyl group at C-16 of the aglycone ring (Janakiram et al., 2015; Jin et al., 2009; Menchinskaya, Pislyagin, et al., 2013; Park et al., 2014; Silchenko et al., 2008). Figure 4 shows the structure of frondoside A.

Most anti-cancer therapies available target one or more of the malignant hallmarks; interfering with cancer cells' survival. Studies have shown frondoside A to target multiple cancer cells characteristics making it an interesting potential compound to be used in cancer therapy. These studies are summarized in Table 5.

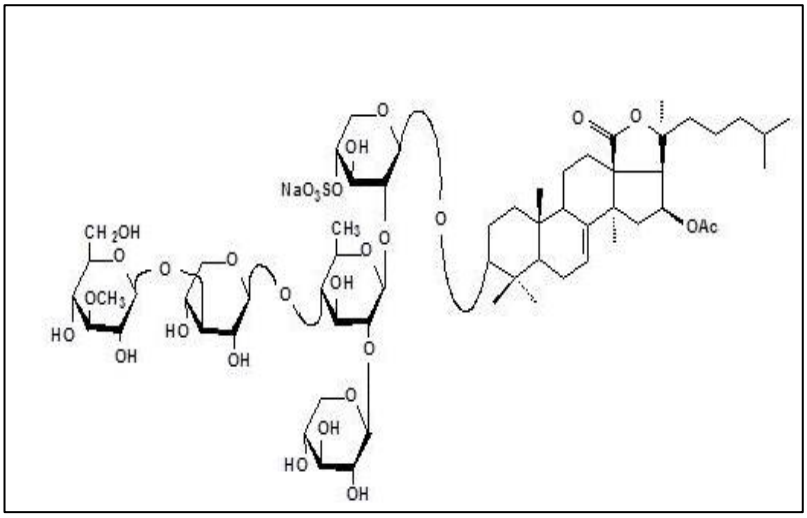

Figure 4: The chemical structure of frondoside A

1.3.2.1 Cancer growth inhibition, anti-proliferation and pro-apoptosis properties

Frondoside A inhibits cancer cell growth via different mechanisms. It causes decrease cell viability in many cell lines including human LNM35 lung cancer cells in a dose dependent manner. Lung cancer growth suppression by frondoside A was also tested *in vivo* in mice injected with this compound alone, cisplatin or combination of both and low concentration of frondoside A (10 µg/kg/day) caused significant decrease in tumor weight and volume ($P < 0.05$). Frondoside A in this study showed to enhance

the effect of cisplatin and treated animals showed no side effects in term of behavioral and body weight changes post treatment (Attoub et al., 2013).

Another study on athymic mice injected with two types of aggressive human pancreatic cancer cell lines AsPC-1 and S2013 showed that combination of the standard treatment (gemcitabine) with frondoside A has statistically higher suppressive effect on cancer growth measured by tumor volume and tumor weight than each compound given alone. Activation of caspase-3 detected by immunohistochemical examination of the tumor developed in xenograft, showed marked apoptosis in the treated groups compared to controls. Those treated with combination therapy showed significantly higher apoptosis rates. In this study it was suggested that decrease tumor growth is mainly due to the activation of apoptosis pathways by both compounds (Al Shemaili et al., 2014). Frondoside A was also found to enhance the effect of paclitaxel in human breast cancer MDA-MB-231 xenograft. Treatment of MDA-MB-231 xenografts with intraperitoneal frondoside A (100 μg/kg) daily for 24 days significantly decreased the tumor volume and weight ($p < 0.001$ and < 0.01, respectively). There was no toxicity recorded in terms of changes in blood counts, creatinine level and liver enzymes post treatment (Al Marzouqi et al., 2011). Furthermore, a study that aimed at investigating the antigrowth effect of salinomycin on MDA-MB-231 cell lines demonstrated an enhanced effect of salinomycin when combined with 1.0 μM frondoside A measured by CellTiter-Glo luminescence assay (Al Dhaheri et al., 2013).

Frondoside A found to increase the expression of cyclin dependent kinase inhibitor; p21 independent from p53 (Li et al., 2008).

Type of study	Cancer type	Cells/animal model	Major finding	Ref
• In vitro • In vivo	Breast	Human MCF-10A MDA-MB-231 Athymic NMRI nude mice	• Decrease cell viability • Induce apoptosis via activation of caspase3/9/8 • Increase p53 expression • Impaired cell migration and invasion • Enhanced the effect of paclitaxel • Decrease tumor volume • No change in CBC/LFT	51
• In vitro • In vivo	Pancreas	Human AsPC-1, S2013 Athymic xenograft mice	• Decrease cell viability • Enhances the effect of gemcitabine • Decrease tumor volume and wieght	52
• In vitro	Leukemia	Human HL-60, NB4, THP-1	• Induces apoptosis via activation of caspase 3/8 after 6h treatment. • No change in mitochondrial membrane potential. • Treatment with caspase 3 inhibitor blocked apoptosis but not caspase 8 inhibitor	54
• In vitro • In vivo	Lung	Human LNM35, A549, NCI-H460-Luc2 NMRI nude mice	• Decrease cell viability • Activates caspase3/7 • Decrease cell migration and invasion • Decrease tumor volume and weight • Decreased capillary-like structures • Enhanced cisplatin activity • No change in mice weight or behavior and no signs of toxicity	56
• In vitro • In vivo	Pancreas	Human AsPC-1 Athymic mice (BALB/c nu/nu)	• Decrease cell proliferation • Induce apoptosis via activation of caspases 3/7/9 • Decrease Bcl-2 and Mcl-1 and increase Bax • Increase p21 expression • Decreased tumor volume and weight	57
• In vitro	Breast	MDA-MB-231	• Enhance the inhibitory effect of salimomycine on cell viability	58
• In vitro	Breast	Human MDA-MB-231	• Inhibit TPA-induced colony formation • Inhibit MMP-9 • Inhibit cancer migration and invasion though PI3K/Akt/ERK pathways	61

Table 5: Studies investigated the anti-cancer effects of frondoside

Type of study	Cancer type	Cells/animal model	Major finding	Ref
• In vitro • In vivo	Prostate	PC3, Du145, VCaP, 22Rv1, LNCaP Human xenograft models of PC3 and Du145 Du145 xenotransplants	• Decrease cell viability, proliferation and colony formation • Cell cycle arrest in PC3 at G2/M phase • Induce caspase-dependent apoptosis in Du145 but caspase-independent in other cell lines • Pro-apoptosis and anti-apoptosis protein expression changes • Inhibit autophagy • Inhibit growth of subcutaneous tumor • Decrease cell metastasis to the lung in PC3 model • Increase spleen size, lymphocytosis and monocytosis • No side effects detected	60
• In vivo	Murine mammary tumor	Balb/cByJ mice with 410.4, 66.1 and 67 cell lines	• Decrease tumor colony formation • Inhibit metastasis in NK cell dependent manner • Restore NK cell capacity for IFN-γ production • Antagonize EP4 receptor	62
• In vitro • In vivo	Breast	Murine mammary 66.1 line Balb/cByJ syngeneic female mice	• High concentration block EP2-mediated cAMP activation • Inhibit ERK1/2 in a dose dependent manner • Pretreatment of the cell lines before injecting to the mice reduced metastasis to the lung • Treatment of the mice with frondoside A 50μg/kg decreased the metastatic potential but didn't affect the tumor size	63
• In vitro • In vivo	Breast	Murine mammary 66.1, 410.4, 67, 410 & human MDA-MB-231, SKBR3, MCF7 Balb/c/SCID mice	• Decrease mammosphere size by antagonizing EP4 but not size • Decrease expression of CD44	65
• In vitro		Mice Ehlich carcinoma ascites cells	Inhibit multidrug resistance via blocking P-glycoprotein pump	68
• In vitro	Lung	Human A549 cell line	• Inhibit PAK1-dependent cell growth • Direct inhibitor of PAK1	102
• In vitro • In vivo	Pancreas	Human AsPC-1, S2-013 Athymic AsPC-1 xenograft	• Inhibit cell growth • Frondoside A more potent than frondoside B • Ip rout decrease tumor volume • Iv bioavailability is longer than ip rout • Oral administration id ineffective • No reported SE after 1 month treatment with frondoside A 100μg/kg/day	129

Table 5 (continued): Studies investigated the anti-cancer effects of frondoside

In vitro testing on human prostate cancer cell lines (PC-3, DU145, VCaP. 22Rv1 and LNCaP) revealed frondoside A to cause reduction in cell viability and colony formation ,with androgen sensitive tumor cell line to be more sensitive to frondoside A (IC_{50} ranged from 0.5 to 2 μM).

It is worth mentioning that the effect of frondoside A was very much lower in the control cell lines compared to the cancer ones at similar concentrations (Dyshlovoy et al., 2016). The same study has demonstrated cell cycle arrest in response to the treatment at 48 h treatment duration in PC-3 cell line but not DU145.

In vivo testing confirmed the *in vitro* results with significant tumor growth inhibition in xenograft models. Again the study reported no side effects in term of stable hemoglobin and platelet count. No change in mice weight was observed and no signs of distress during the treatment. In this study the spleen was reported to be enlarged with higher concentration treatments. Lymphocytosis and monocytosis were seen in the treated group and this can be explained by the immune modulatory effects of frondoside A (Aminin et al., 2008).

Multiple Studies have shown its pro-apoptotic effect to be mediated via decrease expression of anti-apoptotic proteins from the Bcl-2 family and increase in the pro-apoptotic proteins expression (Li et al., 2008). In general, frondoside A was found to activate the cysteine-aspartic proteases caspase 3/7 and 9 in multiple cancer cell lines (Attoub et al., 2013; Dyshlovoy et al., 2016; Jin et al., 2009; Li et al., 2008).

X. Li (2008) and colleagues demonstrated its effect in suppressing cancer cell proliferation in AsPC-1 and S2013 human pancreatic cancer cell lines and inducing apoptosis demonstrated by increased annexin V positive cells after treatment with

different concentrations of the compound. Proteins extracted from treated cells were analyzed by western blot and showed increased bands corresponding to the active forms of caspases-3,-7 and -9. The activation of the apoptotic pathway was suggested to be p21 dependent (Li et al., 2008). Treatment of human estrogen receptor negative breast cancer cell lines (MDA-MB-231) with different concentrations of frondoside A for 24 hours showed higher expression levels of p53 which was dose-dependent and this was associated with increase in caspase-3/7 and 9 activity as well as caspase 8 to a lower extent. In his study, blocking caspase 3 with specific inhibitor was associated with absolute inhibition of apoptosis in the treated cells. This again confirms the caspase dependent apoptosis induced by frondoside A in these cell lines (Al Marzouqi et al., 2011). In human lung cancer cell lines LNM35, A549 and NCI-H460, apoptosis has been confirmed by increase cell population in sub-G1 fraction and was also mediated by the activation of the same caspases (Attoub et al., 2013).

Protein analysis from human breast cancer cell lines treated with the compound showed decrease in anti-apoptotic proteins; Bcl-2 and Mcl-1 with increase in pro-apoptotic proteins such as Bax. Frondoside A was also found to activate p53 in these cell lines (Park , Kim , Kim , & Lee 2012).

One study conducted in human myeloid leukemia cell lines including HL-60, NB-4 and THP-1 showed that apoptosis was induced in these cells after treatment with frondoside A in a dose and time dependent manner. Significant decrease in pro-caspases 3 and 7 expression on western blot and increase in cleaved caspase 3 and 7 was detected after 6 h of treatment. A corresponding change was seen in poly ADP ribose polymerase (PARP) cleavage. In this study, no change in mitochondrial membrane permeability was detected when HL-60 cells were treated and the level of

cytoplasmic cytochrome *c* didn't change which is essential for the activation of the intrinsic apoptosis pathway. To further investigate this, the author used different caspase inhibitors along with frondoside A treatment and observed only partial inhibition of apoptosis. Similar experiments were conducted on other sea cucumber glycosides (holothuria A_2-2 and A_2-4) and complete block of apoptosis was detected with the use of the caspase inhibitors (Jin et al., 2009). From this study, it was concluded that frondoside A might activate apoptosis, at least initially, in a caspase independent manner and alternative pathways may be involved.

Dyshlovoy *et al.* (2016) showed that apoptotic pathways activated in prostate cancer in response to the treatment with frondoside A were cell-type specific. In his study, apoptosis was caspase-dependent in DU145 cell line and caspase-independent in PC-3 and LNCaP. Protein expression analysis on western blot showed down-regulation of anti-apoptotic proteins such as Bcl-2. Up-regulation of the pro-apoptotic protein PTEN was only demonstrated in the caspase-dependent cell line. All cell lines showed up-regulation of p21 which confirms the compound's effect on cell cycle control. Interestingly, frondoside A treatment showed inhibition of survival autophagy in those prostate cancer cell lines (Dyshlovoy et al., 2016).

1.3.2.2 Anti-invasive and anti-metastasis properties

Frondoside A has demonstrated its effect on inhibiting cancer cell invasion by decreasing the intracellular expression of matrix metalloproteinase 9 (MMP 9) which plays a vital role in breaching the extra cellular matrix (ECM) allowing cancer cells to invade adjacent tissue including blood vessels and lymphatics; hence promoting cancer metastasis. This inhibition is mediated through inhibition of PI3k/AKT, ERK1/2, p53

MAPK pathways. Frondoside A also increases the level of tissue inhibitor of metalloproteinase (TIMP 1 &2) (Park et al., 2012).

Frondoside A was found to inhibit AP-1 and NFκβ that are needed to bind the promoter region on MMP gene for activation (Park et al., 2012). This effect was demonstrated in breast and lung cancers by wound healing and matrigel invasion assays (Al Marzouqi et al., 2011; Attoub et al., 2013). In lung cancer, the anti-invasive effect was seen at low concentrations of frondoside A, so the author suggested that it's a specific effect on cell migration and not because of the decrease cell viability as a result of compound's cytotoxic effects (Attoub et al., 2013).

A study on breast cancer metastasis has shown that pretreatment of breast cancer cells with frondoside A before injecting to mice models has led to 30% decrease in the tumor metastatic colonies to the lung but it didn't reach clinical significance (Holt, Ma, Kundu, Collin, & Fulton, 2012). In another study the decrease in lung metastasis from human 66.1 breast cancer cell line has been confirmed. Injecting syngeneic Balb/cByJ mice with cancer cells that has been pretreated with 1.0 μM frondoside A significantly suppressed the number of lung metastasis colonies ($p < 0.0001$) although the direct systemic treatment of the breast cancer mice model with frondoside A in the same study did not reach clinical significance of < 0.05 ($p = 0.06$).

The mechanism of metastasis inhibition involved in such models was further investigated. Ma *et al* (2012) showed decrease cAMP generation and hence blockage of ERK1/2 pathway in cells treated with frondoside A as it antagonizes the effect of prostaglandin E-2 (PGE-2) (Ma, Kundu, Collin, Goloubeva, & Fulton, 2012). Breast cancer cells were also found to inactivate natural killer cells migration and cytokine release via PGE2 receptor 4 (EP4). Since frondoside A has been shown to antagonize

EP4; treatment with low doses were able to restore the function of the NK-cells which added to its contribution to the decrease in metastatic potentials (Holt et al., 2012). Prostate cancer metastasis to the lung in xenograft models were found to be significantly reduced with the treatment of frondoside A (Dyshlovoy et al., 2016).

1.3.2.3 Anti-angiogenic properties

A recently published data showed frondoside A to have an additional anti-angiogenic properties in lung cancer LNM35 xenografts, where it significantly inhibited angiogenesis in chick chorioallantoic membrane assay as well as vascular tube forming assay. The study has shown suppression of basic Fibroblast Growth Factor (bFGF) induced new blood vessel formation by frondoside A when used in non-toxic concentrations which confirms that the observation of reduced vascularity in the tumor is due to specific compound effect and not due to cell apoptosis and death (Attoub et al., 2013). This property might be contributing to its effect in reducing cancer growth and metastasis, since the blood supply is vital for cell growth and can be a mode of influencing distant metastasis (Jia, Zhang, Yuan, & Huang, 2005). Administration of the compound in xenograft lung cancer model showed decrease microvessel density by decrease expression of (CD31/PLT) on immunohistochemistry stained sections from the tumor tissue (Attoub et al., 2013).

1.3.2.4 Effect of frondoside A on cancer stem-like properties

Cancer stem cells are thought to determine the behavior of the tumor including metastasis, relapse, treatment resistance and it is thought that these cells are responsible for cancer growth. Breast cancer stem cells are characterized by the expression of Csf-1, CSF-M, c-met, CXCL12 and CD44 cell markers. A study aiming

at investigating the effect of COX-2 pathway and PGE2 role in breast cancer by blocking prostaglandin E2 receptor 4 (EP-4) either by shRNA or frondoside A which acts as EP-4 antagonist has shown reduction in these stem cells (monitored by the markers' expression) and concomitant decrease in tumor growth and metastatic potentials to the lung. This effect was demonstrated in different murine mammary tumor cell lines including MMT 66.1, MMT 410.4, MMT 67and MMT 410 as well as the human breast cancer cell lines MDA-MB-231, SKBR3 and MCF-7. Mammosphere formation was assessed after blocking EP-4 and showed decreased stem cell frequency ($P < 0.018$). At high doses of frondoside A, the size of the mammospheres were smaller but the number of the mammospheres formed in MDA-MB-231 cell lines didn't decrease. This was associated with decreased expression of CD44 (Kundu et al., 2014).

1.3.2.5 Effect of frondoside A on multi-drug resistant cancer

A major issue that is faced in the field of cancer therapy is the evolution of cancer cells to develop drug resistance. There are different mechanisms by which cancer cells become insensitive to the treatment; these include cancer cells altered metabolism of the drug, inactivation of the drug by the detoxification system in the body and over expression of P-glycoprotein transporter (Kapse-Mistry, Govender, Srivastava, & Yergeri, 2014; Kuete & Efferth, 2015). This is a trans-membrane pump that causes efflux of substances from the intracellular space. Frondoside A extracted from a different sea cucumber strain (*Cucumaria okhotensis*) was found to inhibit this pump in multidrug resistant (MDR) Ehrlich cancer cells extracted from ascetic fluid. The cells were injected with a diffusible fluorescent probe attached to calcein. Resistant cancer cells will pump the calcein out of the cell and hence, will have less intracellular fluorescence detection. Cells treated with sub-cytotoxic concentrations of

frondoside A (range 0.001-0.1 μg/ml) or its complex with cholesterol showed increased levels of fluorescence inside the treated cells indicating the inhibition of P-glycoprotein (Menchinskaya, Aminin, et al., 2013). This might suggest an additional function of frondoside A as an option to use in MDR cancers.

1.3.2.6 Immunomodulatory properties

A study investigating the immune-stimulatory effects of frondoside A on mouse peritoneal macrophages after the treatment with sub-toxic concentrations has shown that it increases macrophage lysosomal number, size and activity measured by acridine orange fluorescence assay after one day of treatment. The maximum stimulatory effect was seen at day 4-5 post-treatment.

Phagocytosis of fluorescein isothiocyanate FITC-labeled bacteria was increased in macrophages treated with 0.0001-0.01 μg/ml of frondoside A. The study also showed increase ROS production in those cells tested by rhodamine production. Using higher (cytotoxic) concentrations of frondoside A in these experiments were associated with immune suppressive effects.

Frondoside A treated animals demonstrated a mild increase in antibody production ($p < 0.05$) (Aminin et al., 2008). Another study on prostate cancer xynografts has also shown enlarged spleen size in the treated group which might also indicate its immune stimulatory effects (Dyshlovoy et al., 2016).

1.3.2.7 Suggested mechanism of action

Frondoside A is thought to interact with the cell membrane leading to an increase in membrane permeability and hence, membrane lyzing (Aminin et al., 2008; Park et al., 2014). It was found that the sulfate group attached to C-4 of the xylose

contributes to the enhanced frondoside A activity (Kalinin, 2000; Park et al., 2014) and for the immune stimulatory effect, compounds with lower number of sulfate groups have higher activity (mono-sulfate compared to di- and tri-sulfate) (Aminin et al., 2008). Intracellular calcium concentration was also found to be enhanced by the number and position of the sulfate group and it contributes to the variable functions of different extracts from sea cucumbers (Janakiram et al., 2015).

The structure of frondoside A highly contributes to its functional efficacy as a pro-apoptotic and cytotoxic agent. Treatment with similar concentrations of frondoside A decreased the leukemia cell line HL-60 viability after 24 h more than the holothuroid glycosides A_2-2 and A_4-2. Apoptosis measured by annexin V was detected in cells treated with 1 μM frondoside A compared to 5 μM need for similar effect to be seen when treated with the glycosides A_2-2 and frondoside A_4-2 (Jin et al., 2009). This confirms the importance and the relation between the structure and the function of the extract. Figure 5 summarizes the suggested mechanism of action of frondoside A.

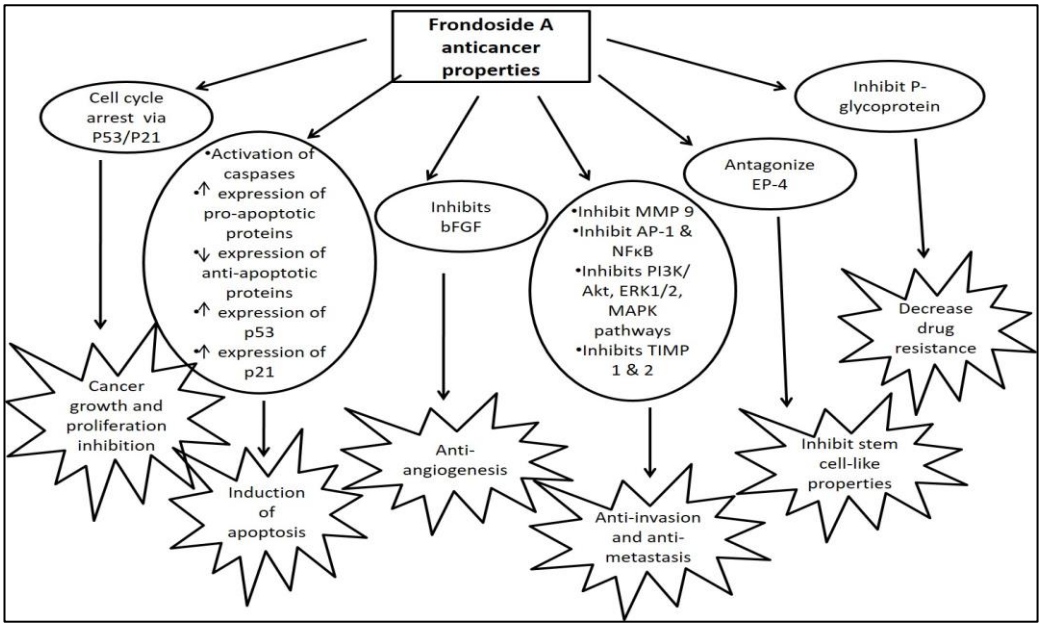

Figure 5: Anti-cancer properties of frondoside A

1.3.3 Cell Cycle

Living cells are classified as somatic cells and germline cells. Somatic cells have a complete set of chromosomes (e.g. 46 chromosomes in humans) and form the multicellular organism while germ cells produce the gametes with a haploid number of chromosomes. These gametes are specific for reproduction.

Cells undergo division to serve the purpose of growth, tissue repair and replacement of dead cells. All cells divide by passing through a specific, highly regulated cell cycle.

Somatic cells divide by mitosis to produce 2 identical diploid cells that are an exact copy of the mother cell. On the other hand, germline cells divide by meiosis to produce germ cells with half the number of chromosomes from the mother cell.

Cell cycle is a process of 4 phases (Figure 6). G1: is the gap 1 phase where cells that are triggered to divide grow in size and increase synthesis of proteins required for the next step. Also during this phase, chromosomes undergo a check to make sure that DNA to be replicated is free of damage. The S phase: is the DNA synthesis phase where DNA strands are replicated. G2: is the second gap phase where the newly synthesized DNA is checked for accuracy. Proteins that are needed for the next step are also synthesized at this stage. M: is the actual cell division (i.e. mitosis) which consists of mitosis and cytokinesis.G0, is a term used to describe cells that are viable but not replicating anymore (resting or senescent).

Many proteins are involved in the control and regulation of cell cycle and these proteins are encoded by genes that will be discussed further in the following sections.

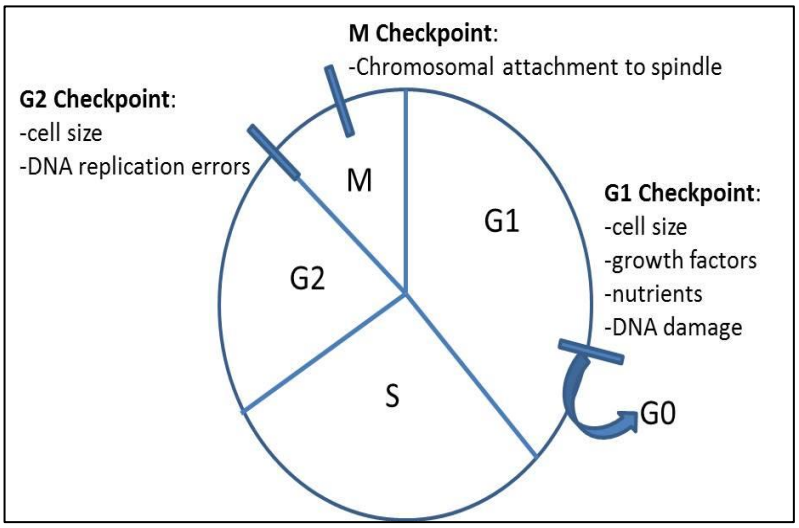

Figure 6: Cell cycle stages and checkpoints

Cell cycle is a highly regulated process (Kreis et al., 2015) by two main molecules; cyclins and cyclin dependent kinases (CDK). These kinases control cell cycle progression through phosphorylating/ dephosphorylating different targets. They have limited kinase activity on their own and get activated when binding to their specific cyclin forming cyclin/CdK complexes. An example of how these complexes control cell cycle, if a growth stimuli binds to a tyrosine kinase receptor of a cell in G1 phase, it signals the cell to inter the cell cycle for mitosis, this activates a cascade of signal transducers such as Ras, Raf, MAP and ERK leading to the accumulation of cyclin D which binds CdK4 forming a complex that becomes phosphorylated by CdK activating kinase (CAK) (Benada & Macurek, 2015), once phosphorylated, the complex is capable of phosphorylating the tumor suppressor, retinoblastoma protein (Rb) which releases the transcription factor E2F. This transcription factor enters the nucleus and binds the promoter region of DNA to activate genes involved in the production of other proteins required for cell cycle progression (Gartel & Tyner, 2002). Cyclin E/Cdk2 also act at this point (G0/G1) to ensure that the cell is ready to enter cell cycle. Another example is at M-phase where cyclin B gets phosphorylated

inhibiting its transport out of the nucleus, cyclin B accumulates and binds CdK1 forming a complex that gets phosphorylated by CAK and also activated by CdC25A phosphatase that removes an inhibitory phosphate from the active substrate binding site. Cyclin B/CdK1 triggers the breakdown of the nuclear membrane driving the cell into mitosis.

Cell cycle is controlled at 4 main checkpoints. First restriction point is at G0/G1 transition. The second checkpoint is at S phase (DNA damage check). The DNA replication checkpoint at G2 ensures that replication has been completed accurately (Shapiro & Harper, 1999). The final checkpoint is in M-phase to make sure proper chromosome attachment to the spindle is achieved, if not, aneuploidy may result. Cyclins and CdK are also involved at these check points. At the G1/S DNA damage check point, a protein called ataxia telangiectasia and Rad3 related (ATR) senses DNA damage and leads to the activation of Chk1 kinase which phosphorylates the phosphatase Cdc25A, this signals the phosphatase to destruction leading to the inactivation of CdKs (Benada & Macurek, 2015). At this point, another protein, ataxia telangiectasia mutant (ATM) encodes Ser/Thr kinase that gets activated in response to dsDNA damage, ATM leads to cell cycle arrest at G1/S phase as well as G2/M phase and activates DNA repair pathways or apoptosis, depending on the extent of damage and repair possibilities (Rossi & Gaidano, 2016). It also stabilizes the tumor suppressor protein p53 (Benada & Macurek, 2015).

Cancer develops due to defect in specific genes that function to prevent such events. These genes are classified as caretaker, landscaper and gatekeeper genes (Soussi & Wiman, 2015). Caretaker genes encode proteins that stabilizes the genome integrity while landscaper genes encode proteins that once cancer develops, they

provide the growth support and the proper stromal environment. The gatekeeper genes such as TP53 encode proteins that prevent the growth and proliferation of genetically abnormal cancer cells and hence, preventing cancer cell survival.

The p53 protein is encoded by TP53 gene located on chromosome 17 (17p13.1) (Hollstein & Hainaut, 2010; Saha, Kar, & Sa, 2015). The gene has several promoters, multiple splice sites and alternative translation initiation sites leading to the transcription of at least 10 isoforms all with similar DNA binding domain but different N and C- terminus. The gene also contains regulatory element (RE) for many molecules such as AP1 and NFκB both suppress p53 promoter decreasing its transcription hence, inhibiting apoptosis while HOXA5 when bound to its RE at the promoter site, it induces the expression of p53 leading to activation of apoptosis (Hollstein & Hainaut, 2010).

The protein is 393 amino acid long consisting of 4 main domains; an N-terminus that has the trasactivation domain, proline rich domain, DNA binding domain and C- terminus with its tetramerization domain. The protein p53 has a complex structure that highly correlates with its complex variable function and its functional structure consists of a homotetramer.

The protein belongs to a family of transcriptional proteins with two other members; p63 and p73. The protein p73 shares common structural features to p53 and get activated by similar signals of cell stress and can initiate apoptosis (Mantovani, Zannini, Rustighi, & Del Sal, 2015; Saha et al., 2015; Soussi & Wiman, 2015). The gatekeeper gene, TP53 has a major role as a tumor suppressor gene. It regulates cell cycle, maintain genetic integrity, involved in DNA repair pathways, initiates cell apoptosis, autophagy, endocytosis, angiogenesis, inflammation and cell differentiation

(Hollstein & Hainaut, 2010; Leroy, Anderson, & Soussi, 2014; Mantovani et al., 2015; Rossi & Gaidano, 2016; Saha et al., 2015). It gets activated in response to DNA damage (as mentioned above), hypoxia, heat shock and other cell stress signals.

Based on cell conditions and multiple other factors, p53 when activated can direct the cell to one of the following paths: cell cycle arrest and DNA repair, apoptosis or senescence. The protein p53 interacts with different pathways and undergo structural modifications leading to different cellular localization (nucleus, mitochondria or cytoplasm) where specific function can be initiated. Acetylation at certain positions on p53 can prolong its half-life ($t_{1/2}$).

As a guardian of the genome and a suppressor of excessive cell growth, in response to severe un-repairable DNA damage or telomerase shortening p53 interacts with different proteins to block cell proliferation and induce apoptosis. Stress like hypoxia leads to the accumulation of p53 in the mitochondria and this promotes the change in mitochondrial outer membrane permeabilization (MOMP). It also directly activates Bcl-2 associated X protein (Bax) and Bcl-2 antagonist/killer (BAK); the pro-apoptosis proteins. The protein also activates the cyclin dependent kinase inhibitor p21 to arrest cell cycle.

Mutations of TP53 are the most common mutations in human cancers (Hollstein & Hainaut, 2010; Leroy et al., 2014; Mantovani et al., 2015). The type and frequency of the mutations are cell type and stage of cancer dependent. For example, TP53 mutations are less frequent in primary leukemias but very much detected in relapse cases. Such mutations are detected in 1-2% of childhood acute leukemia but seen in 19% of B-cell ALL and in 33% of T-cell ALL at relapse (Davies et al., 2011).

When p53 function is lost, cancer evolves with more genetic mutations due to the loss of the protective function of p53 at G1 check point.

TP53 mutations in cancer can be classified based on the site of mutation as contact mutations; affecting the DNA binding site (accounting for > 90% of mutations) (Leroy et al., 2014; Soussi & Wiman, 2015) or structural mutations; affecting the protein stability and transport. They can also be classified as somatic leading to sporadic tumors or germline causing familial cancers. Mutations in p53 can be a resultant of defects in the gene or in the post translational modifications (PTM) process and the effecter pathways it interacts with. Frameshift mutations account for ~11% and lead to loss of p53 expression (null mutations) while silent mutations caused by single nucleotide variant may affect RNA splice sites and protein stability. Splice site mutations account for only 2-4% as reported in the literature (Leroy et al., 2014). The most common type of mutation is missense mutations accounting for 80% of all types. They result in the production of a stable protein that accumulates in the nucleus of the cancer cells forming a stable bound with MDM2 preventing its activation and transport. Wild type p53 interferes with molecules of inflammation and antagonizes NFκB while mutant p53 enhances inflammation and prolongs NFκB transcriptional activity in response to TNF-α in cancer cells.

Another tumor suppressor protein that is involved in the tight and accurate regulation of cell cycle progression is p21. It is a 164 amino acid protein encoded by CDKN1A gene which is located on the short arm of chromosome 6 (6p21.2). The protein p21 has many synonyms including $p21^{Cip1}$ referring to cyclin dependent kinase interacting protein1, $p21^{Waf1}$ (wild type p53 activated fragment1) and Cdk inhibitor 1. Studies have confirmed p21 role in many physiological processes including growth

arrest, cell cycle control, DNA damage repair, control of T-cell proliferation, cell migration, cell senescence and is a major player in cellular stress response (Gartel & Tyner, 2002; Kreis et al., 2015; Ozaki & Hanazawa, 2001). Knockout studies of p21 showed increase potential for tumor development (Davies et al., 2011). It has a short $t_{1/2}$ and is activated either by p53 dependent or independent pathways. The pathway to be activated is cell type, condition and stimulus dependent (Gartel & Tyner, 2002). The protein can be regulated at transcriptional level via p53 where gene analysis of CDKN1A showed the promoter region to have 2 p53 binding sites. Other molecules that regulates p21 expression and activation in a p53-indepenent manner are TNF-α, TNF-β, IFN-γ, epidermal growth factor (EGF), IL-6, Ras, c-myc and histon deacetylase inhibitors (Gartel & Tyner, 2002; Kreis et al., 2015). The protein p21 can also be regulated at post translational level by phosphorylation, acetylation and ubiquitination.

The function of p21 in cell cycle control will be discussed in more detail. Previous studies have demonstrated two peaks of p21 during cell cycle; that is at G1 and G2/M transition phases (Gartel & Tyner, 2002) but recent studies showed that it also acts as a major regulator of mitosis. In response to DNA damage, p21 can suppress the expression of cyclin E/Cdk2 at G1/S phase. The expression of the protein increases in the nucleus after DNA damage where it binds cyclin B/Cdk1 complex leading to the inactivation of the kinase activity of the complex and hence, G2/M cell cycle arrest. The N- terminus of the protein has the Cdk domain with 2 pockets, one binds Cdk and the other blocks the ATP site on the Cdk. The binding of p21 to other cyclin/Cdk complexes depends on the extent of homology in structure between the complexes. To maintain the cycle arrest for DNA repair to take place, p21 also interferes with

Cdk/CAK interaction and this prevents the activation phosphorylation of Cdk1. The C-terminus can bind proliferating cell nuclear antigen (PCNA) and stops DNA synthesis.

Studies in hematopoietic cells showed that abnormalities of p21 protein have been associated with centrosome defects and abnormal nuclear structure as well as polyploidy. In tumor cells, loss of p21 was associated with hyperactive Cdk activities leading to abnormal mitosis (Kreis et al., 2015).

Many recent reviews highlighted the dual function of p21 since it also been shown to have an oncogenic/proliferation effect and research in this field pointed to the importance of cellular compartment localization in determining the function of p21. Cytoplasmic localization was found to be oncogenic were it was associated with inhibition of multiple caspase activities (Kreis et al., 2015).

High levels of p21 were shown to cause cell cycle arrest but also inhibit p53-dependent apoptosis, the mechanism of such dual function is still to be investigated but researchers propose that after DNA damage p21 causes p53-dependent cell cycle arrest and when p53 gets activated and hence activating the caspase cascade (caspase 3 mainly), p21 gets sequestrated by caspase 3 and this is followed by the initiation of apoptosis (Gartel & Tyner, 2002).

In some cancer cells where p53 is mutated, high levels of p21 results in protection of cancer cells from apoptosis. Mutations in p21 are very rare in cancer (Davies et al., 2011) and the protein inactivation is mainly due to epigenetic modifications. Studies in leukemia show that T-ALL cells from patients samples have low levels of p21 due to p53-independent epigenetic suppression while in AML cells

show high basal levels of p21 and in these cases it was associated with poor prognosis (Davies et al., 2011). In leukemias where p53 is mutated, apoptosis is induced in p53-independent manner leading to accumulation of p21 and G2/M cell cycle arrest (Naujokat et al., 2000).

1.3.3.1 Cell Death and Senescence

Adult cells undergo limited number of division which varies depending on the type of the cell (e.g. fibroblasts go through 25-50 cell cycle divisions while bronchial epithelial cells go through 10 cycles only). This is due to a phenomenon known as replication senescence. With each division the end of the chromosomes get shorter because of the difficulties in base copying. To avoid loss of genetic information, the end of the chromosomes (the telomere) are made of non-coding repeated base pairs. If the cell continues to divide indefinitely, genetic material can get lost and end chromosome fusions take place with resultant DNA break and damage. As a protective mechanism; normal cells cease to divide after a critical limit in telomere length is reached (replication senescence). In germline cells, where cell division continues, the telomere length is maintained by the presence of telomerase enzyme. Adult cells lack the expression of this enzyme. During replication senescence, the cells are viable and metabolically active but cannot go further divisions.

Cell death is a process were living cell is not able to function or be metabolically active. Cell dies to maintain homeostasis in the living organism. In an adult organism, number of newly synthesized cells should balance the number of injured, abnormal, destroyed or shedding cells.

1.3.3.2 Types of Cell Death

Cell death can broadly be classified as programmed cell death (PCD), accidental and mitosis catastrophe.

Programmed cell death is usually an active process initiated by the living organism and requires ATP as energy to complete the process. The most common and well-studied form of such death is apoptosis (which will be discussed in detail). Accidental cell death includes cell death by necrosis. Mitotic catastrophic cell death occurs as a default pathway when there is abnormality in cell cycle checkpoints or mitotic failure to protect the organism from faulty cell division.

1.3.3.3 Programmed Cell Death (apoptosis)

Programmed cell death also known as apoptosis and cell death type 1 is a vital physiological process for the body. It maintains cellular homeostasis, protects genome integrity by removing faulty cells, it plays a major part in other processes such as differentiation and healing and it's a way by which the organism removes harmful cells and pathogens without triggering inflammation in the surrounding tissue (Chi, Kale, Leber, & Andrews, 2014; Delbridge & Strasser, 2015; Goldar et al., 2015; Testa & Riccioni, 2007).

Disturbed apoptosis has been associated with many diseases for which a highly regulated balance is required. Excessive apoptosis is a dominant feature of degenerative disorders whereas suppression of apoptosis is seen in cancer.

1.3.3.3.1 Triggers of apoptosis

Apoptosis can be triggered by physiological as well as pathological factors and these can be either intracellular or extracellular triggers. This includes activation of

apoptosis by hormone/growth factor withdrawal such as during menstrual cycle and breast cells regression. Other examples of physiological triggers are loss of cells in the gastrointestinal tract, death of innate immune cells after removing infectious agents and removal of lymphocytes activated against self-cells. Pathological intracellular triggers include un-repairable DNA damage, accumulation of abnormal proteins and increase reactive oxygen species (ROS) beyond cellular adaptation limits.

1.3.3.3.2 Mechanism and pathways

Depending on the type of trigger, different apoptotic pathways can be activated. In general, the activation of apoptotic signal will lead to the activation of pro-enzymes known as caspases (cysteine dependent aspartate-directed proteases) which are able to cleave proteins at specific aspartic acid position through their proteolysis property (Christensen, Jansen, Sanchez, & Waterhouse, 2013). Figure 7 shows the main apoptotic pathways.

Apoptosis is controlled by a tight balance between multiple players including pro- and anti-apoptotic proteins from the B-cell leukemia (Bcl-2) family, caspase proteases and mitochondrial membrane.

The Bcl-2 family proteins share a Bcl homology (BH) domains 1-4 and can be classified either based on the number of domains they have or based on their role in apoptosis. The anti-apoptotic proteins (Bcl-2/Bcl-xL, Bcl-w and A-1) include all the BH domains 1-4 except Mcl-1 protein which lacks BH-2. The effector proteins Bax and Bak contains all the regions while the pro-apoptotic proteins have only BH-3 region and they include Bad, Bik, Bid, Bim, Noxa, Puma and others.

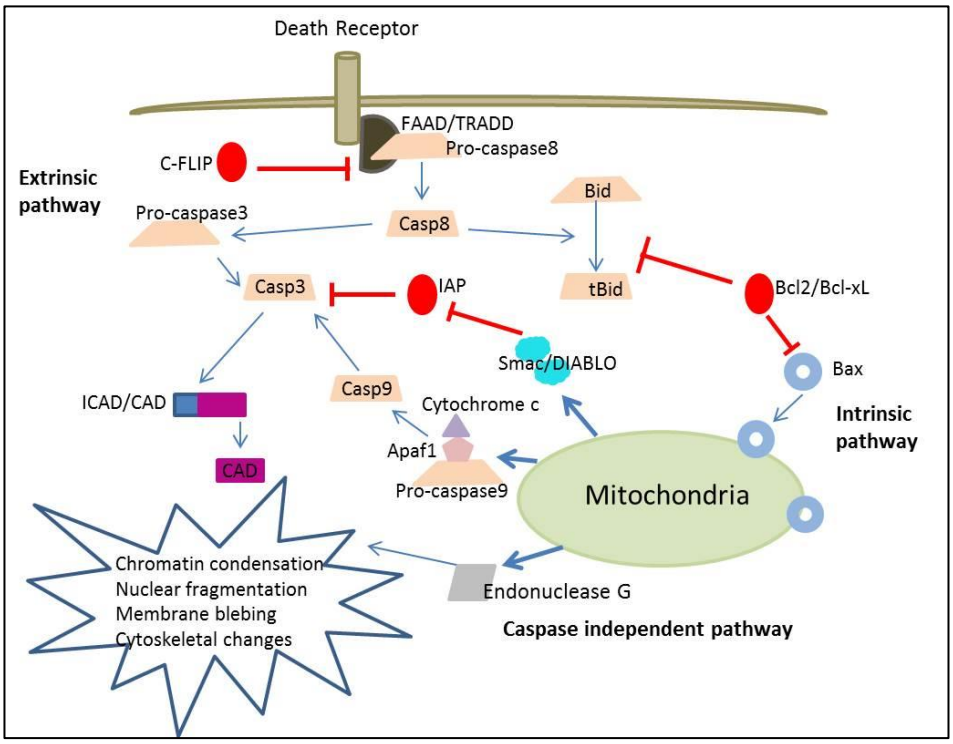

Figure 7: The interaction between apoptosis pathways

The mechanism by which these proteins control apoptosis is very complex and not yet fully clear. In-vivo, in-vitro and structural changes studies have suggested multiple theories to explain how the interaction between these proteins as well as cell receptors and mitochondrial membrane initiate and regulate apoptosis. Evidence from experiments suggest that the mechanism of activating these proteins is cell type and stimulus dependent (Delbridge & Strasser, 2015).

Some studies suggest that the pro-apoptotic proteins except Bid (should be cleaved to be activated) are always active and hence they are required to be kept under the suppressive effect of the anti-apoptotic proteins by binding them. Other studies have demonstrated the importance of interaction with cellular membranes and other kinases to get the active conformational change needed for the proper functioning.

The function of the anti-apoptotic protein Bcl-2 and the effector protein Bax will be discussed in more detail in this section. Bcl-2 protein is encoded by the B-cell lymphoma-2 gene located on chromosome 18. The protein is usually bound to the outer mitochondrial membrane and the endoplasmic reticulum (Chi et al., 2014). On the membrane of the mitochondria it interacts with the effector pro-apoptotic proteins Bax/Bak and prevents their direct activation by other pro-apoptotic proteins such as Bid. Once the cell gets an apoptotic stimuli, Bcl-2 get released by the activated Bid and frees the negative effect on the activation of Bax/Bak which are ready to be activated and oligomerize to form pores in the mitochondrial outer membrane (MOM) increasing its permeability to release cytochrome *c* and other apoptosis related proteins such as Smac/DIABLO (Delbridge & Strasser, 2015; Doerflinger, Glab, & Puthalakath, 2015). Another theory of activation suggests that Bcl-2 is bound to Bid and once activated it releases Bid to further activate Bax/Bak on the MOM (Doerflinger et al., 2015).

Activation of Bax is the rate limiting step in the mitochondrial pathway of apoptosis. Once activated it undergoes conformational changes leading to its deeper insertion into the mitochondrial membrane (Chi et al., 2014). At this stage the cell is obliged to apoptosis and the signal cannot be neutralized.

Caspases are cysteine containing proteases that cleave other proteins at specific aspartic acid position. They are classified into two groups; the initiator caspases 8, 2, 9 and 10 and the executioner caspases 3, 6 and 7 (Goldar et al., 2015; Testa & Riccioni, 2007).

Many cancer types show disrupted balance between pro- and anti-apoptotic proteins either by up- or down regulating the genes involved or by inactivating them

through altered post-translational modifications (Goldar et al., 2015). Some of the altered functions of those proteins have been associated with poor prognosis and more resistance to treatment (Goldar et al., 2015; Jia et al., 2001; Meyer et al., 2006; Testa & Riccioni, 2007).

Intrinsic pathway

Also known as mitochondrial pathway, the intrinsic pathway is activated within the cell (no receptor is involved), usually in-response to intracellular stress as mentioned above. Once activated, it will activate the tumor suppressor p53 protein which will bind the anti-apoptotic Bcl-2 and Bcl-xL proteins leading to their inactivation and the release of the pro-apoptotic protein Bid (which is usually inhibited by being bound to Bcl-2/Bcl-xL). Activated Bid (tBid) translocates to the mitochondrial membrane, activates Bax leading to change mitochondrial membrane permeability, releasing cytochrome *c* to the cytoplasm where it binds apoptosis protease activating factor1 (Apaf1) in the presence of ATP and binds procaspase-9 forming apoptosome, with the resultant of cleavage of procaspase-9 to active caspase-9, this in turns activates the execution pathway caspases 3, 6 and 7. The end result is activation of DNA nucleases, DNA fragmentation and inhibition of polyADP ribose polymerase (PARP) which is a DNA repair enzyme (Goldar et al., 2015; Testa & Riccioni, 2007).

Extrinsic pathway

The extrinsic pathway is activated when the trigger binds a specific death receptor such as Fas ligand binding to Fas receptor, TNF or TNF related apoptosis inducing ligand (TRAIL) binding to TNF receptor. Once activated, the intracellular death domain through FADD or TADD, the adapter proteins, binds caspase 8 forming

death inducing signaling complex (DISC) directly activate caspase 3 that cleaves caspase activating DNas (CAD) from its inhibitor (ICAD) and releases the binding between Bcl-2 and Bid leading to the activation of the mitochondrial pathway (Goldar et al., 2015; Testa & Riccioni, 2007). In some cancers, particularly leukemias, the extrinsic pathway is sufficient to trigger apoptosis without involvement of the mitochondria. In contrast, most solid tumor cells need involvement of the intrinsic pathway with or without the extrinsic pathway (Testa & Riccioni, 2007).

Perforin/granzyme pathway

This is specific for T-lymphocyte mediated cell death. The major apoptotic pathway for T-cell cytotoxicity is via death receptors but another pathway is specially related to T-cells due to the close proximity between activated cytotoxic T-cell and target cells, where perforin molecule forms a junction between the two cells, allowing T-cells to release their proteolytic enzymes into the target cell. These granzymes can activate either the intrinsic or directly the execution pathway.

Execution pathway

These include caspase 3, 6 and 7 which when activated, can cleave proteins and inactivate PARP by cleavage. Caspase 3 also degrades cytoskeletal proteins of the cells leading to their fragmentation and cell death. It is responsible for the morphological features associated with apoptosis as will be discussed later.

All these pathways interact and cross-talk to insure proper control of apoptosis.

1.3.3.3.3 Changes in apoptosis

Cellular changes accompany any form of cell death. Some are general while others are more specific to the type of death. In apoptosis, the cellular change occurs

in individual cells while in accidental cell death, the changes happen in many cells of the tissue, simultaneously. Specifically in apoptosis, there is cytoplasmic shrinking followed by nuclear chromatin changes. The cytoplasmic membrane stays intact but forms blebs due to the biochemical changes in its composition (Delbridge & Strasser, 2015; Goldar et al., 2015).

Many techniques have been developed to confirm cellular apoptosis, some are more specific than others. Apoptosis can be seen morphologically either by light microscopy or electron microscopy. Membrane changes in response to apoptosis, DNA fragmentation, elevated levels of active caspases and expression of proteins/genes involved in apoptosis can be detected.

The choice of the assay used depends on the technique's specificity and timing after the cell is signaled for apoptosis.

Morphological features are not specific for apoptosis especially in late stages where it shares similar findings as in other forms of cell death. Another problem with morphological changes is that they occur fast and without triggering inflammation making their detection difficult.

Light microscopy

Under light microscopy, viable cells are round with intact cell membrane and clearly demarcated nucleus. Once the cells undergo apoptosis, cytoplasm shrinks in size and cells have irregular outer membrane (Goldar et al., 2015).

Phase contrast inverted microscopy will show apoptotic cells with variable degrees of cell membrane blebbing based on being early or late apoptosis. In late apoptosis chromatin changes can also be detected (Figure 8). With Hematoxylin and Eosin (H&E) staining, apoptotic bodies can also be detected.

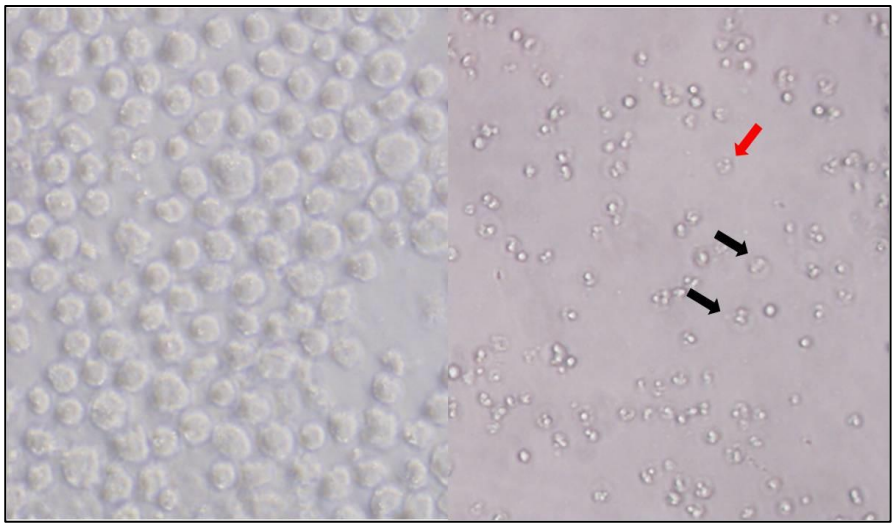

Figure 8: Blast cell morphology. Left: viable blast cells showing regular smooth round cell membrane. Right: shows apoptotic blast cells with variable degrees of membrane shrinkage and blebbing based on stage of apoptosis (indicated by red arrow) and cytoplasmic vacculation (black arrows)

Electron microscopy

Electron microscopy (EM) is considered a better method to detect intracellular as well as membrane morphological changes of apoptosis. These changes include cytoplasmic vacuoles, blebbing of cell membrane, nuclear fragmentation, mitochondrial outer membrane changes (using special cationic dyes) and apoptotic bodies with organelles enclosed within a membrane.

Fluorescent microscopy

Annexin V (fluorescein-isothiocyanate; FITC) stained samples can be used to confirm apoptosis and to accurately quantify it by flowcytometry methods. The principle of this technique is based on the different localization of phosphatidylserine (PS) on cell membrane.

In viable cell, PS is localized on the cytoplasmic side of the cell membrane and when cells undergo apoptosis, it translocates to the extracellular side of the membrane.

Late apoptosis can also be qualified by counter staining of the DNA when the nuclear envelope becomes permeable in late stages. Propidium iodide (PI) is a red fluorescent that binds nucleic acid (7AAD is another dye with high affinity to nucleic acid).

Figure 9 shows early apoptotic cells (stained green) and late apoptosis (stained in green and red) under confocal microscope after special staining.

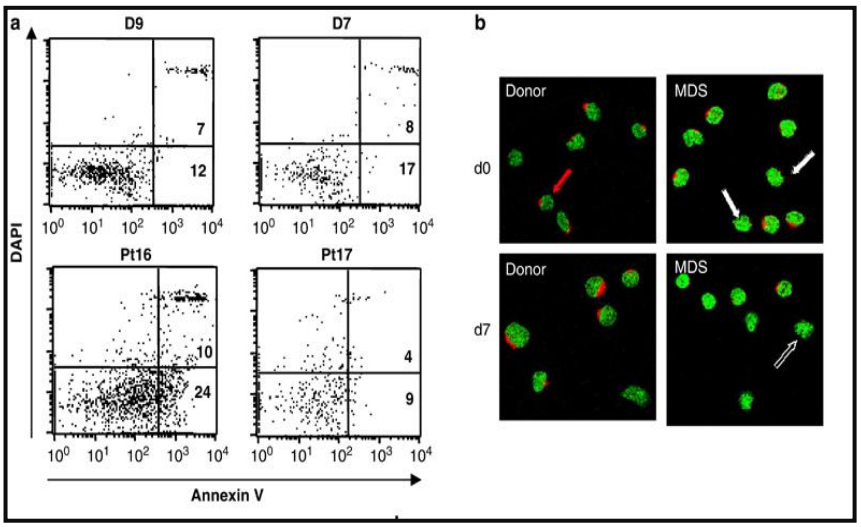

Figure 9: Annexin V test. Annexin V and PI stained apoptotic cells under fluorescent microscope (right) where phosphatidylserine in apoptotic cells is stained green while necrosis is indicated by propidium iodide red staining of the nucleic acid. The quantification on flowcytometry (left)

Flow cytometry can be used to detect specific changes in membrane composition associated with apoptosis. It is also used to detect DNA fragmentation forming a ladder due to activation of DNA endonucleases by caspase 3.

Annexin V

Similar technique to the fluorescence microscope, the stained samples can be analyzed on flowcytometry. This technique allows accurate quantification of apoptotic cells.

Caspase activation assays

Caspase activation is a specific step in apoptosis. Functional assays are available for detecting activated enzymes. Immunohistochemistry tests that detect cleaved caspase substrates such as PARP are another method to detect functional caspases.

Fluorometric as well as colorimetric assays are available to measure active caspases. Different caspases cleave proteins at specific aspartic acid in the protein chain, providing a commercially available protein sequences with fluorochrome or a color dye at the specific aspartic acid and if the sample contains the activated caspase it will cleave the sequence and release the color or fluorescein that can be measure and is proportional to the number of activated caspase enzymes.

Western blots with the use of polyclonal or monoclonal antibodies against active or pro-caspases can give semi-quantitative results when used with appropriate controls. Release of cytochrome *c* into the cytoplasm for the activation of intrinsic pathway can also be detected by this method.

The expression of genes involved in apoptosis such as receptors, transcription factors, intracellular signaling molecules and enzymes, can be detected with accuracy using polymerase chain reaction (PCR) techniques. Decrease expression of anti-apoptotic genes can also be detected with this method.

Chapter 2: Methods

2.1 Research Design

The aim of the study was to investigate the efficacy and mechanism of action of frondoside A as a potential anti-cancer agent for the treatment of acute leukemia. The effect of frondoside A on inhibiting the growth/viability of acute leukemia blast cell lines was compared to the combination with conventional chemotherapeutic drugs. The effect of frondoside A on the expression of apoptosis-related genes as well as proteins in these cells was also investigated.

Objectives:

1) To investigate the effect of frondoside A on viability of leukemic cell.

2) To compare the effect of the currently used chemotherapeutic drugs (asparaginase, vincristine and prednisolone) for the treatment of acute leukemia to their effect when used in combination with frondoside A.

3) To study the changes in expression of apoptosis-related genes after the treatment with frondoside A.

4) To describe the effect of frondoside A on the level of proteins involved in apoptosis and cell cycle control.

Based on the preliminary results, a secondary objective was added and that is to investigate the interaction of frondoside A with NFκB pathway in acute leukemia cell lines.

The study is the first to attempt comparing the efficacy of this compound to conventional drugs. It also describes for the first time the effect of frondoside A on

gene expression in acute leukemia cell lines, including the interaction with NFκB pathway.

2.2 Cell Lines and Cell Culture

Three acute leukemia cell lines were used; T-lymphoblastic leukemia cell line (CCRF-CEM), acute monocytic leukemia cell line (THP-1) and acute promyelocytic leukemia cell line (HL-60), all purchased from American Type Culture Collection Global Biosource Center, ATCC (USA). The cell lines are described in table 6.

Description	**CCRF-CEM**	**THP-1**	**HL-60**
Tissue	PB	PB	PB
Cell type	T-Lymphoblast	Monocyte	Promyeloblast
Diagnosis	T-ALL	AML-M5	AML-M3
Age (years)	4	1	36
Culture media	RPMI-1640 + 10% FBS	RPMI-1640 + 10% FBS + 0.05mM 2-ME	DMEM + 20% FBS
Plating density	2-3x10^5 cell/ml	2-4x10^5 cell/ml	1x10^5 cell/ml
Maintaining density	2x10^5-2x10^6 cell/ml	4-8x10^5 cell/ml	1x10^5-1x10^6 cell/ml
Splitting point	2x10^6 cell/ml	8x10^5-1x10^6 cell/ml	1x10^6 cell/ml
Freezing media	Full culture media + 7.5% DMSO	Full culture media + 5% DMSO	Full culture media + 5% DMSO

Table 6: Acute leukemia cell lines specification and growth requirements

Cells were cultured as per the recommendation of the manufacturer with few modifications. In brief; CCRF-CEM were cultured in RPMI-1640 (HyCloneTM, USA) supplemented with 10% fetal bovine serum (FBS, HyCloneTM-USA). Cells were sub-cultured every 2-3 days; when density reached 1-2x10^6 cells/ml. The THP-1 cells were grown in RPMI-1640 with 10% FBS and 0.05 mM 2-mercaptoethanl (2-ME). The HL-60 cells were maintained in Dulbecco's modified eagle's medium; DMEM (Sigma, USA) with 20% FBS and sub-cultured every 3-4 days. All culture media were also supplemented with 1% penicillin-streptomycin mix antibiotics

(Sigma or HyCloneTM, USA) and 50 mM L-glutamine (Thermo Scientific, USA) was added if not provided already in the media. Cells were grown in humidified incubator supplemented with 5% CO_2 at 37^{o}C.

2.3 Compounds and Drugs

Frondoside A (either purchased from Sigma ≥ 96% pure or provided by Coastside BioResources-USA ≥ 98% pure) was reconstituted in dimethyl sulfoxide (DMSO) initially and further dilutions for different concentrations were made in 1xPBS (phosphate buffered saline) making the final DMSO content < 0.1% in the treated wells. The conventional drugs used in the treatment of acute leukemia and tested in this project in-combination with frondoside A were, vincristine sulphate (2 mg liquid in 2 ml vials) from Hospira-UK, asparaginase 10,000 U/ml vial in powder form (43.2 mg) from Medac-Germany and prednisolone in packages of 20 mg/tablet (Julphar-UAE) dissolved in 1.5% ethanol. Further dilutions were made in 1X PBS so the final concentration of ethanol in the compound treatment concentrations was < 0.1%. Andrographolide (NFκB irreversible antagonist) was purchased from MedChem Express-USA, and the main stock was prepared by dissolving in DMSO. Further dilutions made in serum free media (SFM; RPMI-1640). Table 7 describes the details of the compounds' preparation.

Compound/Drug	**Range of concentrations (μM)**	**Solvent**	**Mass (MW)**
Frondoside A	0.5-5.0	DMSO/PBS	1336.4 g/mole
Asparaginase	0.0005-7.81	PBS	43.2 mg (31731.9 g/mole)
Vincristine	0.0017-1.0625	liquid	2 mg (923.04 g/mole)
Prednisolone	0.446-1393	1.5% Ethanol/PBS	20 mg (358.428 g/mole)
Andrographolide	3-100	DMSO/SFM	350.45 g/mole

Table 7: Range of concentrations and solvents used for the compounds

The concentration ranges used for cell viability experiments were adapted from previously published literature (Chen, Wu, Doerksen, Ho, & Huang, 2015; Cheung et al., 2005; Kristensen, Jonsson, Sundström, Nygren, & Larsson, 1992; Larsson, Fridborg, Kristensen, Sundström, & Nygren, 1993; Larsson, Kristensen, Sandberg, & Nygren, 1992; Manikam, Manikam, & Stanslas, 2009) and subsequently confirmed in our experiments. Initially 5 concentrations of each drug were tested with 5-fold increments in concentration. Inhibitory concentration 50 (IC_{50}) was calculated and extra doses were added to reach as close as possible to the IC_{50} if this was not reached within the initial tested range. Blank wells containing only serum free media were included to determine the baseline luminescence and negative controls were also included; these contain cell suspension treated with 1X PBS or free serum media (the drug carrier).

Trypan blue exclusion test was used with the Neubauer chamber hemocytometer to count the number of viable cells prior to plating the cells.

2.4 Viability and Drug Efficacy Test

Before treatment, cells were transferred to serum free media for 24 h and plated in a 96-well flat sterile optical culture plates with cell density of $5x10^4$ cell/well, in a total volume of 90 µl. Cells were treated with different concentrations of frondoside A, vincristine sulphate (vin), asparaginase (asp) and prednisolone (pred) each compound alone to determine the specific inhibitory concentrations (IC). Experiments were conducted with treatment incubation periods of 24, 48 and 72 h.

The drug-response curve was constructed and the inhibitory concentration IC_{10}, IC_{30}, IC_{50} and IC_{70} for each compound for the 3 cell lines at each time point was

calculated. Treatment duration of 48 h was decided to be used for subsequent experiments.

Concentrations corresponding to approximately IC_{10}, IC_{30} and IC_{50} for each drug were used to treat the cell lines in combination with similar inhibitory concentrations of frondoside A.

Finally, the efficacy of fixed frondoside A IC_{50} concentration in combination with variable concentrations (IC_{10}, IC_{30} and IC_{50}) of conventional drugs was assessed.

All experiments were conducted in duplicate and repeated at least 3 times in independent experiments to confirm reproducibility of the results.

Cell viability after treatment was tested using CellTiter-Glo luminescence kit from Promega, USA as per manufacturer recommendations. The principle of this method is based on the conversion of luciferin to oxyluciferin (luminescence) in the presence of Mg^{+2}, ATP (from viable cells) and the catalyze; luciferase.

The reconstituted reagent (100 µl) was added to each well in the plate including the blank and controls. Protected from light, the plates were kept on a shaking tray for 2 minutes at ~80 rpm, to induce cell lysis. The plates were transferred to the bench and kept in dark for an incubation period of 10 minutes. Luminescence based on ATP produced by viable cells remaining after treatment in the plate was read at 560 nm wavelength in a VictorX3 multiple plate reader (ParkinElmer, USA). Luminescence of the treated groups was expressed as the percentage of viable cells compared to the control groups which were assumed to have 100% viability.

Similar methodology was used to investigate the effect of treating the blast cells with variable concentrations of frondoside A combined with andrographolide fixed IC_{50-70} concentration.

2.5 Apoptosis Assay

Guava Nexin reagent (EMD Millipore Corporation, CA) was used for detecting apoptosis in the treated cells. The reagent contains phycoerythrin (PE) labeled annexin V and 7-AAD dye (7-aminoactinomycin D).

Cells were cultured in 24-well plates with density of $1.0x10^5$ cell/well in a total volume of 180 µl 1% FBS growth media as recommended by the manufacturer. Cells were treated with 20 µl of either PBS (control) or IC_{50} of frondoside A for 1, 3 and 6 hours duration. After incubation, the cells were fixed with 4% paraformaldehyde in 1:1 ratio for 20 minutes. Nexin reagent (100 µl) was added to each well and the cells were incubated for another 20 minutes at room temperature in dark.

After incubation, the cells were visualized on Olympus inverted fluorescent microscope to detect the annexin-PE and the 7-AAD staining.

2.6 RNA Extraction and Gene Expression Profiling

Cells were grown in a T25 culture flasks for 24 h at a density of $3x10^6$ cells in 5.4 ml serum free media. Cells were treated after the incubation with 600 µl of either 1X PBS (control) or IC_{50} frondoside A concentration and incubated again either for 6 h or 24 h (to detect early versus late changes). Cells were transferred from the culture flask to 15 ml falcon tube. Media were removed by centrifugation at 1300 rpm (300 G) for 5 minutes, leaving only small amount not to disturb the cell palette (~250 µl).

One ml of cold 1X PBS (pH 7.4) was added to the pellet and mixed then centrifuged at 1300 rpm (300 G) for 5min. The supernatant was discarded. Cells were re-suspend in the remaining PBS (~150 µl) and transfer into 1.5 ml RNase free Eppendorf tubes. Tubes were washed with an extra 500 µl of cold 1xPBS and the content added to the RNase free Eppendorf. Samples were then centrifuged again and the supernatants were discarded.

Six hundred µl of guanidinium thiocyanate-phenol-chloroform (Trizol, Life technologies-USA) was added to the tubes making sure all cells are covered (Trizol to cell ratio was 1000 µl Trizol to each $5x10^6$ cell). Cells were incubated at room temperature on a shaker for 5 minutes at 100 rpm. Cells were lysed by pipetting up and down several times then vortexed thoroughly. Lysates were stored at -80^oC at this stage until RNA extraction if not done immediately.

For RNA extraction, total RNA isolation system from Promega-USA was used. In the process, 20 µl of chloroform was added per 100 µl of Trizol. Samples were mixed vigorously for 15 seconds on a vortex and left for 2-3 minutes at room temperature. The tubes were centrifuged at 12,000 G for 15 minutes at 4^oC. The chloroform separates the cell components of the homogenate into 3 layers as shown in Figure 10. The aqueous phase was transferred into fresh tube and 50 µl of 95% ethanol per 100 µl Trizol was added and mixed by pipetting up and down several times. The contents were then transferred to a spin column and centrifuged at 12000 G at room temperature. The flow-through fluid was discarded.

Figure 11 shows a flow chart of the process for RNA extraction. In brief, the spin columns were washed with 600 µl RNA wash solution, centrifuged and the flow-through fluid was discarded. DNase incubation master mix was prepared by mixing 40

µl of yellow core buffer with 5 µl 0.09 M $MnCl_2$ and 5 µl of DNase I enzyme per sample.

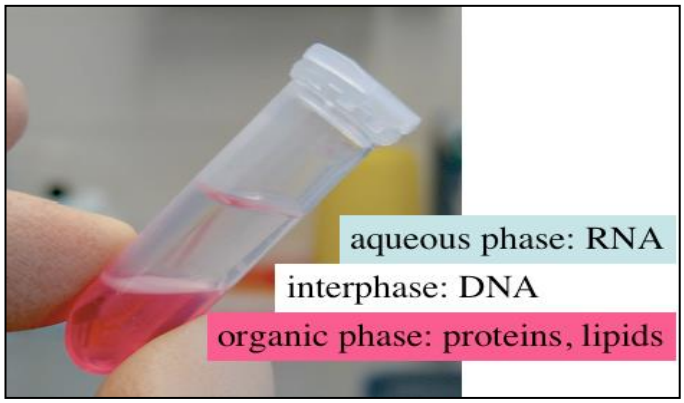

Figure 10: Separation of cellular components

Master mix (50 µl) was added to the spin column and incubated for 15 minutes at room temperature. The DNase stop solution was added and the column was centrifuged then washed twice with RNA wash solution. The spin basket was transferred to 1.5 ml elution tube. Fifty µl of nuclease free water was added and the tube was incubated for 2 minutes on ice, then centrifuged and the flow-through saved labeled as purified RNA.

Once RNA was purified, quantification of the yield was made using NanoDrop spectrophotometer (ND-1000 v3.5.2) reading the nucleic acid based on the wavelengths at 260 nm and 280. The purity of RNA was determined at 260/280 nm and 260/230 nm. RNA was stored in -80^{o}C till later use for reverse transcription and low density array runs.

For reverse transcription, a high capacity cDNA reverse transcription kit was used from Applied Biosystems, USA. The principle of the reaction is based on using target RNA to construct complementary DNA (cDNA).

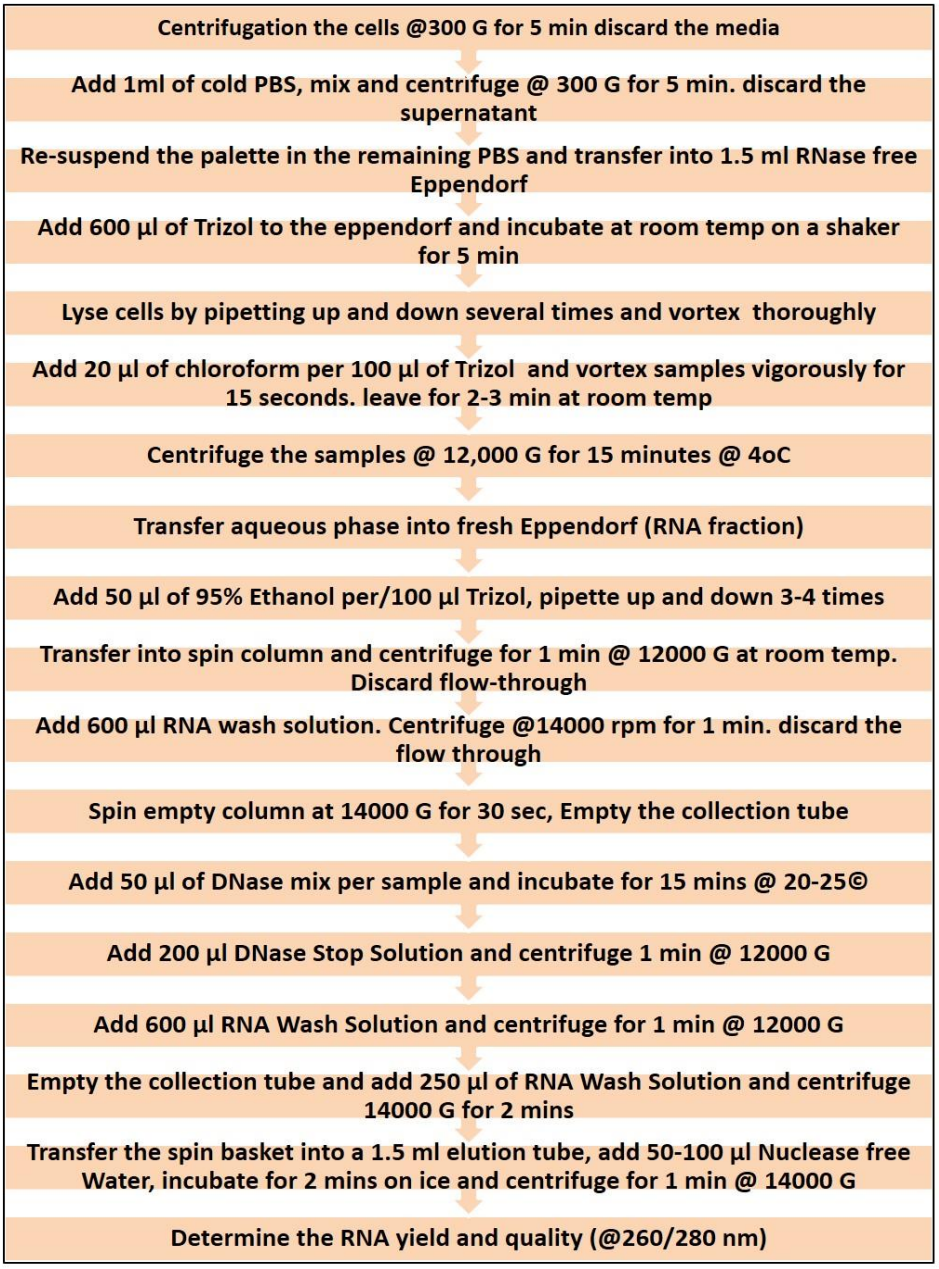

Figure 11: RNA purification method

The purified RNA was used in reverse transcription polymerase chain reaction (PCR) to produce cDNA for the processing on low density apoptosis gene arrays for gene profiling. Twenty five µl reaction volume was prepared with the content of RNA in each sample calculated to be 1 µg per reaction (i.e. 40 ng/µl).

RNA Mastermix was prepared as follows (per sample): 2.5 µl of 10X RNA buffer, 1 µl of 25X dNTP mix, 2.5 µl of 10X RT random mix, 1 µl of 1X multiscribe RT and 1 µl of 1X RNase inhibitor. These were mixed and added to the calculated RNA of each sample in a MicroAmp fast optical 96-well PCR reaction plate (AB Applied Biosystems), then RNase free water was added to make a total volume of 25 µl. The plate was sealed, centrifuged to remove any bubbles and run on AB Applied Biosystems, Veriti 96 well fast thermo-cycler.

The resultant cDNA (total of 40 ng per sample) was saved for later used on apoptosis microarrays.

For low density apoptosis arrays, Taqman human apoptosis arrays from Applied Biosystems were used with 96 genes involved in apoptosis including control genes (listed in table 8). The reaction requires 200 µl of each sample to be loaded on the array in a 1:1 ratio mixed with TaqMan gene expression master mix (AB Applied Biosysttems). Four µg of cDNA was calculated and transferred into a clean 1.5 ml Eppendorf tube. RNase free water was added to make up to 100 µl, then 100 µl of the master mix added (the volumes were adjusted for possible pipetting errors). The samples were mixed and loaded to the arrays and centrifuged then loaded on 7900HT Fast real- time PCR system (AB Applied Biosceiences). The results were analyzed using RQ manager software version 1.2.1 and the fold change comparison was done using DataAssist software, version 3.01.

2.7 Protein Extraction and Western Blot

For protein extraction, radio-immunoprecipitation assay buffer (RIPA) was used to lyse the cells (refer for appendix 1 for the constitution). Protease inhibitor mix

was freshly added as well as phenylmethylsulfonyl fluoride (PMSF), both 10 µl for each 1 ml RIPA buffer. β-Mercaptoethanol 25 µl added to prevent oxidation.

Gene Abbreviation	Gene Name
BIRC2	baculoviral IAP repeat-containing 2
BAK1	BCL2-antagonist/killer 1
BCL3	B-cell CLL/lymphoma 3
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CASP2	caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed)
CASP5	caspase 5, apoptosis-related cysteine peptidase
CASP7	caspase 7, apoptosis-related cysteine peptidase
CASP8	caspase 8, apoptosis-related cysteine peptidase
CASP9	caspase 9, apoptosis-related cysteine peptidase
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
LTB	lymphotoxin beta (TNF superfamily, member 3)
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a
CARD4	caspase recruitment domain family, member 4
NALP1	NACHT, leucine rich repeat and PYD (pyrin domain) containing 1
CASP14	caspase 14, apoptosis-related cysteine peptidase
BCL2L13	BCL2-like 13 (apoptosis facilitator)
TNFRSF21	tumor necrosis factor receptor superfamily, member 21
HTRA2	HtrA serine peptidase 2
TBK1	TANK-binding kinase 1
ESRRBL1	estrogen-related receptor beta like 1
LRDD	leucine-rich repeats and death domain containing
CARD15	caspase recruitment domain family, member 15
CARD9	caspase recruitment domain family, member 9
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
BCL2L14	BCL2-like 14 (apoptosis facilitator)
BIRC7	baculoviral IAP repeat-containing 7 (livin)
CARD6	caspase recruitment domain family, member 6
BIRC8	baculoviral IAP repeat-containing 8
DEDD2	death domain containing 2
APAF1	apoptotic peptidase activating factor
BIRC3	baculoviral IAP repeat-containing 3
BIRC4	baculoviral IAP repeat-containing 4
BIRC5	baculoviral IAP repeat-containing 5 (survivin)
FAS	Fas (TNF receptor superfamily, member 6)

Table 8: Genes amplified on the apoptosis low density arrays

Gene Abbreviation	Gene Name
FASLG	Fas ligand (TNF superfamily, member 6)
BAD	BCL2-antagonist of cell death
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2A1	BCL2-related protein A1
BCL2L1	BCL2-like 1
BCL2L2	BCL2-like 2
BIK	BCL2-interacting killer
BNIP3L	BCL2/adenovirus E1B interacting protein 3 like
BOK	BCL2-related ovarian killer
CASP3	capsase 3, apoptosis related cysteine peptidase
CASP6	caspase 6, apoptosis related cysteine peptidase
CASP10	caspase 10, apoptosis-related cysteine peptidase
DAPK1	death-associated protein kinase 1
HIP1	huntingtin interacting protein 1
BIRC1	baculoviral IAP repeat-containing 1
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α
RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65
TNF	tumor necrosis factor (TNF superfamily, member 2)
IKBKG	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
PEA15	phosphoprotein enriched in astrocytes 15
TRADD	TNFRSF1A-associated via death domain
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1
HRK	harakiri, BCL2 interacting protein (contains only BH3 domain)
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
FADD	Fas (TNFRSF6)-associated via death domain
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
CFLAR	CASP8 and FADD-like apoptosis regulator
DEDD	death domain containing
BCL2L10	BCL2-like 10 (apoptosis facilitator)
BCL2L11	BCL2-like 11 (apoptosis facilitator)
BBC3	BCL2 binding component 3
PYCARD	PYD and CARD domain containing
DIABLO	diablo homolog (Drosophila)
BIRC6	baculoviral IAP repeat-containing 6 (apollon)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ACTB	actin, beta
CHUK	conserved helix-loop-helix ubiquitous kinase
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
RIPK2	receptor-interacting serine-threonine kinase 2
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
BCAP31	B-cell receptor-associated protein 31
ICEBERG	ICEBERG caspase-1 inhibitor
TA-NFKBH	T-cell activation NFKB-like protein
BID	BH3 interacting domain death agonist
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
CASP4	caspase 4, apoptosis-related cysteine peptidase
LTA	lymphotoxin alpha (TNF superfamily, member 1)
TNFRSF25	tumor necrosis factor receptor superfamily, member 25
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
BCL10	B-cell CLL/lymphoma 10
CASP8AP2	CASP8 associated protein 2
18S	18S ribosomal RNA

Table 8 (Continued): Genes amplified on the apoptosis low density arrays

Cells were grown in a serum free media for 24 h in T25 culture flasks with cell density of $3x10^6$ cells in a 5.4 ml media. Cells were treated after the incubation with 600 µl of 1X PBS or IC_{50} frondoside A concentration and incubated again either for 6 h or 24 h (to detect early versus late changes). After the incubation, the contents of the culture flasks were transferred to 15 ml falcon tubes. Figure 12 demonstrates the process for the protein extraction.

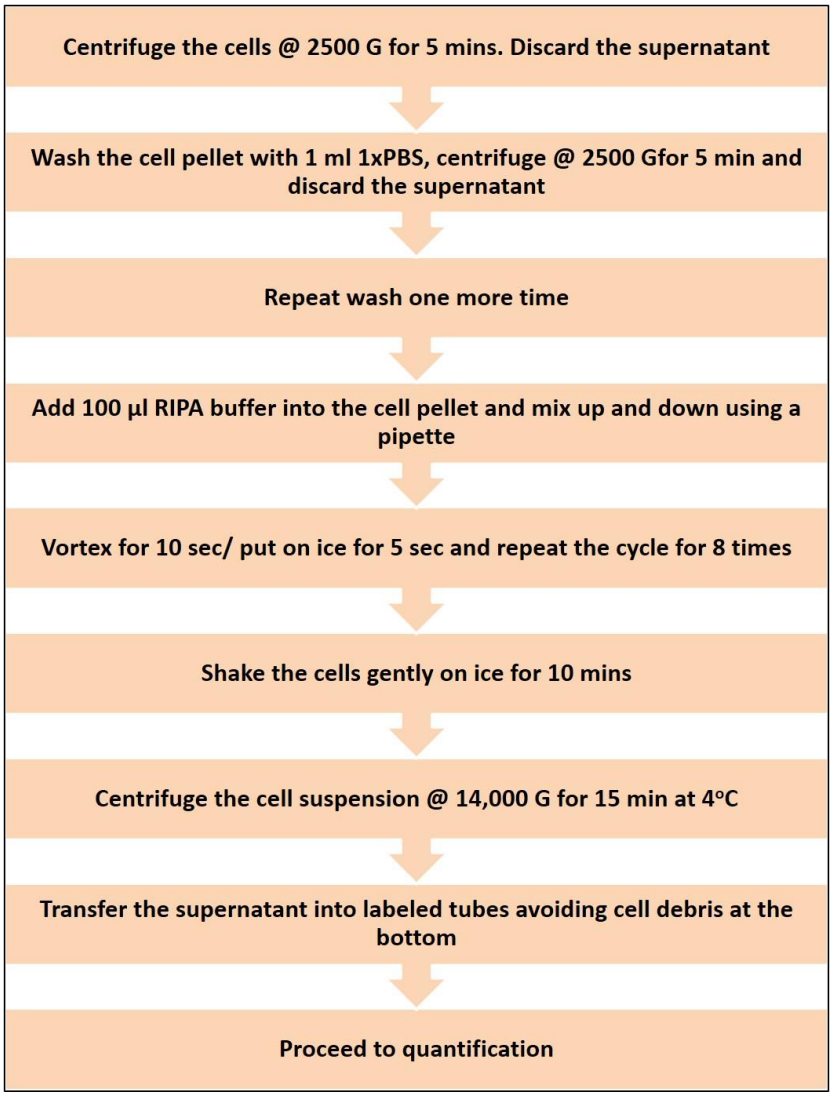

Figure 12: Protein extraction method

Once the proteins were quantified using either BCA protein assay kit or Coomassie plus protein assay kit, both from Thermo Scientific-USA. Fifty µg of each sample was loaded on the SDS-PAGE gel after mixing with 7 µl 5X loading dye and making the total volume loaded up to 20 µl using RIPA buffer. Western blots were run for Bcl-2, Bax and p53 all purchased from Abcam-USA as well as p21, caspase 3, RelB, c-IAP2 and NFkB2 from Cell Signaling. Beta actin was used as a protein loading control (Abcam-USA). All the secondary horse-radish peroxidase polyclonal antibodies were from Abcam. The detail of the antibodies specifications are listed in Table 9.

Protein	Primary antibody/dilution	Secondary antibody/dilution
Bcl-2	Mouse monoclonal/ 1:1000	Rabbit anti-mouse HRP polyclonal/ 1:2000
Bax	Rabbit monoclonal/ 1:1000	Goat anti-rabbit HRP polyclonal/ 1:2000
Caspase 3	Mouse monoclonal/ 1:1000	Rabbit anti-mouse HRP polyclonal/ 1:2000
p53	Mouse monoclonal/ 1:1000	Rabbit anti-mouse HRP polyclonal/ 1:2000
p21	Rabbit monoclonal/ 1:250	Goat anti-rabbit HRP polyclonal/ 1:2000
NFκB2	Rabbit monoclonal/ 1:500	Goat anti-rabbit HRP polyclonal/ 1:2000
Relb	Rabbit monoclonal/ 1:500	Goat anti-rabbit HRP polyclonal/ 1:1000
cIAP	Rabbit monoclonal/ 1:500	Goat anti-rabbit HRP polyclonal/ 1:1000
ACTB	Mouse monoclonal/ 1:1000	Rabbit anti-mouse HRP polyclonal/ 1:2000

Table 9: Western blot antibody specifications

Samples were thawed on ice then pre-heat on a heating block to 99ºC for 5 min. The running and stacking gels were prepared as described in table 10. 10% SDS-PAG was used for the Bcl-2, Relb, cIAP2 and p53 while 12% SDS-PAG was used for Bax and p21 proteins. For NFκB2 protein, 7.5% SDS-PAG was used (see Table 10; for gel preparation). When the gels had solidified, they were assembled in the electrophoresis tank and running buffer (1X SDS, see appendix 2A) was added to fill the tank.

Ten µl of multicolor broad-range protein ladder (Thermo Scientific-USA) was mixed with 7 µl loading dye and run alongside the samples. The gels were run at 100 V for 30 min. Once the samples appear in straight line, the voltage was increased to 160 V for another 40-45 min.

Component	7.5% gel	10% gel	12% gel	Staking gel
dH_2O	6.2 ml	5.25 ml	4.5 ml	3.25 ml
Tris 8.8	5.7 ml	5.7 ml	5.7 ml	-
40% acrylamide (29.1)	2.8 ml	3.75 ml	4.5 ml	0.5 ml
1xSDS	150 µl	150 µl	150 µl	40 µl
Tris 6.8	-	-	-	0.5 ml
10% APS	150 µl	150 µl	150 µl	40 µl
TEMED	15 µl	15 µl	15 µl	4 µl

Table 10: Western blot gel composition

Once the run had finished, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific) in a transfer cassette (shown in Figure 13) after the membrane was activated by soaking in 100% methanol for 3 min. The cassette was placed in a transfer tank filled with cold 1X transfer buffer (100 ml of 10X transfer buffer with 200 ml of 100% methanol and 700 ml water). Refer to appendix 2B for the constitution of the 10X transfer buffer.

The transfer was run for 80 minutes at 100 V in a cold room with ice pack in the tank (4^oC). Once the transfer was finished, the membrane was washed with 1X TBS (refer to appendix 2C) for 3 times each lasting for 5 minutes on a shaker and the membrane was blocked using 5% non-fat milk (NFM) in 1X TBST (refer to appendix 2D) for 1 h at room temperature with shaking.

Once the blocking was finished, 1:1000 diluted primary monoclonal antibody (refer to Table 9 for the specific antibody dilutions) in 5%NFM/TBST was added and the membrane was incubated on a shaker overnight at 4^oC in dark.

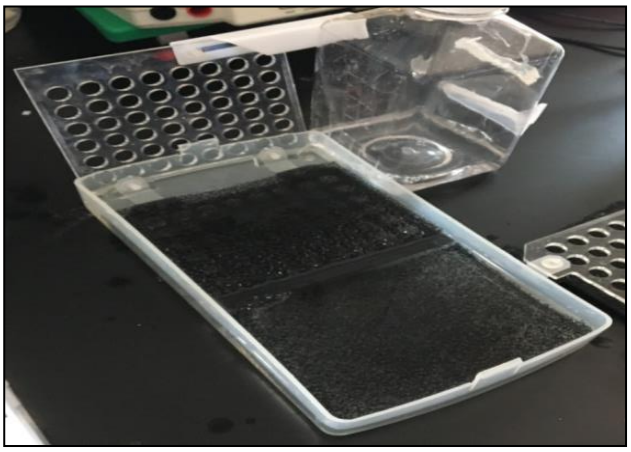

Figure 13: Western blot transfer assembly cassette

After the incubation, the primary antibody was removed and the membrane washed with 1X TBST for 3 times each lasting 5 minutes on a shaker at room temperature. Meanwhile secondary antibodies were prepared diluted in 5%NFM/TBST. The blots were incubated with the secondary antibodies for 1:30 h with shaking. Once incubation is completed, the blots were washed with 1X TBST for 5 times each for 5 minutes with shaking.

The Pierce enhanced chemiluminescence (ECL) plus western blot detection substrate (Thermo Scientific) was prepared as per the recommendation of the manufacturer. The substrate was added to the membrane and incubated in dark for 5 minutes, then the blots were scanned for protein bands on Typhoon FLA 9500 reader. The bands were quantified using image Quant TL software.

The bands corresponding to the protein MW were measured and normalized against the control protein (ACTB) on the same sample. The protein bands were then expressed as percentage area and the IC_{50} treated compared with the control samples at each specific time of treatment (6 h vs 24 h).

2.8 Data Collection and Statistical Analysis

For comparing multiple groups of different treatments and different compound concentrations, the multi-variant analysis two-way ANOVA was used with Bonferroni correction as appropriate for multiple comparisons. P-value of < 0.05 was considered statistically significant. For gene expression analysis, a fold-change of 2 or more was considered significant. The GraphPad Prism program (v 5.01) was used for the analysis and to construct the graphs.

Chapter 3: Results

3.1 Efficacy Comparison

Inhibitory concentration for each compound at each treatment duration was calculated and the treatment duration of 48 h was decided to be used for all subsequent experiments. Frondoside A purchased from Coastside BioResources was more potent where the IC_{50} for frondoside A in CCRF-CEM cells was 1.5 μM at 48 h treatment duration. For HL-60 cells, the frondoside A IC_{50} was 2.5 μM, while for THP-1 cells, 3.0 μM inhibited cell viability by 50%. The stock purchased from Sigma was less potent with CCRF-CEM IC_{50} of 2.5 μM and 3.5 μM for THP-1 cells. This suggests the monocytic leukemia cells are more resistant to the compound (Figure 14 shows frondoside A from Coastside BioResources response curve for all cell lines 48 h treatment.

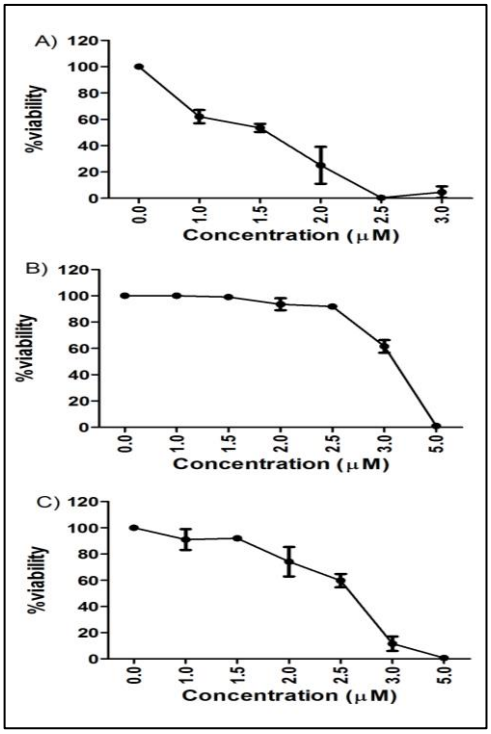

Figure 14: Frondoside A treatment response curve at 48 h. A) Showed the response of CCRF-CEM cells with IC_{50} ~1.5 μM. B) The response of THP-1 cells with IC_{50} 3.0 μM. C) HL-60 cells response to frondoside A (IC_{50}~2.5 μM)

When different concentrations corresponding approximately to IC_{10}, IC_{30} and IC_{50} of the conventional drugs where tested alone and in combination with similar inhibitory concentrations of frondoside A, the CCRF-CEM cell line showed the higher concentration combinations to be more effective in decreasing cell viability than the same concentration of each drug given alone. Asparaginase 0.0025 μM and 0.0075 μM concentrations combined with frondoside A at 1.0 μM and 1.5 μM respectively, were more effective than asparaginase alone ($P < 0.001$). Similar findings were seen with vincristine ($P < 0.01$). For prednisolone, 0.0558 μM and 0.1115 μM concentration combinations with frondoside A 1.0 and 1.5 μM respectively gave statistically higher suppression of cell viability ($P < 0.01$ and < 0.05 respectively). CellTiter-Glo viability results were confirmed morphologically as shown in Figure 15. The statistical analysis is shown in Figure 16.

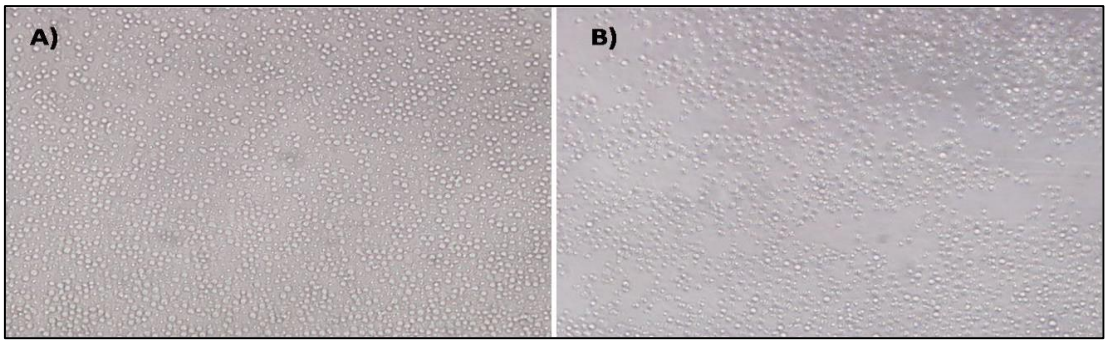

Figure 15: CCRF-CEM cell viability. A) Cells treated with asparaginase 0.0075 μM alone. B) Cells treated with combination of asparaginase 0.0075 μM and Frondoside A 1.5 μM

Interestingly, the combination effect of asparaginase 0.0025 μM with frondoside A 1.0 μM and asparaginase 0.0075 μM with frondoside A 1.5 μM resulted in significantly greater reduction in cell viability than accounted for by the calculated additive effect (P-value < 0.01). For both vincristine and prednisolone, the

concentrations combined with frondoside A 1.0 μM were also showing synergistic effect rather than additive effects (P-value < 0.01) as shown in Figure 17.

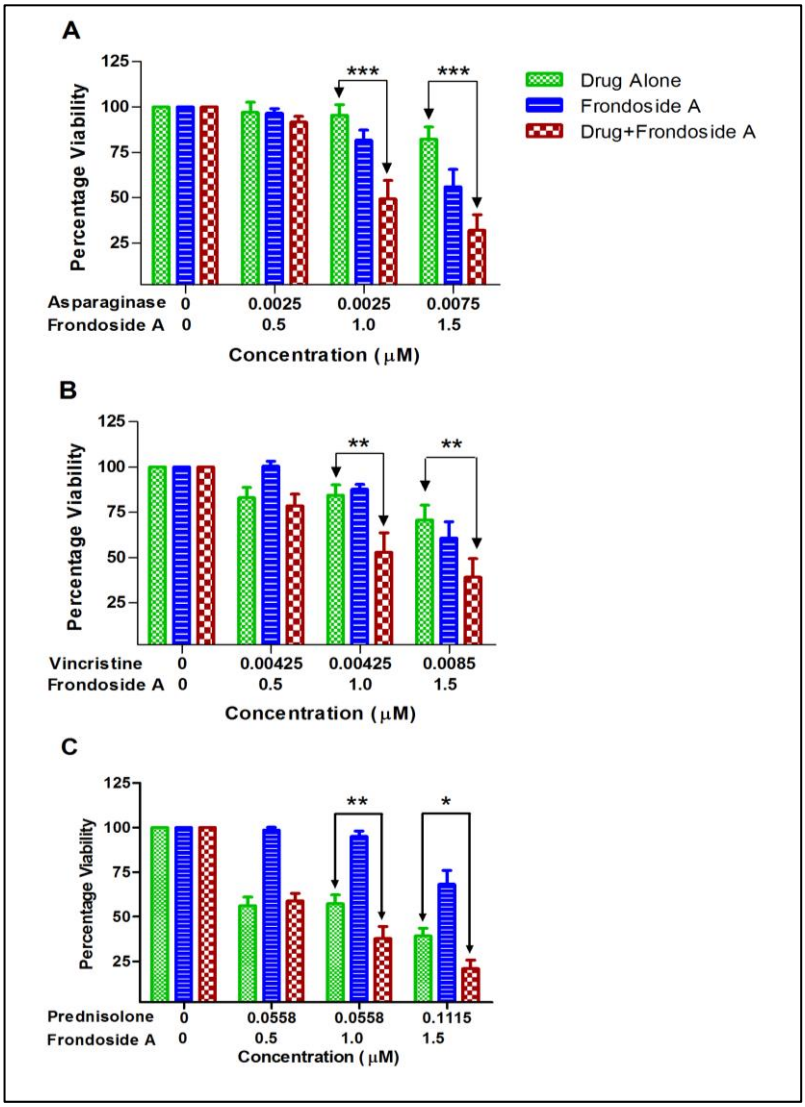

Figure 16: CCRF-CEM response to variable compound concentrations. A) The effect of different concentrations of asparaginase and frondoside A alone and in combination. The first group shows the control (untreated) cells with 100% viability. Second group represents the viability in response to the treatment with 0.0025 μM asparaginase alone, frondoside A 0.5 μM alone and both concentrations in combination (the 3rd column). The 3rd and 4th groups represents the concentrations of asparaginase 0.0025 and 0.0075 μM and frondoside A 1.0 and 1.5 μM respectively, both showed statistical significant suppression in viability in the combination groups compared to asparaginase alone (P < 0.001). B) Similar data was seen with vincristine: when combination of 0.00425 μM vincristine and 1.0 μM of frondoside A and 1.5 μM were tested (P < 0.01). C) The response to prednisolone with the higher two combinations showing a more pronounced decrease in viability in the combination groups (P < 0.01 and < 0.05 respectively). * = < 0.05, **= < 0.01, ***= < 0.001

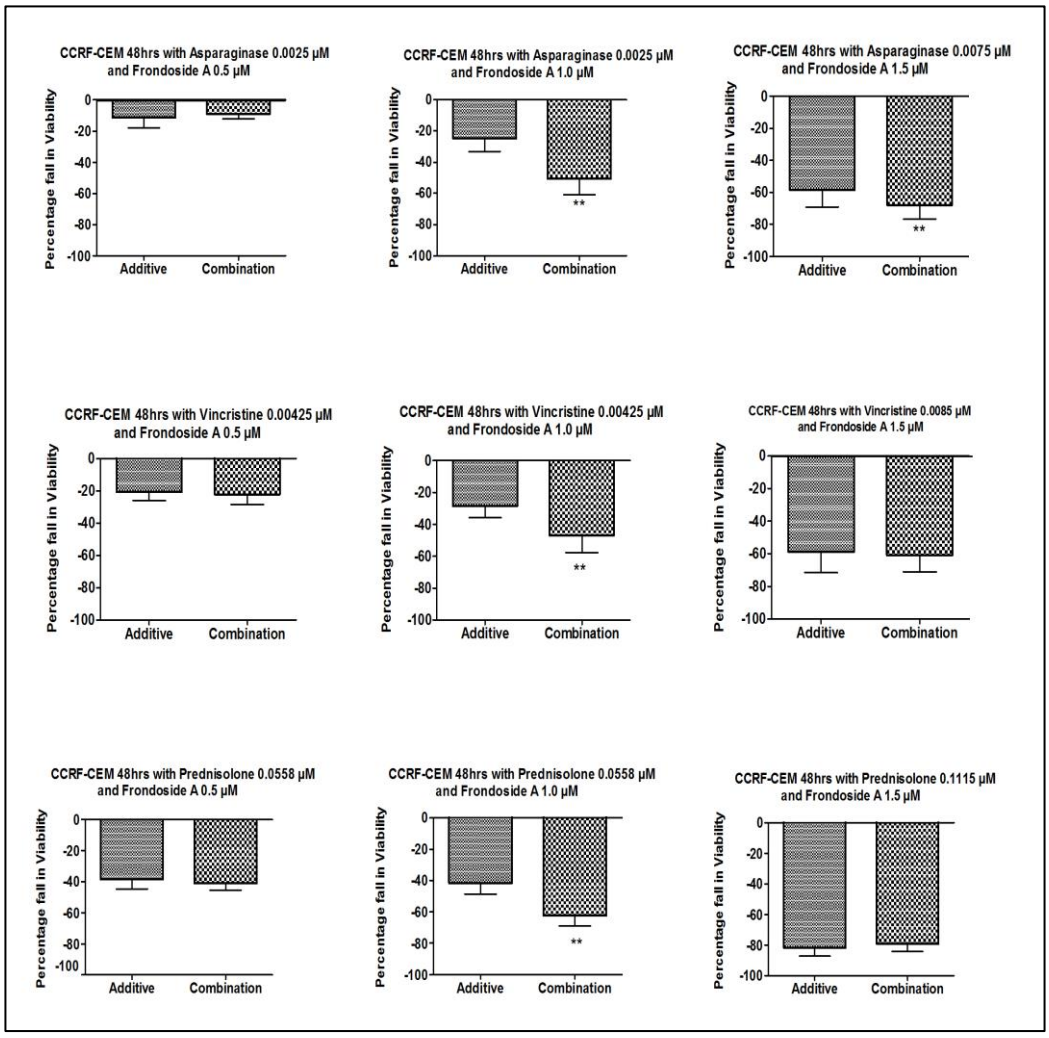

Figure 17: Compounds synergistic compared to additive effect in CCRF-CEM. The top row represents the observed effect of the combination treatments with asparaginase and frondoside A compared to the calculated additive effects. The highest two concentration combinations showed statistically significant reductions in cell viability compared to the expected calculated additive effect ($P < 0.01$). The middle and bottom rows represent the data for vincristine and prednisolone. Once again, the middle concentrations show statistically significant reductions in cell viability compared to the expected calculated additive effect ($P < 0.01$)

In THP-1 cells, treatment with asparaginase and frondoside A was more effective in suppressing cell viability than asparaginase alone in all combinations ($P = < 0.01$, < 0.001 and < 0.001 for the three combinations) (Figure 18). Vincristine treatment combined with frondoside A showed similar pattern of effect as the combination with asparaginase (Figure 18). Only the combination of the highest

concentration of prednisolone and frondoside A showed a statistically greater effect than either drug alone ($P < 0.001$) as shown in Figure 18. In this cell line, significant synergistic effects were observed in all the combinations of asparaginase and vincristine with Frondoside A compared to the calculated additive effect, as shown in Figure 19. While enhanced effects of the combinations with prednisolone were only seen at the two higher concentrations (Figure 19).

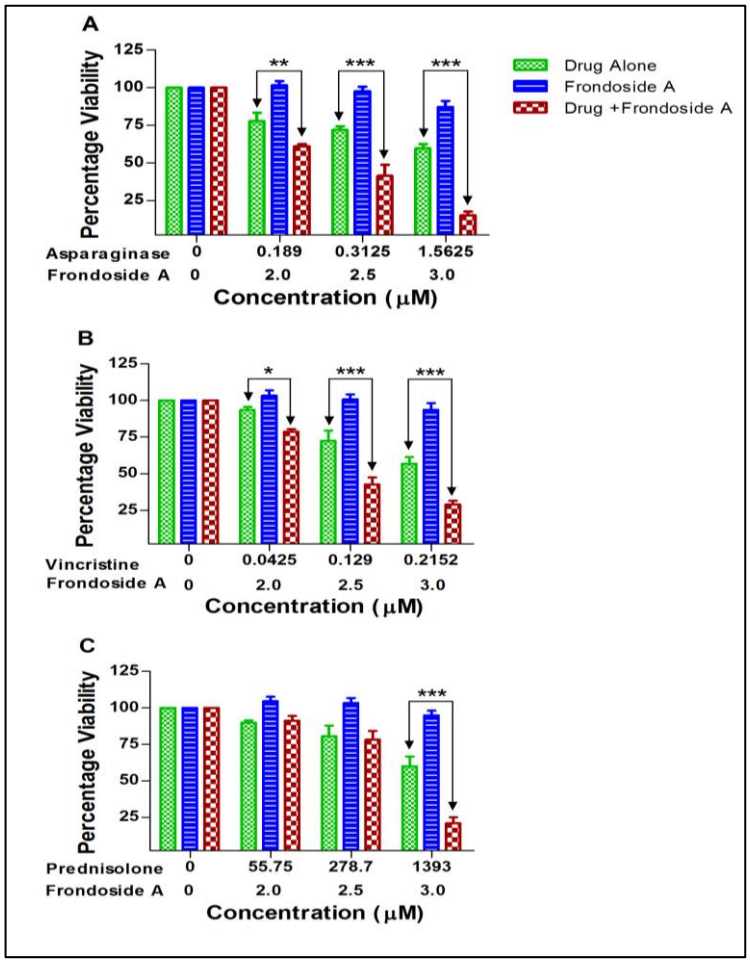

Figure 18: THP-1 response to variable compound concentrations. A) Asparaginase in all combinations with frondoside A showed more marked suppression of viability compared to asparaginase alone ($P < 0.01$ for the lowest concentration and < 0.001 for the higher two). B) Vincristine in all combinations showed statistically greater reduction in viability ($P < 0.05$ at lowest concentration and $P < 0.001$ for the higher two concentrations). C) In response to prednisolone treatment, only the highest concentration combination caused statistically significant suppression of cell viability ($P < 0.001$)

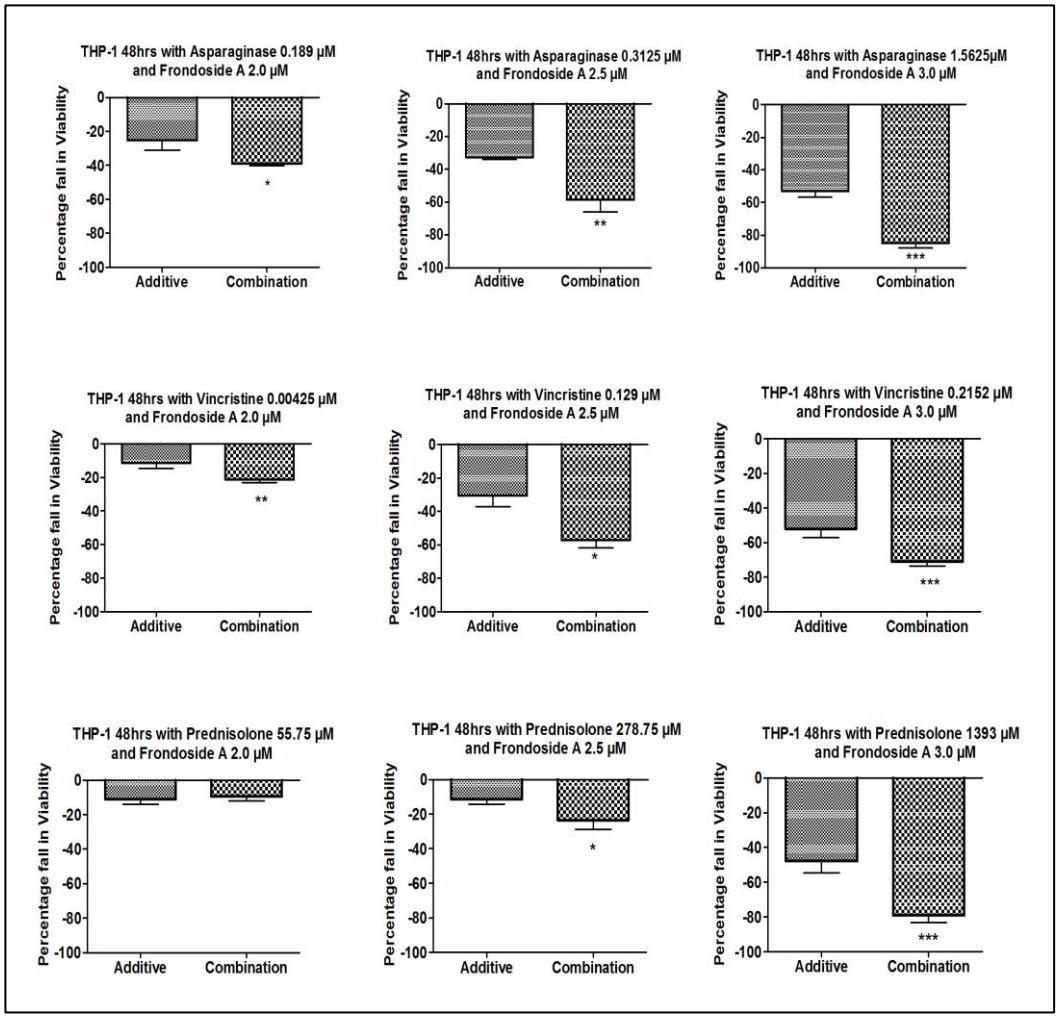

Figure 19: Compound synergistic compared to additive effect in THP-1. Top and middle rows show asparaginase and vincristine combination at all tested concentrations were able to suppress cell viability more than the calculated additive effect (P * = < 0.05, ** = < 0.01, *** = < 0.001). For prednisolone (bottom row), the highest two concentration combinations showed statistical significant differences

The promyelocytic cell line, HL-60, showed greater resistance to combination treatment and only the highest concentration combinations of all three drugs tested showed greater suppression of cell viability than the individual drugs alone (Figure 20). Although combination effect on cell suppression was higher than the calculated additive effect, none reached statistical significance.

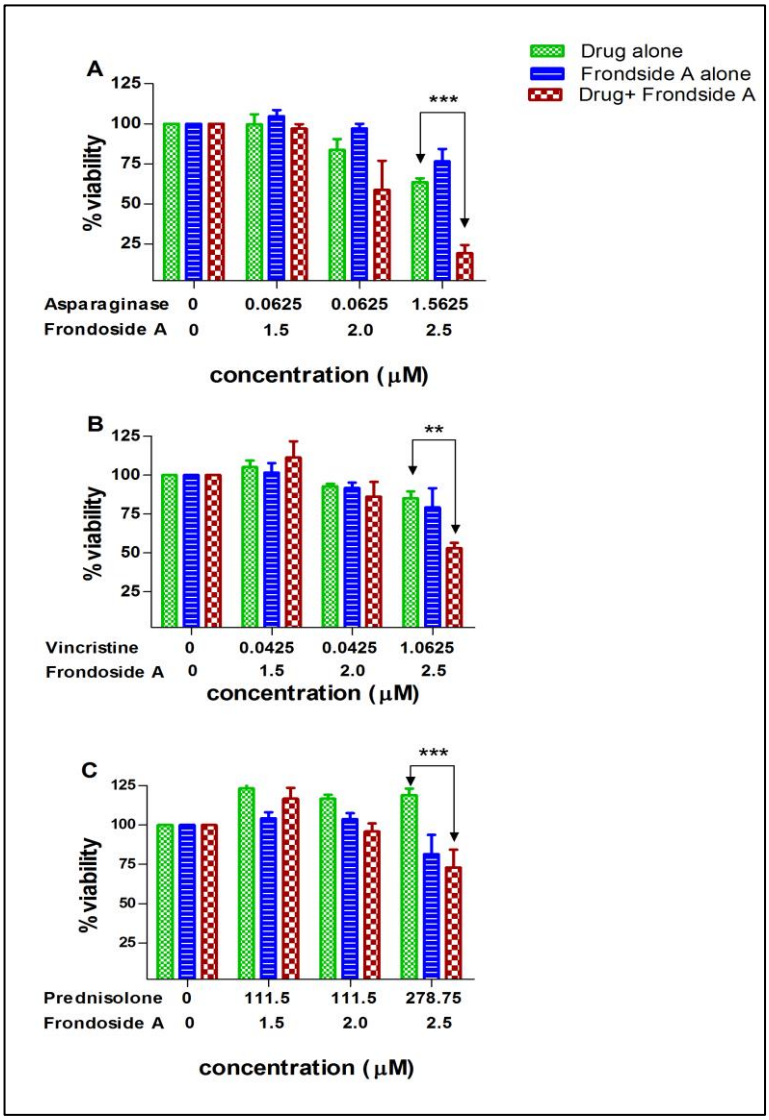

Figure 20: HL-60 response to variable compound concentrations. A) Asparaginase at its highest combination with frondoside A 2.5 μM showed significant suppression of viability ($P < 0.001$). B) The combination of vincristine and frondoside A was significant compared with vincristine agent alone at the highest concentration ($P < 0.01$). C) Combination of prednisolone and frondoside A also significantly suppressed the viability compared to prednisolone alone at the highest concentration combination ($P < 0.001$)

To determine if the results observed are due to frondoside A and not to the variation in the concentration of all compounds used, frondoside A at a fixed IC_{50} concentration was used with different concentrations of the other drugs in this set of experiments. Using the multi-variant two-way ANOVA to analyze the effect of

different compounds combinations in one arm and different concentrations combinations in the other arm, it was shown that asparaginase combination treatment in all concentrations with frondoside A 1.5 μM in CCRF-CEM cells was statistically more effective in decreasing leukemia cell viability than asparaginase alone. Similar findings were seen with vincristine but prednisolone combination with frondoside A concentration was better than the drug alone only at the lowest concentration ($P < 0.01$). Detailed analysis at each concentration is shown in Figure 21.

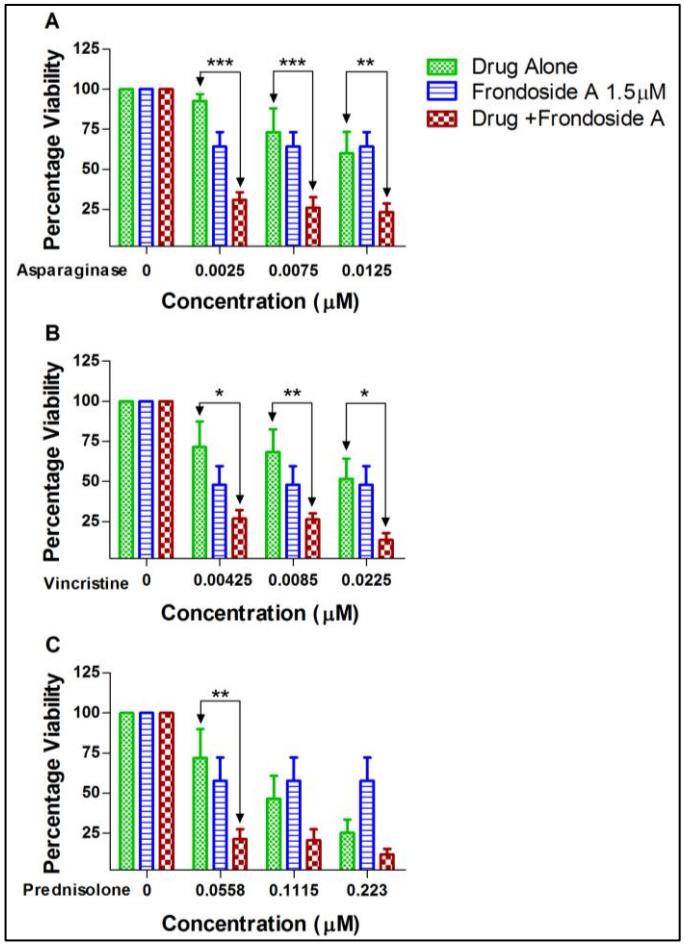

Figure 21: CCRF-CEM treatment in combination with IC_{50} frondoside A. A) The combinations of frondoside A with asparaginase at all concentrations showed significantly greater suppression in cell viability than asparaginase alone ($P < 0.001$, < 0.001 and < 0.01 respectively). B) The combinations of frondoside A and vincristine treatment was more effective than vincristine alone ($P < 0.05$, < 0.01 and < 0.05 respectively). C) Prednisolone at the lowest concentration decreased cell viability more effectively when combined with frondoside A, compared to prednisolone alone ($P < 0.01$)

In the THP-1 cell line, asparaginase with frondoside A IC_{50} treatment was more effective than asparaginase alone in the lower two concentrations (P-value < 0.001 & < 0.01 respectively). Vincristine also at lower concentrations combined with frondoside A 3.0 μM was more effective in suppressing cell viability than vincristine alone (P < 0.001). The combination of prednisolone with frondoside A showed only the highest concentration to be more effective than prednisolone treatment alone (P < 0.001). Data of the different therapy effects on THP-1 combined with frondoside A IC_{50} are shown in Figure 22.

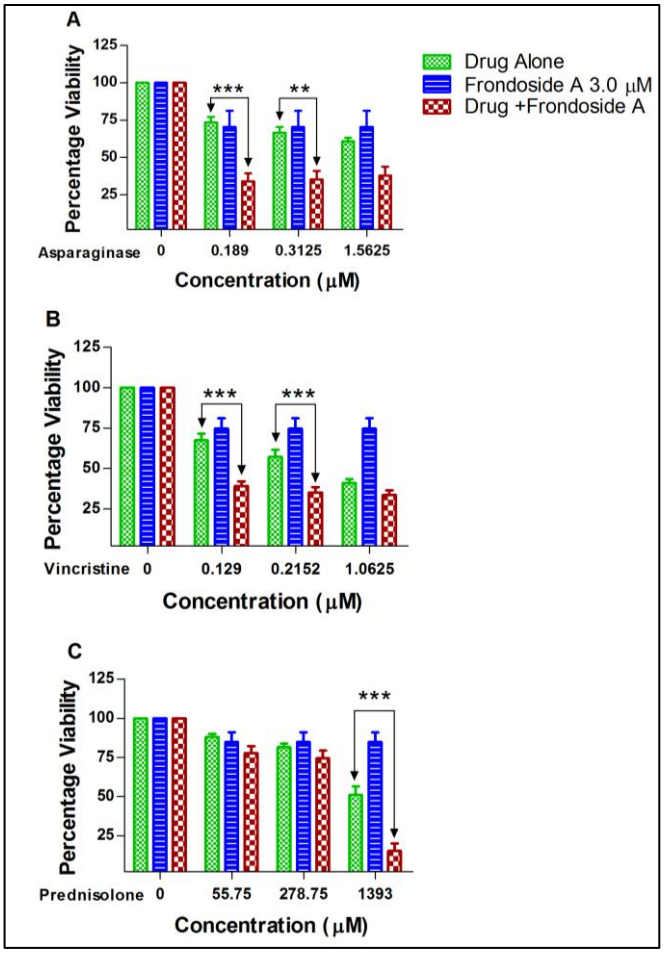

Figure 22: THP-1 treatment in combination with IC_{50} frondoside A. A) and B) The first two combinations of both asparaginase and vincristine with frondoside A decreased cell viability more effectively than either drug alone. C) Treatment with combinations of frondoside A with different concentrations of prednisolone show a statistically significant change only at the highest concentration (1393 μM) (P < 0.001)

Asparaginase combined with frondoside A IC_{50} treatment was more effective than asparaginase alone at low concentrations in the HL-60 cell line ($P < 0.01$). There was no significant difference between the combination treatment and the treatment alone at any of the tested concentrations with both vincristine and prednisolone, as shown in Figure 23.

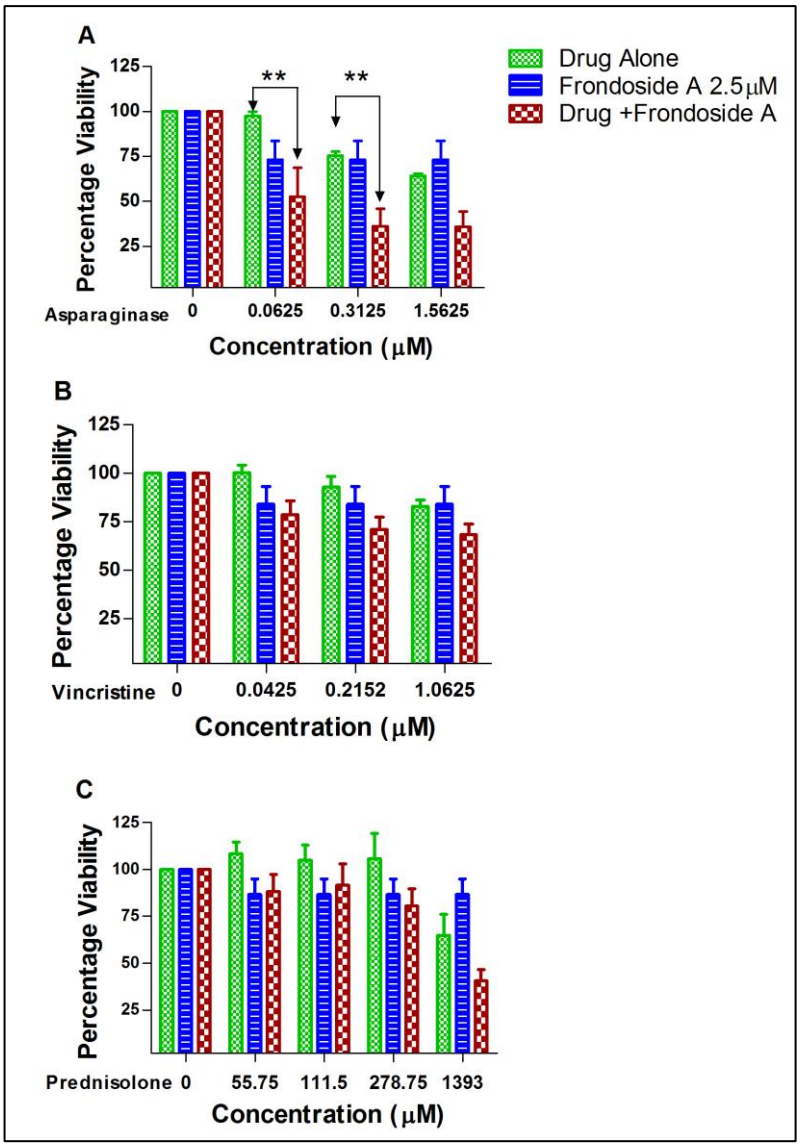

Figure 23: HL-60 treatment in combination with IC_{50} frondoside A concentration. A) Asparaginase at lower concentrations combinations with frondoside 2.5 μM decreased viability significantly compared to asparaginase alone ($P < 0.01$). B) and C) Combination treatments of both vincristine and prednisolone with frondoside A did not show any significant change compared to each of the drugs alone

From these experiments, frondoside A induced cell death in different leukemia cell lines measured by decrease in ATP production (as well as morphologically, showing features of cell death) in the treated groups. The efficacy of frondoside A was variable, being more effective in acute T-cell ALL (CCRF-CEM) and less effective in the monocytic acute leukemia cells (THP-1). Frondoside A also enhanced the effect of conventional chemotherapeutic drugs used in the treatment of this malignancy.

Further investigations were performed on CCRF-CEM as an example of frondoside A sensitive cells and on THP-1 as an example of more resistant cells. The HL-60 cell line was excluded from further investigation because of showing minimal response to combination treatment.

3.2 Apoptosis Assays

At least 10 consecutive fields were screened in both, control and treated wells. Frondoside A induced apoptosis in both cell lines. Annexin positive cells were significantly higher in the IC_{50} treated group compared to controls. Figure 24 shows THP-1 annexin stained cells at 1 and 3 h treatment points. Results from experiments in CCRF-CEM cells (at 1, 3 and 6 h) are shown in Figure 25.

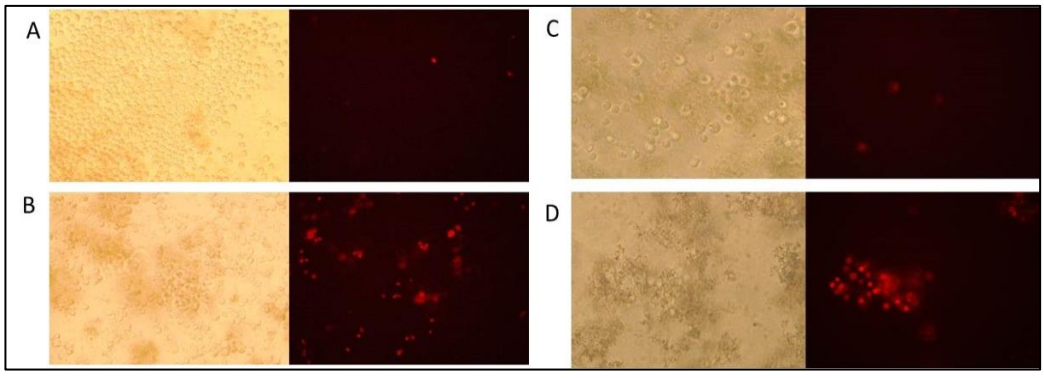

Figure 24: THP-1 cells annexin staining. A) Control cells treated with PBS for 1 h and B) Frondoside A IC_{50} treated cells at 1 h showing increase in cell numbers that are annexin-PE stained. C) and D) represents control and frondoside A treated at 3 h respectively with marked increase in annexin staining compared to the control and to 1 h treatment duration ($P < 0.05$)

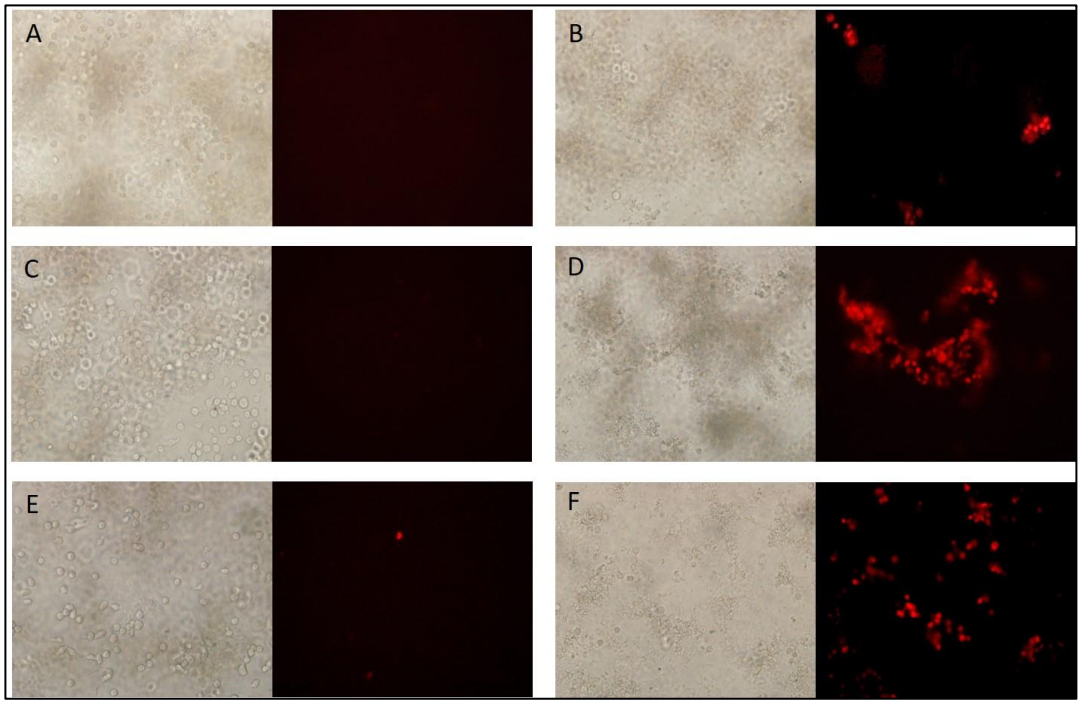

Figure 25: CCRF-CEM cells annexin staining. A) Control cells treated with PBS for 1 h and B) Frondoside A IC_{50} treated cells at 1 h showing increase in cell numbers that are annexin-PE stained. C) and D) represents control and frondoside A treated at 3 h respectively with marked increase in annexin staining compared to the control and to 1 h treatment duration. E) and F) represents control and frondoside A treated at 6 h respectively with marked increase in annexin staining compared to the control ($P < 0.001$ at all-time points)

3.3 Gene Expression Analysis

For gene expression profiling, β-actin mRNA (ACTB) was chosen as endogenous control to which all the other gene amplifications were normalized for all the samples. The choice of ACTB was made because its expression was consistent among all samples (controls vs treated) at both treatment duration points (6 h vs 24 h) (Naora, 1995). It also amplifies at Ct value close to the samples and not earlier (as did the 18S gene) which can give false higher fold increase in expression as shown in Figure 26.

Control samples (untreated) were used as reference for the analysis of the treated samples, each control at its time point; i.e. control 6 h was used to compare treated samples at 6 h treatment duration and control 24 h was used to compare treated samples at 24 h treatment duration. Then the 6 h treated samples were compared to the 24 h treated ones to detect the trend of change in the gene expression in response to different time durations. Increase in gene expression more than 2-fold from the reference sample or decrease of more than 0.5 fold was considered significant.

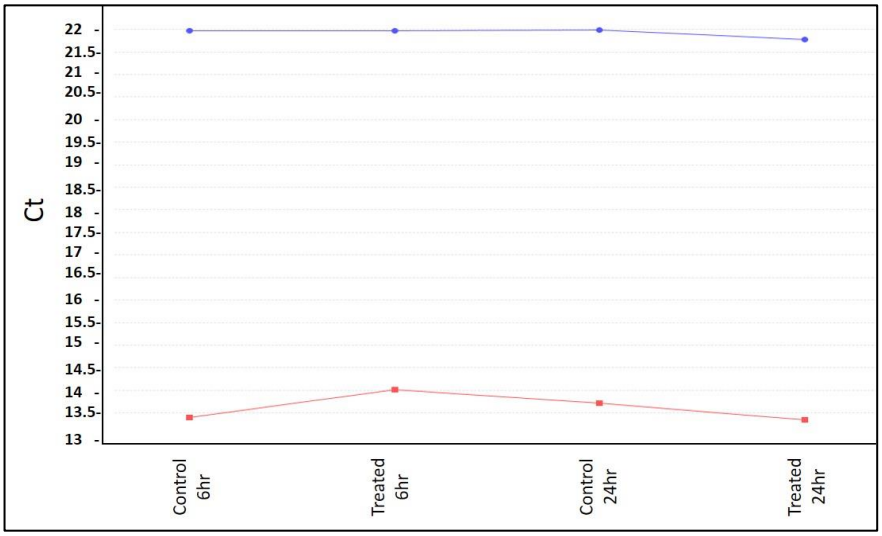

Figure 26: The choice of control gene in CCRF-CEM cells. Comparison between ACTB (in blue) and 18S (in red). Control samples at 6 and 24 hr time points as well as frondoside A 1.5 μM treated sample show no variation in ACTB expression which amplified at Ct value of around 22

In order to simplify the analysis, the expression of genes on the arrays were grouped into 4 groups; TNF pathway genes, apoptosis pathway genes, NFκB pathway genes and NFκB inhibitor genes.

3.3.1 Gene expression in CCRF-CEM cells

In the CCRF-CEM cell line, frondoside A induced significant increase in expression of tumor necrosis factor C (TNF-C, also known as lymphotoxin β, LTB) at

both time points. Expression of tumor necrosis factor β (TNF-β, also known as lymphotoxin α, LTA) and tumor necrosis factor receptor 1 (TNF-R1, also known as tumor necrosis factor receptor superfamily member 1A, TNFRSF1A) were up-regulated after 24 h treatment duration while expression of death receptor 3 (DR3, also known as tumor necrosis factor receptor superfamily member 25, TNFRSF25) and death receptor 6 (DR6, also known as tumor necrosis factor receptor superfamily member 21, TNFRSF21), although not significantly increased from controls, they were more than 2-fold higher in the longer treatment duration compared to 6 h treatment.

Frondoside A caused a marked increase in expression of caspase-6, NoxA (phorbol-12-myristate-13-acetate-induced protein 1; PMAIP1), leucine rich repeat and pyrin domain containing 1 (NALP1), death effector domain (PEA15) and BCL2 like 1 (BCL2L1) which are pro-apoptosis genes encoding pro-apoptosis proteins in CCRF-CEM cells. The pro-apoptosis genes caspase 1, 2, 6, 7 , 8, 10, CASP8AP, leucine-rich receptor and death domain (LRDD), tumor necrosis factor receptor superfamily 1A associated death domain (TRADD) and death associated protein kinase 1 (DAPK), although not significantly changed from the control samples, there was ≥ 2 fold increase at 24 h compared to 6 h treatment duration. Frondoside A caused a minimal down-regulation of the anti-apoptosis gene B-cell CCL/lymphoma 2 (Bcl-2) at 6 h treatment duration.

Frondoside A increased expression of the nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFκB2), v-rel reticuloendotheliosis viral oncogene homolog B (RelB, also known as nuclear factor of kappa light polypeptide gene enhancer in B-cells 3) and inhibitor of kappa light polypeptide gene enhancer in

B-cells kinase beta (IκBKβ) genes significantly in this cell line. The gene encoding cIAP2 (BIRC3) was also markedly up-regulated. In response to frondoside A treatment, the NFκB pathway inhibitors; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFκBIA), T-cell activation NFκB-like protein (TA-NFκBH, also known as NFκBID) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta (NFκBIZ) were also markedly up-regulated. The details of the genes affected by the treatment with frondoside A in CCRF-CEM cell line is shown in Figure 27 and Table 11.

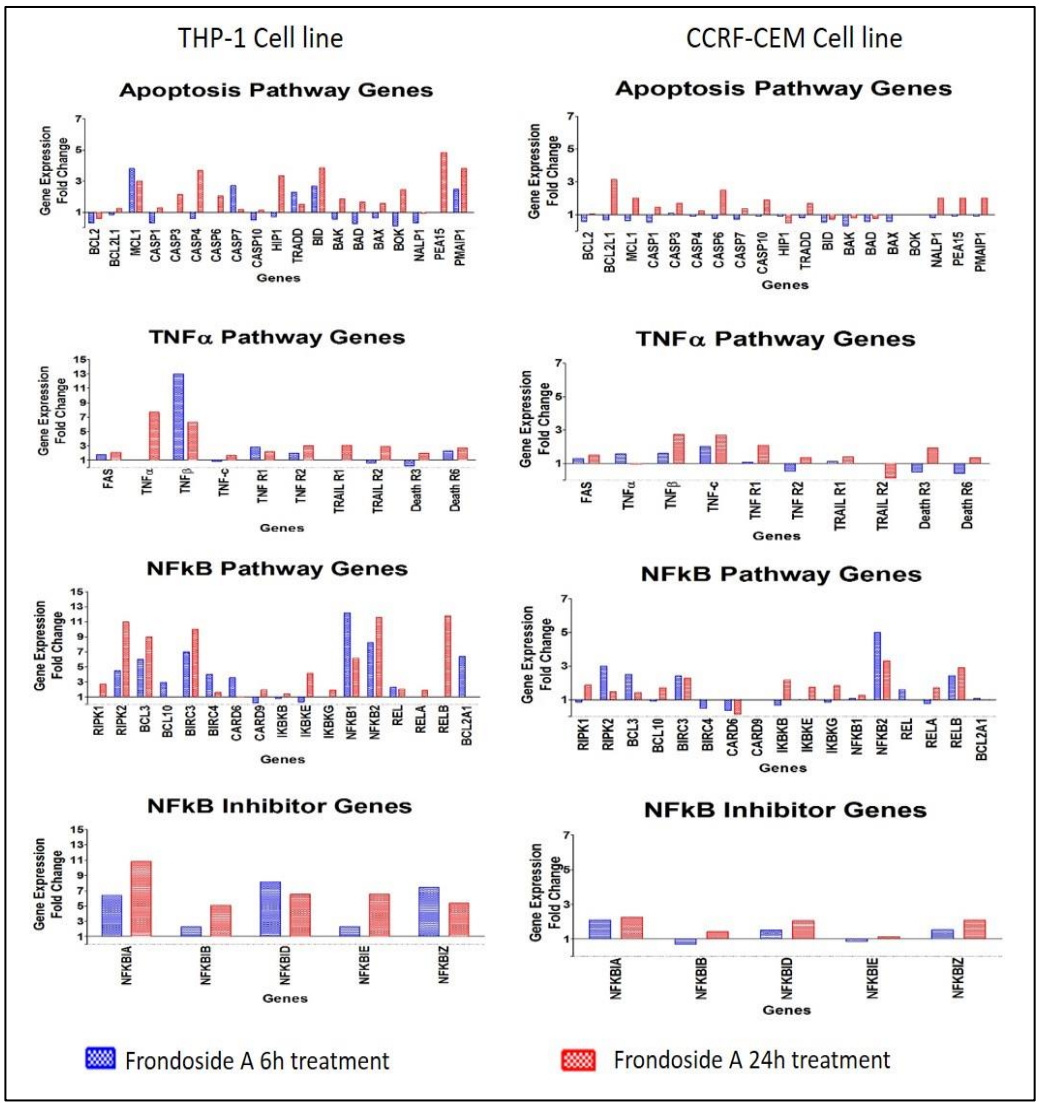

Figure 27: Gene expression fold change in response to frondoside. In THP-1 and CCRF-CEM cell lines at 6 and 24 h treatment duration

Gene	Pathway	Fold Change 6 h/24 h	Direction of Change
APAF1	Apoptosis	0.57/1.19	No change, increased at 24h
BCL2	Apoptosis	0.59/1.04	No change
BCL2L1	Apoptosis	0.66/3.13	Up-regulated at 24 h
MCL1	Apoptosis	0.63/1.74	No change, increased at 24 h
CASP1	Apoptosis	0.55/1.47	No change, increased at 24 h
CASP2	Apoptosis	0.53/1.54	No change, increased at 24 h
CASP6	Apoptosis	0.77/2.49	Up-regulated, increased at 24 h
CASP7	Apoptosis	0.71/1.37	No change, increased at 24 h
CASP8	Apoptosis	0.38/1.46	Increased at 24 h
CASP8AP2	Apoptosis	0.45/1.13	Increased at 24 h
CASP10	Apoptosis	0.91/1.89	No change, increased at 24 h
DEDD	Apoptosis	0.88/1.88	No change, increased at 24 h
DEDD2	Apoptosis	0.65/1.49	No change, increased at 24 h
LRDD	Apoptosis	0.57/1.34	No change, increased at 24 h
HIP1	Apoptosis	0.88/0.48	Down-regulated at 24 h
TRADD	Apoptosis	0.81/1.67	No change, increased at 24 h
DAPK1	Apoptosis	0.55/1.49	No change, increased at 24 h
BAK	Apoptosis	0.32/0.78	Increased at 24 h
NALP1	Apoptosis	0.81/2.01	Up-regulated at 24 h
PEA15	Apoptosis	0.91/2.01	Up-regulated at 24 h
PMAIP1	Apoptosis	0.91/2.01	Up-regulated, increased at 24 h
TNFRSF1A	TNF	1.09/2.09	Up-regulated at 24 h
TNFRSF10A	TNF	-/0.13	Down-regulated at 24 h
TNFRSF21	TNF	0.41/1.34	No change, increased at 24 h
TNFRSF25	TNF	0.49/1.92	No change, increased at 24 h
LTA	TNF	1.61/2.73	Up-regulated at 24 h
LTB	TNF	2.0/2.7	Up-regulated
RIPK1	NFκB	0.85/1.87	No change, increased at 24 h
RIPK2	NFκB	3.0/1.48	Down-regulated, decreased at 24 h
BCL3	NFκB	2.5/1.42	Up-regulated, decreased at 24 h
BCL10	NFκB	0.91/1.71	No change, increased at 24 h
BIRC3	NFκB	2.42/2.29	Up-regulated
BIRC4	NFκB	0.47/-	Down-regulated
CARD6	NFκB	0.36/0.13	Down-regulated
IKBKB	NFκB	0.65/2.17	Up-regulated at 24 h
NFKB1	NFκB	1.07/1.26	No change
NFKB2	NFκB	4.99/3.3	Up-regulated
RELA	NFκB	0.76/1.7	No change, increased at 24 h
RELB	NFκB	2.44/2.9	Up-regulated
IKBKG	NFκB	0.84/1.84	No change, increased at 24 h
NFKBIZ	NFκB inhibitor	1.53/2.1	Up-regulated at 24 h
NFKBIA	NFκB inhibitor	2.1/2.25	Up-regulated
TA-NFKBH	NFκB inhibitor	1.51/2.06	Up-regulated at 24 h

Table 11: CCRF-CEM cell line gene changes. The fold change in CCRF-CEM cell line gene expression in response to frondoside A treatment at 6 and 24 h time points, categorized based on gene function

Nine genes failed to amplify in this cell line at both time points and were excluded from the analysis. These included BOK, CARD9, caspase-5, caspase-14, BIRC7, BIRC8, HRK, BCL2L10 and ICEBERGE.

3.3.2 Gene expression in THP-1 cells

The THP-1 cell line showed more changes in gene expression in response to frondoside A treatment compared to CCRF-CEM cell line.

Frondoside A caused a significant up-regulation in caspase-3, caspase-4, caspase-6, Noxa, BCL-2 ovarian killer (Bok), huntingtin interacting protein 1 (HIP1), BH3 interacting domain death agonist (Bid) and phosphoprotein enriched in astrocytes 15 (PEA15). Caspase-7 was up-regulated early after 6 h treatment duration. Although there was no significant increase compared to controls, caspase-1, caspase-10, BCL2-antagonist/ killer 1 (Bak), BCL2-antagonist of cell death (Bad) and BCL2-associated X protein (Bax) show more than 2-fold increase in expression at 24 h compared to 6 h treatment. The anti-apoptosis gene Bcl-2 was down-regulated at both time points while myeloid cell leukemia sequence 1 (MCL1) showed up-regulation.

Frondoside A-treated THP-1 cells showed marked up-regulation of the tumor necrosis factor (TNF) pathway genes including TNF receptor superfamily member 6 (FAS), TNF receptor superfamily member 2 (TNF), tumor necrosis factor receptor superfamily member 10B (TNFRSF10B, also known as TRAIL-R1), tumor necrosis factor receptor superfamily member 10A (TNFRSF10A, also known as TRAIL-R2), death receptor 3 (DR3, also known as tumor necrosis factor receptor superfamily member 25, TNFRSF25) and death receptor 6 (DR6, also known as tumor necrosis factor receptor superfamily member 21, TNFRSF21), tumor necrosis factor receptor

superfamily member 1A (TNFRSF1A, also known as TNF-R1), tumor necrosis factor receptor superfamily member 1B (TNFRSF1B, also known as TNF-R2) and lymphotoxin α (TNFβ).

Treatment with frondoside A was associated with massive up-regulation of the expression of NFκB pathway genes such as nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFκB1), nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFκB2), v-rel reticuloendotheliosis viral oncogene homolog B (RelB, also known as nuclear factor of kappa light polypeptide gene enhancer in B-cells 3), v-rel reticuloendotheliosis viral oncogene homolog (Rel, also known as c-Rel), B-cell CLL/lymphoma 3 (BCL3) and inhibitor of kappa light polypeptide gene enhancer in B-cells kinase epsilon (IκBKE). Frondoside A also affected the expression of multiple genes involved in NFκB inhibition, these genes were markedly up-regulated (nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor alpha; NFκBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor beta; NFκBIB, NFκBID, nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor epsilon; NFκBIE and nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor zeta; NFκBIZ). The genes encoding cIAP2 and XIAP (BIRC3 and BIRC4 respectively) were markedly up-regulated at both time points. The details of the gene changes in THP-1 cell line in response to frondoside A treatment is shown in Figure 27 and Table 12.

In this cell line 6 genes failed to amplify at both time points and were excluded from the analysis. These included BCL2L14, caspase-14, FASLG, DAPK1, HRK and ICEBERG.

Gene	Pathway	Fold Change 6 h/24 h	Direction of Change
APAF1	Apoptosis	0.16/0.69	No change, increased at 24 h
BCL2	Apoptosis	0.3/0.6	Down-regulated
BCL2L1	Apoptosis	0.18/1.46	Down-regulated at 6 h
MCL1	Apoptosis	3.8/3.0	Up-regulated
CASP1	Apoptosis	0.3/1.3	No change, increased at 24 h
CASP3	Apoptosis	-/2.16	Up-regulated at 24 h
Casp4	Apoptosis	0.58/3.68	Up-regulated at 24 h
CASP6	Apoptosis	-/2.04	Up-regulated at 24 h
CASP7	Apoptosis	2.4/1.17	Up-regulated at 6 h
CASP8	Apoptosis	0.99/-	No change
CASP8AP2	Apoptosis	1.13/1.09	No change
CASP10	Apoptosis	0.5/1.12	No change, increased at 24 h
DEDD2	Apoptosis	0.36/1.69	Down-regulated, increased at 24 h
LRDD	Apoptosis	0.74/1.24	No change, increased at 24 h
HIP1	Apoptosis	0.72/3.36	Up-regulated at 24 h
TRADD	Apoptosis	2.3/1.53	Up-regulated at 6 h
BID	Apoptosis	2.69/3.85	Up-regulated
BAK	Apoptosis	0.56/1.86	Increased at 24 h
BAD	Apoptosis	0.26/1.66	Increased at 24 h
BAX	Apoptosis	0.63/1.58	Increased at 24 h
BOK	Apoptosis	0.12/2.45	Up-regulated at 24 h
NALP1	Apoptosis	0.3/0.92	Increased at 24 h
PEA15	Apoptosis	-/4.83	Up-regulated at 24 h
PMAIP1	Apoptosis	2.47/3.8	Up-regulated, increased at 24 h
FAS	TNF	1.79/2.06	Up-regulated at 24 h
TNF	TNF	-/7.67	Up-regulated at 24 h
TNFRSF1A	TNF	2.79/2.19	Up-regulated at 24 h
TNFRSF1B	TNF	2.0/3.0	Up-regulated
TNFRSF10A	TNF	0.65/2.9	Up-regulated at 24 h
TNFRSF10B	TNF	-/3.05	Up-regulated at 24 h
TNFRSF21	TNF	2.28/2.75	No change, increased at 24 h
TNFRSF25	TNF	0.2/2.0	No change, increased at 24 h
LTA	TNF	13.02/6.22	Up-regulated at 24 h
LTB	TNF	0.85/1.68	Up-regulated
RIPK1	NFκB	-/2.68	Up-regulated at 24 h
RIPK2	NFκB	4.49/10.95	Up-regulated
BCL3	NFκB	6.0/9.0	Up-regulated
BCL10	NFκB	2.94/-	Up-regulated at 6 h
BIRC3	NFκB	7.0/10.0	Up-regulated
BIRC4	NFκB	4.0/1.6	Up-regulated at 24 h
CARD6	NFκB	3.56/0.97	Up-regulated at 24 h, Down-regulated at 6 h
CARD9	NFkB	0.36/-	Down-regulated at 6 h
IKBKB	NFκB	0.76/1.4	No change, increased at 24 h
IKBKG	NFkB	-/1.9	No change
NFKB1	NFκB	12.18/6.12	Up-regulated
NFKB2	NFκB	8.24/11.55	Up-regulated
REL	NFkB	2.27/2.03	Up-regulated
RELA	NFκB	-/1.89	No change
RELB	NFκB	0.97/11.72	Up-regulated
BCL2A1	NFκB	6.36/-	Up-regulated at 6 h
NFKBIZ	NFκB inhibitor	7.47/5.41	Up-regulated
NFKBIA	NFκB inhibitor	6.4/10.84	Up-regulated
NFKBIB	NFκB inhibitor	2.27/5.07	Up-regulated
NFKBIE	NFκB inhibitor	2.27/6.6	Up-regulated
TA-NFKBH	NFκB inhibitor	8.16/6.54	Up-regulated

Table 12: THP-1 cell line gene changes. The fold change in THP-1 cell line gene expression in response to frondoside A treatment at 6 and 24 h time points, categorized based on gene function

3.4 Protein Expression Analysis

A minimum of 3 western blot experiments were performed for each cell line for each protein and reproducibility of the results was confirmed.

3.4.1 Bcl-2

The anti-apoptotic protein Bcl-2 showed a small but significant decreased in expression in both cell lines at 6 h in response to frondoside A treatment. This finding was in accordance with the gene expression as discussed in section 3.3. The western blot membranes are described in Figure 28.

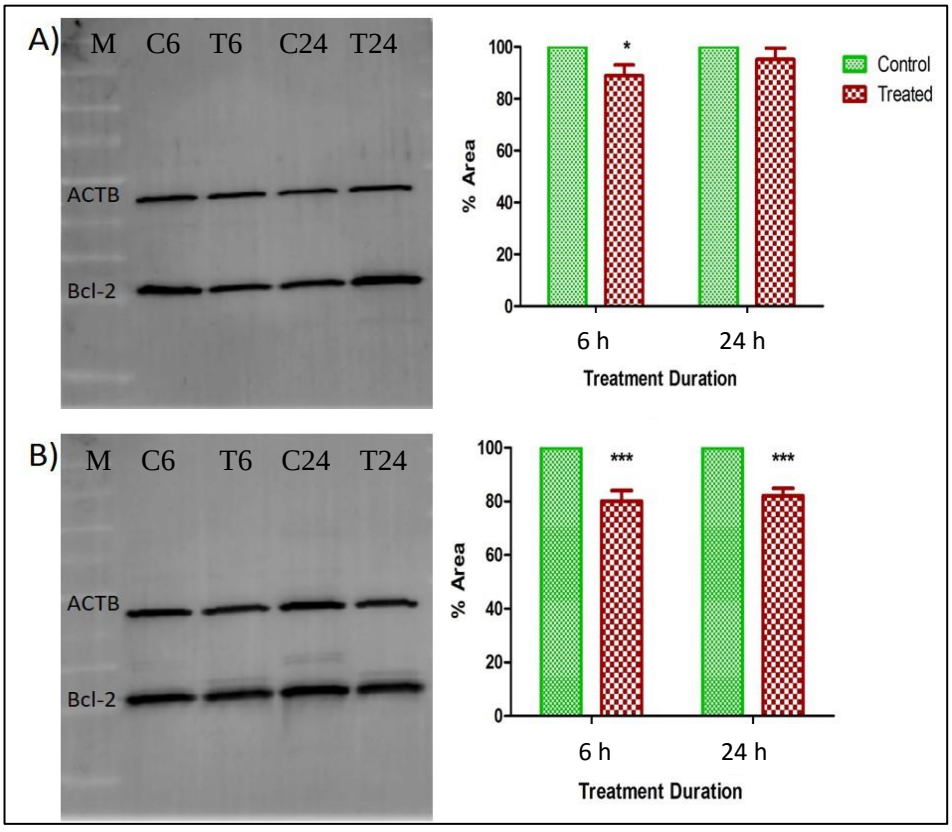

Figure 28: Western blot of Bcl-2 protein. A) Western blot of CCRF-CEM showing decrease in Bcl-2 expression after 6 h of frondoside A treatment (left). On the right is the statistical quantification of the bands. B) Western blot of THP-1 showing decrease in Bcl-2 expression after 6 h and 24 h of frondoside A treatment (left). Right panels show the statistical analysis of the quantification of the bands. (M= protein ladder, C6= control samples at 6 h, T6= frondoside A-treated sample at 6 h, C24= control samples at 24 h, T24= frondoside A-treated samples at 24 h)

3.4.2 Bax

In THP-1 cells, although the expression of the pro-apoptotic gene, Bax was not significantly altered in response to frondoside A treatment compared to the control, at the protein level, Bax showed marked decrease in expression level in the frondoside A treated groups at both time points. Similar findings were observed in the CCRF-CEM cell line. Figure 29 showed the analysis and level of significance for the protein in both cell lines.

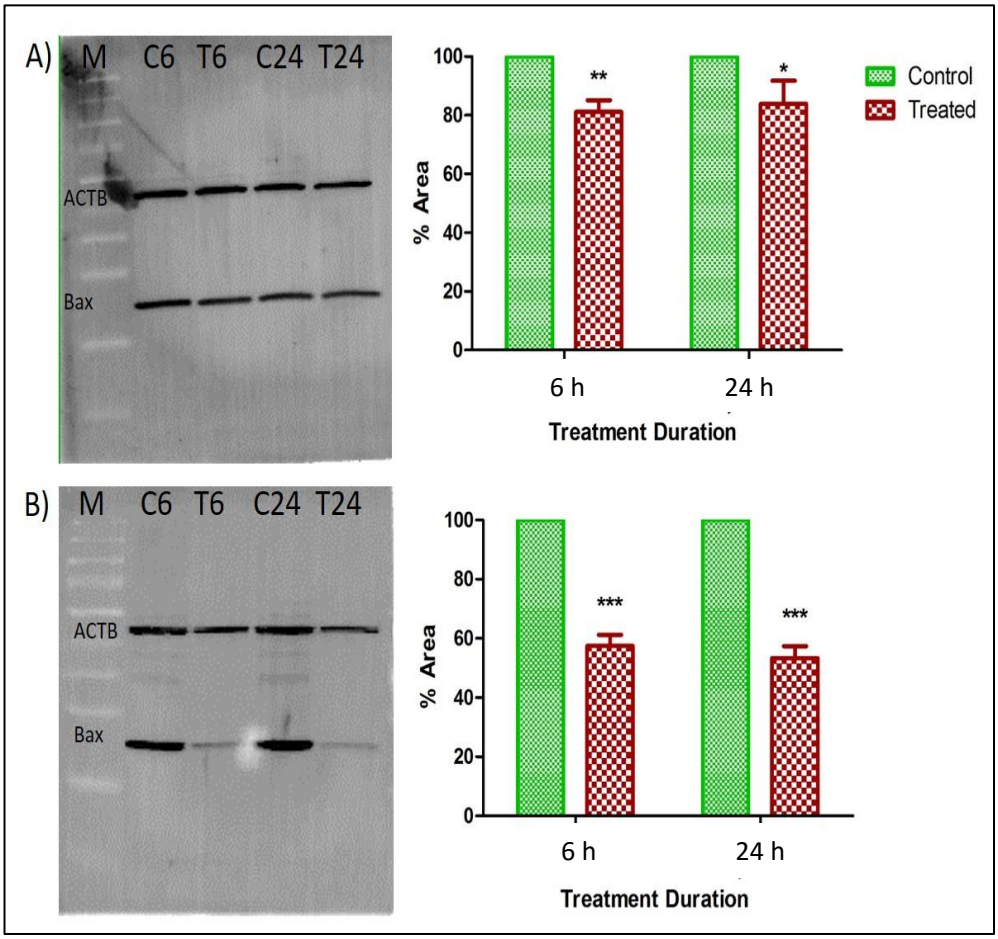

Figure 29: Western blot of Bax protein. A) Bax protein showing decrease expression in the frondoside A treated groups in CCRF-CEM cells being more significant at 6 h. B) THP-1 cells show marked reduction in Bax expression post frondoside A treatment at both time points. Right panels show the statistical analysis of quantification of the bands

3.4.3 Caspase-3

The cysteine-dependent aspartate- directed protease 3 is the main player in the execution apoptosis pathway where its activation leads to the fragmentation of cell cytoskeletal proteins, DNA fragmentation and PARP inactivation. This protein in its full length inactive form has a MW of 35 KDa, when it is activated, it gets cleaved to a smaller active form with a MW of 17/19 KDa.

Analysis of this caspase on WB has shown marked reduction in the expression of pro-caspase-3 (inactive full length form) in both the CCRF-CEM and THP-1 cell lines in response to the treatment with frondoside A at 6 h and 24 h (see Figure 30). This suggests that the protease has been activated.

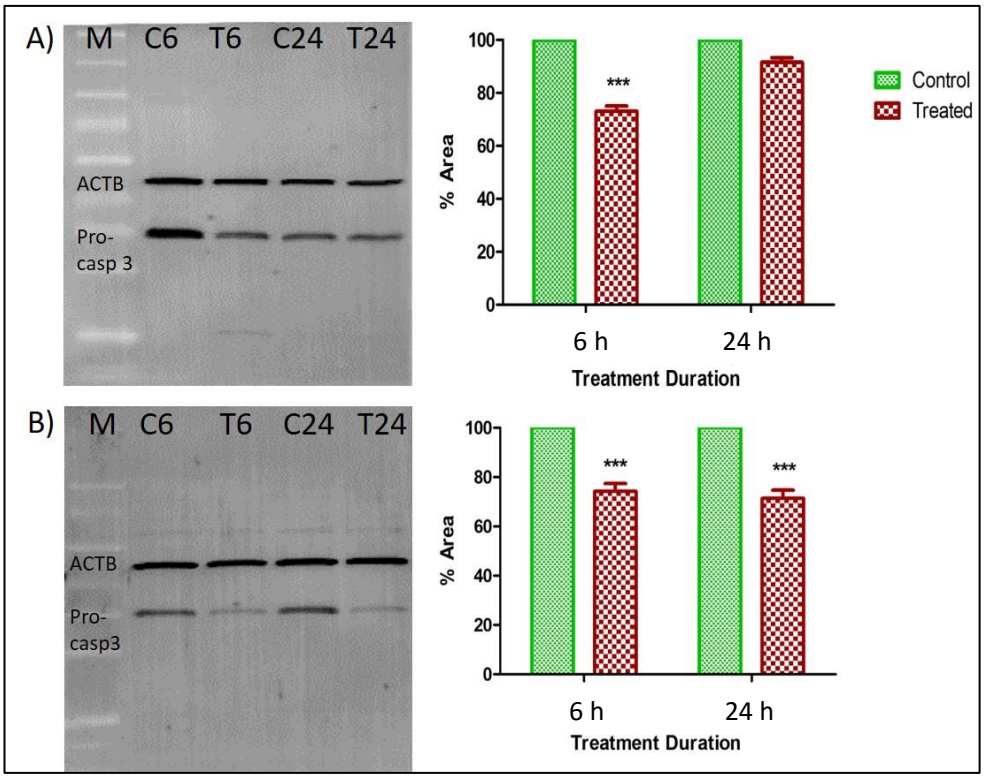

Figure 30: Western blot of pro-caspase-3. A) Shows significant reduction in pro-caspase-3 expression at 6 h frondoside A treatment duration in the CCRF-CEM cell line (left). B) Shows significant reduction in pro-caspase-3 expression at both 6 and 24 h frondoside A treatment duration in the THP-1 cell line. Right panels show the statistical analysis of the quantification of the bands

3.4.4 p53

The p53 protein is known to be mutated in the CCRF-CEM cell line. It has a missense mutation at 524 changing guanine to adenine leading to arginine amino acid substitution by glutamine at position 248 (R248Q). In this cell line, expression of the mutated p53 was decreased in the frondoside A treated group at 6 h treatment duration compared to untreated cells as shown in Figure 31.

The THP-1 cell line has a deletional mutation resulting in complete lack of expression of the protein (Guerrouahen et al., 2010), hence, the protein was not detected on WB using p53 monoclonal antibodies.

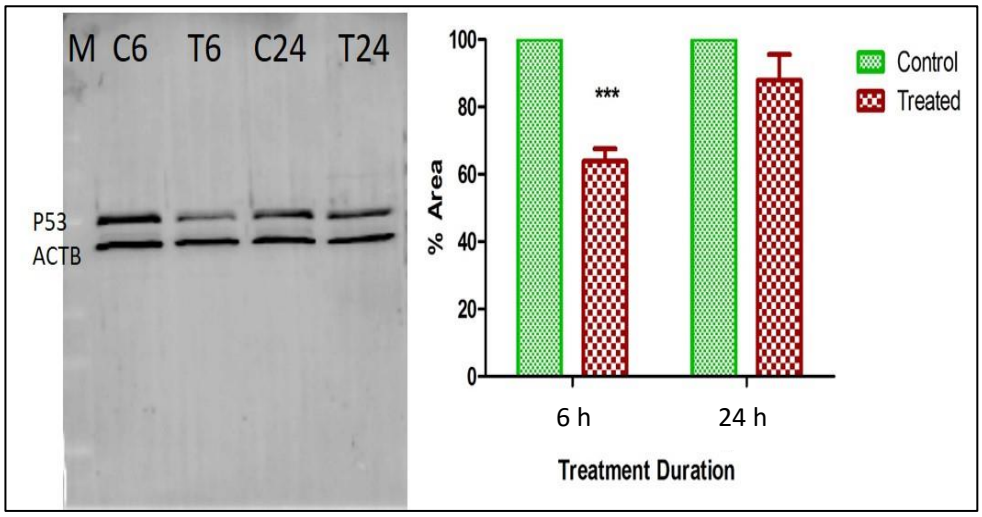

Figure 31: Western blot of p53 protein. The expression of mutated p53 protein in CCRF-CEM cells showing a significant decrease after 6 h treatment with frondoside A, but not after 24 h. The right panel shows the statistical analysis of quantification of the bands

3.4.5 p21

In the monocytic leukemia cell line, THP-1, frondoside A caused increased expression of p21 protein (CDK inhibitor 1) after 6 h of treatment. Milder significant change in the expression was seen at 24 h in the treated groups compared to the control.

The CCRF-CEM cells lack expression of p21, hence it was not tested. The protein expression and statistical analysis id shown in Figure 32.

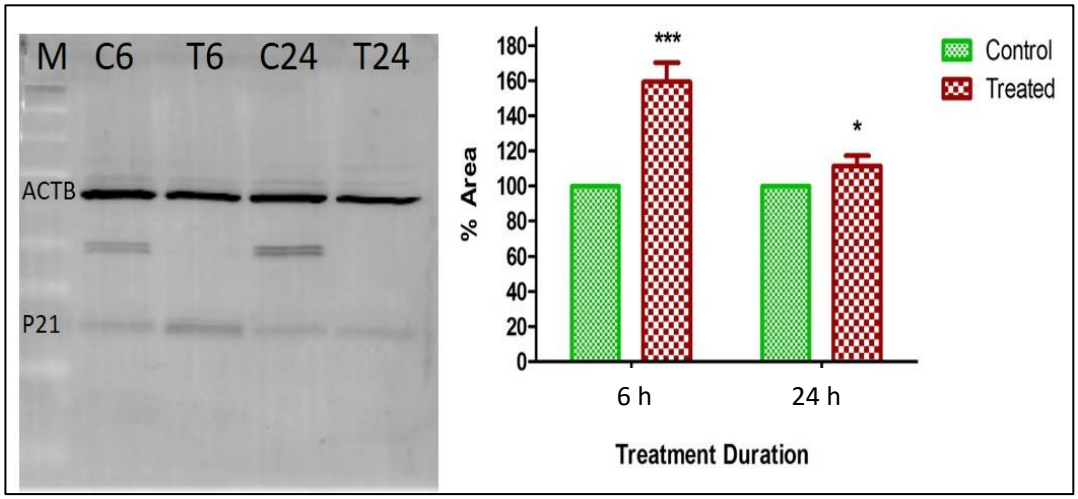

Figure 32: Western blot of p21 protein. THP-1 cells show a marked increase in p21 protein expression after 6 and 24 h treatment with frondoside A. The change in expression compared to controls was more significant at early treatment point. The right panel shows the statistical analysis of the quantification of the bands

3.5 Frondoside A Interaction with NFκB Pathway

From gene expression results, we found that in both cell lines, the frondoside A-treated groups showed marked change in the expression of multiple members of the NFκB pathway. We hypothesized that blast cells activate NFκB pathway as a survival mechanism in an attempt to escape the apoptotic effects of frondoside A.

To investigate the interaction of frondoside A with NFκB and its effect on CCRF-CEM and THP-1 cell lines, cells were treated with variable concentrations of frondoside A in combination with IC_{50} of andrographolide (an irreversible NFκB antagonist). Note that the frondoside A used in these experiments was from the stock purchased from Sigma-USA. Andrographolide IC_{50} was around 50 µM in both cell lines.

We found that andrographolide enhanced the anti-leukemia effect of low dose frondoside A in both cell lines where it significantly potentiated the effect of frondoside A in all tested concentrations. Data are shown in Figure 33.

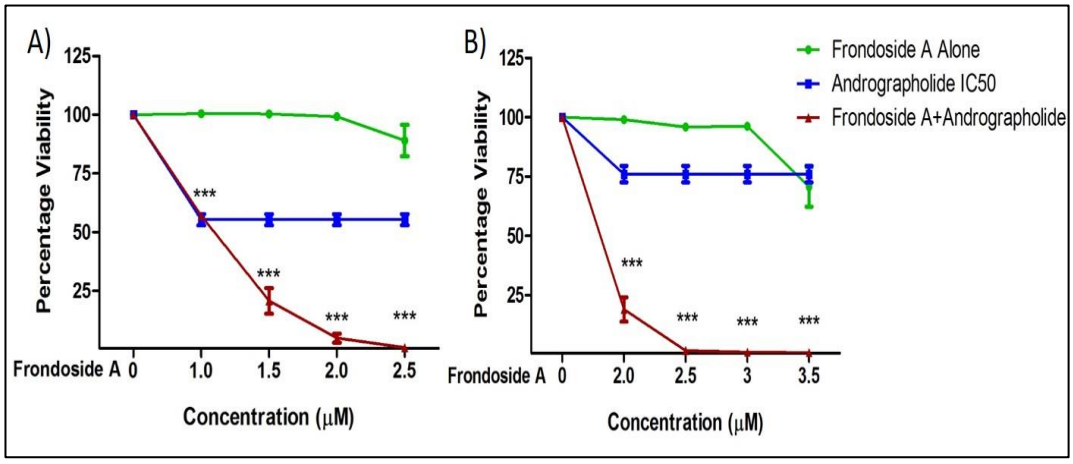

Figure 33: Frondoside A effect in-combination with andrographolide. A) The effect of low dose frondoside A was markedly enhanced when combined with andrographolide IC_{50} in the CCRF-CEM cell line in all tested concentrations. B) Similar effects were seen in the THP-1 cell line ($P < 0.001$)

The protein expression of selected NFκB pathway members was further investigated. These proteins were chosen based on the results of the gene expression profiling findings. Expression of RelB, cytoplasmic inhibitor of apoptosis protein 2 ($cIAP_2$ encoded by BRIC3 gene) and NFκB2 proteins was investigated using the western blot technique.

3.5.1 RelB

Frondoside A increased expression of RelB (v-rel reticuloemdotheliosis viral oncogene homolog B) gene in the frondoside A-treated groups after 6 and 24 h in CCRF-CEM cells. In contrast it was initially not changed in THP-1 after 6 h, but increased in the frondoside A-treated group at 24 h. However, none of the changes on

the protein level reached statistical significance. The images of the western blots are shown in Figure 34.

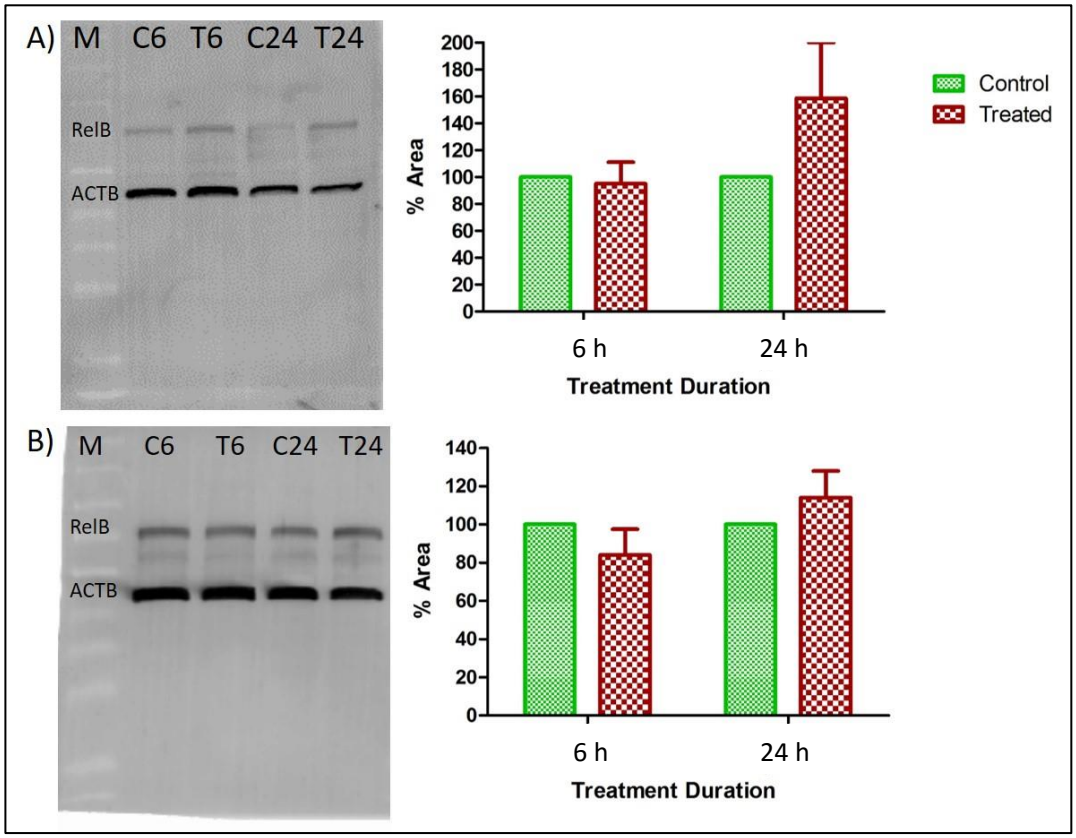

Figure 34: Western blot showing RelB protein expression. In CCRF-CEM cells (A) RelB protein expression is increased in the frondoside A-treated groups (T6, T24) compared to controls (C6, C24) at both treatment points. B) RelB protein expression in response to frondoside A treatment in THP-1 cells is unchanged. Beta Actin was used as a control for the protein loading. Right panel shows the statistical analysis of the quantification of the bands

3.5.2 cIAP2

Expression of $cIAP_2$ (cytoplasmic inhibitor of apoptosis protein 2), which is the protein product of BIRC3 gene, was not changed in response to frondoside A in CCRF-CEM cells. In contrast, expression of $cIAP_2$ was increased after 24 h frondoside A treatment in THP-1 cells but did not reach clinical significance. See Figure 35.

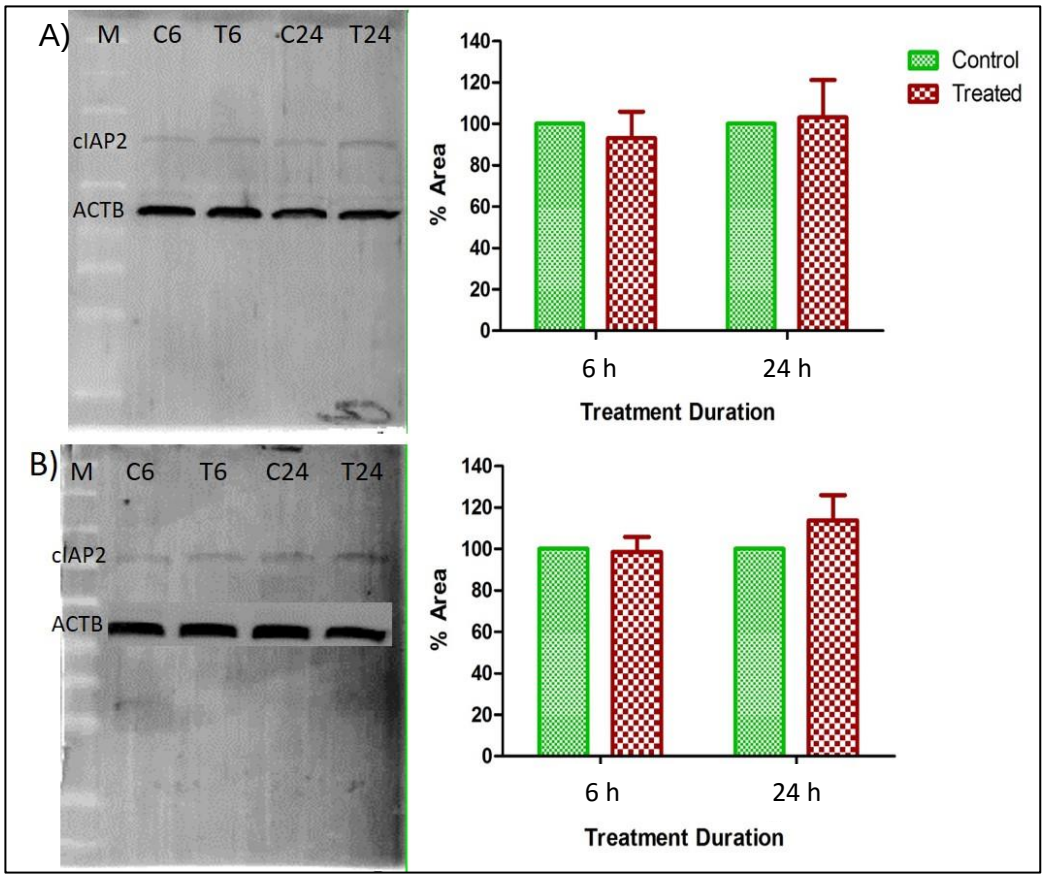

Figure 35: Western blot showing $cIAP_2$ protein expression. A) $cIAP_2$ protein expression in CCRF-CEM cells was not significantly changed by frondoside A treatment in the treated groups (T6, T24) compared to controls (C6, C24) at either treatment point. B) In contrast, $cIAP_2$ protein expression in THP-1 cells was increased by frondoside A in the 24 h treated group. Right panel shows the statistical analysis of the quantification of the bands

3.5.3 NFκB2

NFκB2 (Nuclear factor kappa light polypeptide enhancer in B-cell 2) expression was investigated for both, the full-length protein (p100) which is a 120 kDa protein and for the active (cleaved/ p52) form with MW of 52 kDa. There was an increase in the full-length protein expression following frondoside A treatment at 6 h in CCRF-CEM cells. In contrast, there was a small decrease in the p52 form that didn't reach statistical significance. Both forms of the NFκB2 protein were increased in the

frondoside A-treated group at 24 h but this did not reach statistical significance. Figure 36 shows NFκB2 protein expression in the western blot.

In THP-1 cells, NFκB2 protein expression was decreased at 6 h following frondoside A treatment ($p < 0.05$) but there was increase in expression of the full-length form after 24 h treatment duration (See Figure 36).

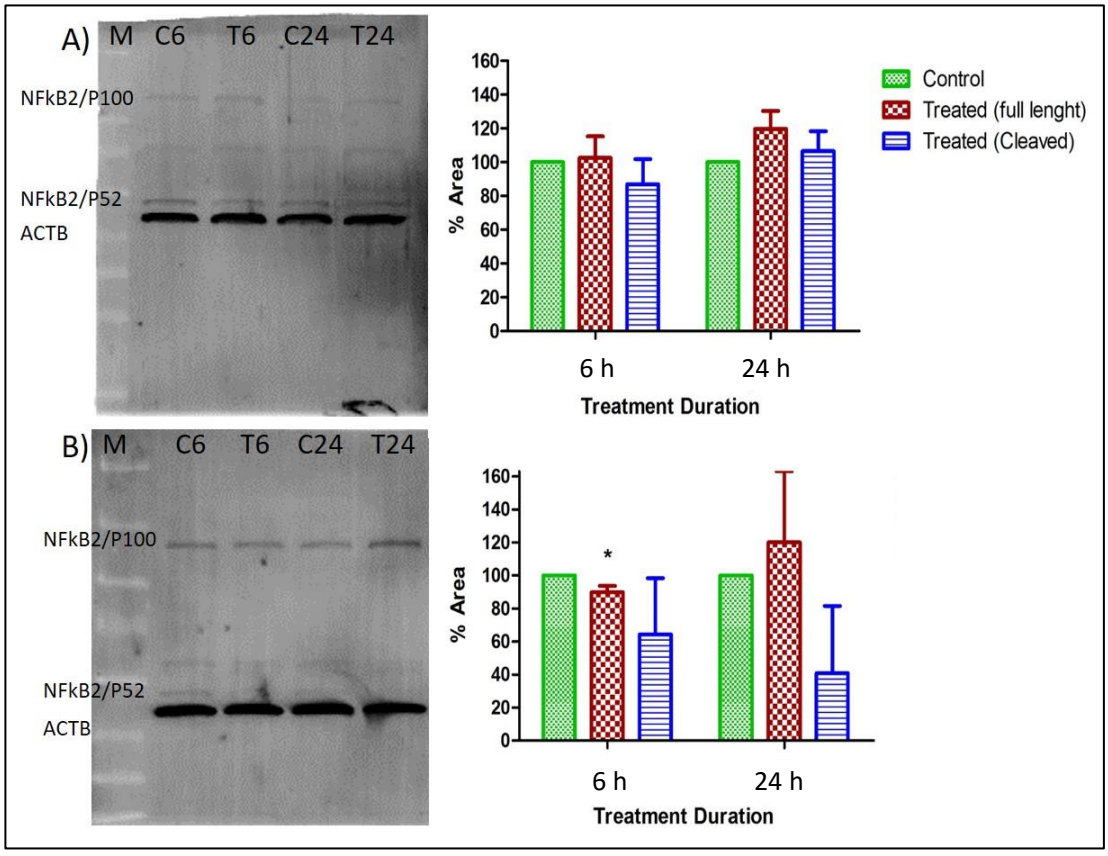

Figure 36: Western blots showing NFκB2 protein expression. A) Increased expression of the full-length NFκB2 protein in frondoside A-treated CCRF-CEM cells after 6 h treatment. In contrast, no significant change seen at 24h treatment duration. B) Decreased expression of the full-length (p100) NFκB2 protein in frondoside A-treated THP-1 cells after 24 h treatment. Right panels show the statistical analysis of quantification of the bands

Chapter 4: Discussion

Acute leukemia is a common hematological malignancy and is a major health concern. Although complete remission is achievable with the conventional chemotherapeutics (Cisterne et al., 2014; Davies et al., 2011) this is not without debilitating side-effects. The long-term side-effects of the current available synthetic drugs are difficult for patients to cope with and add burden on the health system. To address this problem, scientists have turned to the naturally available compounds to investigate their potential anti-leukemic properties (Park et al., 2012). One such compound is frondoside A, which is a triterpenoid glycoside from the sea cucumber *C. frondosa*.

Frondoside A has been known for its anti-bacterial, anti-inflammatory and immune-modulatory effects (Bordbar et al., 2011; Janakiram et al., 2015; Kalinin, Aminin, Avilov, Silchenko, & Stonik, 2008) but its anti-cancer properties have only been explored in the last 10 years. Studies have shown its potent anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects in solid tumors (Gomes, Dasari, Chandra, Kiss, & Kornienko, 2016; Li et al., 2013). Only one study has shown the pro-apoptotic effect of frondoside A on some leukemia cell lines (Jin et al., 2009). The current study is the first to investigate in detail the anti-leukemic effects of frondoside A and compare it to conventional chemotherapeutic drugs.

Frondoside A was effective in reducing leukemic blast cell viability in all the tested acute leukemia cell lines in this project; including acute T-cell lymphoblastic leukemia (CCRF-CEM), acute monocytic leukemia (THP-1) and the acute promyelocytic leukemia (HL-60) cell lines. The sensitivity of these leukemic cells to

frondoside A treatment varied, with the most sensitive being CCRF-CEM and the least sensitive was THP-1.

4.1 Frondoside A Potentiates the Effect of Other Drugs

Combinations of low concentrations of frondoside A with conventional therapeutic drugs used to treat acute leukemia (asparaginase, vincristine and prednisolone) led to more pronounced suppression of cell viability than the conventional drugs used as single agents. The CCRF-CEM and THP-1 cell lines showed increased suppressive effects on cell viability in all the combination treated groups, while the HL-60 cell line showed enhanced effects of conventional drugs only when higher compound concentrations were used.

L-asparaginase acts on leukemia blast cells by depriving the cells of the amino acid asparagine which is crucial for leukemic cell function. The impairment of the blast cell function, leading to cell death following treatment with asparaginase was enhanced by the addition of frondoside A, which also induced cell death through the induction of apoptosis (as will be discussed later). A similar explanation applies to the enhanced effect of vincristine when combined with frondoside A. Vincristine eradicates leukemia blast cells by interfering with cell division. It prevents chromosomal segregation in metaphase which in turn triggers apoptosis as a default mechanism. Prednisolone when given as a single agent for the treatment of ALL, was found to induce a short-lived remission in 50% of cases (Inaba & Pui, 2010). Its anti-cancer effect is thought to be mediated through its regulation of gene expression by interaction with activated protein 1 (AP-1) and NFκB. Frondoside A has also shown in previous studies on solid tumors to inhibit AP-1 and NFκB (Park et al., 2012). In our study (as will be explained later) frondoside A treatment resulted in the up-

regulation of multiple NFκB inhibitors and this may possibly explain the reduction in cellular viability when frondoside A is combined with prednisolone.

Studies have shown p21 activated kinase 1 (PAK1) to be highly involved in the oncogenic transformation of many tumors including acute leukemia (Kumar, Gururaj, & Barnes, 2006). This protein with a kinase activity was found to be over-expressed in tumors and was associated with more aggressive phenotype. PAK1 is involved in many physiological functions in the cell including cytoskeletal and actin modifications, cell motility, proliferation, survival and metabolism. Activation of PAK1 leads to the phosphorylation of survival pathways such as MAPK/JNK and ERK (Kumar et al., 2006; Pandolfi et al., 2015). PAK1 also activates the NFκB survival pathway through NFκB inducing kinase (NIK). A recently published study showed frondoside A to inhibit PAK1 at concentrations identical to those that exhibit anti-cancer effects (Nguyen, Yoshimura, Kumazawa, Tawata, & Maruta, 2017). It would appear that this has unraveled a new mechanism of action of this drug. The pro-survival NFκB pathway promotes proliferation, metastasis, angiogenesis and cell survival through activating multiple gene targets involved in these cellular activities (Pahl, 1999). Frondoside A through its direct inhibition of PAK1 (Nguyen et al., 2017) and hence, NFκB pathway, showed the ability to inhibit these activities, which are major characteristics of cancer cells. Frondoside A promotes cell death through the intrinsic apoptotic pathway where the pro-apoptotic protein expression was increased and the anti-apoptotic proteins decreased in response to the treatment (Li et al., 2008). Those apoptotic proteins are the product of genes that are targets of the NFκB pathway (Pahl, 1999). PAK1 has shown the ability to inhibit activation of the pro-apoptotic protein Bad on the mitochondrial outer membrane that leads to inhibition of cell death, while frondoside A promotes the activation of Bad. Frondoside A also inhibited breast cancer

migration and invasion through inhibiting MMP-9 (Al Marzouqi et al., 2011; Park et al., 2012), which is also a target gene product for NFκB activation (Pahl, 1999). Angiogenesis is required for the survival and metastasis of cancer cells and this is also inhibited by the treatment with frondoside A (Attoub et al., 2013) and the growth factors required for this process are products of genes under the influence of NFκB pathway. Inflammation is triggered through the activation of NFκB, which binds the DNA promoter genes and up-regulates multiple pro-inflammatory molecules such as cytokines and eicosanoids (Pahl, 1999). On the other hand, frondoside A has been shown to enhance immunity by stimulating the lysosomal and phagocytic activity of macrophages (Aminin et al., 2008).

Inhibition of PAK1 by frondoside A, inhibits PAK1 activation of NFκB pathway. As mentioned before, PAK1 activates NFκB through the activation of NFκB inducing kinase (NIK) that is required for the non-canonical pathway induction. It was also found to activate NFκB via activating inhibitor of kappa B kinase beta (IKKβ) that is needed to release NFκB from its inhibitor (IKB) (Kumar et al., 2006).

4.2 Frondoside A Effect on Apoptosis Genes and Proteins

Frondoside A changed the expression of many genes and proteins in acute leukemia cell lines that are involved in apoptosis. In the CCRF-CEM cell line, there was up-regulation of Bcl-2 like-1 (BCL2L1) gene which encodes the pro-apoptosis protein Bcl-Xs and the anti-apoptotic protein Bcl-xL by an alternative splicing of the transcript. Both isoforms are located on the mitochondrial outer membrane and regulate the voltage channels controlling the release of cytochrome *c* and hence, the intrinsic apoptosis pathway. The gene is also a transcriptional target for NFκB pathway and the up-regulation might have been a direct effect of NFκB pathway activation.

Although the gene expression was increased, the protein product of the gene was not examined. Interestingly, PAK1 is known to increase the nucleus translocation of signal transducer and activator of transcription 5 (STAT5) which is a transcription factor targeting Bcl-xL gene (Chatterjee et al., 2014) and studies have shown frondoside A to inhibit this action.

Treatment with frondoside A in CCRF-CEM cells led to the up-regulation of phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1). This gene encodes Noxa, a member of the Bcl-2 family proteins that promotes apoptosis by enhancing the change in the mitochondrial membrane potential. Noxa also known to degrade the pro-survival protein Mcl-1 (myeloid cell leukemia sequence 1). Noxa is regulated by p53 in normal cells and since in CCRF-CEM cells, p53 is mutated, the up-regulation of Noxa must be p53 independent. Noxa expression is also regulated by other genes of the p53 family, like p73 and p63. However, p73 is activated only in the presence of NFκB (Martin, Trama, Crighton, Ryan, & Fearnhead, 2009).

Although Bax gene expression was not altered by the treatment, both cell lines showed significant decrease in the protein expression on western blot analysis. Since we have demonstrated the induction of apoptosis as early as 1h after frondoside A treatment (Annexin V assay), this decrease in Bax might be due to consumption or degradation after activating the mitochondrial pathway (Guerrouahen et al., 2010).

There was also a marked up-regulation of caspase 6, the executioner caspase. The expression of caspase 1, 2, 7, 8, 10 and Bcl-2 antagonist/ killer 1 (Bak) genes were more than 2-fold increased in the 24 h frondoside A treated cells compared to 6 h treated, indicating the activation of those pro-apoptosis genes transcription by frondoside A. When comparing these gene changes to the untreated (control) cells, the

up-regulation was less than 2-fold, hence, frondoside A might have activated the protein products of those genes rather than significantly affecting the gene transcription. This is in support of the early morphological changes consistent with apoptosis after 1h of frondoside A treatment. Another explanation might be that the increase in expression of the pro-apoptosis genes in the treated groups occurred before 6 h and since our gene profiling experiments were investigating the changes only at two time points (6 h and 24 h), earlier changes might have been missed.

Our experiments showed evidence to support activation of caspase 3 without an effect on its mRNA level. We have shown frondoside A to induce apoptosis in CCRF-CEM cell line (where annexin positive cells were present in the treated groups) as early as 1 h post-treatment. Western blot analysis for caspase 3 showed decrease in the pro-caspase 3 protease expression at 6 h and 24 h treatment durations. We could not detect a band corresponding to the active cleaved form on western blot and one explanation for this might be that caspase-3 was activated early after treatment, but the active form was consumed or degraded at time of protein extraction (Hu, Li, Liu, Miao, & Yao, 2015) since our western blot experiments were performed only at two time points (6 and 24 h) post frondoside A treatment. This might indicate that frondoside A activates those proteases and proteins that are present in the cell without inducing the transcription and hence the translation of new proteins (Davies et al., 2011; Soussi & Wiman, 2015) as is the case with other chemotherapeutic agents (Hu et al., 2015). Frondoside A also might have initiated cell death in these cells in a caspase independent manner (Dyshlovoy et al., 2016; Goldar et al., 2015; Jin et al., 2009) such as the apoptosis-inducing factor (AIF) and endonuclease G pathway, then later, the intrinsic pathways get activated post 24 h treatment which has been reported in other types of chemotherapy drugs (Guerrouahen et al., 2010) and couldn’t be detected here

due to the time experiments not investigating changes beyond 24 h. In the CCRF-CEM (T-cell ALL) cells, other mechanisms might have activated the executionary pathway (Caspase-3 and 7) directly without much involvement of other caspases from the intrinsic or extrinsic pathways. In lymphocytic cells, the granzyme/perforin apoptosis pathway might have been activated (Goldar et al., 2015) and this pathway was not investigated. Activation of the initiator caspase 12 can also directly activate the executionary pathway (Goldar et al., 2015) and was also not investigated.

Studies on the effect of frondoside A in solid tumors confirmed the induction of the intrinsic apoptosis pathway by the compound but in CCRF-CEM cells, treatment with frondoside A also up-regulated the gene expression of multiple death receptors and adaptors that are involved in the extrinsic apoptosis pathway such as the death effector domain containing 2 (DEDD2), the death associated protein kinase 1 (DAPK1), the leucine-rich repeats and death domain (LRDD) which encodes for a protein that activates death domains by binding to Fas associated death domain (FADD) and the NATCH leucine-rich repeats and pyrin domain containing 1 (NALP1). The later gene encodes for caspase recruitment domain (CARD). Frondoside A also up-regulated phosphoprotein enriched in astrocytes 15 (PEA15) that encodes Pea15 protein which contains death effector domain (DED). These findings suggest that frondoside A induced apoptosis in CCRF-CEM cells might involve both, extrinsic and intrinsic apoptosis pathways.

The pro-survival protein Bcl-2 is usually overexpressed in leukemia and is associated with inferior outcome (Goldar et al., 2015; Testa & Riccioni, 2007). The Bcl-2 protein was mildly down-regulated in frondoside A-treated CCRF-CEM cells at 6 h. The protein as mentioned before binds the pro-apoptosis proteins Bad, Bik, Puma,

Bid and Noxa leading to inhibition of apoptosis. Its decreased expression after frondoside A treatment resulted in attenuation of its inhibitory effect on the pro-apoptosis proteins (Delbridge & Strasser, 2015; Goldar et al., 2015). A limited number of studies have shown that lowering Bcl-2 level using antisense to Bcl-2 improve patient response to treatment in acute leukemia (Testa & Riccioni, 2007). The myeloid cell leukemia sequence 1 (Mcl-1) gene, which is also an anti-apoptotic member of the Bcl-2 family, was more than 2- fold increased after 24 h frondoside A treatment compared to 6 hr.

Although p53 is mutated in the CCRF-CEM cell line (Davies et al., 2011), the function of this mutation has not been investigated. Recently, many studies have demonstrated that some cancer associated p53 mutations are causing an oncogenic gain of function which can drive the leukemic blast cells survival (Mantovani et al., 2015; Soussi & Wiman, 2015). In the present study, treatment with frondoside A resulted in the decrease of the mutated protein in response to treatment at both time points being more significant at 6 h treatment, which might have contributed to its anti-leukemic effect. It is well demonstrated in previous studies that a functional p53 is important for achieving the desired response to chemotherapeutic drugs, while cancers with dysfunctional p53 are known to be more resistant to treatment (Davies et al., 2011; Mantovani et al., 2015). The tumor suppressor p21 protein is repressed in this cell line due to epigenetic alterations (Davies et al., 2011).

In cells where p53 is non-functional, other members of the same family of proteins, i.e. p63 and the more structurally and functionally similar p73 proteins and their isoforms may act to induce apoptosis as substitute for p53 (Hollstein & Hainaut, 2010; Mantovani et al., 2015; Menchinskaya, Pislyagin, et al., 2013). The protein p73

can be activated by DNA damage (that is caused by conventional drugs in treated cells) leading to the phosphorylation of p38/MAPK (Mantovani et al., 2015; Saha et al., 2015). This leads to an increase in p73 transcriptional function promoting the promoters of pro-apoptosis genes (Mantovani et al., 2015). See above relationship between these proteins and Noxa.

Many p53 mutations in cancer are missense mutations that affect the DNA binding site but keep the trans-activation site intact. This might explain how p53 acts in CCRF-CEM cells in transcription-independent manner (Naujokat et al., 2000) by affecting the balance and activation/inactivation of different pro- and anti-apoptosis proteins rather than affecting their expression (Mantovani et al., 2015). Frondoside A being functional in p53-independent manner makes it an attractive compound since many cancers are characterized by loss of p53.

In the monocytic acute leukemia cell line (THP-1), there was a similar up-regulation of Bcl2 like 1 (BCL2L1) and phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1 encoding Noxa) genes. In contrast to CCRF-CEM, THP-1 cells showed marked up-regulation in additional pro-apoptosis genes involved in the intrinsic and executioner pathways, including the BH3 interacting domain death agonist (BID), which encodes the Bid protein, which upon apoptosis activation enhances the insertion of Bax protein (Bcl-2 associated X protein) into the mitochondrial membrane, leading to the opening of the mitochondrial voltage-dependent anion channels and the release of cytochrome *c*. The Bcl-2 related ovarian killer (Bok) gene was also significantly up-regulated in these cells post frondoside A treatment. It encodes Bok protein, which is another pro-apoptotic BH1-4 containing Bcl-2 protein family member.

In THP-1 cells there was marked up-regulation of caspase 4, 3, 6 and 7. At the protein level, analysis of caspase 3 on western blot showed similar changes as seen in CCRF-CEM cells with the activation of pro-caspase 3. Unlike CCRF-CEM cells, frondoside A treatment of THP-1 cells up-regulated caspase 3 gene expression as well as activating the protease at the protein level.

Other pro-apoptosis genes were more than 2-fold increased after 24 h treatment compared to 6 h treatment but were not significantly changed compared to the control cells. These included caspase 1, caspase 10, Bak, Bad and Bax. Again, when the Bax protein level was investigated, we showed decreased expression of the protein in frondoside A-treated groups. This might be due consumption or degradation after activating the mitochondrial pathway post 6 and 24 h of treatment (Guerrouahen et al., 2010).

In THP-1 cells, although there was no change in the gene expression of caspase 8, caspase 10 showed more than 2-fold up-regulation after 24 h treatment compared to 6h. Studies have shown that in some acute leukemia cells, activation of caspase 10 can substitute for caspase 8 in triggering apoptosis. Caspase 10 can cleave Bid and activate the mitochondrial apoptosis pathway (Cisterne et al., 2014).

As seen in CCRF-CEM cells, treatment with frondoside A in THP-1 cells was associated with marked up-regulation of death/effector receptors and domains. These included PEA15 gene and tumor necrosis factor receptor superfamily 1A associated via death domain (TRADD) that encodes for an adaptor molecule, which interacts with TNF-R1 and mediates apoptosis through decreasing the recruitment of inhibitor of apoptosis proteins (IAPs) and activates pro-caspase 8. Further detail of this interaction will be discussed in section 4.3. The THP-1 cells also showed up-regulation of leucine-

rich repeats and death domain containing (LRDD), death effector domain containing 2 (DEDD2) and Huntingtin interacting protein 1 (HIP1). The later gene encodes for a protein similar in structure in death effector domains and might promote cell death via activation of caspase 3 but its exact role is not clear.

The Bcl-2 gene was significantly down-regulated at both treatment points in THP-1 cells and the protein level change was consistent with this down-regulation. In acute myeloid leukemia, the pro-survival Bcl-2 family protein, Mcl-1 is found to be up-regulated (Delbridge & Strasser, 2015; Testa & Riccioni, 2007) and in our study Mcl-1 gene was up-regulated in THP-1 cells following frondoside A treatment. The protein Mcl-1 has a similar carboxy terminus to Bcl-2 and it also controls the intrinsic apoptosis pathway by inhibiting the change in mitochondrial membrane potential. Many studies have failed to demonstrate a correlation between Mcl-1 level and outcome in leukemia (Testa & Riccioni, 2007).

In the monocytic leukemia cell line (THP-1), p53 is not expressed because of a mutation that causes deletion (Eder et al., 1992) but studies have shown p21 to be functional in these cells and causes cell cycle arrest independent of p53 (Guerrouahen et al., 2010; Kreis et al., 2015). Here we saw that frondoside A markedly increased the expression of p21which undoubtedly contributes to the anti-leukemia effect in this cell line. A possible mechanism by which frondoside A increases the expression of p21 cyclin dependent kinase inhibitor is through its inhibitory effect on PAK1. PAK1 is known to suppress p21 and its inhibition by frondoside A removes its suppressive effect on p21 (Nguyen et al., 2017).

In HL-60 cells (promyelocytic leukemia cell line), both p53 and p21 are non-functional because of deletion (Eder et al., 1992) and the effect of frondoside A on cell

viability in these cells must be due to other mechanisms independent of the proteins. The deletions might also explain the reason why those cells were more resistant to frondoside A.

Frondoside A acts as a PAK1 inhibitor in leukemia cells but the response of the cells is cell-type and circumstances dependent (Yun, Shin, Stonik, & Park, 2016). In THP-1 cells frondoside A not only activated caspase-3 but also affected many apoptosis- related genes, including modest up-regulation of caspase-3, caspase-4, caspase-6, Bok and HIP1 post 24 h. Caspase-7 which is an executioner protease showed early up-regulation (at 6 h). The pro-apoptosis Bcl-2 family proteins genes Bid and PMAIP1 (encoding Noxa) were also up-regulated early, indicating frondoside A to be acting in THP-1 cells in a caspase-dependent manner, activating the mitochondrial pathway and affecting gene transcription as well as activating the proteins. In CCRF-CEM cells, expression of the Bid and caspase 3 genes did not change in response to the treatment. The apoptotic protease activating factor 1 (APAF1) is essential for the formation of apoptosome in the mitochondrial pathway (Jia et al., 2001) and the corresponding mRNA was down-regulated in THP-1 cells but not changed in CCRF-CEM cells. Studies have shown that 25% of leukemia cell lines and 42% of primary blast cells to have low expression of APAF1 mRNA due to gene silencing (Jia et al., 2001; Testa & Riccioni, 2007).

Previous studies have demonstrated the activation of the intrinsic apoptosis pathway in solid tumors in response to frondoside A treatment (Al Marzouqi et al., 2011; Li et al., 2008). In our leukemia cell lines, it is clear that frondoside A significantly affects and activates the transcription of many genes involved in the extrinsic pathway as well. A study conducted in THP-1 cells showed that despite

blocking caspases, frondoside A was still able to induce apoptosis in those cells but to a lower extent. The study suggested that frondoside A induces cell death in THP-1 cells, at least initially, in a caspase-independent manner (Jin et al., 2009) but in this study, the suppression of caspase activity might have been partial.

Frondoside A concentrations that induced apoptosis in our study were comparable to those reported in solid tumors (Al Marzouqi et al., 2011; Al Shemaili et al., 2014; Attoub et al., 2013).

4.3 Frondoside A Interaction with TNF Pathway

The tumor necrosis factor (TNF) superfamily is known to regulate inflammatory responses, cell differentiation, proliferation and cell death, depending on the state of the cell (Etemadi et al., 2013). The ligands of the TNF family can bind multiple receptors with different affinities leading to different biological responses. In response to frondoside A, both T-cell ALL and the monocytic leukemia cell lines showed marked involvement of the TNF pathway, which gets activated following the binding of a ligand to one of the pathway specific receptors (Etemadi et al., 2013; Goldar et al., 2015). Both cells showed marked gene up-regulation of multiple receptors, adapter proteins and death effector domains. Many TNF pathway gene members are also transcriptional targets of NFκB pathway (the interaction of both pathways is discussed in section 4.4).

The CCRF-CEM cells showed down-regulation of the tumor necrosis factor superfamily 10A (TNFRSF10A, also known as death receptor 4; DR4 and TRAIL-R1), which upon the binding of TRAIL-1 (TNF related apoptosis inducing ligand) induces apoptosis in the cells (Wang & El-Deiry, 2003). The gene expression of

TNFRSF25 (DR3) and TNFRSF21 (DR6) was more than 2-fold increased post 24 h frondoside A treatment. The up-regulation of DR6 is known to favor apoptosis through its interaction with TRADD (TNFRSF1A associated via death domain) (Pobezinskaya & Liu, 2012). Depending on the cell conditions the up-regulation of DR3 can interact with FADD, and this interaction leads to the activation of caspases and hence, apoptosis (Luqman & Pezzuto, 2010). Interaction of DR3 with the adaptor molecule TRADD usually favors inflammatory response and the activation of NFκB pathway, as shown in Figure 37. The tumor necrosis factor β (lymphotoxin alpha, LTA) and TNF-C (lymphotoxin beta, LTB) genes were up-regulated and binding of the ligands encoded by these genes to the up-regulated TNFRSF1A (TNF-R1) promotes inflammatory cytokine production (Luqman & Pezzuto, 2010). Studies have shown that upon binding to its receptor TNF-β promotes degradation of the inhibitor of nuclear factor kappa B- alpha (IKB-α) leading to the release of RelA/p50 and its translocation to the nucleus for its transcriptional activity which includes up-regulation of inflammatory cytokines and their receptors and many other proteins (Etemadi et al., 2013).

The involvement of the TNF pathway in physiological processes in the cell is complex. Activation of ligands and receptors of this pathway can either induce apoptosis or promote inflammation through the interaction with NFκB pathway (Etemadi et al., 2013). Reciprocally, NFκB pathway activation can activate the transcription of multiple TNF pathway ligands, receptors and other pathway member proteins (Hoesel & Schmid, 2013).

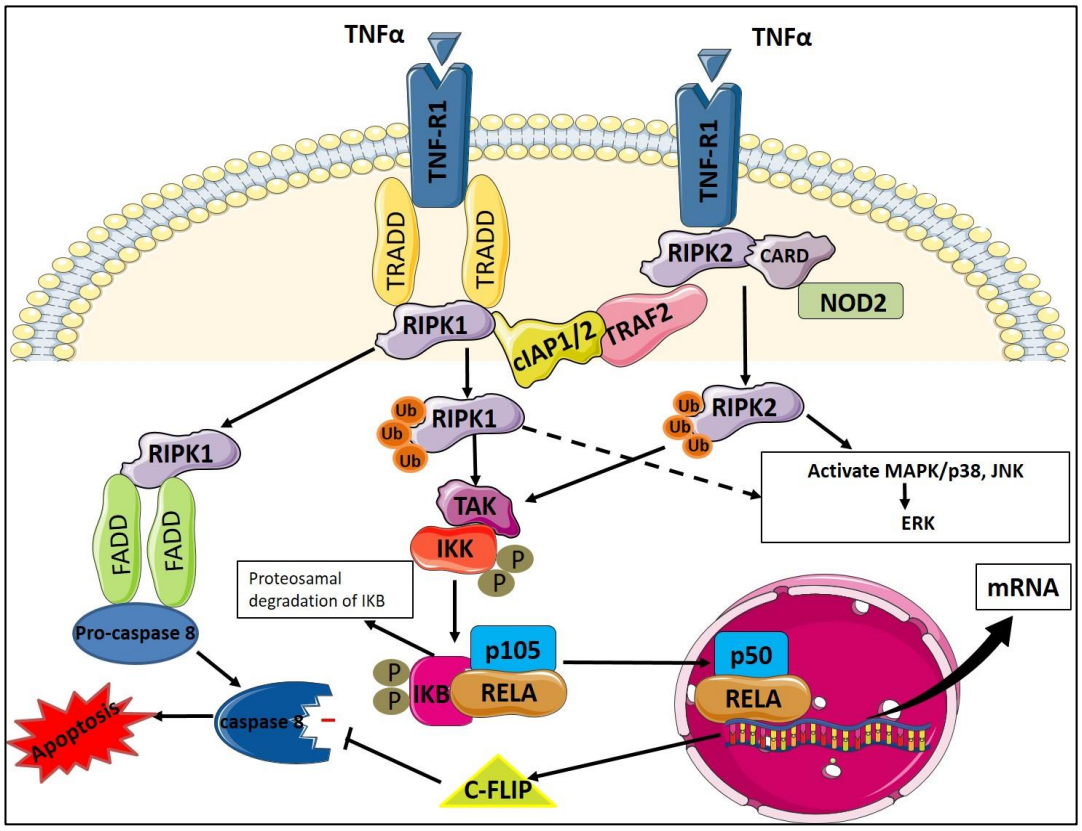

Figure 37: Pathways interaction. When TNF-α binds TNF-R1, RIPK1 phosphorylates TRADD and recruits TRAF2 which in the presence of c-IAP1/2 results in the polyubiquitination of RIPK1 leading to activation of IKK and hence, the degradation of IKB and the translocation of p50/RelA (NFκB canonical pathway) into the nucleus. Polyubiquitinated RIPK1 can also activate the MAPK/P38/JNK/ERK pathways. When RIPK1 is not ubiquitinated, it binds FADD and activates caspase 8 and apoptosis occurs. Activation of TNF-R1 can also activate the canonical NFκB pathway through the interaction of CARD domain of RIPK2 with NOD2. Activation of NFκB leads to the production of inflammatory cytokines and inhibitors of apoptosis such as c-FLIP

In THP-1 cells in addition to the up-regulation of TNFRSF1A (TNF-R1), DR3, DR6 and TNF-β (LTA) there was a significant up-regulation of TNFRSF1B (TNF-R2), DR4, TNFRSF10B (DR5 also known as TRAIL-R2), TNF (TNF-α) and Fas (TNFRSF6). The Fas gene encodes a death receptor that when activated, induces apoptosis in the cell through the formation of DISC (death induced signaling complex), this is followed by the internalization of the complex to interact with FADD (the adaptor molecule) leading to the activation of caspase 8/10 (Goldar et al., 2015).

The role of the cytokine TNF-α in acute leukemia is controversial, some studies reported its elevation to be associated with blast cells suppression (Cisterne et al., 2014) while others demonstrated its association with increased leukemia cell proliferation (Cisterne et al., 2014) depending on the state of the cells and the receptor involved (activated). Binding of TNF-β (LTA) or TNF-c (LTB) to LTB receptor (LTB-R) mediates lymphoid organ development while binding of the same ligands to TNF-R1 can either lead to apoptosis or activation of NFκB with promotion of inflammatory cytokine production (Etemadi et al., 2013). TNF-α binds TNF-R1 and phosphorylates MAPK (p38), ERK and c-JNK signaling pathways that eventually lead to the activation of NFκB (Etemadi et al., 2013). Both TNF-α and TNF-β also degrade inhibitor of nuclear factor kappa B alpha (IKBα) leading to the activation of RelA/p50 and its translocation into the nucleus (Etemadi et al., 2013) as mentioned above.

The fate of the cell after TNF-R1 activation is controlled by a complex interaction with other molecules, enzymes/ligase and adaptor proteins. The receptor-interacting serine-threonine protein kinase group of genes (RIPKs) encode a family of kinases of 7 members (RIPK1 to RIPK7), which are known to be key players in the decision of the cell response (Etemadi et al., 2013). The CCRF-CEM cells showed marked up regulation of RIPK2 gene after 6 h of frondoside A treatment while THP-1 cells showed significant up-regulation of RIPK1 as well as RIPK2. Both kinases contain a kinase domain and an intermediate domain. The RIPK1 has a c-terminal death domain while the c-terminal of RIPK2 contains a caspase activation and recruitment domain (CARD) (Humphries, Yang, Wang, & Moynagh, 2015).

When TNF-α binds TNF-R1, it activates the receptor and this leads to the interaction with the adaptor TRADD and RIPK1 (Humphries et al., 2015). This results

in the recruitment of E3 ubiquitin ligases such as the tumor necrosis factor receptor associated factor 2 (TRAF2) and further recruitment of the inhibitors of apoptosis, c-IAP_1 and c-IAP_2. The RIPK1 gets polyubiquitinated. This results in the phosphorylation, and hence, the activation of inhibitor of kappa B kinase (IKK) by ubiquitin-dependent kinase (TAK1). The inhibitor of kappa B kinase further phosphorylates the IKB which subjects it to degradation by proteasomes releasing NFκB to be translocated to the nucleus (Figure 37). Activation of the NFκB pathway is known to increase the expression of cellular c-FLIP which is a negative regulator of apoptosis. The c-FLIP inhibitory protein inhibits apoptosis induced via TNF-R1, death receptor 4 (DR4) and DR5 by interacting with death inducing signaling complex (DISC) and preventing the activation of pro-caspase 8 and 10 (Goldar et al., 2015; Hoesel & Schmid, 2013; LaCasse et al., 2008). The activation of NFκB also inhibits the apoptotic effect of the TNF pathway through the up-regulation of multiple cytoplasmic inhibitors of apoptosis proteins (cIAP) including c-IAP_2 (Goldar et al., 2015).

Ubiquitinated RIPK1 can also activate MAPK/p38 and JNK pathways with subsequent activation of ERK signaling pathway (Humphries et al., 2015). If the activation of TNF-R1 is associated with RIPK1 and the latter is not ubiquitinated, this favors the binding of RIPK1 to FADD adaptor molecule with subsequent activation and cleavage of pro-caspase 8 with further activation of the executioner apoptosis pathway (Humphries et al., 2015). RIPK2 kinase can also either induce apoptosis through caspase 8 or activate NFκB through the interaction with nucleotide-binding oligomerization domain-containing protein 2 (NOD2), depending on the ubiquitination and the kinase status of RIPK2 (Etemadi et al., 2013; Humphries et al., 2015). The RIPK's can also activate necroptosis pathway (Humphries et al., 2015).

4.4 Frondoside A Interaction with NFκB Pathway and Its Inhibitors

In cancer, NFκB can be activated by direct mutations of related genes but this is not very common (Hoesel & Schmid, 2013). Mutations of Bcl3 and inhibitor of kappa kinase complex (IKK) are also seen which can affect the NFκB signaling pathway (Hoesel & Schmid, 2013). The activation usually happens in response to the secretion of activating cytokines from the tumor cells or its microenvironment (Hoesel & Schmid, 2013). Interaction of NFκB with other transcription factors, either by direct binding or co-binding to the DNA promoter region can activate or suppress NFκB function (Hoesel & Schmid, 2013). Such interaction is seen between the tumor suppressor protein p53 and NFκB; p53 inhibits the transcriptional activity of NFκB and reciprocally NFκB inhibits p53 (Hoesel & Schmid, 2013). One of the normal functions of wild-type p53 is to inhibit inflammation by antagonizing the NFκB pathway (Hoesel & Schmid, 2013). Loss of this protein either by deletional mutation (such as in THP-1 cells) or other missense mutation (as in CCRF-CEM cells) might be an explanation for the increased expression of NFκB pathway genes in these cell lines. Further increase in the gene expression of the NFκB pathway in cancer cells can be due to activation in response to endogenous survival triggers to counteract and resist the apoptosis inducing drugs/ compounds (Mercurio & Manning, 1999). Since leukemias arise from cancerous stem cells, those have a constitutively activated NFκB pathway (Rinkenbaugh & Baldwin, 2016).

Studies have shown that loss of p53 function by mutation can lead to the activation of tumor-promoting inflammation and enhance NFκB activity in response to TNF-α stimulation (Mantovani et al., 2015). In the acute leukemia cell lines used in this project, the elimination of the inhibitory effect of the wild-type p53 protein can be

the cause of the up-regulation of NFκB signaling pathway (Hoesel & Schmid, 2013). As mentioned before, NFκB pathway once activated, can activate the TNF pathway creating a loop of feed-back activation rendering the NFκB pathway constitutively active.

In the T-cell lymphocytic acute leukemia cell line (CCRF-CEM), there was a marked up-regulation of $NF\kappa B_2$ and Relb (v-rel reticuloendotheliosis viral oncogene homolog B) indicating the activation of the alternative/ non-canonical NFκB pathway (shown in Figures 1 and 38).The expression of the gene encoding inhibitors of kappa B kinases β and γ (IKBKB and IKBKG) were also increased, which enhances IkB degradation and the release of NFκB to be translocated into the nucleus (Hoesel & Schmid, 2013). When the protein expression of $NF\kappa B_2$ (both, the full length p100 and the cleaved active form p52) and Relb were investigated, there was no significant change compared to the control cells at either time point.

We have also observed the up-regulation of Bcl-3 gene. Studies have shown that when the inhibitory ankyrin repeats of $NF\kappa B_1$ (p105) and $NF\kappa B_2$ (p100) are cleaved they produce the active forms p50 and p52 respectively (Gilmore, 2006; Gilmore & Herscovitch, 2006). These two proteins are transcriptional suppressors in the homodimeric form since they lack the transactivation domain but when bound to Bcl-3 (with its intact transactivation domain) they turn into transcriptional activators (Hoesel & Schmid, 2013) as is the case when they are bound to RelA or RelB respectively. The activation of NFκB pathway through Bcl-3 binding is known as the atypical activation pathway (Gilmore & Herscovitch, 2006).

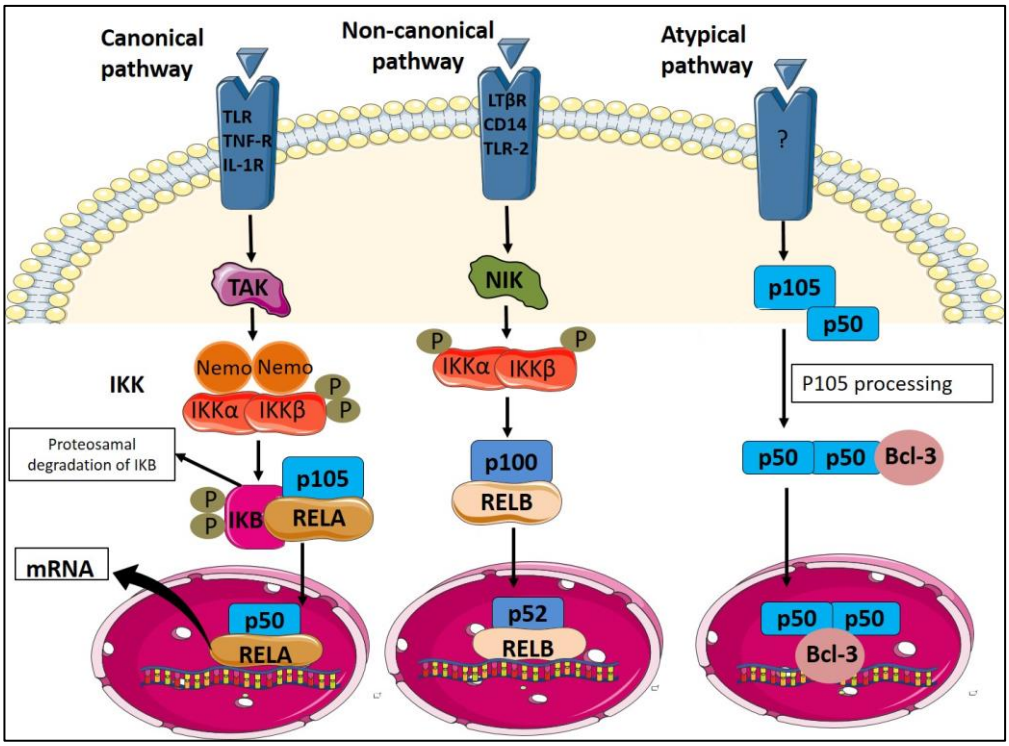

Figure 38: The NFκB activation pathway. The canonical pathway is activated through Toll-like receptor (TLR), TNF-R and IL-1R. It involves the activation of IKK by TAK and the phosphorylated mediation of IKB degradation with the translocation of p50 to the nucleus with RelA after cleavage of p105. The non-canonical pathway involves the activation of LTB receptor by LTB (TNF-c) or the activation of DR14 and TLR-2 by LTA (TNF-β). It requires the activation of NIK and the translocation of p52/RelB complex to the nucleus. The alternative pathway activation mechanism is unknown and requires Bcl-3 to carry processed p50 to the nucleus

In response to frondoside A treatment, CCRF-CEM cells showed up-regulation of the expression of BIRC3 gene that encodes the cellular inhibitor of apoptosis protein 2 (c-IAP$_2$). As mentioned in the previous section (see Figure 37), c-IAP$_2$ inhibits apoptosis through TNF-R1 and augments the activation of NFκB pathway when binding to RIPK1 and TRAF2 (Humphries et al., 2015). Analysis of c-IAP$_2$ protein expression showed no significant change. The gene encoding X-linked inhibitor of apoptosis protein (XIAP), BIRC4 was down-regulated in the treated groups. XIAP can

directly bind caspase 3 and inhibit apoptosis and its down-regulation by frondoside A favors the enhancement of apoptosis (LaCasse et al., 2008).

The gene expression of caspase activation and recruitment domain 6 (CARD6), which encodes a protein involved in signal transduction and activation of NFκB pathway (Stehlik, Hayashi, Pio, Godzik, & Reed, 2003) was markedly reduced.

Treatment with frondoside A resulted in the up-regulation of the NFκB inhibitors; NFKBIA, NFKBID (TA-NFKBH) and NFKBIZ that code for IKBα, IKBδ and IKBZ, respectively (Gilmore & Herscovitch, 2006).

The monocytic acute leukemia cell line (THP-1) showed marked changes in the genes of the NFκB pathway in response to frondoside A treatment. Not just a greater number of genes were affected but also the magnitude of the changes in expression was higher. This might explain our finding of THP-1 cells being more resistant to the treatment compared to CCRF-CEM cells (THP-1 cells frondoside IC_{50} was 3.0 μM compared to 1.5 μM).

Again in THP-1 cells, the non-canonical pathway genes were markedly up-regulated including $NF\kappa B_2$ and Relb. The protein expression analysis for these gene products showed little change. Only a modest decrease in the protein expression of the full length $NF\kappa B_2$ was observed after 6 h treatment ($p < 0.05$). In these cells, although RelA gene expression didn't change in response to frondoside A treatment, $NF\kappa B_1$ and Rel (c-Rel) genes were highly up-regulated which might indicate the activation of the NFκB through the classical/canonical pathway along with the alternative pathway.

There was no change in the gene expression of the inhibitors of kappa B kinase complexes (IKK) while looking to BIRC3 and BIRC4 genes encoding c-IAP_2 and

XIAP respectively, the cells showed significant up-regulation of c-IAP_2. At the protein level, the expression of c-IAP_2 was slightly increased after 24 h treatment but didn't reach statistical significance. For XIAP, interestingly, the gene was up-regulated initially but dramatically down-regulated post 24 h frondoside A treatment. In the presence of up-regulated RIPK1 and RIPK2 in this cell line, these changes might favor the activation of NFκB pathway but with the later changes in XIAP, cells might favor the shift in the direction of apoptosis enhancement. Inhibitors of apoptosis protein are frequently over-expressed in acute leukemias and this is usually associated with poor prognosis (Hu et al., 2015).

The effect of frondoside A on other gene expression might also be delayed as seen in the expression of CARD6. The gene was up-regulated but post 24 h it was greatly down-regulated which might interfere with the signal transduction and the activation of NFκB. Caspase activation and recruitment domain 9 (CARD9) and B-cell leukemia/lymphoma 10 (Bcl10) gene products are functionally connected. The Bcl10 protein requires the presence of CARD9 to be functional and assembled (Bertin et al., 2000). Although the expression of CARD9 was decreased, there was an up-regulation of Bcl10. Both gene products play a role in apoptosis and NFκB pathway activation depending on the cell status and stimuli (Bertin et al., 2000).

Again in THP-1 cells, like CCRF-CEM cells, the expression of the Bcl-3 gene was up-regulated and this promotes cleavage of NFκB p105 form and the translocation of p50 active form into the nucleus in an atypical way of activation (Gilmore & Herscovitch, 2006). The Bcl-2 related protein 1 (BCL2L1) was up-regulated. This gene encodes a protein that is capable of decreasing the release of cytochrome *c* from the mitochondria and inhibiting caspase activation, depending on splicing-the short

form is an apoptosis activator and the long form is an apoptosis inhibitor. NFκB is known to target this gene by binding to its promoter region on the DNA and activating its transcription. Here, the up-regulation of this pro-survival gene might be a result of NFκB pathway activation rather than a direct effect of frondoside A treatment.

Comparing the effect of frondoside A on CCRF-CEM and THP-1 cells, we can see a similar pattern of action but detailed examination of each cell type showed some specific changes that were not seen in the other cell line indicating that the effect of frondoside A has a general pattern but it is further tuned based on the cell type.

Activation of the NFκB pathway is physiologically essential for cell growth and differentiation. It also plays a role in acute and chronic inflammation. The signaling pathway maintains the survival of immune cells at site of infection and enhances the secretion of cytokines to attract cytotoxic cells at sites of abnormal (pre-malignant) cells to eliminate them (Hoesel & Schmid, 2013). We can say that NFκB pathway activation is important for preventing cancer development but studies have shown that its constant activation leads to cancer promotion. This have led researchers to attempt using NFκB inhibitors for cancer treatment. Given the complexity of the signaling pathway, multiple compounds have been developed, some are general inhibitors while others are more specific. The NFκB pathway can be blocked at the stage of initiation of signaling activation, at the level of activation in the cytoplasm, at the stage of nuclear translocation or at the stage of binding the κB site on DNA promoter region (Gilmore & Herscovitch, 2006).

In our study we attempted investigating the effect of NFκB inhibitor (andrographolide) when combined with frondoside A on acute leukemia cells. Treating CCRF-CEM and THP-1 cells with different frondoside A concentrations in-

combination with andrographolide IC_{50} concentration markedly enhanced the anti-leukemic effect of frondoside A. Andrographolide is a diterpenoid, a natural product extracted from the leaves of *Andrographis paniculata* (Low, Khoo, Münch, Govindaraghavan, & Sucher, 2015). The mechanism of action by which andrographolide inhibits NFκB pathway activation have been studied in different type of cells and showed variable mechanisms that are cell-type and concentration dependent (Chen et al., 2015; Cheung et al., 2005; Hidalgo et al., 2005; Lee et al., 2012; Luqman & Pezzuto, 2010). Andrographolide inhibits NFκB activation through covalent modification of reduced Cys62 of the p50 sub-unit (Levita, Nawawi, Mutalib, & Ibrahim, 2010). It binds to NFκB and prevents the transactivation.

Studies have demonstrated multiple biological properties of andrographolide including anti-bacterial, anti-inflammatory, anti-platelet aggregation, neuro-protective, gastro-protective, immune-stimulant and hypolipidemic functions (Chen et al., 2015; Hidalgo et al., 2005; Low et al., 2015). Multiple studies showed that andrographolide induces apoptosis in cancer cells including acute leukemia cells. In a study conducted in HL-60 cells (acute promyelocytic leukemia cell line); treating the cells with andrographolide induced significant suppression in cell viability with morphological changes consistent with apoptosis (Cheung et al., 2005). Cell cycle analysis showed G_0/G_1 arrest and protein expression analysis showed decrease in the pro-survival proteins Bcl-2 and Bcl-xL while there was a significant increase in the pro-apoptosis protein, Bax (Cheung et al., 2005), all of which are products of NFκB target genes. The study also demonstrated release of cytochrome *c* in the treated cells which indicates the activation of the intrinsic mitochondrial pathway in these cells (Cheung et al., 2005). In another study conducted on the same type of cells, an additional mechanism was demonstrated by which andrographolide induced apoptosis

(Cheung et al., 2005). It was found to suppress phosphorylation of Akt/ERK pathways, hence, decreasing their activation which led to apoptosis (Chen et al., 2015). It was demonstrated that andrographolide suppresses NFκB activation and that leads to apoptosis (Chen et al., 2015). In the hepatocellular cancer cell line, HepG, andrographolide caused cell cycle arrest and the apoptosis was associated with reduction in the levels of glutathione (GSH) (Li, Cheung, Zhang, Chan, & Fong, 2007), which is again a target gene product for NFκB pathway. A similar mechanism was proposed in the THP-1 cell lines, were treatment with the compound caused cell cycle arrest in the G_2/M phase and was associated with decreased GSH level due to inhibition of GSH transferase enzyme (Raghavan, Cheriyamundath, & Madassery, 2014).

The effect of andrographolide as a potent anti-inflammatory agent has been extensively studied. Its effect on a major pro-inflammatory pathway (the NFκB pathway) is well demonstrated since it acts as a specific inhibitor of the NFκB pathway. This inhibition might result in a different pattern of change in gene expression depending on the particular cell line, the state of the cells and on environmental factors.

In the HL-60 cell line, treatment with andrographolide led to decrease phosphorylation of the inhibitor of NFκB (IκB) and hence, its degradation. This stabilizes the NFκB-IκB complex in the cytoplasm, preventing NFκB activation and translocation to the nucleus (Chen et al., 2015). Andrographolide decreases the phosphorylation of PI3K/Akt and MEK/ERK pathways, leading to decreased activation of NFκB through these signaling cascades (Chen et al., 2015). Another study showed that andrographolide inhibited the binding of NFκB to its DNA consensus sequence in HL-60 derived neutrophilic cells but IκB-α degradation was not affected

(Hidalgo et al., 2005). In the same study no effect of andrographolide on PI3K/Akt and MEK/ERK pathways was demonstrated and it was concluded that the compound does not inhibit the p38/MAPK/ERK pathway in HL-60 derived neutrophilic cells (Hidalgo et al., 2005).

In the THP-1 cell line, the effect of andrographolide on TNF-α/ lipoplolysaccaride (LPS)-mediated NFκB activation was through the suppression of TNF-α interaction with IκB-α rather than a direct effect on IκB degradation. In this study, andrographolide inhibited NFκB activation and translocation to the nucleus but did not affect its binding to DNA, or the MAPK/ERK pathway (Lee et al., 2012). Treatment with andrographolide was associated with the inhibition of the release of TNF-α in LPS- activated macrophages as well as inhibition of the p38 MAPK/ERK1/2 pathway and the downstream activation of NFκB (Low et al., 2015).

In the present study, the effect we observed from combining the treatment of frondoside A and andrographolide in acute leukemia cell lines was synergistic. As we proposed earlier, that the treatment of frondoside A triggered the NFκB survival pathway in THP-1 cells and CCRF-CEM cells, the potent effect of andrographolide on inhibiting NFκB sensitized the cells to frondoside A. When the acute leukemia cell lines were treated with frondoside A combined with andrographolide; a potent NFκB pathway inhibitor, the suppression of cell viability was significantly enhanced compared to the treatment with frondoside A alone. This can be explained by the fact that resistant blast cells in these cell lines, when exposed to frondoside A they activate NFκB pathway as a survival mechanism and when this pathway is inhibited, even resistant cells undergo cell death. However, since andrographolide has been shown to have other biological effects, including the inhibition of cyclooxygenase 2 (COX-2)

activity, synthesis of inducible nitric oxide synthase (iNOS) and platelet activating factor (PAF)-induced platelet aggregation (Hidalgo et al., 2005), which are also targets for NFκB activation. It is necessary to confirm the effect of this compound in enhancing the effects of frondoside A using drugs which are structurally and mechanistically unrelated to andrographolide (Levita et al., 2010).

Chapter 5: Conclusion

Acute leukemia is a major cause of death among hematological malignancies. Current chemotherapeutic drugs can cure the majority of patients but there still remain some who will not respond, develop resistance, or suffer from long-term side-effects of the drugs. Because of that, there is a need to search for effective new anti-leukemia agents with reduced side-effects.

Natural products have been found to have a wide range of bioactive components that can serve as alternatives to established chemotherapeutic drugs, or to potentiate their anti-leukemia effects thus allowing reduced doses of drugs, which will decrease side-effects without compromising the effectiveness of the leukemia blast eradication therapy. Natural product derived from marine sources that are in current clinical use for such purposes are limited (Dyshlovoy et al., 2016; Schwartsmann, Brondani da Rocha, Berlinck, & Jimeno, 2001).

Frondoside A is a triterpenoid glycoside, extracted from the skin of the Atlantic sea cucumber; *Cucumaria frondosa*. Studies have shown its potent effect as anti-bacterial, anti-cancer and immune-modulatory agent. Its anti-cancer properties in solid tumors have been explored. Frondoside A induces cell cycle arrest and induces apoptosis. Frondoside A also prevents migration, invasion and the development of metastasis as well as exhibiting anti-angiogenic effects. These anti-cancer functions have been demonstrated *in vivo* as well as in *in vitro* studies. Since frondoside A has been shown to be a potent PAK1 inhibitor, it is most likely this mechanism that is responsible for the biological effects of this agent.

The effect of frondoside A as anti-leukemia agent (i.e. its effect in hematological malignancies) has not been fully explored. This project is the first to extensively study the effect of frondoside A in acute leukemia cell lines, alone and in-combination with other conventionally used chemotherapeutic drugs for the treatment of acute leukemia.

In the present study, frondoside A significantly suppressed the viability of an acute T-cell lymphoblastic leukemia cell line, CCRF-CEM and the acute monocytic leukemia cell line, THP-1. It also decreased the cell viability in acute promyelocytic leukemia cells (HL-60). CCRF-CEM cells were the most sensitive to frondoside A treatment while THP-1 cells were the least. Frondoside A showed marked enhancement of the anti-leukemia effect of asparaginase, vincristine and prednisolone when the cells were treated in-combination. The combinations showed synergistic effects in multiple concentration combinations in CCRF-CEM and THP-1 cells.

The mechanism by which frondoside A exerts its effect on those acute leukemia cell lines was further investigated and frondoside A was found to induce apoptosis as early as one hour post treatment.

Analysis of apoptosis and cell cycle related proteins showed significant decrease in the expression of the pro-survival Bcl-2 protein in both cell lines. Treatment with frondoside A also led to a significant decrease in expression of the Bax protein and we attributed this finding to the early activation of apoptosis (1 h post treatment) and the degradation/consumption of Bax protein by the time protein extraction was made (6 h and 24 h post treatment). Analysis of caspase-3, the main executioner caspase in the apoptosis pathway, showed a marked decrease in pro-

caspase-3 concentrations in the treated groups, which indicates the activation of apoptosis in CCRF-CEM and THP-1 cells.

The acute T-cell lymphoblastic leukemia cell line is known to have a mutated p53 protein and the treatment with frondoside A significantly decreased the expression of the mutated tumor suppressor at 6 h of treatment.

The cyclin-dependent kinase inhibitor p21 was assessed in THP-1 cells. Frondoside A treatment resulted in increased expression of this tumor suppressor protein. This might be a result of frondoside A inhibitory effect on PAK1 (p21 activated kinase 1) and is p53-independent.

Further analysis was done to study the effect of frondoside A treatment on the mRNA /gene expression level of multiple genes involved in apoptosis/survival pathways using low density arrays. In general, the changes in gene expression induced by frondoside A were more marked in THP-1 cells compared to CCRF-CEM cells.

Acute leukemia cell lines used showed marked up-regulation of the pro-apoptosis genes; Bcl-2 like 1 (BCL2L1), Caspase-4, Caspase-6, Caspase-7, BH3 interacting domain death agonist (Bid), death effector domain (phosphoprotein enriched in astrocytes 15, PEA15), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1 also known as Noxa), leucine rich repeat and pyrin domain containing 1 (NALP1) and TNFRSF1A-associated via death domain (TRADD).

Frondoside A also resulted in marked up-regulation of genes of the TNF pathway, including multiple ligands and receptors.

Interestingly, gene expression analysis showed massive increases in expression of genes of the NFκB pathway as well as inhibitors of the pathway. Nuclear factor

kappa light polypeptide enhancer in B-cell 2 ($NF\kappa B_2$), RelB and $cIAP_2$ proteins were selected to be further analyzed at protein expression level since they were markedly up-regulated at the mRNA level in both cell lines. Analysis show modest changes in expression between the treated groups and the controls that did not reach statistical significance. The NFκB activation is suggested to be mainly through the non-canonical pathway in these cells. PAK1 is known to activate NFκB pathway in acute myeloid leukemia cells. We also proposed that the treatment with frondoside A might have triggered the survival NFκB pathway in the treated cells to overcome the anti-leukemia effect of frondoside A but on the other hand, frondoside A inhibited the protein synthesis at the translation level and that might be through its inhibitory effect on PAK1. Interaction between frondoside A and NFκB pathway was confirmed when the anti-leukemia effect of frondoside A was enhanced in these cells when tested in-combination with the NFκB inhibitor, andrographolide.

In conclusion, this study has demonstrated that frondoside A has marked anti-leukemic effects. It decreased the viability of acute leukemia blasts by inducing apoptosis. The apoptosis appeared to be due to the activation of both extrinsic and intrinsic pathways. Resistance to frondoside A can be due to the activation of the NFκB survival pathway in the treated cells and combining the treatment with NFκB pathway inhibitors results in dramatic enhancement of the anti-leukemic effect of frondoside A. Frondoside A affected different genes and pathways in leukemia blast cells and inhibiting malignant cells by targeting multiple pathways might be more beneficial in the treatment strategy. The addition of frondoside A to acute leukemia conventional therapeutic protocols currently used in the treatment might prove beneficial in high risk patients, while sparing the side effects of high dose therapy and bone marrow transplantation.

5.1 Managerial Implications

This project highlighted the possible use of frondoside A in the treatment of acute leukemia.

Preliminary experiments to explore the possible mechanism of action were started in this project but further investigations are needed. Future work is required to study the exact apoptosis pathway involved. Inducing cell death through death receptors specifically should be examined and the release of cytochrome *c*, which is a hallmark of intrinsic pathway activation should also be tested.

The effect of frondoside A on other p53 family members such as p63 and p73 should be explored in leukemia cells were p53 is mutated or deleted.

The interaction of frondoside A with NFκB pathway members should be further examined. Protein synthesis experiments will help to explain the discrepancy between the gene expression and the protein expression in this pathway after the treatment with frondoside A.

Limited studies have investigated the pharmacokinetics of frondoside A (Al Shemaili et al., 2016) and further investigations are needed in leukemia animal models.

Finally, comparing the current findings with experiments that test frondoside A effect on primary acute leukemia blast cells from patients diagnosed with the disease can be the first step to start *in vivo* experiments in animals to mimic the physiological settings when frondoside A is given.

5.2 Research Implications

Data from the current project highlighted the potential use of frondoside A for the treatment of acute leukemia. Its use in high risk patients might prove beneficial since it markedly potentiates the effect of chemotherapeutics currently included in the treatment protocols.

From previous studies in solid tumors, frondoside A showed its marked effect on inducing apoptosis and arresting cell cycle in cancer cells. There we demonstrated similar effect on leukemia blast cells, in addition, an unexpected finding was discovered and that is the interaction of frondoside A with NFκB pathway. The gene expression profiling on RNA low density arrays have shown frondoside A treatment to induce multiple genes in the NFκB survival pathway as well as many NFκB pathway inhibitors.

One of the well-known mechanisms by which cancer cells resist anticancer treatment is through the activation of NFκB pathway and here we have shown that using frondoside A in-combination with andrographolide (NFκB antagonist) can overcome this mechanism and induces cell death in treatment-resistant leukemia cells.

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Appendix

Buffers and solution reconstitution.

1) Protein extraction:

Radioimmunoprecipitation assay (RIPA) buffer:

1M TRIS-HCl pH 7.4	5 ml (final concentration will be 50m M TRIS-HCl pH 7.4)
5M NaCl	3 ml (final concentration will be 150 Mm NaCl)
Triton X-100	1 ml (final concentration will be 1%Triton X-100)
Soduim Deoxycholate	1 gm (final concentration will be 1% Sodium Deoxycholate)
10% SDS	1 ml (final concentration will be 0.1% SDS)

The solution is made up to **100 ml** with autoclaved distilled water and filtered.

2) Western blot reagents:

2A) 10X Sodium Dodecyl Sulfate (SDS) buffer:

Trizma base	30 g
Glycine	72 g
SDS	10 g

Dissolved in 1 L dH_2O.

For 1X SDS buffer, 100 ml of 10X SDS is diluted in 900 ml dH_2O.

2B) 10X Transfer buffer:

Trisma base	15.15 g

Glycine 72 g

Dissolved in 500 ml dH_2O

For 1X Transfer buffer (1 L): 100 ml of 10X Transfer buffer added to 200 ml absolute methanol and 700 ml dH_2O.

2C) 10X TBS, pH 7.2-7.5 (1 L)

Trisma base 24.2 g

NaCl 87.7 g

Dissolved in 1000 ml H_2O.

For 1X TBS (1 L): 100 ml of 10X TBS is diluted in 900 ml of dH_2O.

2D) 10X TBST, pH 7.2-7.5 (1 L):

Trisma base 24.2 g

NaCl 87.7 g

Tween20 10 ml

Dissolved in 1000 ml dH_2O.

For 1X TBST (1 L): 10X TBST 100 ml is added to 900 ml H_2O.