Doxorubicin Combined with Phytochemicals as Pro-Apoptotic Agents and Protein Kinase Inhibitors for the Treatment of Acute Myeloid Leukaemia

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Dedication

To the Lord God almighty for his love, grace, favour and faithfulness all throughout the program. To my parents Mr. Albert Osemeke and Mrs. Esther Osemeke for their support, guidance and overwhelming love. Thank you both for believing in me and making my dream come true.

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Declaration

I hereby declare that the thesis content is original and have not been submitted in part or in whole to any university for any qualification or degree. The results in this thesis are my own work and not any results of work done in collaboration. Approximately 61,056-word count is contained in this thesis including references and appendices. This thesis contains 41 figures and 9 tables.

Uzoma Cynthia Osemeke

March, 2020

Signature: _____

Date: _____

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ABSTRACT

Acute myeloid leukaemia is one of the most common form of adult leukaemia with several kinase mutations. The FLT3-ITD mutation is a common kinase mutation observed in (20-30%) of AML patients and this mutation is also the most involved in the prognosis of AML. It confers poor treatment outcome in patients despite intensive chemotherapy due to an increased relapse rate. Kinases are crucial in controlling different cellular activities, and their mutations are well known to signal cancer development and progression. FLT3 inhibitors have been evaluated in clinical trials leading to the approval of Midostaurin for the treatment of AML. However, drug resistance and kinase selectivity are still challenges faced by current FLT3 inhibitors. The aim of this study was to investigate *in vitro*, the anti-leukaemic effect of phytochemicals that are commonly consumed and widely distributed in plants and fruits (α-mangostin, gallic acid and vitamin C), singly and in combination with doxorubicin (Dox) in the relapsed AML cell line MOLM-13. CyQUANT proliferation assay was used to determine their effects on cell viability. Flow cytometric analysis of cell apoptosis and cell cycle were conducted. Expression of various proteins (Bax, Bak, Bcl-2, caspase 3, 8 and 9, p53, p21, p16, cdc25s and FLT3) were determined using Western blot technique, and ELISA to confirm FLT3-ITD phosphorylation. Statistical analysis including ANOVA, Student T-test were chosen for comparing the effects of the standard chemotherapeutic drug (Dox) with the phytochemicals, either singly used or combined. Results showed that α-mangostin at the concentration 5-50 µM inhibited cell growth in a dose-dependent manner in MOLM-13 cell line. Gallic acid and vitamin C inhibited cell growth at 5-30 µM. More apoptotic effects were observed when α-mangostin (20 µM) was combined with Dox (1 µM), with more TUNEL positive cells and increased expression of the pro-apoptotic protein Bak. Treatments of vitamin C (15 µM) combined with Dox (1 µM) induced early apoptotic cell death when compared to Dox only. Gallic acid (15 µM) showed synergistic effect with Dox (1µM) in suppressing cell growth and induced apoptosis was associated with the expression of Bak ($p<0.01$). However, Dox only and vitamin C single treatment significantly inhibited Bak ($p<0.01$) in this study. Furthermore, combination of either α-mangostin, gallic acid or vitamin C and Dox blocked MOLM-13 cells at G_2/M phase, indicating irreversible cell cycle arrest. Induction of senescence, an anti-tumorigenic mechanism that inhibit cancer was determined by p16 expression to suggest senescence. Reactivation of senescence pathway results in cells that should not undergo repair in

the cell cycle and will not divide even in the presence of mitotic factors. In this study, expression of p16 protein by combination of doxorubicin with the phytochemicals was not significant when compared to Dox only, while vitamin C and Dox combination was significant compared to negative control ($p<0.05$). In addition, doxorubicin combined with α-mangostin suppressed phosphorylation of FLT3-ITD and cdc25s phosphatases significantly ($p<0.05$). In conclusion, results from this study suggests that the mechanism of drug action of Dox, when combined with phytochemicals is through the induction of apoptosis resulting in irreversible programmed cell death Further studies revealed that α-mangostin had a synergistic effect with doxorubicin in the inhibition of phosphorylated FLT3-ITD which could have promising potential to be developed as FLT3 inhibitors. Therefore, AML cells treated with a combination of α-mangostin and Dox promotes the induction of apoptosis that could be effective to overcome resistance in AML and therefore require further study to understand its mechanism as a targeted FLT3-ITD therapy.

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List of Abbreviations

AML	Acute Myeloid Leukaemia
FLT3	FMS-like Tyrosine Kinase 3
FLT3-ITD	FLT3- Internal Tandem Duplication
FLT3-TKD	FLT3-Tyrosine kinase Domain
FLT3-WT	FLT3-Wild Type
FL	FLT3 Ligand
DNA	Deoxyribonucleic Acid
MDS	Myeloid Dysplastic Syndrome
CD33	Cluster Differentiation of cells 33
CR	Complete Remission
FDA	Food and Drug Administration
ATO	Arsenic Trioxide
ATRA	All Trans Retinoic Acid
FAB	French American British
JM	Juxtamembrane Domain
KD	Kinase Domain
Cdc25	Cell Division Cycle 25
Dox	Doxorubicin
DSB	Double Strand Break
SSB	Single Strand Break
ATM	Ataxia Telangiectasia mutated

ATR	AtaxiatelangiectasiaandRad3-relatedprotein
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
E2F	E2 transcription Factor
pRB	Retinoblastoma Protein
ERK	Extracellular signal Regulated Kinase
STAT5	Signal Transducer and Activator of Transcription 5
PKB	Protein kinase B
PI3K	Phosphoinositide 3-kinase
MDM2	Mouse Double Minute 2
MDM4	Mouse Double Minute 4

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CHAPTER ONE

1.1 Executive Summary

Acute myeloid leukaemia (AML) is an aggressive haematological malignancy with diverse prognostic factors such as mutations, complex karyotype, anticedent haematological disease such as myeloid dyspalstic syndrome (MDS) and prior treatment from other malignancies due to exposure to chemotherapy and/or radiation (Aberger *et al*., 2017). AML attracts considerable attention due to its high incidence rate compared to other common types of leukaemia (about two-fold increase compared to chronic myeloid leukaemia and acute lymphocytic leukaemia) and also it has one of the worst survival prospects (more than 12 months survival after diagnosis in less than 40% of patients) (Chen *et al*., 2017). In addition, treatment failure in all ages, due to increasing resistance to therapy, results in 50% of patients experiencing relapse after complete remission (Medeiros, 2018) and relapse as cause of death after stem cell transplant (Saultz & Garzon, 2016). Standard therapy for decades (30-40 years) in AML has been with the same chemotherapy drugs (a combination of cytarabine and anthracycline antibiotic such as doxorubicin or daunorubicin in a 7+3 days therapy regime) (Aberger *et al*., 2017) or stem cell tranasplant for resistant AML (Hamilton & Copelan, 2012). Recent advancement has led to the discovery of some new drugs for AML. Effective diagnosis, using cytogenetics and molecular studies has improved detection, established prognostic factors and identified molecular targets that could determine treatment selection for each patient and reduce resistance and relapse in patients (Larrosa-Garcia & Baer, 2017). This has led to the World Health Organisation (WHO) revisiting AML classification in 2016 and the recently concluded RATIFY study, resulting in new drug for AML (Midostaurin) approved by Federal Drug Agency. However, despite recent development, overcoming chemoresistance, understanding its complex genetics, cellular pathways and mechanisms that promote survival and proliferation of leukaemic cells are still a challenge in AML (Saultz & Garzon, 2016). One major reason for these challenges is the development of new mutations due to the heterogeneity of AML and molecular studies have been developed to detect specific gene mutations of interest, as well as to detect new mutations within the shortest time possible for treatment to commence (Merino *et al*., 2018 ; Patnaik, 2017). For instance, FLT3 kinase mutation is present

in 20-30% of AML patients and can lead to poor prognosis and treatment outcome, confers resistance, high relapse rate and short overall survival (Larrosa-Garcia & Baer, 2017). Since FLT3 mutation which has been reported as the key driver of AML (Swords *et al*., 2012), is now recommended for early detection in all newly diagnosed AML patients for urgent targeted therapy to overcome resistance and subsequent relapse (Patnaik, 2017). However, due to its involvement in haematopoiesis, may not be detected in some cases during diagnosis but after relapse or resistance. Therefore, FLT3 mutation is emerging as a diagnostic and prognostic biomarker, as well as promising therapeutic target in AML.

Some chemotherapeutic drugs have been developed from phytochemicals such as vinca alkaloids (vincristine and vinblastine) (Lobert *et al*., 1996), taxol analogues (Rimoldi *et al*., 1993), camptothecins (Pommier, 2006), artemisinin (Nakase *et al*., 2008) and podophyllotoxin (Hosseini & Ghorbani, 2015). Some phytochemicals with anticancer properties have been reported to reduce toxicity of chemotherapy drugs to normal cells, overcome chemorsistance or sensitize resistant cells to chemotherapy drugs. One common chemotherapeutic agent for cancers and in the treatment of leukaemia is doxorubicin (Mahbub *et al*., 2015). Doxorubicin, similar to daunorubicin are used in AML and function as topoisomerase II inhibitors and act by inhibiting cell division (Mahbub *et al*., 2015). However, it is associated with side effects that can be severe and cause drug resistance (Suzuki *et al*., 1997). Although novel liposomal formulation CPX-351 vyxeos (daunorubicin/cytarabine) has been approved for MDS and therapy related AML to minimize toxicity to non-leukaemic cells, the use of doxorubicin as a targeting agent against mutations in AML (which has numerous mutations) is yet to be explored.

Some cancer patients being treated with chemotherapy drugs may choose to co-administer certain phytochemicals due to their reported anticancer effects, often without informing their doctors. Such combinations may lead to possible drug interactions which are not yet fully understood and may be of concern (Cheng *et al*., 2010). It is worth noting that some of the combination could be synergism, additive or antagonistic.

Some phytochemicals when combined with established chemotherapeutic agents could enhance the efficacy and tolerance of established chemotherapy agents (Gu *et al*., 2018). Such combinations could result in simultaneous targeting of signaling pathways that enhance cell survival and proliferation caused by gene mutation. For instance, the phytochemical

α-mangostin (the main active compound in mangosteen fruit) has been studied extensively as an anticancer agent, singly and in combination with established chemotherapeutic agents (Chapter 4). In addition, phytochemicals abundant in nature such as gallic acid and vitamin C have been reported to have potent anticancer effects (Chapter 5 and 6, respectively). However, combinational studies of α-mangostin, vitamin C or gallic acid with doxorubicin in AML has not yet been reported.

The determination of the effect and molecular mechanism of action of a drug is crucial in evaluating the process of cell death and inhibition of proliferating cancer cells. A possible mechanism of action could be the ability to cause DNA damage via the DNA damage response pathway. This mechanism often involves the action of the tumour suppressor protein p53 (known as the guardian of the genome) and cellular process such as cell apoptosis, cell cycle arrest and senescence (which when activated by chk1/ATR or chk2/ATM kinase result in its separation from its inhibitor MDM2). p53 has been reported to be inactive in over 50% of all human cancers (Ozaki & Nakagawara, 2011). p53 regulates the cell cycle through the cell cycle inhibitors, p21, p27 and p57 (Figure 1). The cell cycle is a coordinated series of events that leads to DNA replication and cell division, which it is regulated by complexes of cyclin dependent kinases and cyclins (cdk/cyclin) (Tyson *et al*., 2002). Expression of cell cycle inhibitors after activation by p53 and inhibition of certain cdc25 phosphatases can result in cell arrest at either G_0/G_1, S or G_2/M phase (Tyson *et al*., 2002).

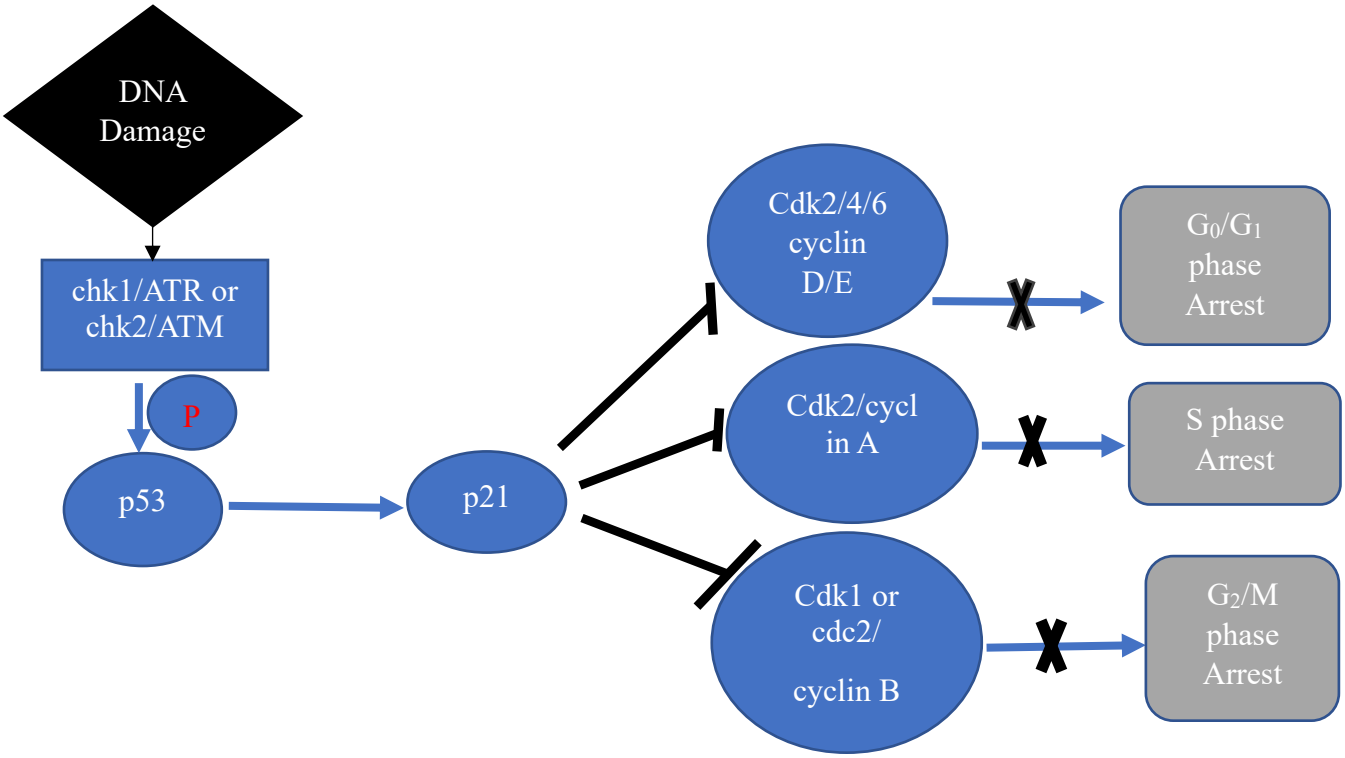


Figure 1: p53 and Cell Cycle Regulation during DNA Damage. ATR/ATM kinases phosphorylate p53 to activated p21 which inhibit cdk/cyclin complex to arrest cells at different phases of cell cycle.

Cell cycle dysregulation is a major cause of abnormal cancer cell proliferation and its effective regulation by drug treatment is a strategy for cancer treatment. DNA defect is thereby detected, especially in G_1/S transition, that is commonly deregulated in human cancers (Zhang *et al*., 2017). Apoptosis is regulated by p53 and is activated either through intrinsic or extrinsic pathways involving the activation of caspases (caspase 3, 8 and 9) and apoptotic proteins (Bax, Bak and Bcl-2). Apoptosis or anti-inflammatory cell death is induced by most anticancer drugs. However, necrosis may cause toxicity to neighboring cells due to inflammation. Therefore, induction of cell death either through necrosis or apoptosis after cell treatment is a way to determine cytotoxicity of the drug to both cancer and normal cells. Senescence (also known as irreversible cell cycle arrest) is another cellular process which is activated by p53 through the p21 and stabilized by p16 proteins. Senescence is an anticancer mechanism which suppress cancer development (Vargas *et al*., 2012).

α-Mangostin has been reported to activate p53 and regulates apoptosis through increased expression of Bax (Kaomongkolgi *et al*., 2011), caspase 3 and 9 (Aisha *et al*., 2011), and decrease the expression of antiapoptotic protein, Bcl-2 (Kaomongkolgi, *et al*., 2011). It has been reported to induce G_0/G_1 arrest via p21 in a p53 mutant cell line (Kurose *et al*., 2012) and G_0/G_1 arrest via p38 MAPK/p16 pathway (Korm *et al*., 2015). Therefore, α-mangostin has the potential to mediate important components or gatekeepers (p53 and p38) of the tumour surveillance network which are inactivated by mutation in cells (Korm *et al*., 2015; Zhang *et al*., 2017). Gallic acid has been reported to induce DNA damage (Weng *et al*., 2015), activate p53 and induce cell cycle arrest via p21 and p27 (Yeh *et al*., 2011). Vitamin C has also been reported to activate p53 and induce cell arrest at G_0/G_1 phase (An *et al*., 2011; Park *et al*., 2004). Therefore, α-mangostin, gallic acid and vitamin C could synergize with doxorubicin to minimize toxicity and improve treatment efficacy in AML.

Targeting FLT3 mutation is important in AML as it is reported to have high incidence of the mutation, with the FLT-ITD mutation being the most prevalent and associated with poor clinical outcomes (Larrosa-Garcia & Baer, 2017). Currently, the approved drug midostaurin for newly diagnosed AML with FLT-ITD mutation still has the challenge of developing resistance despite showing significant efficacy and prolong survival (Fakih *et al*., 2018 ; Perl, 2017). Therefore, drug combination studies in AML are important in developing novel targeted therapy that may overcome resistance and may also result in increasing our understanding of their anticancer effect in AML, leading to improved clinical outcome.

1.2 Aims and Objectives of the Study

The aim of this study was to evaluate the anti-leukaemic potential of the phytochemicals α-mangostin, gallic acid and vitamin C singly or in combination with the established chemotherapy drug doxorubicin in a relapsed acute myeloid leukaemic cell line.

This aim was achieved using the following objectives:

- To determine the therapeutic potential of the phytochemicals to sensitise MOLM-13 cells to the cytotoxic effect of doxorubicin on cellular processes such as apoptosis, cell cycle arrest and senescence
- To evaluate the potential of the drug as FLT3 kinase inhibitors to overcome abnormal phosphorylation of ITD mutation in chemoresistance AML cell line

1.3 Thesis Outline

In this thesis, doxorubicin, a chemotherapy drug (an anthracycline antibiotic) used in AML treatment and known to be associated with dose-dependent cardiotoxicity was studied in AML cell line (MOLM-13) singly and in combination with three phytochemicals (α-mangostin, vitamin C and gallic acid). These phytochemicals are commonly consumed, widely distributed in fruits and reported to have anticancer properties. This study determines potential synergistic and antagonistic effect with doxorubicin in AML. Executive summary includes rational for AML, FLT3-ITD, phytochemicals and cellular process studied.

Chapter 2

This chapter comprises of introduction and literature review of relevant subject topics, mechanism and proteins studied. Introduction to doxorubicin was included in this chapter.

Chapter 3

The method section with detailed description of each method employed and assays examined in this research. To address the issue of interaction, the combination index software CompuSyn was used to access the type of interaction observed in each combination. Statistical tools used were included in this chapter.

Chapter 4

Introduction to α-Mangostin, background information on AML and FLT3-ITD mutation, aim and objectives, results and detailed discussion on effect singly and in combination with doxorubicin on MOLM-13. In addition, detailed discussion on potential FLT3-ITD inhibition by combination treatment is found in this chapter.

Chapter 5

Background on gallic acid, aim and objectives, results on anticancer effect singly and in combination with doxorubicin in MOLM-13 inducing DNA damage and determining effect on biological mechanisms (cell cycle, apoptosis and senescence).

Chapter 6

Vitamin C in cancers, aim and objectives, results on anticancer effect singly and in combination with doxorubicin in AML MOLM-13.

Chapter 7

Overall discussion, what is not known, concluding comments, limitation of the research and recommendation for future work.

CHAPTER TWO

2. INTRODUCTION AND LITERATURE REVIEW

2.1 Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is an expansion of clonal myeloid blasts in the bone marrow, peripheral blood or other tissues resulting in abnormal proliferation and arrest in differentiation and maturation (Merino *et al*., 2018). AML is the most common adult leukaemia, predominant in men, with incidence increasing with age and peaking between 80 and 84 years (Merino *et al*., 2018). There is constant investigation of new drugs in AML which is a daunting challenge due to its biological heterogeneity and high risk of relapse (Merino *et al*., 2018 ; Kadia et al., 2016).

For several decades, there was no substantial therapeutic advancement (Short *et al*., 2018), despite increasing understanding of the pathogenesis of AML, resulting in over three decades of stagnation and the use of the same standard therapies known as the backbone of AML treatment (Yang & Wang, 2018). In recent years, there has been major breakthroughs with discovery of novel therapies for AML treatment that includes modification of conventional chemotherapies, epigenetic and genetic target drugs, and Immunotherapies (Yang & Wang, 2018). This has led to the approval of four novel drugs in 2017 by the US Food and Drug Administration (FDA) that includes; midostaurin for newly diagnosed AML patients with FLT3 mutation, enasidenib for refractory or relapsed AML with IDH2 mutation, gemtuzumab ozogamicin for newly diagnosed or refractory/relapsed CD33 positive patients and a novel daunorubicin/cytarabine liposomal formulation CPX-351 (Vyxeos) for myeloid dysplastic syndrome (MDS) or therapy related AML (Perl, 2017). Treatment of AML patients with Acute Promyelocytic Leukaemia has been modified to include Arsenic trioxide (ATO) alongside All Trans Retinoic Acid (ATRA) previously administered (Zhang, 2017). In 2018, two more drugs (Daurismo and venclexta) were approved for patients that are not candidate of intensive therapy or cannot stand standard AML therapy like the elderly. In addition, there has been development of diagnostic assays (Merino *et al*., 2018) for the early detection of the disease. Although induction chemotherapy is deemed suitable for the treatment of AML patients, complete remission (CR) is not documented in most patients with >75% CR rates in younger patients less than 60 years and in older patients 60 years and above >50% CR rates (Medeiros, 2018). Unfortunately, most patient relapse in the space of 3 years after complete remission and cases of relapse are a common cause of mortality in AML patients

(Medeiros, 2018). The risk of relapse is linked to minimal residual disease that is persistent post chemotherapy and defined by low levels (>5% bone marrow blasts) of leukaemic cells which are beyond morphological detection but are sensitive to detection by molecular techniques, such as immunophenotyping and flow cytometry for routine monitoring of AML patients (Ravandi *et al*., 2018). Therefore, a major clinical obstacle in AML therapy remains relapsed and primary refractory AML, including resistance to chemotherapy drugs such as doxorubicin and cytarabine (Medeiros, 2018). According to the World Health Organisation (WHO), specific AML subgroup (Figure 2) are classified according to significant molecular genetics and cytogenetic features (Merino *et al*., 2018), which are important in the diagnosis of AML, as well as clinical, morphological (Table 2) and immunophenotyping (Table 2.1) (Arber, 2018). However, techniques for cytogenetics have limited sensitivity, such that minor defects such as small duplicates, microdeletions and point mutations are not detected (Merino *et al*., 2018).

Table 2: FAB classification of AML

Classification	Name
M_0	AML with no Romanowsky or cytochemical evidence of differentiation.
M_1	Myeloblastic leukaemia with little maturation
M_2	Myeloblastic Leukaemia with Maturation
M_3	Acute promyelocytic leukaemia (APL)
M_4	Acute Myelomonocytic Leukaemia (AMML)
$M4_{eo}$	AMML with dysplastic marrow eosinophils
M_5	Acute monoblastic leukaemia (AMoL)
M_{5a}	**AMoL poorly differentiated*** ⬅
M_{5b}	AMoL differentiated
M_6	Acute erythroid leukaemia
M_7	Acute megaloblastic leukaemia (AMkL)

French American British (FAB) classification of AML based on morphological features.
➡ ***M5a** subtype is one of the subtypes poorly differentiated and with poor prognosis, therefore a target for therapy. This classification has been incorporated into WHO classification Adapted from (Abdul-Hamid, 2011)

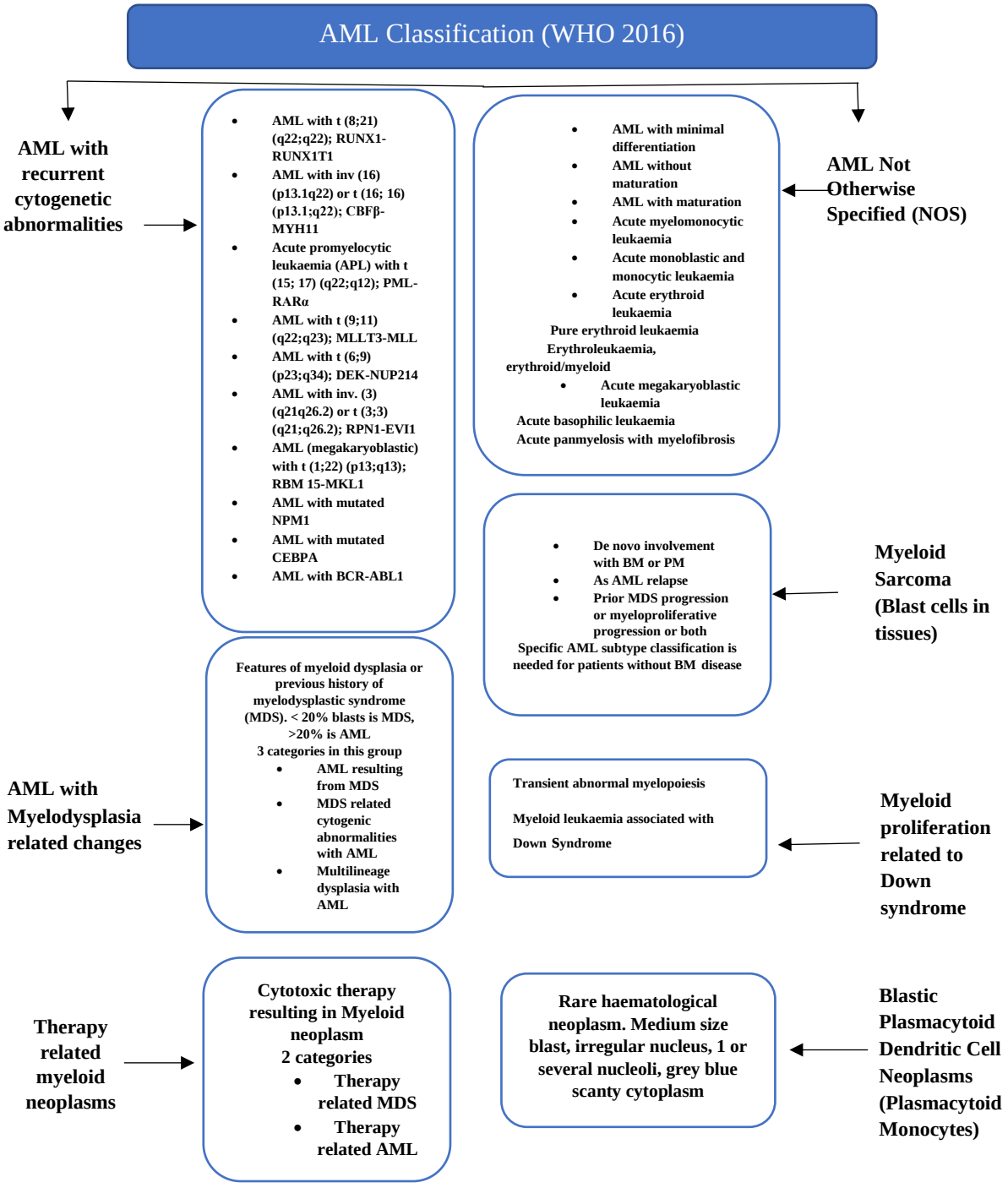


Figure 2: AML classification by WHO. Initially classified in 2001 and then revised in 2008 then 2016 (Arber et al., 2016; Arber, 2018; Merino et al., 2018).

Table 2.1: European group of the immunological characteristics of acute leukaemia (EGIL)

MIC Group Acute Myeloid leukaemia (AML)	**FAB**	**Immunological Markers** **CD13**	**CD33**	**Karyotype**
M2/t (8;21)	M2	+	+	t (8;21) (q22; q22)
M3/t (15;17)	M3, M3v	+	+	t (15;17) (q22; q12)
M5a/del(11q23)	M5a (M5b, M4)	+	+	t (del (11) (q23)
M4E0/ inv (16)	M4E0	+	+	del/inv (16) (q23)
M1/t (9;22)	M1(M2)	+	+	t (9;22) (q34; q11)
M2/t (6;9)	M2 or M4 with basophilia	+	+	t (6;9) (p21-22; q34)
M1/inv (3)	M1(M2, M4, M7) with thrombocytosis	+	+	Inv (3) (q21q34)
M5b/t(8:16)	M5b with Phagocytosis	+	+	t (8;16) (p11; p13)
M2 Baso / t (12p)	M2 with basophilia	+	+	t/del (12) (p11-13)
M4/+4	M4(M2)	+	+	+4

Adapted from (Behm, 2003) EGIL group in 1995 classified AML based on Immunological marker expression and change in appearance and number of chromosomes (karyotype). The table shows cell surface and cytogenetics (translocation (t), inversion (inv) and deletion (del) which changed the requisite >20% blast percentage for diagnosis of AML to <20% if there is associated markers and karyotype present. Cluster differentiation CD34, CD33, CD117, CD13 and HLA-DR are myeloid precursor markers. This classification has been incorporated in to the WHO classification (Merino *et al*., 2018; Muniraj, 2015).

Molecular analysis is essential for diagnosis as it enables detection of prognostically important mutations that have no clear or exclusive mutant specific immunophenotype or morphology. Such mutations are present in about 50% of AML patients with no cytogenetic abnormalities and such prognostic mutations include FLT3, RUNX1, NPM1 and/or CEBPA (Table 2.2) (Döhner *et al*., 2017). Studies on the pathogenesis of AML has shown the involvement of at least three mutations that are involved in cellular pathways such as survival/proliferation, differentiation, and are required for the disease process to progress (Tangpong *et al*., 2011). For haematopoietic cells to become leukaemic cells at least one mutation has to act in concert with FLT3 mutation, to increase growth and survival signals, while the other mutation act to block differentiation and transform the cells to a leukaemic blast cells (Tsapogas *et al*., 2017). Therefore, FLT3-ITD mutation has been reported to be frequently found with point mutations and genetic rearrangements in leukaemias (Gilliland & Griffin, 2002). It was estimated that an average of 13 mutations are contained in each case of AML with some of them as driver mutations (Ley *et al*., 2013). Furthermore, mutations in epigenetic regulators (ASXL1 and DNMT3A) has been shown to occur in early leukaemogenesis and maybe present also in apparently healthy elderly subjects hence the term "clonal haematopoiesis of indeterminate potential" was proposed (Ley *et al*., 2013). Therefore, a prognostic and genomic classification of AML using a panel of 111 myeloid cancer genes with mutations has been proposed recently and an understanding of this AML classification can improve clinical outcome (Merino *et al*., 2018), perhaps through the development of targeted drugs.

Table 2.2: Genetic abnormalities in AML

Mutations	***Occurrence in AML***
Nucleophosmin 1 (NPM1)	25%-30%
***Feline McDonough Sarcoma (FMS)-like tyrosine 3 (FLT3) (FLT3-ITD)**	**23%** ⬅
DNA methyltransferase 3A (DNMT3A)	18%-22%
Isocitrate Dehydrogenase (IDH)	15%-20%
Mixed lineage leukaemia (MLL)	11%
Ten Eleven Translocation 2 (TET2)	9%-23%
Tumour protein p53 (TP53)	8%-14%
CCAAT enhancer Binding Protein α (CEBPA)	6%-10%
Runt-Related transcription factor (RUNX1)	5%-13%
Additional Sex Comb-like 1 (ASXL1)	5%-11%
c-KIT	<5%

Information from (Saultz & Garzon, 2016). Genetic abnormalities in order of prevalence observed in AML patients. AML with mutated NPMI is most frequent, associated with normal karyotype and shows good response to chemotherapy. Poorer prognosis is associated with the coexistence of FLT3-ITD and NPMI in young adult patients. Particular poor outcome has been associated with co-occurrence of FLT3-ITD, NPM1 and DNMT3A. ➡*****FLT3-ITD is a common mutation in AML patients, associated with adverse prognosis and the only mutation that is most involved in AML prognosis therefore called the key driver of AML due to its effect on haematopoiesis. Therefore, an important therapeutic target and choice of mutation to study.

2.2 Treatment of AML

In the last 50 years remarkable advances have been made in the management of haematological malignancies (Anderson *et al*., 2014). Treatment approaches in AML includes anthracycline antibiotics such as doxorubicin or daunorubicin consolidated with cytarabine (Burnett *et al*., 2011). Stem cell transplantation is a treatment of choice for patients at high risk (Burnett *et al.*, 2011). The cytarabine and anthracycline antibiotic treatment consist of 7 days of cytarabine infusion and 3 days of anthracycline known as 7+3 regimen for patients with favourable prognosis (Kouchkovsky & Abdul-Hay, 2016). Daunorubicin or doxorubicin at 60 or 90 mg/m^2 is recommended (Rowe & Tallman, 2010) followed by cytarabine at 100-200 mg/m^2 daily dose (Kouchkovsky & Abdul-Hay, 2016). Higher dose of the anthracycline can be given to patients with poor prognostic markers such as DNMT3A mutation (Kouchkovsky & Abdul-Hay, 2016).

The outcome of AML for adults is poor, with 40-50% long term overall survival (OS) for younger patients (Pollyea *et al*., 2014) and relapse in more than 50% of patients (Ravandi et al., 2018).

While in the elderly, median OS is less than one year despite treatment (Pollyea *et al*., 2014). This has been linked to leukaemic stem cells (LSCs) maintaining and perpetuating AML by producing identical daughter and undifferentiated cells. LSCs are therefore a source of relapse or refractory AML (Pollyea *et al*., 2014). Patients who cannot withstand intensive chemotherapy and are over 60 years old have just 5-10 month average survival (Döhner *et al*., 2015). However, about 35%-40% of patients under 60 years of age and 5%-15% of those above 60 years go into remission with AML treatment (Saultz & Garzon, 2016). Current treatment of a subtype of AML known as acute promyelocytic leukaemia (M3) (according to FAB classification and under AML not otherwise specified in WHO classification) is ATRA and ATO (Dayton *et al*., 2017). Treatment outcome with ATRA has improved in the last two decades with above 80% remission rate (Lo-Coco *et al*., 2016). However, patients are at a risk of developing secondary leukaemias and severe infections. The combination of ATRA and ATO have shown to be more effective than the single treatments with less toxic effect on normal cells and more manageable, but the long term results are still awaited (Lo-Coco *et al*., 2016). The recently concluded C10603/RATIFY trial have led to the approval of Midostaurin/Rydapt for AML patient with FLT3 mutation (Pratz & Levis, 2017). The treatment strategy for FLT3 positive cells includes the backbone of AML treatment which is the 7+3 regimen (cytarabine 200 mg/m^2 for 7 days and daunorubicin 60mg/m^2 for 3 days), 8-21 days with midostaurin 50mg twice daily consolidated with cytarabine, and after consolidation stage, midostaurin is given as a single agent for 12 months (Levis, 2017). Other combinations of FLT3 inhibitors and chemotherapy which have also been reported to work synergistically and are more effective than the individual drugs include: CEP-70 and cytarabine, daunorubicin, etoposide, or ormitoxantrone (Levis *et al*., 2004), SU11248 and cytarabine or daunorubicin, (Yee *et al*., 2004). ATO and MEK inhibitor, as well as ATO in combination with a FLT3 inhibitor (AG1296) has also been reported to show synergistic effect on FLT3-ITD cells (Takahashi *et al*., 2006).

Establishing FLT3 oncogenic signalling in AML may improve AML therapy and identifing phospho FLT3 interacting proteins may provide more pathway coverage and identify other druggable targets (Chan, 2011). In addition, identifing phosphatases and kinases that mediates FLT3 oncogenic signalling may provide insight into the mechanism of FLT3 mutation (Chan, 2011).

2.3 FLT3 kinase structure and function

FLT3 kinase is also known as stem cell tyrosine kinase 1 (STK-1), fetal liver tyrosine kinase 2 (FLK2) or CD135. FLT3 is expressed in haematopoetic stem cells, primarily in commited myeloid progenitors, as well as in lymphoid progenitors with irregular expression in more mature monocytic lineage (Meshinchi & Appelbaum, 2009). Expression in haematopoeitic organs (spleen, liver, placenta and thymus) due to the presence of haematopoietic cells has been reported (Stirewalt & Radich, 2003). FLT3 is not expressed in megakaryocyte, erythroid and mast cells (Stirewalt & Radich, 2003). Therefore, FLT3 ligand stimulation does not affect these cells. Although FLT3 ligand is expressed in both haematopoietic organs and other tissues/organs (ovary, kidney, lung, heart, testis, small intestine and colon except the brain), FLT3 kinase expression is limited to only haematopoietic or progenitor cells (Stirewalt & Radich, 2003). Activation of FLT3 by binding of FLT3 ligand induce production of early progenitor cells (Stirewalt & Radich, 2003).

FLT3 regulates cellular process (including phospholipid metabolism, proliferation, transcription and apoptosis) and play a crucial role in controlling haematopoiesis and cell growth (Grafone *et al*., 2012). For optimum function of activated FLT3, it requires growth factors (including SCF and IL3) to promote the proliferation of haematopoietic progenitor cells and early commited myeloid and lymphoid precussors (Meshinchi & Appelbaum, 2009). Marked proliferation was not observed in cells stimualted with FLT3 ligand without growth factor but vigorous proliferative response was observed when growth factors (IL-3, Kit ligand, GMCSF, CSF1, G-CSF and Epo) stimulate with FLT3 ligand (Stirewalt & Radich, 2003). Combination of FLT3 ligand, IL-3 and Kit ligand increase proliferative effect in myeloid progenitors and FLT3 stimulation also promote lymphoid development (Stirewalt & Radich, 2003). During haematopoietic stress, *in vitro* data has confirmed the role of FLT3 in normal haematopoiesis (Mackarehtschian *et al*., 1995). Transplantation of early haematopoietic progenitor cells with a mixture of wild type FLT3 and non functional FLT3, resulted in the bone marrow reconstituted with cells expressing mostly wild type FLT3 (Stirewalt & Radich, 2003). Suggesting growth advantage of the cells in all cells lineage and indicating global distrubance in the haematopoietic system if there is disruption of FLT3 (Stirewalt & Radich, 2003). Kit and FLT3 knockouts in mice revealed lethal haematopoietic defieciency. Therefore, the role of FLT3 in normal haematopoiesis is important but not absolute and stimulation with growth

factors induce cell proliferation and also differenciation in both myeloid and lymphoid cells (Stirewalt & Radich, 2003).

FLT3 is found on chromosome 13q12 and belongs to the class of receptor tyrosine kinase (RTK) type 3 which all have similar structure with four regions; immunoglobulin-like extracellular region with domains for ligand/substrate binding, a transmembrane domain, a regulatory juxtamembrane region and intracellular protein tyrosine kinase domain (Lemmon & Schlessinger, 2010). All RTK subclass 3 members play crucial roles in survival, proliferation and differentiation of haematopoietic cells (Stirewalt & Radich, 2003). FLT3 receptor in an inactive state is unphosphorylated and upon binding to a FLT3 ligand conformational changes in the receptor leads to the unfolding of the receptor, resulting in the dimerisation (forming a single dimer by joining two molecular subunits) of the domain or the exposure of the juxtamembrane (Meshinchi & Appelbaum, 2009). Binding of a ligand to FLT3 receptor results in autophosphorylation to produce phosphotyrosine residues, creating docking sites to bind downsream proteins involved in cell proliferation (Chan, 2011). The activated receptor develops a number of complex protein–protein interactions in the intracellular domain (Meshinchi & Appelbaum, 2009). Such proteins, also known as phosphotyrosine domain proteins, include GRB2, SHC proteins, SHIP, CBLB, SHP2 and CBL (Meshinchi & Appelbaum, 2009). Once these proteins bind to the complex, they are activated resulting in phosphorylated reaction cascade that accumulate to activate secondary mediators such as STAT, P13K/PKB, ERK and MAP kinase signal transduction (Wander *et al*., 2014). Once these secondary mediators are activated they are chaperoned by HSP90 to the nuclear interphase and once in the nucleus, they activate signalling cascade that promotes gene transcription regulating cell survival, proliferation, differentiation of cells, and apoptosis (Meshinchi & Appelbaum, 2009; Larrosa-Garcia & Baer, 2017).

2.3.1 FLT3 mutation and its function

There are two distinct types of FLT3 mutations observed in AML patients. Mutations of internal tandem duplication (ITD) in FLT3 receptor tyrosine kinase (FLT3-ITD) is not only the most common mutation observed in 20% of AML patients but also involved the most in AML prognosis and it is associated with poor prognosis (Smith & Shah, 2013). Most scientific publications with FLT3-ITD mutation identify this mutation as an unfavourable prognostic factor with reduced

overall survival (Prada-Arismendy *et al*., 2017). In ITD mutatiom, 69.5% occur in the juxtamembrane region and 30.5% in the kinase domain (Figure 2.1) (Patnaik, 2017). FLT3-ITDs occurs when a fragment of the coding sequence of the juxtamembrane is duplicated (Stirewalt & Radich, 2003). This duplication is inserted with an orientation of head to tail and this could be insertions of about 3 to > 400 base pairs (Patnaik, 2017). The juxtamembrane region with varying length of ITD insertion of head to tail orientation and preserved reading frame. The transcript reading frame of ITD is always preserved by either frame duplication or nucleotide insertion at the ITD junction, so the original reading frame is maintained (Stirewalt & Radich, 2003). The conformational change (constitutively active) due to its 3 to > 400 base pairs in frame duplication in the juxtamembrane region is responsible for the activation and dimerization of the receptor (Takahashi, 2011). Therefore, the ITD mutation causes FLT3 receptor to undergo constitutive activation (Parcells *et al*., 2006). The cause of duplication is unknown, however the mutation has been reported to be due to general failure in mismatch or slippage repair mechanism in DNA replication process (Parcells *et al*., 2006). Absence or low expression of FLT3-ITD suggest increased overall survival (Ugo & Pelosi, 2013). FLT3–ITD are not diagnosed through secondary AML but through de novo and are conferred to have high relapse and the worse overall survival (Kayser & Levis, 2014). If detected in MDS it makes the leukaemic cells progress more rapidly to AML with shorter survival, indicating a driver mutation from MDS to AML (Ugo & Pelosi, 2013). The second common type of FLT3 mutation is the mutations observed in tyrosine kinase domain (TKD), specifically at the activation loop. The mutations in the tyrosine kinase domain involves substitution of aspartate to tyrosine at codon 835. However other substitutions have been observed (Takahashi, 2011). TKD activating mutation occurs in about 14% of patients, with 90.5% occuring within the tyrosine kinase domain 2 (TKD2) activation loop and 9.5% within the TKD1 (Figure 2.2) (Patnaik, 2017). FLT3 activation loop missense mutation has been observed (Meshinchi & Appelbaum, 2009). Both mutations in the juxtamembrane (ITD) and tyrosine kinase domain (TKD) result in constitutively active FLT3 receptor signalling that promote cell survival and proliferation in AML patients (Larrosa-Garcia & Baer, 2017).

FLT3-ITD has been detected in MDS (3%) and occasionally in CML, ALL and CLL (Meshinchi & Appelbaum, 2009). In FLT3 mutations, the receptor is constitutively active due to disruption of its autoinhibition (Takahashi, 2011).

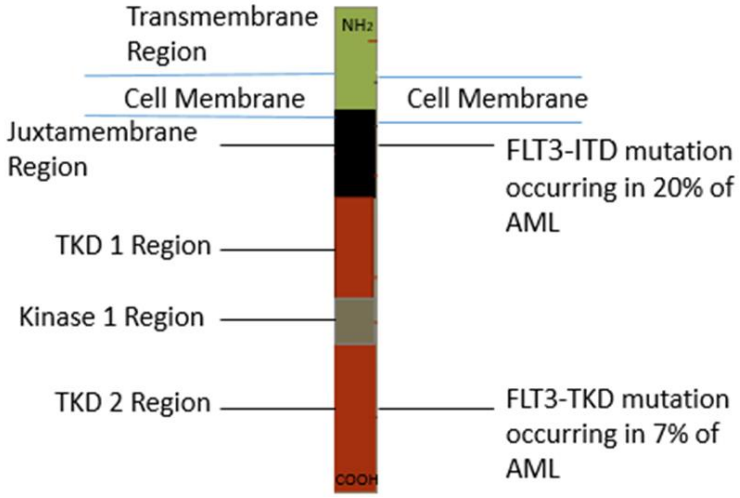


Figure 2.1: FLT3 mutations. Muations of FLT3 surface kinase receptor occuring as Internal Tandem Duplications (FLT3-ITD) near or in the juxtamembrane region. Mutation occuring as point mutations due to substitution of single amino acid in the activation loop located in the tyrosine kinase domain region known as FLT3-TKD. FLT3 is expressed mostly in haematopoietic progenitor cells and regulates proliferation, differentiation, cell survival and apoptosis (Prada-Arismendy *et al*., 2017). Hence, duration of remission with FLT3 mutations is short and associated with high relapse rate (Lim *et al*., 2017).

2.3.1.1 Differences between FLT3-WT and FLT3- ITD mutataion

FLT3-WT (normal FLT3) is expressed on CD34+ cells and its binding to ligands like other tyrosine kinases phosphorylate and activate signalling cascades such as STAT5, PI3K /PKB/mTOR, MAPK/ERK and RAS/MEK pathways that act on the progression of the cell cycle, activation of cell differentiation and inhibition of apoptosis (Wander *et al*., 2014). In the case of mutation, higher levels of mutant FLT3 is expressed that is not associated with CD34+ cells and ligand independent despite being ubiquitous and phosphorylating proteins involved in different signalling cascades (Wander *et al*., 2014). FLT3 mutation can still respond to ligand binding and in its presence (ligand), there is more phosphorylation of P13K and activation of MAPK increases (Grafone *et al*., 2012). Exogenous FLT3 ligand has been reported to increase blast proliferation in both mutant and WT-FLT3 (Bruserud *et al*., 2003) and therefore, important for triggering FLT3 signalling (Takahashi, 2011). The FLT3-ITD juxtamembrane domain undergoing conformational change promote dimerisation that is ligand-independent, constitutively activated and autophosphorylation of the receptor, leading to proliferation that is cytokine-independent and

inappropriate signal pathways activation, blocking haematopoietic progenitors from myeloid differentiation (Grafone *et al*., 2012). FLT3-ITD signalling to some degree represses differentiation (through repressing PU.1 and C/EBP transcription factors) and it has been identified that blocking differentiation promote cancer progression (Parcells *et al*., 2006). Kinase receptors of ITD and WT regulates signalling pathway in different ways, specificly the activation of signal transducer and activation of transcription 5 (STAT5). FLT-WT does not bind phosphorylated STAT5 to DNA while high level of STAT5 phosphorylation, haboured by FLT-ITD cells binds to DNA (Grafone *et al*., 2012). FLT3-ITD may accelerate cell growth through STAT5 and Pim serine threonine kinases (Pim-1 and Pim-2) (Takahashi, 2011). RAS and P13K signalling pathways are activated in parallel in both ITD and WT (Grafone *et al*., 2012). The expression of FLT3-ITD has been reported to activate PKB and concomitant Forkhead family member (FoxO3a) phosphorylation, which does not only promote translocation of FoxO3a to the cytoplasm from the nucleus but inhibit FoxO3a mediated apoptosis. Therefore, suggesting negative regulation of FoxO transcription factors by oncogenic tyrosine kinase and suppression of its role to mediate apoptosis, resulting in proliferation and survival of AML cells (Takahashi, 2011). In addition transcription factors (PU.1 and C/EBP) that are expressed in normal myeloid development are repressed in cells expressing FLT3-ITD therefore inhibit differentiation (Larrosa-Garcia & Baer, 2017).

Activation of downstream signalling by FLT3-ITD may be increased due to inhibition of cellular phosphatases (SHP-1) that can amplify anti-apoptotic and proliferative effect (Grafone *et al*., 2012). Increased expression of SHP-1 correlate with inhibition of FLT3-ITD phosphorylation and loss of SHP-1 function result in factor-independent growth (Parcells *et al*., 2006). In addition, deregulation of the activity of Wnt signalling pathway through expression of Frz-4 receptor by FLT3-ITD result in active mutant receptor in the absence of natural Wnt ligand (Grafone *et al*., 2012). The Wnt signalling pathway is implicated in haematopoietic stem cell proliferation and renewal. Its deregulation promote carcinogenic processes through its down stream molecules that act as either proto-oncogenes or tumour suppressors (Grafone *et al*., 2012). FLT3-ITD promote genomic instability through induction of reactive oxygen species (ROS) that promote double strand breaks and error in repair (Lim *et al*., 2017).

Taken together, four major features distinguish the wild type FLT3 from FLT3-ITD. Firstly, wild type FLT3 activate down stream effectors (RAS, PKB and STAT5) in a manner different from

mutant FLT3 (Stirewalt & Radich, 2003). WT-FLT3 after binding of FLT3 ligand cannot bind to the p85 subunit of PI3K directly but instead phosphorylates SHC protein that form complex with other proteins (GRB2, GAB2, SHP, SHP2, CBL, SHIP, CBLB) which then act on p85 subunit of PI3K to activate the PI3K pathway and also RAS via GRB2 (Figure 2.3) (Stirewalt & Radich, 2003). PI3K and RAS activities are regulated by interaction of FLT3 with SH2 (Src-homology 2) containing sequence (SHC) proteins (Grafone *et al*., 2012). FLT3 mutant activation of P13K and RAS pathway dose not require SHC adaptor proteins, thus it recruits its pathogenic pathhway that upon phosphorylation there is direct contact with the p85 subunit of PI3K and RAS pathway is activated without GRB2 (Stirewalt & Radich, 2003;Grafone *et al*., 2012). Loss of autoinhibitory effect is associated with the ITD duplication resulting in conformation change to catalytic active state (Stirewalt & Radich, 2003). Therefore, regulation or inhibition of PI3K and RAS pathways is lost in FLT3 mutant due to its constant active state and absence of SHC adaptor protein.

The second distictive feature is expression of STAT5 trancription factor. High expression of phospho STAT5 and increased DNA binding was observed with FLT3-ITD, which was not observed with FLT3-WT. There is no significant activation of STAT5 by FLT3-WT which indicates that unique downstream phosphorylation by mutant FLT3 is targeted to induce abnormal phenotype (Parcells *et al*., 2006). STAT5 activates PIM-1 which increases cell survival and mitogenesis when induced by FLT3 ligand related cytokines (Parcells *et al*., 2006). Activity of PIM-1 phosphorylate cdc25A and Bad which are cell cycle progression and antiapoptotic agents, respectively (Parcells *et al*., 2006).

The third feature is the repression of differentiation markers Pu-1 and c/EBPα in FLT-ITD but expressed more in normal FLT3 (Grafone *et al*., 2012). Finally the fourth distinctive feature is the expression of the Frizzled- 4 (Frz-4) receptor which is activated in the absence of Wnt ligand by FLT3-ITD. Frz-4 activation regulates β-catenin a transcriptional coactivator for TCF/LEF transcription factors, cyclin D1 and c-myc, and genes for transcription. Activation of β-catenin and TCF/LEF signalling cascade without Wnt ligand by FLT3-ITD promote leukemic progression due to significant cell proliferation (Grafone *et al*., 2012). High expression of β-catenin induced by FLT3-ITD increases stability which promotes dependent transcriptional activity with TCF/LEF (Grafone *et al*., 2012). The Frz-4 pathway was not observed with FLT-WT (Grafone *et al*., 2012).

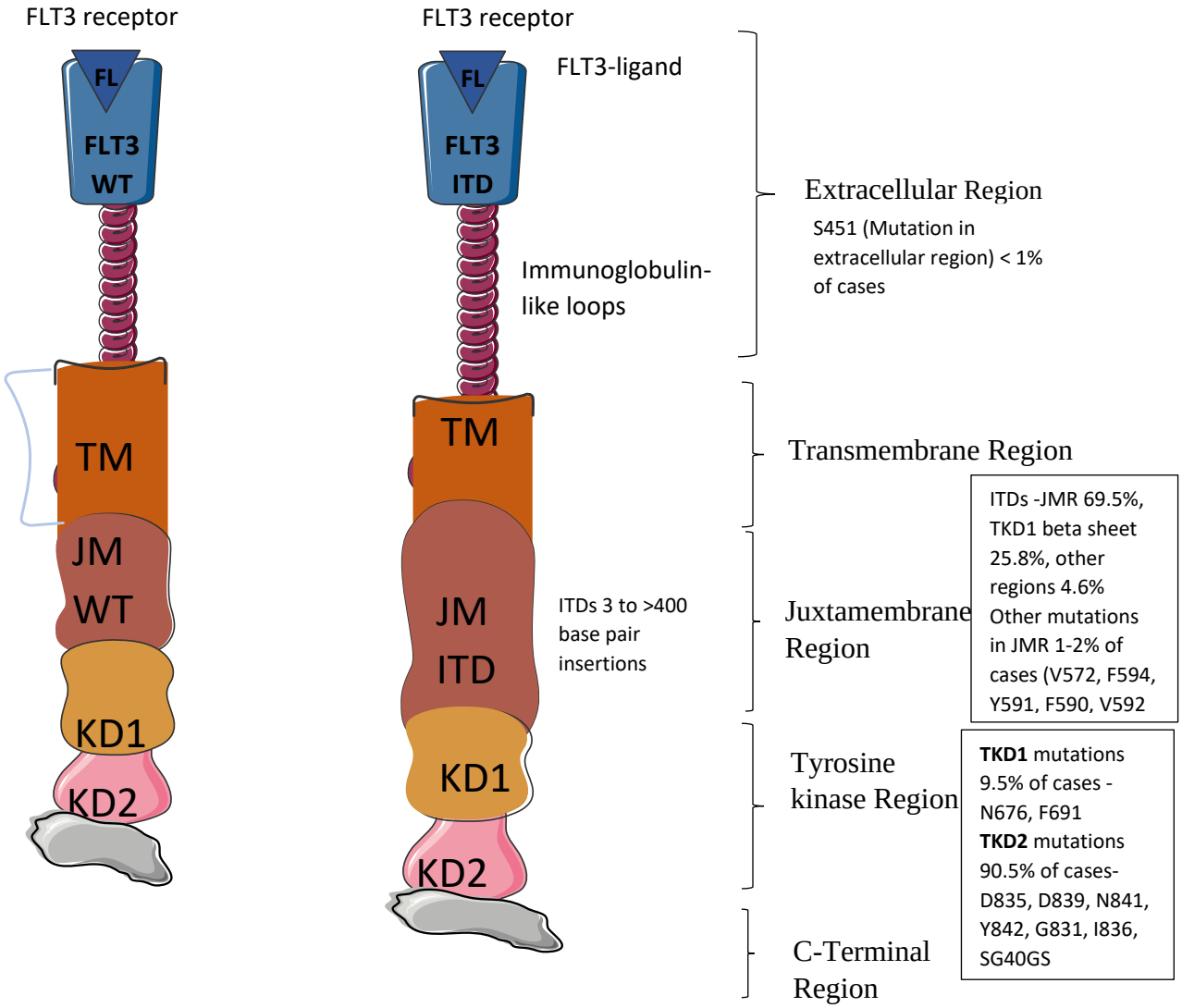


Figure 2.2: Schematic diagram of wild type FLT3 and FLT3-ITD receptor structure

consisting of extracellular region, transmembrane region, juxtamembrane region, kinase region and C-terminal region. Internal tandem duplications ITD is the most common FLT3 mutation and could consist of 3 to >400base pair (bp) insertion. ITD mutation can occur in JM (69.5%). Beta1 sheet pair of TKD1 (25.8%) and other regions in TKD1 (4.6%). Activating mutation in TKD or TKR occurs mostly in TKD2 (90.5%) and less in TKD1 (9.5%). Most mutations in the TKD are point mutations due to amino acid changes. Other activating mutations in the TKD due to insertions and deletions have been identified. Additional activating mutations within the extracellular region (<1%) and JMR (<1-2%) have been identified. Additional point mutations within the extracellular region, transmembrane region, JMR, TKD1 and TKD2 have also been identified in AML ptaients (Patnaik, 2017).

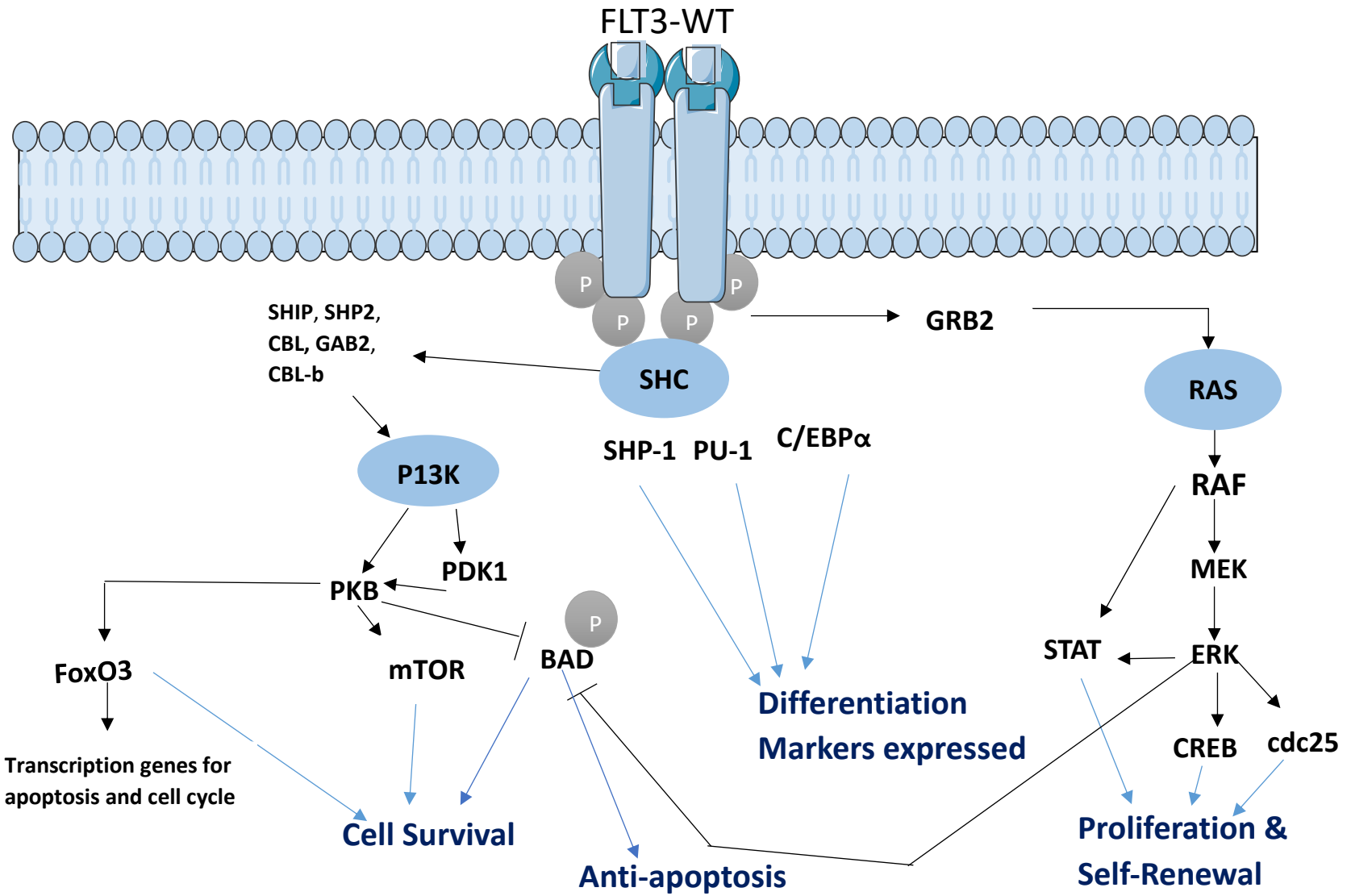


Figure 2.3: FLT3-WT signaling cascade (downstream effect) and biological mechanisms that regulates haematopoietic cells.

FLT3-WT interact with SHC proteins upon activation and form complexes that activate PI3K and RAS pathway. STAT5 is activated via RAF and it prevent gene expression that apoptosis (Bax). P13K is regulated by FLT3 via SHC adaptor proteins. P13K is involved in proliferation and phospholipid metabolism. P13K activate PKB either directly or indirectly through PDK1 and PKB activate mTOR. mTOR activate key transcription gene such as p70S6 kinase that promote survival of cells and protein synthesis. PKB prevents apoptosis by phosphorylating BCL2 antagonist (Bad). PKB activate FoxO3 which is associated with transcription of apoptosis and cell cycle regulation. Fork head transcription through transcription factors control survival, apoptosis and cell cycle. Activation of PI3K and PKB promote cell survival in all cell types. Antiapoptotic signal is induce in haematopoietic progenitor cells via PKB that is dependent on cytokine (Stirewalt & Radich, 2003 ; Grafone et al., 2012). Ras via ERK inhibit. phosphorylation of Bad to prevent apoptosis. P53 targets Bad to release Bax for apoptosis.

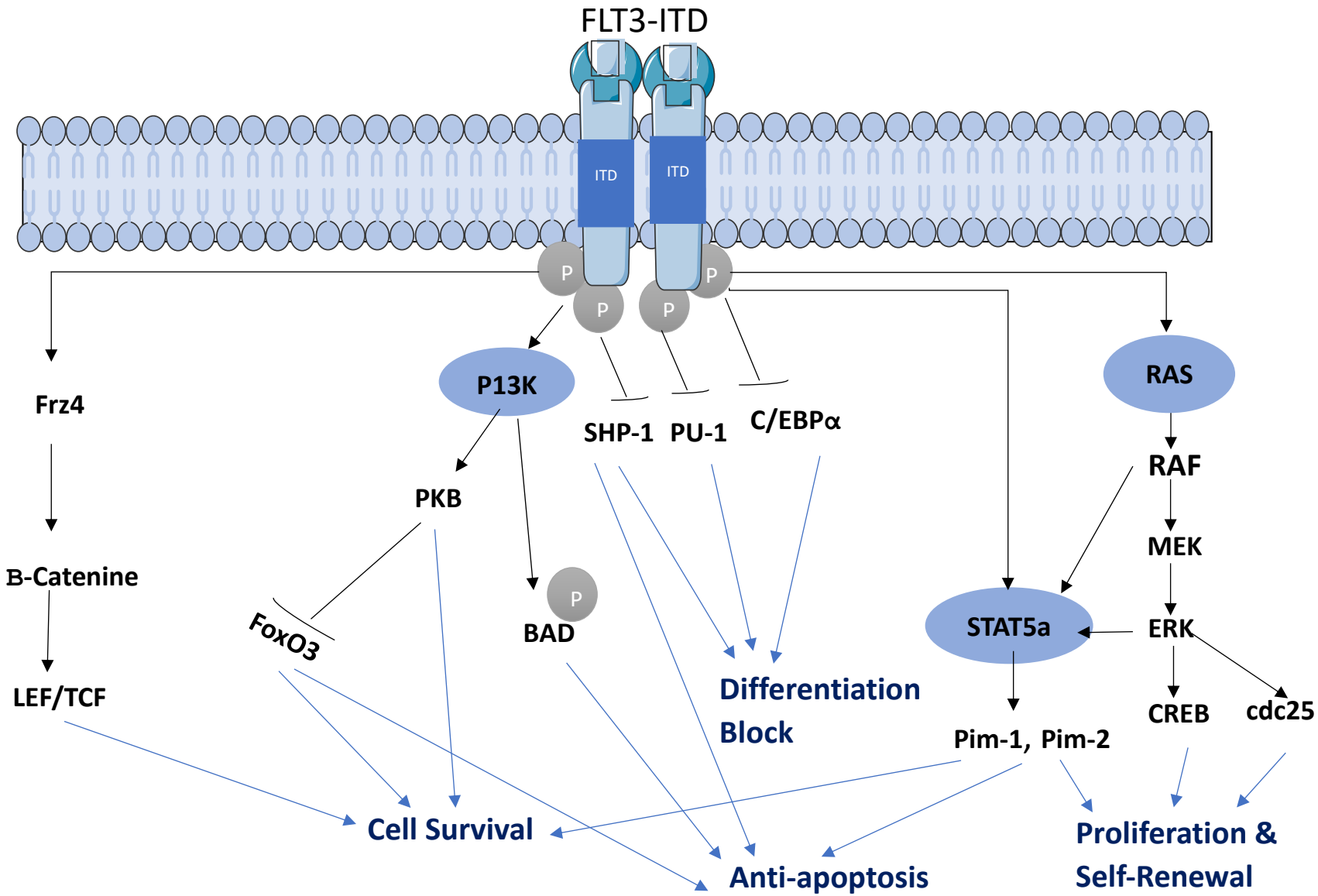


Figure 2.4: FLT3-ITD signaling cascade (downstream effect) and biological mechanisms that promote AML cells during mutation. Binding of FLT3 ligand triggers activation of PI3K (phosphatidylinositol 3-kinase) and RAS pathways resulting in increased cell proliferation and anti-apoptosis. PI3K stimulates downstream effector protein kinase B (PKB) to induce cell survival. P13K inhibit apoptosis by phosphorylating BAD.RAS activation stimulates activation of RAF, MAPK/ERK kinases (MEK), extracellular signal regulated kinase (ERK) and cyclic adenosine monophosphate response element binding protein (CREB) which result in transcription of gene that regulates cell proliferation. Signal transducer and activators of transcription 5 (STAT5) regulates proliferation via RAF from RAS pathway. FLT3 mutant activate mitogen signaling pathway and proliferation factor RAS to induce self-renewal (Prada-Arismendy *et al*., 2017).

2.3.1.2 Differences between FLT3-ITD and FLT3- TKD mutation

FLT3-ITD and/or TKD mutations are found in AML patients with normal karyotype as well as karyotypic abnormalites, such as those associated with core binding factor and t(15;17)/PML-RARA (Patnaik, 2017; Wander *et al*., 2014). FLT3-ITD is commonly (up to 90%) associated with t(6;19)(DEK-NUP214) abnormalities (Patnaik, 2017). ITD proteins are localised in the perinuclear region (space between the outer and inner nuclear membrane) while WT proteins is present in the plasma membrane (also known as cell or cytoplasmic membrane) (Chan, 2011).

About 5-10% of patients express point mutations at residues D835, I835 and Y842 of the activating loop on the tyrosine kinase domain TKD1 (FLT3-TKD) which is the second most common mutation in exon 20 (Smith & Shah, 2013). The FLT3-TKD represent a mechanism of resistance (observed after relapse) (Levis, 2013). To a lesser extent, mutation at TKD2 (N676 and F691), and other smaller imsertion/deletion and point mutations occurs at TKD1, extracellular and juxtamembrane domains (2%) (Patnaik, 2017).

Mutations in either ITD or TKD leads to constitutive activation of the receptors but via different mechanisms and therefore with different biological consequences (Levis, 2013). Differential clinical presentations of ITD and TKD may be due to the difference in substrate specificity and hence differential activation of signalling pathways (Chan, 2011). Both FLT3-ITD and TKD mutations promote AML proliferation and survival through ligand- and cytokine-independent signalling pathways (Larrosa-Garcia & Baer, 2017). FLT3-ITD activates FLT3 through PI3 kinase (PI3K/PKB and MEK/ERK), STAT5 signalling and its effector Pim-1 kinase. TKD signal FLT3 through the PKB/ERK pathway but not STAT5 (Larrosa-Garcia & Baer, 2017). FLT3 ITD induces Pim-1 and STAT5 serine threonine kinases which may accelarate the growth of AML cells (Takahashi, 2011). FLT3-ITD mutation inhibit myeloid differntiation through the suppression of transcription factor as well as Pu-1 and CCAAT/estradiol-binding protein (C-EBpalpha) (Grafone *et al*., 2012). However, in FLT3-TKD mutataion, expression of Pu-1 and C/EBPA transcription factors are not suppressed (Larrosa-Garcia & Baer, 2017).

Within the FLT3 gene are internal tandem duplications which occur mostly in exon 14, encoding the juxtamembrane (JM) domain. Loss of autoinhibition result in constitutively active FLT3. There

are variable sizes of ITDs (C3-1236 nucleotides) and the size of the duplication in the receptor is independent of the loss of inbitory effect of FLT3 (Larrosa-Garcia & Baer, 2017). Long ITD length compared to shorter lenght has been reported to correlate with reduced overall survival and more likely for the TKD mutation to develop resulting in resistance to tyrosine kinase inhibitors (Grunwald & Levis, 2013).

Allelic ratios and ITD location vary in AML patients. Lower complete remission (CR) is associated with higher allelic ratios and shorter overal survivial (OS) (Larrosa-Garcia & Baer, 2017). Stem cells with FLT3-ITD mutation is CD34+/CD38- and are likely to result in relapse (Larrosa-Garcia & Baer, 2017). Relapse FLT3-ITD present new cytogenetic abnormalities and genomic instability due to increased double stranded DNA breaks, with increased generation of reactive oxygen species from error prone double strand DNA break repair (Larrosa-Garcia & Baer, 2017).

2.3.1.3 FLT3-ITD and other mutations in AML

FLT3-ITD is associated with normal karyotype, high blast counts (leukocytosis), high relapse rate, short overall survival and short relapse free survival (Larrosa-Garcia & Baer, 2017). Prefered treatment for patients in remission is HSCT but high early relapse rate results in poor outcome, suggesting targeted FLT3 signalling treatment after transplant (Larrosa-Garcia & Baer, 2017). FLT3-TKD mutation has no leukocytosis and is only modestly associated with negative impact on treatment outomes, which may be due to downstream signalling that differs between FLT3-TKD and ITD mutation (Larrosa-Garcia & Baer, 2017).

FLT3-ITD mutation is the most common mutation with decreased overall survival. Low survivial rate with increased length of ITD mutation could be due to higher intracellular phospho-JM kinase load and more substrates accessible due to efficient autophosphorylation (Chan, 2011). There is controversy over prognosis associated with FLT3-ITD fragment size (Arruda *et al*., 2017). It has been reported that a worse outcome is associated with 48-60 base pair duplications while other authors report that FLT3-ITD length alone cannot confirm this relationship due to varied clinical characteristics of patients, therefore, difficulty in correlating set standards to data (Arruda *et al*., 2017). Co-existence of nucleophosmin 1 (NPM1) with FLT3-ITD mutation may counter balance or reduce the poor prognosis of FLT3 to improve outcome (Wander *et al*., 2014). However, NPMI has been reported to have good prognosis and overall treatment outcome (85% of cases exhibit

complete remission), only when FLT3-ITD is not associated with it (Prada-Arismendy *et al*., 2017). However, some studies has suggested good prognosis with co-existence of ITD and NPMI while others have suggested negative impact by coexistence. NPMI mutation in both the young and elderly respond to intensive chemotherapy hence, associated with improved outcome and overall survival (Saultz & Garzon, 2016). In addition, co-existence of ITD and DNA methyltransferase 3A (DNMT3A) mutation suggest that there is functional cooperation between FLT3 mutations and other molecules for the transformation of leukaemic cells in AML patients (Takahashi, 2011). Poor outcome has been associated with co-occurrence of FLT3-ITD, DNMT3A and NPMI (Merino *et al*., 2018). NPM1 is considered a reliable therapeutic marker of MDR because during disease progression it is stable and correlates with response to treatment (Patnaik, 2017).

2.3.1.4 AML Kinase inhibitors

Tyrosine kinase inhibitors compete with available ATP binding sites on kinases thereby preventing phosphorylation of protein substrate which occur through the transfer of terminal phosphate of ATP (Kayser & Levis, 2014). Attempts to effectively target mutation in FLT3 were initially unsuccessful leading to only reduction in peripheral blast and slight response on the bone marrow as seen with first generation inhibitors lacking selectivity, favourable pharmacokinetic properties and potency (Smith & Shah, 2013). These first generation inhibitors were also called multikinase inhibitors targeting not only FLT3 but other receptor tyrosine kinases (RTKs) such as KIT, JAK2, PDGFR and VEGFR (Petrushev *et al*., 2016). These first generation inhibitors include sunitinib (SU11248), midostaurin (PKC412), lestaurtinib (CEP-701) and sorafenib (Bay43- 9006) (Wander, et al., 2014). Molecular insight into poor response observed, broadened the understanding of pathobiology of FLT3 which prompted improved second generation inhibitors (Wander *et al*., 2014).

The second generation inhibitors of FLT3 are quizartinib, crenolanib, PLX3397 and other inhibitors such as P13K, mTOR, AKT and MEK under development and may prevent emerging FLT3 TKI resistanace (Wander *et al*., 2014). KW-2449 is also a unique and potent inhibitor of FLT3 (Kiyoi, 2015). FLT3 mutation has moved from being prognostic marker to diagnosis marker since the approval of kinase inhibitors, therefore diagnosis of FLT-ITD and FLT3-TKD mutation is done in all AML patients and results available within 48-72h, indicating FLT3-ITD$^-$, FLT3-

TKD^{-}, FLT3-ITD^{+}, FLT3-TKD^{+}, FLT3-ITD^{low}, FLT3-TKD^{low}, FLT3-ITD^{high}, FLT3-TKD^{high} (Patnaik, 2017). FLT3 testing can be done using multiplex targeted therapy which is highly sensitive and has a short turn around time (Patnaik, 2017). The gene panel can also be used, and this can detect rare abnormalities and is suitable for patients enrolling for trials. However,use of the gene panel has long turn around time of 3-20 days (Patnaik, 2017).

Kinase mutations in AML such as C-KIT and JAK2 have inhibitors developed (Table 2.3). However, combination of kinase inhibitors with chemotherapy could either lower likelihood of relapse or improve remission rates since most potent inhibitors such as AC220 which is an FLT3 inhibitor can only achieve approximately 50% remission rate as a single agent (Smith & Shah, 2013), therefore has limited efficacy as single agents and rapid development of drug resistance. Mechanism of resistance to TKIs include appeareance of new mutations such as TKD in previously diagnosed ITD mutation, protective effects mediated by bone marrow stroma resulting in cell cycle arrest and not apoptosis (Kazi *et al.*, 2017). Only inappropriate FLT3 activation is blocked by FLT3 inhibitors in leukaemic cells with minimal haematopoietic side effect (Stirewalt & Radich, 2003).

Table 2.3: AML kinase and inhibitors

Mutation	**Target Inhibitors**
FLT3	Midostaurin (multi-kinase inhibitor) AC220 (quizartinib) Sorafenib PLX397 Crenolanib
KIT	Imatinib SU5416
JAK2	Ruxolitinib
mTOR	Rapamycin Everolimus Deforolimus
MEK	Trametinib
Aurora Kinase	AZD1152 MLN8237
CDK	Dinaciclib
PLK1	Volasertib

Kinase mutations and common inhibitors developed. Midostaurin has been approved for newly diagnosed FLT3-ITD patients. Adapted from (Smith & Shah, 2013).

Combination of FLT3 inhibitor and chemotherapy drug has been reported to be synergistically effective, and such combinations include CEP-70 and cytarabine or daunorubicin, etoposide, ormitoxantrone (Levis *et al*., 2004). SU11248 and cytarabine or daunorubicin, (Yee *et al*., 2004). Arsenic trioxide (ATO) and MEK inhibitor, as well as ATO in combination with FLT3 inhibitor has been reported to show synergistic effect on FLT3-ITD cells (Takahashi *et al*., 2006).

Recently, midostaurin a multi kinase inhibitor that showed no significant effect on FLT3 mutation as single agent, when combined with standard therapy for AML has shown promising effect and has therefore been approved by FDA in 2017 for FLT3 mutant AML patients (Perl, 2017). However increase in rashes has been observed in patients treated with midostaurin and may be the probability of long term effect.

FLT3 is expressed primarily on commited myeloid progenitors as well as lymphoid progenitors with irregular expression in more mature monocytic lineage (Meshinchi & Appelbaum, 2009). It regulates cellular processes (phospholipid metabolism, proliferation, transcription and apoptosis) and plays crucial role through these process in controlling haematopoiesis and cell growth (Grafone *et al*., 2012). Inhibition of abnormal biological response by FLT3 inhibitors could regulates and induce proper function affected by mutation. Regulation of cell proliferation, apoptosis, cell cycle and senesscence could inhibit cell survival promoted in AML cells. Therefore, the need to study induction of apoptosis, cell cycle arrest, senescence (inhibit tumoriogensis) and inhibition of FLT3-ITD phosphorylation.

2.3.2 Programmed Cell Death

Apoptosis is programmed cell death characterized morphologically by cell shrinkage (Kuno *et al*., 2012), nuclear fragmentation, chromatin condensation, cell volume reduction and membrane blebbing (Koff *et al*., 2015). Biochemical characteristics includes caspase activation, breakdown of protein and DNA molecules, and modification of membrane surface which allows the recognition of apoptotic cells to be engulfed by phagocytes (Koff *et al*., 2015). Programmed cell death also plays crucial roles in development of embryo and tissue homeostasis (Millimouno *et al*., 2014). Cysteine-dependent aspartate directed proteases (caspases) regulates apoptosis at cellular level and cause cell death through a cascade of events (Palai & Mishra, 2015). They activate each other and amplify apoptotic signalling pathway that result in rapid cell death (Elmore, 2007). Activation of caspases indicates an irreversible commitment to cell death (Elmore, 2007). There are different types of caspases (Table 2.4).

Table 2.4: Caspases involved in apoptosis

Cysteinyl aspartic acid-protease (caspase)		
Type	name	synonyms
Initiator (or Apical)	Caspase-2	ICH1, Nedd2
	Caspase-8	FLICE, MACH1, MCH5, FADD-like Ice
	Caspase-9	MCH6, ICELAP6
	Caspase-10	FLICE2, MCH4
Effectors (or executioner)	Caspase-3	CPP32, YAMA
	Caspase-6	MCH2
	Caspase-7	MCH3, CMH, ICELAP3
Inflammatory	Caspase-1	ICE
	Caspase-4	ICH2, TX, ICErII
	Caspase-5	ICErIII, TY
	Caspase-11	-
	Caspase-12	-
	Caspase-13	ERICE
	Caspase-14	MICE

Adapted from (Kuno *et al*., 2012) Caspasess are expressed in most cells as inactive proenzyme form. Active procaspase inducing proteolytic cascade when activated (Elmore, 2007). Initiator caspase induce effector cascade to initiate cell death.

Dysregulated apoptosis can lead to variety of diseases and the development of drug-resistance by cancer cells and/or tumour formation (Millimouno *et al*., 2014). Necrosis is an inflammatory cell death due to uncontrolled release of cellular contents leading to damage of surrounding cells and corresponding tissues (Khoo *et al*., 2010).

Apoptosis is a non-inflammatory cell death which occurs via two pathways; the intrinsic or extrinsic pathway. The mitochondria or intrinsic pathway is activated due to structural changes upon detecting internal stimuli such as growth factor deprivation or DNA damage, resulting in upregulation of Bax and Bak (Koff *et al*., 2015). Bax and Bak are pro-apoptotic Bcl-2 proteins which line the mitochondria membrane to form oligomers and they control permeability of the mitochondria membrane (Chaabane *et al*., 2013). This results in the release of certain proteins including cytochrome C (Koff *et al*., 2015). Apoptosome complex is formed when cytochrome C binds to Apoptotic Protease Activating Factor 1 (ApaF1) in the presence of ATP to activate caspase 9 which joins the apoptosome complex and then activate effector caspase such as caspase 3 leading to cell death (Deegan *et al*., 2014; Chaabane *et al*.,2013). The activities of caspases result in DNA fragmentation, nuclear fragmentation by binding to nuclear lamins, cell fragmentation and fragment golgi apparatus thereby resulting in internal killing of the cells (Chaabane *et al*., 2013). The mitochondria is involved in key events that includes release of caspases activators, loss of

mitochondria membrane potential, participation of proapoptotic and anti-apoptotic Bcl2 family of proteins (Millimouno *et al*., 2014).

External signals activate the extrinsic pathway and such signals include tumour necrosis factor α (TNFα) and Fas ligand (FasL) (Suliman et al., 2001). Apoptotic signals and be mediated through p53 alongside other proteins (TNF, TRAIL and Fas) which are highly specific mediator of the extrinsic pathways of apoptosis (Millimouno *et al*., 2014). Binding of stress ligand to death receptors containing intracellular death domain (Death Inducing Signalling Complexes (DISCs) initiates the extrinsic pathway (Millimouno *et al*., 2014). FasL binds to a death receptor (Fas receptor) and Fas associated death domain receptor (FADD) which activates formation of DISC that converts procaspase 8 to active caspase 8 (Palai & Mishra, 2015). Caspase 8 can either directly cleave or activate caspase 3 and /or involve the intrinsic pathway by cleaving Bid (which is a Bcl-2 homology 3 (BH3) protein) to t-Bid, the active form that translocate to the mitochondria and activate Bax and Bak to initiate the intrinsic apoptotic pathway (Kurokawa & Kornbluth, 2009). Through promoting Bax and Bak activation, it triggers mitochondria outer membrane permeabilization (MOMP) and proapoptotic mitochondria constituents are released into the cytoplasm (Kurokawa & Kornbluth, 2009). In some cells the direct activation of caspase 3 by caspase 8 is sufficient to induce cell death (Type 1 cells) while in some other cells caspase 8 must involve the mitochondria (Type II cells) (Parrish *et al*., 2013). Type I cells are thymocytes while type II cells are liver cells (Ozoren & El -Deiry, 2002).

The third apoptotic pathway involves cytotoxic T lymphocytes and natural killer cells; and both delivers to the target cells proteases that belong to the granzyme family (Charles & Rehma, 2014). The granzyme/perforin pathway induces apoptosis either via granzyme A which is caspase independent and cause death through single stranded DNA damage or via granzyme B which is caspase-dependent and directly activate caspase 3 or through activation of caspase 10 (Palai & Mishra, 2015).

2.3.3 p53 and Cancer

The underlying hallmark of cancer is genome instability or mutation which results in several biological capabilities such as sustained proliferative signal, resisting cell death, activating invasion, inducing angiogenesis and ensuring replicative immortality (Hanahan & Weinberg, 2011). Activating DNA damage, triggers the relaese of the protein p53 known as the guardian of the genome which is involved in several processes such as cell cycle, apoptosis and senescence pathways (Blanpain *et al*., 2011). The uncoupling of p53 from mediated degradation by MDM2 results in its induction, which is the main focal point of every initiating stimulus that leads to its induction or activation (Loughery & Meek, 2013). Therefore, MDM2 restrain p53 and act as its regulator, as well as MDM4 that support the effect of MDM2 and it is targeted during induction of P53 (Loughery & Meek, 2013). Aside from response to DNA damage, p53 can be induced in response to oncogenes activated, ribosomal stress and MDM2 targeted drugs (Loughery & Meek, 2013). DNA damage can be induced by heat shock, oxidative stress, irradiation, feed deprivation and chemotherapy drugs (Fulda *et al*., 2010).

Aberrant p53 genes are haboured frequently in human neoplasms with multiple mutations involved in disease progression since it is non functional in inhibition of cancer cells (Thompson *et al*., 2010). Inactivation of p53 due to mutation in over 50% of cancers in humans, is a major dysfuctional mechanism of p53 which sometimes result in chemoresistant phenotype (Ozaki & Nakagawara, 2011). p53 not affected by mutation could be inactive due to overexpression of p53 inhibitors such as MDM2 and MDM4 (Henry *et al*., 2012). Some tumours have p53 pathway partially inactive of signalling component (Amaral *et al*., 2010). About 95% of p53 mutations is found within the genomic region for DNA binding therefore lack the ability for sequence specific transactivation (Ozaki & Nakagawara, 2011). p53 is also a transcription factor and its transactivation ability is linked to the proapoptotic function of p53 (Ozaki & Nakagawara, 2011). p53 also known as wild type (wt) p53 are bound to the mutant p53 to form inactive tetramers and certain mutant p53 act on normal cells to transform them neoplastically by inhibiting the function of endogenous wild type p53 in a negative dominant fashion (Watanabe & Sullenger, 2000). The acquired oncogenic function of dominant mutation p53 is independent of endogenous wt p53 (Muller & Vousden, 2013). The mutant p53 through different pathways can increase genomic instability, induce metastasis, chemoresistance, survival and proliferation (Muller & Vousden,

2013). The wild type p53 is short lived (20 mins) while mutant p53 has a long half life (2-12 h) (Ozaki & Nakagawara, 2011). It is important for efficient chemotherapy to eliminate this negative dominant effect on wild type p53 by mutant p53 (Ozaki & Nakagawara, 2011). p53 comprises of three functional domains that include the NH_2 terminal domain, DNA binding domain and COOH terminal domain (Ozaki & Nakagawara, 2011).

2.3.3.1 p53 and Apoptotic Proteins

Most chemotherapeutic and radiotherapeutic agents target the intrinsic pathway of apoptosis to induce DNA damage (Mobahat *et al*., 2014). Regulators of the intrinsic pathway include Bcl2, Bcl-Xl, Bcl-W, MCL-1 and A1 which are antiapoptotic (inhibitors of apoptosis) family members of Bcl-2 that inhibit the release of cytochrome C. Proapoptotic (promoters of apoptosis) family members of Bcl-2 such as Bax, Bak, Hsp70, Puma, Noxa and Bok release cytochrome C. BH-3 only proteins such as Bad, Bid, Hrk, Blk, Bim, (regulators of apoptosis) help regulates the balance between antiapoptotic and proapoptotic bcl-2 family members (Parrish *et al*., 2013; Koff *et al*., 2015). In cancer, dysregulation of apoptotic proteins affects the cell cycle leading to the over-proliferation of cells. Overexpression of anti-apoptotic protein Bcl-2 has been reported to be implicated in AML and induce chemoresistance, hence an oral inhibitor of Bcl-2 (Venetoclax) has been introduced in AML treatments (Lim *et al*., 2017).

p53 is essential in the activation of apoptosis associated genes such as Noxa, Bax, Bak, Pidd, Puma, DR5, Bid, perp and p53AIPI (Schwartz, 2005). p53 protein activates apoptosis through the mitochondria or intrinsic pathway by acting as a pro-apototic BH3-only factor (Loughery & Meek, 2013). Bcl-2 homologous 3 (BH3) protein activate proapoptotic protein and inhibit antiapoptotic proteins causing outer membrane permeability of the mitochondria (MOMP) (Youle & Strasser, 2008). Puma is BH3 protein that is tightly regulated by p53, localised exclusively in the mitochondria and interacts with Bcl-XL and Bcl-2 to promote mitochondria cell death and multimerisation of Bax through BH3 domain it posses (Schwartz, 2005). The p53 targets Bad, and Bax is released and translocated to the mitochondria (Maximov & Maximov, 2008). p53 can induce Apaf-1 expression directly and has been reported to antagonize Bcl -2 and Bcl-XL by binding directly to them at the outer membrane of the mitochondria (Bharatham *et al*., 2011)

Apoptotic signals can be mediated through p53 alongside other proteins (TNF, TRAIL and Fas receptors) which are highly specific mediators of the extrinsic pathways of apoptosis (Millimouno *et al*., 2014). p53 activating apoptotic proteins such as Bax, Puma and Naxa can also trigger apoptosis by suppressing survivin, an antiapoptotic gene to promote the activation of caspase (Amaral *et al*., 2010). p53 enhances levels of Fas on cell surface when overexpressed, activate death domain receptor for TRAIL and promote caspase 8 cell death (Amaral *et al*., 2010). In addition, apoptosome activation through the induction of Apaf-1 expression involves the p53 (Amaral *et al*., 2010). In AML, p53 has been reported to inhibit self-renewal of cancer cells as its anticancer effect (Zhao *et al*., 2010). Loss of its function in AML promote aggressive cancer and has been linked to drug resistance and unfavourable outcome (Zhao *et al*., 2010).

2.3.3.2 The p53 Protein and the cell cycle

p53 transactivates genes (p21 and PUMA) as a master transcription factor that drives cellular responses such as cell arrest and apoptosis due to DNA damage (Zhao *et al*., 2016). The protein is short lived and is regulated by changing its protein stability (Hu *et al*., 2012). The regulatory region of p53 undergo several post translational modifications such as phosphorylation, ubiquitylation, methylation and SUMOylation (Dai & Gu, 2010). p53 attched to the E3 ligase of MDM2, is usually kept at reduced level by MDM2 through proteasomal and ubiquitin degradation known as ubiquitylation (Loughery & Meek, 2013). Proteasome and ubiquitin are molecules that degrade unwanted or damaged protein through ubiquitin proteosome system (Ross *et al*., 2015). MDM2/p53 at high levels becomes polyubiquitylated resulting in degradation and at low levels monoubiquityled (Li *et al*., 2003). p53 and its negative regulator MDM2 operate a negative feedback whereby p53 maintains the level of MDM2 by stimulating its expression (Wu *et al*., 1993). MDM4 which is related to MDM2 and known as defective E3 ligase act to suppress p53 mediated transcription and an MDM2 stimulatory partner that favours p53 polyubiquitylation (Loughery & Meek, 2013).

Induction and activation of p53 involves uncoupling p53 from MDM2 (Hu *et al*., 2012). Phosphorylation events uncouple p53 from MDM2 and this phosphorylation is activated by Ataxia telangiectasia and Rad3-related protein (ATR) and Ataxia telangiectosia mutated (ATM) protein kinases in response to any damage on the DNA caused by either single or double stand break

(Loughery & Meek, 2013). ATM responds to double strand break while ATR respond to single strand break (Loughery & Meek, 2013). The outcome of the uncoupling result in the recruitment of certain transcription factors (initially suppressed by MDM4) which leads to remodelling of the chromatin and transcriptional activation (Loughery & Meek, 2013).

Activated p53 in the cell cycle binds with DNA to activate different proteins including p21 an inhibitor of cyclin-dependent kinase (Appavu & Mohan, 2016). p53 activation block cells at G_1 and G_2 phase (Tu *et al*., 2012). Due to the role of p53 in preventing genome instability and prevent damaged DNA cells from proliferating, it is regarded as the guardian of the genome (Toufektchan & Toledo, 2018).

Cell cycle arrest is induced by p53 through cdk inhibitor p21 (Blanpain *et al*., 2011). The activation of P53 due to DNA damage induce p21 which act as a cyclin dependent kinase inhibitor (Schwartz, 2005). p21 acts on cdk/cyclin complex to inhibit its function. G_1 arrest is primarily due to p21 while G_2 arrest is due to p21 and 14-3-3 induction, thus the arrest via p53 and p21 pathway (Schwartz, 2005). This ensures that the DNA is in the proper amount without mutation or damage, otherwise cells will undergo arrest and DNA repair (Schwartz, 2005). From the oncology point of view the mechanism of cell arrest and DNA repair provide healthy cells a protective covering from the effect of chemotherapy since they are not affected and tumour cells are protected because they will keep undergoing repair and limit the effect of chemotherapy (Schwartz, 2005).

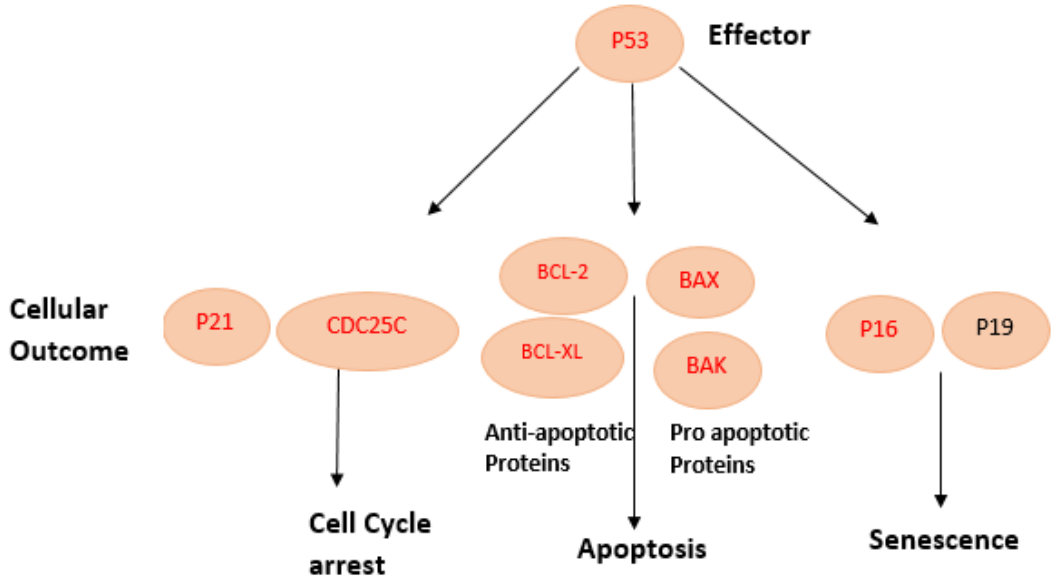


Figure 2.5: DNA damage response pathway (DDR) involving p53 and cellular outcomes (cell cycle,apoptosis and senescence). Adapted from (Blanpain *et al*., 2011).

2.3.4 CELL CYCLE

The cell cycle plays an important role in normal cells and disease conditions through its involvement in growth, repair, development and reproduction (Gérard *et al*., 2015). The process of DNA replication and cell division involves series of coordinated events in the cell cycle (Collins *et al*., 1997). The cell cycle refers to events that comprises of sequential actions leading to cell proliferation such as DNA synthesis (S phase), cell division (M phase), intervening gap phases for cell growth (G_1 and G_2 phase) and genomic integrity check point (G_2 phase) (Hindley & Philpott, 2013) and cell division (cytokinesis) (Tyson *et al*., 2002). The S and M phases are two distinct phases required for the progression of cell cycle (Gérard *et al*., 2015). A cell replicating in a day would spend about 42% of its time in the G1 phase (6-12 h), 38% in S phase (6-8 h), 16% in G_2 phase (3-4 h) and 4% in mitosis phase (1 h) resulting in a total of 24-25 h (Gehringer, 2010). The periodicity of DNA replication depends on the type of cell involved, for instance liver cells replicate once every one or two years, while normal gut cells replicate twice every day (Gehringer, 2010).

The cell cycle checkpoints takes place at late G_1 preventing entry into the S phase and late G_2 phase preventing entry into mitosis (Dipaola, 2002). The G_1 phase contains newly generated cells and these cells spend most time in this phase (Reynolds & Schecker, 1995). Non-dividing cells enter the resting or quiecent phase known as G_0 and at this phase the cell cycle is dismantled partially and the cells can differentiate or acquire specialized characteristics (Reynolds & Schecker, 1995). Quiecent cells are nondividing cells that has the capacity to divide but lack the nutrients to do so while senescent cells are nondidvidng cells that can never divide despite the presence of nutrients. Quiecent cells are not considered to be in cell arrest (Blagosklonny, 2011). A cell can leave the G_0 phase and enter the G_1 phase (prereplicative phase) thus begining the cell cycle (Yanagida, 2015; Reynolds & Schecker, 1995).

The S phase (DNA synthesis phase) comprises of replicating DNA double stranded molecule to produce sister chromatid pairs held by cohesins proteins (Tyson *et al*., 2002). G_2 or DNA replication checkpoint ensures that there is accurate duplication of genome and DNA is repaired by replication when damaged before a cell progresses to mitosis (Yanagida, 2015). For the onset of M phase, there is activation of protein kinases such as cdk1, polo and aurora (Yanagida, 2015). The M phase consist of 4 subphases; prophase, metaphase, anaphase and telophase. In the prophase there is condensation of the chromosomes into compact structures and there is alignment of the condensed chromosomes on the mitotic spindle which is the mid plane (Tyson *et al*., 2002). Anaphase is delayed until spindle assembly checkpoint allow proper alignment of all chromsomes (Lim & Kaldis, 2013). In anaphase cohesins are degraded and partitioning of sister chromatids into two bundles separately occurs and in telophase the cell divides when the daughter nuclei is formed (Tyson *et al*., 2002). At the end of the M phase duplicated genetic material in the cell is divided into two daughter cells that are identical (Suryadinata *et al*., 2010). There is guaranteed progression of the cell cycle due to strict dependency on the completion of the previous stage before intiation of a new stage (Urrego *et al*., 2014).

2.3.4.1 Cell Arrest And Cancer

Cell cycle deregulation underlies uncontroled cell proliferation in cancer (Williams & Stoeber, 2012). In normal cells, damaged DNA is repaired during G_1 arrest or the damage cells commit suicide via apoptosis. However in cancer cells there is deficient G1-S checkpoint, therefore they

depend on G2-M checkpoint for repair of DNA (Matheson *et al*., 2016). The retinoblastoma protein (pRB) gene inactivation due to mutation is a frequent occurance in cancer (Williams & Stoeber, 2012). In addition tumour suppressor genes (p15, p27 and p16) that encode cdk kinase inhibitors (cdkIs) are inactivated in common tumour types and this drives cell progression without checkpoint control mechanism (Williams & Stoeber, 2012). It has been observed that proteins in G_1 phase can promote cancer development (Reynolds & Schecker, 1995), and can drive proliferation by cdk6 or cdk4 complexes (Asghar *et al*., 2015). In addition pivotal tumorigenic events can occur through cdk2 and cdk1 which mediates control of S and G_2M phase, respectively (Asghar *et al*., 2015). $p27^{kip}$, cyclinA and cyclin E are regulators of G1-S and switching off of G_1-S is due to degradation of p27kip controled by cdk2 protein (Kar, 2016). The timing of the transition of G1-S is controlled by cdk2-cyclinE1 complex and only in cells where there is absence of cyclin E1 does cyclin A alter the G1 phase length of time (Kacar, 2016).

In cancer, inhibition and expression of CDKs are deregulated (Kacar, 2016). Therefore targeting cell cycle kinases could be promising in cancer therapy (Kollmann *et al*., 2013), since arrest of cell cycle is a barrier in cancer (Malumbres, 2011). The first inhibitor drug for cdk, is IBRANCE (palbociclib) apporved by US FDA (for the treatment of breast cancer), P1446A-05 for the treatment of leukaemia (Kacar, 2016). cdk inhibition are of interest in cancer research and more specific inhibitors of cdks at different checkpoints are required. (Kacar, 2016). Cancer occurs due to uncontrolled cell division and understanding of cell cycle regulation can be of relevance to the activity of drugs in cancer. There are specific proteins that contribute to the regulation of the cell cycle.

2.3.4.2 *CDK, CYCLINS AND CKIs IN CELL CYCLE*

2.3.4.2.1 Cyclin Dependent Kinase (cdk)

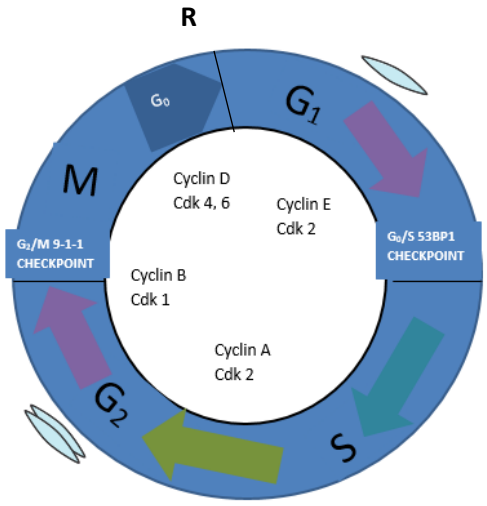
Protein kinase complexes stimulate cell cycle progression and they consists of a cyclin dependent kinase (cdk) and a cyclin (Stewart *et al*., 2003). The regulation of the cell cycle is through phase specific Cdks and cyclin subunit (Hindley & Philpott, 2013). cdk is a serine/threonine kinase catalytic unit while the activating subunit is the cyclin (Suryadinata *et al*., 2010). Ubiquitin mediated degradation and transcriptional regulation of cyclin encoding genes restrict cyclin levels in the cell cycle, whereas the cdks are expressed throughout the cycle except inhibited by cdk

inhibitors (Stewart *et al*., 2003). cdk is activated by binding to a cyclin partner and site specifiic phosphorylation occurs (Stewart *et al*., 2003). cdk activities regulates cell cycle progression through gene expression and the level of gene expression regulates the accumulation of cyclins which is degraded once the phase is over (Bertoli *et al*., 2013). There are over 20 cdk family members, each with an ATP binding pocket, cyclin binding domain and activation-loop (Lim & Kaldis, 2013). Most members have inhibitory and activating phosphorylation sites (Lim & Kaldis, 2013). Four cdks; cdk1, 2, 4 and 6 control cell cycle and cdk 7-11 controling transcription (Malumbres, 2011).

2.3.4.2.2 Cyclins

Cyclins are short lived regulatory proteins that induce progression of cell cycle by activating cyclin dependent kinases (cdks) (Appavu & Mohan, 2016). Cyclin proteins are unstable and they vary in their levels throughout the cell cycle and controled by ubiquitin/proteasome pathway degradation (Sandal, 2002).

Eight types of cyclins have been described (cyclin A-H) and it is known that cyclin A-E affect the cell cycle directly. There are 3 general classes of cyclin based on the phase of the cell cycle. G_1 phase cyclins (cyclin D and E); S phase cyclins (Cyclin E and A) and the G_2/Mitosis cyclins (Cyclin A and B) that drive and promote mitosis (Behl & Ziegler, 2014); (Appavu & Mohan, 2016). In addition, the phosphatase cell division cycle 25 (cdc25) is required to dephosphorylate cdks at each phase for the cell cycle to progress (Appavu & Mohan, 2016). Members of the cyclin and cdk families can function in maintaning genome integrity and DNA repair mechinery (Lim & Kaldis, 2013). In the cycle, progression from one phase to another is dependent on the enzyme proteasome which degrades cdks, cyclin and cdc25 (Appavu & Mohan, 2016). Inhibition of proteasome blocks the degradation of cdks, cyclin and cdc25 after a phase is completed, hence result in cell arrest (Appavu & Mohan, 2016).



Phases	End Result
G_1 Phase	Growth and chromosome preparation for replication
S phase	DNA synthesis
G_2 Phase	Preparation for mitosis
M Phase	Mitosis
G_0 Phase	Temporary or permanent state for terminally differentiated cells

Figure 2.6: Cyclins, cdk and phases involved in cell cycle. **R** is the restriction line that commit cells to complete the cycle

2.3.4.2.3 CKIs

Cyclin kinase inhibitors (CKIs) restrict cdk activity and are divided into classes based on their structure and the specificity of cdk. They include the Ink4 (inhibitors of kinase cdk4) family members (p16, p15, p18, p19) and the Cip/kip (checkpoint or kinase inhibitor proteins) family members (p27, p21, p57) (Lim & Kaldis, 2013). The Inks inhibit cdk4 and also cdk6, and are involved in DNA repair, senescence, and apoptosis. They prevent G1-S phase transition. The Kips inhibit all cdks. The cdks consist of the head, neck and body. While the Ink family bind to the back of the head of cdk to inactivate cdk and prevent binding to cyclin the Kip family bind to the neck of cdk/cyclin complex once formed to inhibit the complex. CKIs are therefore negative regulators of the cell cycle inhibiting cdk/cyclin after a phase is completed.

The initial understanding that cyclin activates cdks when they bind together to form a complex and the complex is inhibited by CKIs have changed recently with demonstrations that subunits of cdk and cyclin, function without the complexes being formed and are implicated in diverse independent roles in cell cycle (Lim & Kaldis, 2013).

2.3.4.3 Cell Cycle Regulation

Cell cycle regulation involves the DNA structure checkpoints (G1 and G2) and the commitment or restriction point (R) (Figure 2.6) The interdependency of cell cycle events is due to the control checkpoint mechanism, which minimises the production of genetic inaccuracies (Urrego *et al*., 2014). The DNA structure checkpoints arrest cells in response to incomplete replication (G2) or DNA damage while commitment point also known in animal cells as restriction point is the point whereby a cell commit to cell cycle and progress to completion (Bertoli *et al*., 2013). The checkpoints are feedback control pathways that safeguard the transition of key cell cycle stages (G_1/S and G_2/M) (Urrego *et al*., 2014). Mutations of regulatory proteins involved in commitment point and DNA checkpoints occurs during oncogenesis and signifies the importance of these checkpoints (Bertoli *et al*., 2013). One of such proteins is the tumour suppressor RB protein which inhibit G1-S transcription that is initiated during G1 and inactivated during S phase (Bertoli *et al*., 2013). The cdk, cyclin, CKIs and Cdc25 phosphatases are regulatory molecules that undergo ubiquitin mediated proteolysis, a key mechanism underlying control of cell cycle, whereby two major complexes of ubiquitin ligase which are anaphase promoting complex/cyclosome (APC/C) and SKPi-cullin F box protein (SCF) are involved in irreversible transition of cell cycle (Tu *et al*., 2012).

The cycle entails transduction of tightly regulated mitogenic signals to biochemical mechineries which control DNA duplication and its division to daughter cells (Malumbres, 2011). There is an increase in specific cyclins within the nucleus from G_1 phase to distinct phases in the cell cycle (Figure 2.6) which regulates each phase activity and progression (Reynolds & Schecker, 1995).

2.3.4.3.1 Cyclin and cdk complexes

The cyclin/cdk complexes are central players in cell division with each complex acting in each cell cycle phase and are degraded for the next phase to begin (Rastogi & Mishra, 2012). The cyclin/cdk complex undergo numerous control mechanisms such as action of inhibitory proteins p16, p27 and p21, activating phosphorylation by cdk activating kinases (CAK), activating dephosphorylation by cdc25 phosphatase and inhibitory phosphorylation by Myt1 and Wee1 kinase (Kristjansdottir & Rudolph, 2004).

The retinoblastoma protein (pRB) is a substrate of activated cyclin-cdk complexes and is involved in G1 phase (Stewart *et al*., 2003). The pRB substrate is phosphorylated by cyclin E-cdk2 and cyclin D-cdk4, cdk6 during G1 phase and it either activate or inactivate transcription factor (such as the E_2F family) based on its phosphorylated state (Stewart *et al*., 2003). The interaction of the pRB protein with the E2F protein prevents the function of E2F as transcription factor (pRB/E2F) (Sandal, 2002). Hypophosphorylated pRB inactivate E2F family when they bind together, and because the transcription gene for DNA synthesis is mediated by E2F family, binding of E2F to hypophosphorylated pRB results in cell arrest in G1 phase. Progression from G1 to S phase occurs when hyperphosphorylation of pRB mediated by cdk results in pRB and E2F dissociation (Stewart *et al*., 2003). Therefore, phosphorylation of pRB by cdk/cyclin complexes inhibit pRB and this releases E2F to carry out its function and ensure the phase is completed. After the phase is completed, dephosphorylation of pRB by hypoxic stress activate pRB and it binds back to E2F to inhibit E2F and arrest occurs.

pRB protein can bind and interact with E2F when in hypophosphorylated or unphosphorylated state thereby preventing the function of E2F. In a hyperphosrylated state, there will be no interactions between pRB and E2F. (Figure 2.7B) (Sandal, 2002). Therefore, phosphorylation of pRB results in cell progression while pRB hypophosphorylation results in cell arrest (Todd *et al*., 2002).

The cyclins are however considered the regulator of G1 and S phase transition. They activate cdk and dissociation of E2F and pRB will not occur by cdk/cyclin complex for progression of cell cycle (Reynolds & Schecker, 1995). In addition, highly active or overexpressed cyclins would lead to premature pRb phosphorylation, release of E2F early and abnormal G_1 to S phase progress (Reynolds & Schecker, 1995). E2F function is frequently dysregulated in cancer (Bertoli *et al*., 2013). Typically, cyclin is degraded when a phase ends (Reynolds & Schecker, 1995). G_2 progression into mitosis is regulated by cyclinB-cdk1 (Williams & Stoeber, 2012).

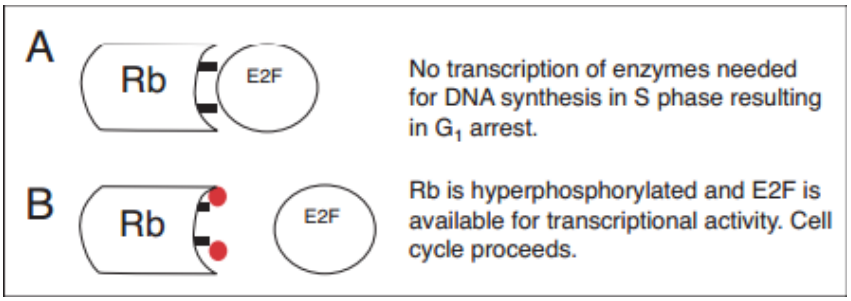


Figure 2.7: pRB activity on cell cycle. Adapted from (Sandal, 2002).

In summary, the cell cycle phases are grouped as G_0/G_1, S and G_2/M. It can be considered that cdks are the engine of the cell cycle, cyclins can be referred to as the activators, checkpoints (G_1 and G_2) are the gears to allow transition from one phase to the other, and to halt the cell cycle during unfavourable conditions are the CKIs which act as the brake (Lim & Kaldis, 2013).

2.3.4.3.2 The ChK1 and ChK2 activity in cell cycle

Serine/threonine kinases Ataxia telengiectasia mutated (ATM) and Ataxia telangiectasia and Rads related(ATR) are kinases that regulates checkpoints and repair of DNA due to breakage of DNA double strand (DSBs) and single strand break (ssDNA), respectively (Wang *et al*., 2015). The signalling kinases ATR and ATM that respond to damage, activate and phosphorylate the transducer kinases chk1 and chk2, respectively (Deckbar *et al*., 2010). The checkpoint pathways (chk1 and chk2) via ATR and ATM respond to DNA damage and cell cycle event errors such as replication errors or chromosomal segregation (Jiri & Jiri, 2003). They facilitate DNA repair by delaying cell progression and remove damaged cells by induction of cell death. They sense damage and send signals to downstream effector proteins (Jiri & Jiri, 2003).

The two functionally similar and structurally unrelated protein kinases (chk1 and chk2) mediate cell cycle checkpoints due to genotoxic stress (Xiao *et al*., 2006) and are genome integrity checkpoint critical messengers (Jiri & Jiri, 2003). Genotoxic stress activates ATR and ATM which inturn activate and phosphorylate chk1 and chk2 (Xiao *et al*., 2006). Chk1 and chk2 are key components of DNA damage response and they mainly influence diverse aspect via transcription but not exclusively, with their largely distinct functions that includes DNA repair, cell cycle progression and apoptosis (Stracker *et al*., 2009). Checkpoint kinases (chk1&2) are upregulated by ATM and ATR whereby they phosphorylates chk1 at ser 345 and ser 317, and chk2 at Thr 68

and other amino terminal domain sites during breakage of the DNA double strand (Wang *et al*., 2015). However, chk2 and chk1 solely activated by ATM due to double strand DNA break (DSB) or ATR single DNA strand break, respectively, has now been reconsidered due to cross talk between these kinases (ATM-chk2, ATR-chk1). With reports of chk1 activated by ATM and chk2 activation independent of ATM (Jiri & Jiri, 2003; Stracker *et al*., 2009). Chk2 protein is stable, expressed through out the cell cycle but only active during DNA damage (Jiri & Jiri, 2003). While chk1 protien is always active, further activated by stalled replication or DNA damage and restricted to S and G2 phases mainly (Jiri & Jiri, 2003). Hence, the association of S and G_2/M phase arrest to chk1 irrespective of single strand or double strand DNA break.

Chk1 is the main effector checkpoint kinase of S and G_2/M phase while chk2 partially influence and play accessory role in G1 and G_1/S phase checkpoint (Stracker *et al*., 2009). Mechanism of activated chk1 & 2 is exerted on cell cycle by regulation of cell division cycle 25 (cdc25) through phosphorylation (Wang *et al*., 2015). The key substrates for chk1 and chk2 are Cdc25A, B and C which regulates activation of cdks at the transition of G_1-S and G_2-M (Xiao *et al*., 2006). Chk1 and Chk2 respond to incomplete DNA synthesis or DNA damage by phosphorylation of Cdc25c at ser216 and bind it to 14-3-3 protein (Thomas et al., 2005; Xiao *et al*., 2003).

Phosphorylation of activated chk2 inactivate Cdc25C phosphatase with Cdc2 or cdk1 maintained in its inactive form in G_2/M arrest, which is the phosphorylated form (Wu *et al*., 2013). Elevated cyclin B1 levels (a G_2/M arrest marker) inactivate phosphotyrosine 15 of Cdc2, form complex with G_2 arrest marker cyclin B1 which accumulate and induce G_2 phase arrest (Pavey *et al*., 2001). Other substrates of chk1 and chk2 are Rb, E2F-1, PCNA, TLK1, Asf1, BRACA2 and Rad51 (Stracker *et al*., 2009).

2.3.4.4 The Cell division cycle 25 (Cdc25)

Phosphorylation and dephosphorylation of protein is important for regulating protein activity and this can be seen with many proteins involved in essential events such as cell cycle progression, intracellular signalling, proliferation, differentiation and apoptosis (Hunter, 1995). Protein phosphorylation is catalysed by protein kinase and protein dephosphorylation is catalysed by protein phosphatase (Hunter, 1995). The protein phosphatase are of 2 groups, one group hydrolyses serine/threonine phosphoesters and the other group hydrolysis phosphotyrosine and are called tyrosine phosphatase (Brenner *et al*., 2014). Within the phosphatase group are phosphatase

with dual specificity that hydrolyse both serine/threonine phosphoesters and phosphotyrosine and they are called cell division cycle 25 (Cdc25) which consist of Cdc25A, Cdc25B and Cdc25C (Brenner *et al*., 2014). These three enzymes play impotant regulatory steps in the cell cycle and could be involved in the development of various malignancies therefore possible therapeutic strategy should include Cdc25 inhibition. Cdc25A and Cdc25B have oncogenic properties but not Cdc25C (Galaktionov *et al*., 1995). Cdc25A act on G_1/S phase transition while Cdc25B and Cdc25C play important roles in G_2/M progression (Sur & Agrawal, 2016). However, all three has been reported to be involved in G_2/M phase with cdc25B playing key role and the most abundant at late G_2/M phase (Brenner *et al*., 2014). Their involvement in malignacies has been reported to correlate with aggressive cancer and poor prognosis.

2.3.4.4.1 Funtion of Cdc25 Phosphatase in the cell cycle

The Cdc25 dual specificity phosphatases family are important and critical cell cycle regulators (Frazer & Young, 2012). They are the final critical activator of cdk/cyclin complex by removing inhibitory phosphate on cdks after DNA damage or during the cell cycle (Stracker *et al*., 2009). They activate cdks to initiate key transitions in the cell cycle and they represent key points for regulation of pathways monitoring DNA integrity, replication, signalling of growth factor and extracellular stress (Frazer & Young, 2012). Misregulation of Cdc25 could allow cells in genetically unstable state and result in cancer cell progression (Frazer & Young, 2012). Cdc25 can be activated by cdks, FLT3, STAT3 and E2F (E2F1, E2F2, E2F3) transcription factors (Brenner *et al*., 2014) and are regulated haevily by phosphorylation (Frazer & Young, 2012). Phosphorylation sites influence substrate specificity, stability, catalytic activity and subcellular localization (Frazer & Young, 2012).

2.3.4.4.2 Cdc25A

Cdc25 family proteins are expressed more than usual especially, Cdc25A and Cdc25B which has been reported to correlates with poor prognosis and more aggressive disease (Wan *et al*., 2009). As such they are reported to have oncogenic properties but not Cdc25C (Galaktionov *et al*., 1995). A critical substrate of Chk1/2 is the phosphates Cdc25A and it regulates inhibiton of cdk1/cdk2 phosphorylation (Deckbar *et al*., 2010) leading to cell arrest at S and G_2 phase. Cdc25A has more general role in the cell cycle, controlling G_1/S and G_2/M transitions (Kristjansdottir & Rudolph, 2004). This is due to the report that upon DNA damage timely inactivation of Cdc25A activate the

cell cycle checkpoint and its hyperphosphorylation by ATR-Chk1 degrades Cdc25A (Wan *et al*., 2009). Which contribute to cell cycle delay allowing either repair of DNA or induction of apoptosis depending on how severe the damage on DNA that occurred (Wan *et al*., 2009). All three Cdc25 phosphatase are required for cell cycle execution however Cdc25A has been reported to be sufficient in initiating each step of the cycle alone (Brenner *et al*., 2014). Therefore, loss of Cdc25A will enhance cell arrest. Cdc25A when upregulated enhances cell proliferation and P13K-PKB-mTOR pathway which seems to control its expression and is reported to be a possible pathway mediating AML chemoresistance (Brenner *et al*., 2014). Overexpression of Cdc25A by phosphorylation, deregulates the events of G_1/S and G_2/M including checkpoint of G_2 (Sur & Agrawal, 2016)

2.3.4.4.3 Cdc25B

The phosphatase Cdc25B is amongst the three phosphatases in humans that activate the cyclin/cdk complexes trigering progression of the cell cycle (Schmitt *et al*., 2006). Cdc25B binding to 14-3-3 protein helps to regulates localization and activity of Cdc25B (Sur & Agrawal, 2016). It has been reported that Cdc25B is involved in initiating the activity of centrosomal cdk1/cyclinB (G_2/M phase) (Schmitt *et al*., 2006). Cdc25B phosphorylation allow entry into mitosis by being the first to stimulate cdk1 activity (Bansal & Lazo, 2007). Increased Cdc25B is important for resumption of the cell cycle after DNA damage and increased Cdc25B reduces the cell cycle resumption time (Bansal & Lazo, 2007). However overexpression of Cdc25B permit premature mitotic entry (Bugler *et al*., 2006). Serine/threonine phosphatase PPMID and polo like kinase 1 regulates resumption of the cell cycle induced G2 arrest in a process called 'recovery' (Bansal & Lazo, 2007). High expression of Cdc25B is related to resistance against the effect of inhibitors of P13k-AKt-mTor that is suppose to stimulate antiproliferative effect in primary AML cells (Brenner *et al*., 2014). In addition inhibition of Cdc25B reduces proliferation of AML cell line by affecting P300 and NfkB (Brenner *et al*., 2014).

2.3.4.4.4 Cdc25C

The 14-3-3 proteins isolate or sequestrate Cdc25C (Bonnet *et al*., 2008). Cdc25C needs to be dissociated from 14-3-3 protein for it to be involved in G_2/M transition and contribute to mitotic pathway factor (MPF) activity in the nucleus and after G_2/M transition it counteract the activity of Wee1 kinase which is an inhibitor of cyclin/cdk complex (Bonnet *et al*., 2008). Mitotic activation at G_2/M transition by Cdc25C occurs when dissociated from 14-3-3 proteins (Sur & Agrawal,

2016)**.** Cdc25C dephosphorylate cdk1 in late G_2 phase leading to cdk1/cyclinB complex activation and cell cycle progression (Ozen & Ittmann, 2005). Checkpoint kinases (chk1 or chk2) phosphorylate cdc25C to inhibit cdc25c, blocking cdk1 activation and progression to M phase (Ozen & Ittmann, 2005).

The cdc25 phosphatases have been reported to be involved human malignant pathogenesis including myeloid leukaemia (Perner *et al*., 2016 ; Bertoli *et al*., 2015). There has been report that continous phosphoylation by mutant FLT3 affect the activity of the cell cycle (Perner *et al*., 2016). Through continous phosphorylation of cdc25C by FLT3-ITD at Thr48 allowing it constant localization in the nucleus to promote continuous activation of cdc25C and its inhibition at Ser216 was switched on when inhibitor of FLT3 was used (Perner et al., 2016). Therefore, aberrant FLT3-ITD signalling induces abnormal Cdc25C phosphorylation that allow progression of mutant cells at G_2/M phase of the cell cycle. There has been report of Cdc25 inhibitors triggering inhibition of cell proliferation and induction of cell death on FLT3-ITD cells and resistant cells to FLT3 inhibitor AC-220 (Bertoli *et al*., 2015). In addition, inibition of FLT3 phosphorylstion in cells with FLT3-ITD mutation reduced cdc25A level and this regulation was STAT5 dependent. Cdc25 inhibitior triggered cell arrest and cell death in ITD and resistant cells but WT FLT3 were not affected and proliferation of ITD cells was reduced with knockout of Cdc25A (Bertoli et al., 2015). Primary ITD cells in the bone marrow were reduced with Cdc25 inhibitor without normal CD34+ and FLT-WT cells affected (Bertoli *et al*., 2015). This suggest that Cdc25 may indicate early cell cycle deregulation by FLT3-ITD and promising target in inhibition of proliferating ITD cells without affecting normal cells.

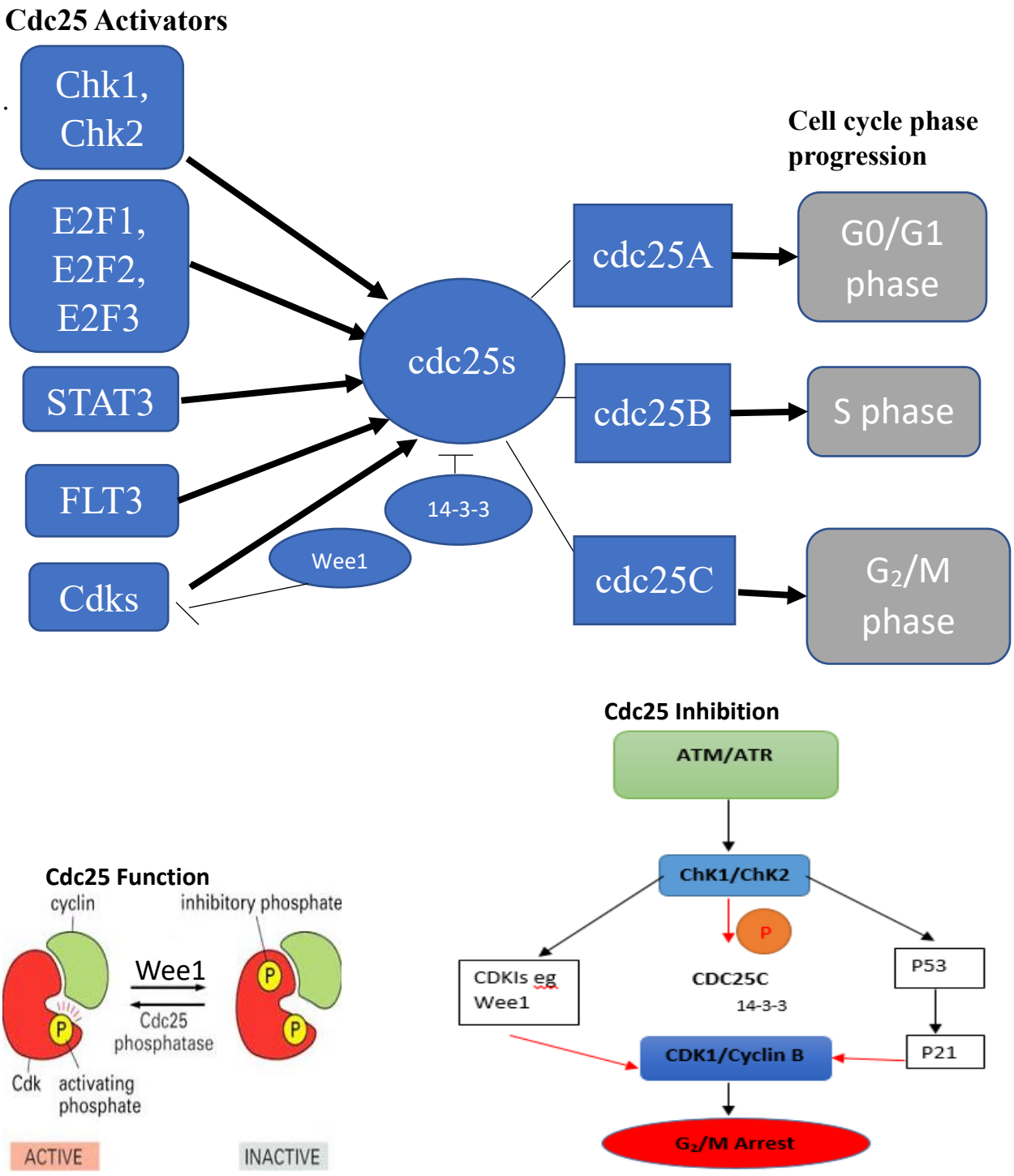


Figure 2.8: Cdc25s and Cycle Arrest due to DNA Damage. Inducer activate p53 which inturn activate p21 a cdkI which act on cdk/cyclin complex and result in cell arrest at specific phase (Schwartz, 2005). Cdks are most commnon activators of cdc25 and without cdc25 the cell cycle may not progress from one phase to the other.

2.3.5 Cellular senescence

Cells undergo senescence as a physiological process in response to DNA damage, telomere malfuction or oncogenic alterations (Kuilman *et al*., 2010). Senescence is also a stress response, whereby cells are withdrawn from the cell cycle due to inability to proliferate even in the presence of growth factors or mitogens (Rufini *et al*., 2013). Senescence cells are viable, active metabolicly and consist of complex phenotypic changes (Petrova *et al*., 2016). Recent studies have demonstrated that senescence induced by p53 can limit progression of tumour *in vivo* (Yingjuan & Xinbin, 2013), and it has been refered to as an intrinsic barrier against tumouriogenesis (Besancenot *et al*., 2010). It has been reported that phosphorylated p53 causes senescence while unmodified or unphosphorylated p53 causes quiescence. Therefore, the understanding of the mechanism of p53 dependent senescence is important in cancer therapies (Yingjuan & Xinbin, 2013).

Cellular senescence is a key driver of aging (Soto-Gamez & Demaria, 2017) due to DNA damage and removal of senescence cells increases lifespan by 25% (Faragher *et al*., 2017). Late in life, senescent cells accumulate at sites known for age related pathogenesis, contributing to progression or onset of diseases (Soto-Gamez & Demaria, 2017). Till date distinction of senescence, quiescent and differentiated cells by a single universal marker has not been established (Soto-Gamez & Demaria, 2017).

Senescence observed in physiological aging and cancer lesion was initially called cell culture artifact *in vitro* (Rufini *et al*., 2013). This was due to their dramatic cellular morphology such as flattened or enlarge cell size (Gire & Dulic, 2015). They also exhibit intracellular vacuolization, nuclear enlargement, inability to synthesize DNA, altered chromatin structure, senescence associated β- galactosidase (SA-βgal) expressed, increased p53, p21, p16 expression and other cyclin dependent kinase inhibitors (cdkIs) such as p15 and p27 (Yingjuan & Xinbin, 2013; Rufini *et al*., 2013). These are markers for senescence including trimethylation of H3k9 (Milanovic *et al*., 2017).

The life timing of senescence is regulated by a number of repeats of telomere cycling, a nucleoprotein structure found at the tips of chromosome which during cell division undergoes progressive loss overtime and can no longer enter the cell cycle and proliferate (Yingjuan &

Xinbin, 2013). Therefore, insufficient telomere after several cell doublings or fused ends of chromosome trigger signals for DNA damage also known as telomeric stress signals which leads to senescence (Yingjuan & Xinbin, 2013).

2.3.5.1 Induction or Triggers of Senesecence (cellular replicative senescence, Premature senescence and Senescence like state)

Progressive telomere attrition (replicative senescence) is the most common trigger of cellular senescence (Burton & Faragher, 2015). Other factors that trigger senescence state entry include oncogenes such as RAS and BRAF resulting in oncogene induced senescence (OIS), stress induced premature senescence (SIPS) due to elevated ROS and cell-cell fusion (Burton & Faragher, 2015). Factors that triggers DNA damage resulting in DNA damage response (DDR) could result in senescence (Burton & Faragher, 2015). Replicative cellular senescence is physiological erosion of telomere after repeated replication resulting in shortening of telomere that can no longer divide and therefore induction of senescence (Gire & Dulic, 2015). Premature senescence is not as a result of telomere shortening due to repeated replication but occurs due to several factors such as stress, oncogene induced and tumour supressor induced senescence (Kuilman et al., 2010). Reversible senescence like state is a term refering to cells exhibiting classical senescence hallmarks but they re-enter the cell cycle once the stress inducing the senescence state is removed after prolong arrest (Maya-Mendoza *et al*., 2014). Cells in reversible senescence like state preserve sufficient chromatin and maintain genetic integrity to resume proliferation after arrest is removed (Maya-Mendoza *et al*., 2014). Inhibition of pRb and p53 pathways as well as balance of suppression of apoptosis induction and checkpoints in the cell cycle has been linked to this mechanism of reentring the cell cycle (Maya-Mendoza *et al*., 2014). Chemotherapy resistance in AML has been linked to reversible senescence like state which maintained the leukaemic populating potential after several months or years of treatment (Duy *et al*., 2016).

Permanent cell cycle exit remains the characteristic feature that identifies senescence cells however it has been reported these cells produce pro-collagenase and are therfore active and not passive in tissue (Burton & Faragher, 2015). DNA damage can result in replicative senescence (Kuilman *et al*., 2010). DNA damage such as double strand break (DSBs) that evokes DNA damage response (DDR), that is persistent and irreparable has been related to cellular senescence.

DDR can be activated by detection of oncogenes, genotoxic agents or dysfunctional telomere (Burton & Faragher, 2015).

2.3.5.2 p53 and Senescence

p53 through cell arrest via p21 induce senescence and suppress senescence by inhibiting mTOR pathway. The ATM/ATR kinases promote p53 activation which inhibit progression of the cell cycle upon detection of DNA damage. This results in the activation of cell cycle inhibitor $p21^{CIP1}$ which binds to cdk/cyclin complexes to inhibit thier activities in the cell cycle. Resulting in dephosphorylation (activation) of retinoblastoma (pRB) which prevents the release of E2F gene expression responsbile for transcription. DNA damage also results in activation of $p16^{INK4a}$ after $p21^{CIPI}$ cell cycle inhibitor, thus acting as a stabilizer and guardian of senecence state but not inducer (Gire & Dulic, 2015). While $p21^{CIPI}$ initiate cellular senescence, $p16^{INK4a}$ enforces and sustain the senescence state to ensure it is irreversible (Maya-Mendoza *et al*., 2014). G_2M arrest has been reported to be the onset of senescence. Therefore, induction of cellular senescence involve activation of $p16^{INK4}$, $p21^{CIP1}$, RB and p53 as key signalling components (Kuilman *et al*., 2010). The expression of SA-β-galactosidase, cell cycle inhibitors (p16 and p21), loss of DNA synthesis, and trimethylation of H3K9 demonstrate senescence.

2.3.5.3 Short and Long term Senescence cells

In somatic cells, several cell division accumulate DNA mutations that could result in cancer if division contiues, therefore the cells go into senescence and then obtain immunogenic phenotype that allows the immune system to eliminate them (Burton & Faragher, 2015). Senescence cells remain in the tissue for a short time to faciliate important functions that include tumour suppression, wound healing and development of the placenta (Burton & Faragher, 2015). Senescence cells exhibit secretory phenotype that attract immune cells which recognise upregulation of ligands on the surface of senescence cells (Burton & Faragher, 2015). Therefore, the secretory phenotype of senescence cells induce clerance by the immune system.

Long term senescence cells accumulate *in vivo* due to aging and impaired immune clearance resulting in age related proinflammatory pathological conditions (Burton & Faragher, 2015). Senescence cells undergoing immune clearance and are not sensitive to apoptosis due to

upregulation of Bcl-2 as observed in fibroblast. However, not all senescence cells express resistant to apoptosis. Senecence state rather than apoptosis is thought to preserve tissue integrity and dose not result in cell loss (Burton & Faragher, 2015).

2.3.5.4 Senescence cells re-entering the Cell Cycle

Cellular senescence is a cell cycle arrest mechanism that prevents further expansion of malignant cells (Milanovic *et al*., 2017). The senescence process is a physiologically irreversible mechanism but not withstanding, some cancer cells still escape from this process and re-enter the cell cycle (Besancenot *et al*., 2010). Senescence prevent oncogenic transformation and should efficiently limit oncogenic transformation through suppression of proliferation (Besancenot *et al*., 2010). It has been reported to inhibit Ras/Raf-driven pro-tumorigenic potential in cancerous lesions through p53 and p16 activation (Serrano *et al*., 1997). This has been shown to be eliminated by inhibition of MEK/ERK pathway (Kochetkova *et al*., 2017). However, some studies have reported escape senerio from permanent cell cycle arrest state (senescence) and refered to it as failsafe condition (Kuilman *et al*., 2010). Factors that could lead to cells re-entering the cell cycle include p53 and PRb inactivation, some interleukins inactivated, tumour suppressor protein levels and strength of oncogenic signal can contribute to senescence entering the cell cycle (Kuilman *et al*., 2010). In activation or absence of p53 and p21 resulted in senescence cells entering the cell cycle with acquired resistant to chemotherapy.

Senescence associated with stemness (stem cell function) in haematological malignancies (ALL and AML) has shown senescence cells going back into the cell cycle with enhanced potential to promote clonogenic growth than other cells equally exposed to genotoxic agent that have never been senescence (Milanovic *et al*., 2017). In human haematological malignancy, senescence stemness is found in most relapse tumours (Milanovic *et al*., 2017). Human mammary epithelia cells and primary human mammary fibroblasts respond to senescence arrest differently based on p16 expression status (Maya-Mendoza *et al*., 2014).

Breast epithelia cells have shown more efficient recovery after prolonged S phase arrest and proliferate compared to fibroblast escaping from senescence and breast epithelia cells that escaped from telomere erosion induced senescence (Maya-Mendoza *et al*., 2014). On avoidance of the senecence pathway, it has been reported in melanomas arising from melanocytic nevi. However,

its sensecence state can be ractivated incompletely by inhibiton of P13k pathway, probaly due to genetic alteration in melanomas (Vredeveld *et al*., 2012). Therefore, there are cell lines that could support re-entery of senesecence cells into the cell cycle such as haematological malignancy and human mammary cells while some cells prevent activation of senesecence such as the melanomas.

p53 and p21 activation induces reversible senescence arrest, with limited growth observed with oncogenic Ras and extensive growth observed with inactivation of p53. However, activation of p53, p21 and subsequent activation of guardian of senescence p16 result in irreversible senescence arrest with inactivation of p53 and pRb with or without oncogenic Ras resulting in S arrest and no growth afterwards. Hence, activation of p16 is a determining factor for irreversible senescence to occur.

2.3.5.5 Senescence cell paracrine signalling

Senescence cells secrete chemokines, proinflammatory cytokines and growth factors which are collectively called senescence associated secretory phenotype (SASP) (Gonzalez-Meljem *et al*., 2018). SASP has been shown to mediate paracrine signalling that can induce protomourogenic activities that includes promoting tumour initiation and enhancing malignant phenotypes (Gonzalez-Meljem *et al*., 2018). Paracrine signalling is when a cell (senescence cell) produces signals that effect changes on another cell (nonsenescence cells) thereby, the cell shares information with another cell (Handly *et al*., 2015). It appears that while senescence suppress cancer by limiting early malignant cell proliferation, they can also promote cancer by signalling incipient cancer cells in their microenvironment to proliferate (Kuilman *et al*., 2010). This has been refered to as senescence messaging secretome (SMS) due to its communicative fuctions and complex signalling network, which affect both neighbouring cells (paracrine effect) and surrounding cells (autocrine effect) around the cells producing the signal (Kuilman *et al*., 2010). SASP is involved in senescence cell immunological clerance and also affects neighbouring cells with proliferative potential (Galluzzi *et al*., 2018). Therefore, reduction of secretory SASP phenotype and its effect on cancer cells will be of beneficial effect in anticancer mechanism (Soto-Gamez & Demaria, 2017).

Cellular senescence could be seen as a major physiological process which requires further investigation for cancer treatment with SASP modulating or senolytic anticancer compounds such

as chemotherapy drugs that induce DNA damage (Gonzalez-Meljem *et al*., 2018). Most agents that induce DNA damage (senescence trigger) induce senescence. In conclusion, anticancer mechanisms through senescence induce activation of p16 along side p53 and p21 to prevent the likelihood of developing reversible senescence like state that could return back into the cycle. Reduction of secretory SASP inhibits the SMS communicative effect that has been reported to promote cancer.

In this study, the established chemotherapy drug doxorubicin singly and in combination with the phytochemicals (α-mangostin, gallic acid and vitamin C) was studied to determine anticancer effect on cell viability, apoptosis, p53, cell cycle and its proteins (p21 and cdc25), senescence (p16) and inhibition of FLT-ITD phosphorylation in AML cell line MOLM-13.

2.4. Doxorubicin

Doxorubicin which is an anti-tumour drug that causes DNA damage and induces DNA double strand breaks (DSBs) by inhibition of topoisomerase II was used in this study (Sapio *et al*., 2015). Topoisomerase II enzyme is involved in several DNA metabolic process such as DNA replication, chromosome condensation, DNA transcription, recombination, de-condensation and untangling replicated chromosomes (Thaker, 2011). Doxorubicin is widely used as an anti-tumour drug (Table 6), including treatment of AML as part of its standard regimen, however it is associated with dose dependent cardiotoxicity (Sapio *et al*., 2015 ; Pan et al., 2002 ; Tydeman-Edwards & Elizabeth, 2015). Doxorubicin side effects such as drug resistance and dose-dependent cardiotoxicity limit its clinical use (Volkova & Russell, 2011). In addition, Dox neuronal toxic effect has been observed in a cell line (H19-8/IGF-IR) and it was dose dependent (Alhowail, et al., 2019). Several studies have reported that combination of chemotherapy drug and phytochemicals has shown significant effect compared to single treatment alone, and since their mechanism of action are different, there could be potential synergistic or additive effect (Chuang *et al*., 2013 ; Adina *et al*., 2014). Some phytochemicals with anticancer properties have been reported to reduce toxicity of chemotherapy drugs to normal cells, overcome chemorsistance or sensitize resistant cells to chemotherapy drugs. This study will explore the combination effect of doxorubicin with the phytochemical, α-mangostin, doxorubicin with gallic acid and doxorubicin with vitamin C to determine potential enhanced or suppressed anticancer effect on cellular processes such as cell growth, apoptosis, tumour suppressor protein p53, cell arrest and senescence.

Table 2.5: Anticancer effect of Doxorubicin in different Cancers

From Bacterium Streptomyces Peucetius

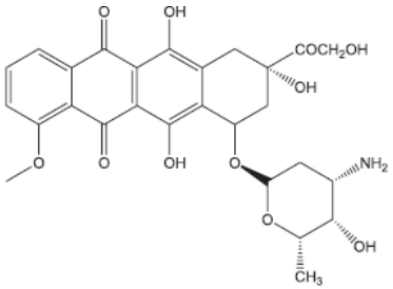
Topoisomerase II an enzyme that regulates DNA topology during replication, transcription and recombination. DNA intercalation result in transcription blockage.

Doxorubicin induced Apoptosis- Fas, Bcl-2, p53, AMPK

Apoptosis of Cardiac cells- MAPK

Inhibition of apoptosis- ERK

Doxorubicin



Mechanism of Action

DNA Damage- DNA intercalation

Topoisomerase II inhibitor

Mitochondria targeting

Generating free radicals

Iron dependent oxidative damage

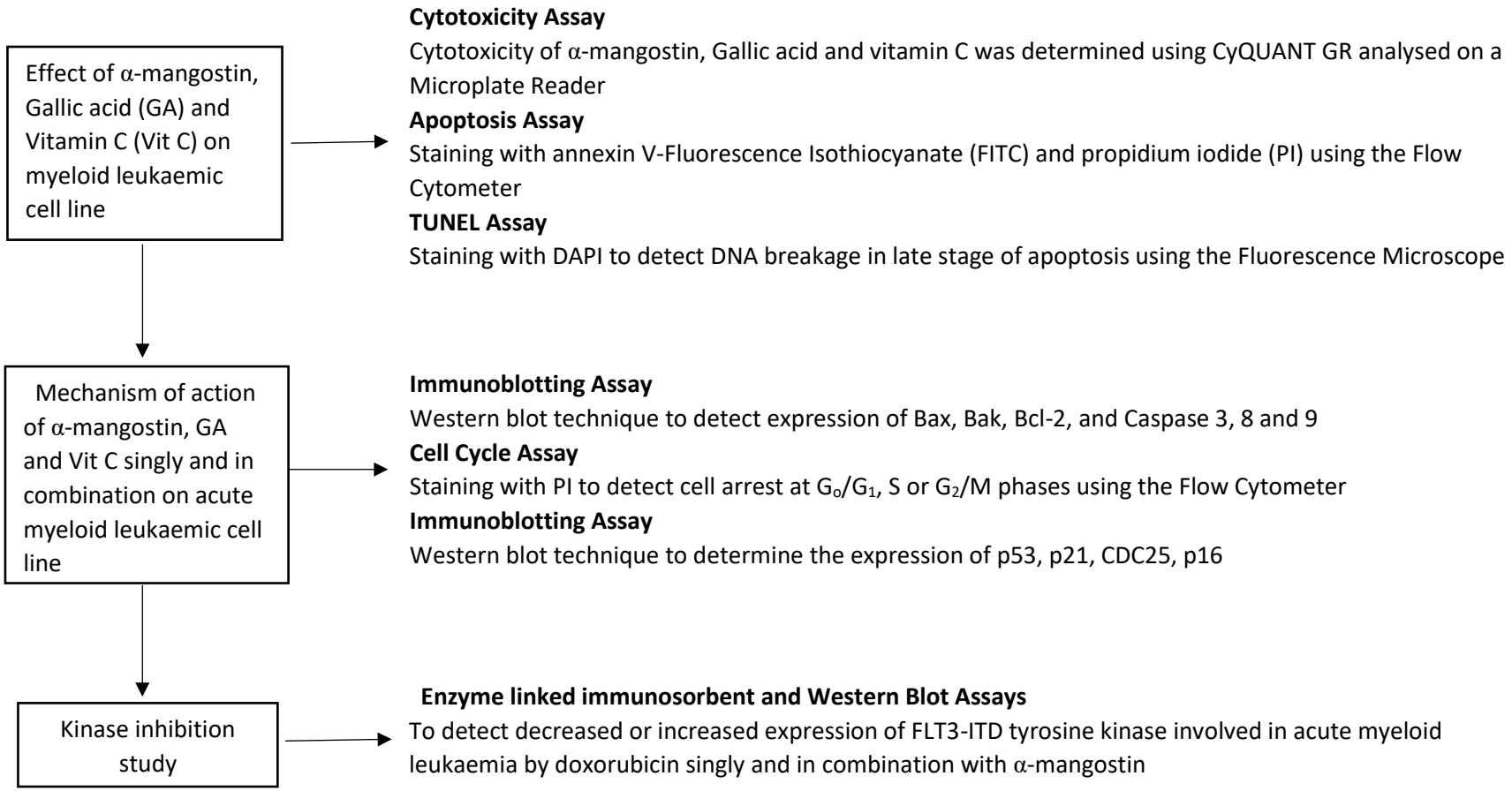
Cancer cell line	Concentration studied	Anticancer Effect	Human/Animal studies	References
Leukaemia K562 cell line	0.5, 5 and 10 µM	G_2M arrest, ↑cyclin A expression		(Grzanka *et al*., 2005)
Prolymphocytic leukaemic (PLL) derived cell line JVM-2	1.5 µM	Induced G_2/M arrest		(Fahrmann & Hardman, 2013)
Breast cancer cell line BT483	0.25, 0.5, 1, 2 and 4 µM	Cell arrest after 48 h with 0.5 µM at G2 phase by 35%, no decrease in cyclin A, B, E, cdc25, p21 and p27 after 48 h with 0.25, 0.5, 0.125, 0.25 and 0.5 µM		(Chuang *et al*., 2013)
Breast cancer cell line MCF-7	200 nM	Cell cycle arrest was at G_2/M phase after 48h ↑p52, Bcl-2.		(Adina *et al*., 2014)
Breast cancer cell line MDA-MB-231 cells	0.1, 1 and 5 µM	Increased in G_2 phase by 80% at 24h and 90% in G_2 at 48h, ↑cleaved caspase 3 and Stat3 after 48 h ↓Erk		(Sapio *et al*., 2015)

Hela Cells and A549	1 µM	More DNA damage at G2 phase than in G1 and S phase. In A549 DNA damage in G_2/M and less in S phase but more in S than in G1 phase. Induction of DNA repair (maximum time of DNA damage is 6 h) after 24 h, failed to repair in Hela cells but repaired in A549 cells, 12h in G1, 15 h in G2M and S phase.		(Potter & Rabinovitch, 2005)
Human lung cancer cell line H1299	500nM after 24 h	G_2/M arrest, ↑Chk1, ↓(degrades) cdc25A and no effect on cdc25C		(Xiao *et al*., 2003)
Proximal tubular cell line HK-2 cells derived from normal kidney	1, 2, 4 and 8 µM after 24 h	Dose dependent inhibition, increased cells at Sub G_1 and G_2M phases, ↓ERK, cytochrome C ↑Nitric oxide and tumour necrosis factor (TNF-α) in a dose dependent manner, ↓PKA and Bcl-2, ↑Phospho STAT3, phospho ERK, ATF3, p38, p53 mRNA, IL-6 proteins. In ATF3 KO cells, S and G2M phase arrest by doxorubicin		(Park *et al*., 2012)
Gastric cancer cell line BGC-823, SGC-7901	0.3 µM after 12 h	Inhibited cell growth, ↑AKT or PKB, PI3K after 12 h		(Yu *et al*., 2008)

CHAPTER THREE

3. MATERIALS AND METHOD

The research plan for this study is seen on the flow diagram addressing the aim and objectives using different assays.



3.1 Cell Lines and Materials

Leukaemic cell line used in this study was purchased from European Collection of Cell Cultures (ECACC) Public Health England. AML cell line (MOLM-13) derived from the peripheral blood of a relapsed acute myeloid leukaemic patient with the classification FAB M5 (Acute Monoblastic leukaemia) which was originally a myelodysplastic syndrome (MDS) (Matsuo *et al*., 1997). Control cell line (SC-ATCC CRL-9855), peripheral blood macrophage was purchased from American Type Culture Collection (ATCC).

Penicillin/Streptomycin, L-glutamine, Roswell Park Memorial Institute (RPMI) 1640 medium, CyQUANT cell assay kit, halt protease inhibitor cocktail, Radioimmunoprecipitate assay buffer, Tween-20, Alexa Flour 488 annexin V and Propidium Iodide (PI) for Flow Cytometry dead cell apoptosis kit were purchased from Thermo Fisher Scientific (UK).

Trypan blue, Iscove's modified Dulbecco's medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), dimethyl sulphoxide (DMSO), doxorubicin, Vitamin C, gallic acid, α-mangostin, Bradford assay and FLT3 ligand were purchased from Sigma Aldrich (UK).

Phospho-FLT3 Tyr591 was bought from Cell Signaling Technology (UK). VECTASHIELD + DAPI was from Vector Laboratories Inc. (UK). DeadEnd Fluorometric TUNEL system kit was purchased from Promega Corporation (UK). Path Scan Phospho-FLT3 (panTyr) Sandwich ELISA kit #7761C was purchased from Cell Signaling Technology (UK). The primary antibodies for Western Blotting and Propidium iodide flow cytometer kit (ab139418) for cell cycle analysis were purchased from Abcam (UK). Secondary antirabbit and antimouse antibodies, SDS-PAGE gel, nitrocellulose membrane, enhanced chemiluminescent (ECL), horseradish peroxidase (HRP) were from Bio Rad (UK).

3.2 Cell Culture

Roswell Park Memorial Institute (RPMI) 1640 medium, has been reported to culture neoplastic leukocytes in suspension. RPMI medium containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin antibiotics was used for culturing MOLM-13 cells at 37^{o}C in a humidified incubator with 5% CO_2. MOLM-13 cells were maintained at 0.4 -2.0 × 10^6 cells/ml, and frozen for storage at 2.5 × 10^6 cells/ml in 70% complete medium, 20% fetal bovine serum (FBS) and 10% dimethyl sulphoxide (DMSO). Control cells were subcultured in Iscove's modified Dulbecco's medium-containing 10% fetal bovine serum (FBS), 1% L- glutamine and 1% penicillin/streptomycin antibiotics. Media was changed every 2-3 days. Control cells were maintained at 2×10^5 or 1×10^6 cells/ml and frozen in complete medium with 5% DMSO.

Although the use of antibiotics prevents contamination and bacteria growth, recent studies have implicated its use in changing gene expression and regulation (Ryu, et al., 2017). Aseptic condition and sterile techniques are encouraged in cell culture as the use of antibiotics can affect drug response, cell cycle regulation, growth and differentiation of cells (Ryu, et al., 2017). However, there is a debate that the impact of antibiotics on gene expression is negligible and further evaluation across cell lines on the biological impact or molecular consequences of antibiotics is highly warranted, for caution with the use of antibiotics in cell culture (Ryu, et al., 2017). This information was not apparent to me at the start of my research and when I became aware of the debate, I had already conducted several experiments. Therefore, for consistency, antibiotics was used for cell culturing throughout this project. However, due to lack of reports on the benefits of antibiotics on gene expression and few reports on its side effects (Ryu, et al., 2017), (Llobet, et al., 2015), (Varghese, et al., 2017), (Relier, et al., 2016), (Nygaard, et al., 2015), (Singh, et al., 2014) future work will include thorough investigation of antibiotics in cell culture for optimal culture condition and to evade erroneous results.

3.3 Cell Counting using Trypan blue

Cell counting with Trypan blue was conducted on the cells by mixing equal volumes of cells in suspension with Trypan blue dye and 20 µl of the mixture was loaded into a chamber of the haemocytometer and covered with a cover slip. Cell counts were performed under a ×10 objective lens according to the standard method of counting on a phase-contrast microscope. This determines number of cells/ml and done over a period of time indicates or determines the doubling time of cells. Cell counting with Trypan blue can also distinguish live cells (viable cells) from dead cells through uptake of the blue dye by dead cells and not by live cells. Cell counting to determine percentage of viable cells was done before assays were conducted in this study. Cells at log phase (cells multiplying exponentially) and free from mycoplasma was used in this study. The Trypan blue dye is a simple, inexpensive and colour dead cells blue within seconds.

3.4 Cytotoxicity Assay

3.4.1 Determination of Dimethyl Sulfoxide (DMSO) concentrations on cell viability using CyQUANT GR

A cytotoxicity assay was used to determine the effect of DMSO solvent used in the preparation of the compounds α-mangostin, gallic acid, vitamin C and the chemotherapy drug doxorubicin in this study. Various concentrations of DMSO in RPMI medium (0.05%, 0.1%, 0.2%, 0.25%, 0.5%, 0.7%, 0.8%, and 1%) was used to determine effect on MOLM-13 and control cell lines using CyQUANT GR detection dye. The CyQUANT cell assay kit is a fluorescence-based cytotoxicity and proliferation assay using the microplate readers. CyQUANT GR or CyQUANT Direct consist of a green fluorescent nucleic acid dye which exhibits strong fluorescence when bound to nucleic acids in live cells. It provides sensitive, rapid and convenient procedure for determining cell density in culture. The CyQUANT DNA binding dye binds to highly regulated DNA content on live cells but do not bind to dead cells or cells with compromised cell membranes in order to estimate accurate live cell number. It does not rely on metabolic activity of the cell, therefore minimises false positive results, and there are no washing steps, change in growth media and long incubation period before reading.

Cells were suspended at a density of 1.5×10^5 cells/ml and plated in 24 well plates, with different concentrations of DMSO (total volume of 1000 ml containing 1 in 20 dilutions of DMSO)

incubated at 37ºC for 72 hours. Negative control (cells without DMSO) and positive control (Dox 5 μM) were included. CyQUANT GR detects viable cells that has been centrifuged in an Eppendorf tube after treatment period (48 or 72 h) and cell pellets frozen at -70ºC overnight. The cell pellets were thawed at room temperature, suspended in the volume (1000 μL) used during treatment with CyQUANT GR buffer/dye and briefly vortexed. The suspended cells in CyQUANT GR buffer/dye was plated in 96 well plate at 100 μL per well and allowed to incubate for 2-5 minutes protected from light, read in a plate reader with fluorescence wavelength of 480 nm excitation and 520 nm emission.

3.4.2 Determination of doxorubicin on cell viability using CyQUANT Direct

The effect of doxorubicin (Dox) on viability of MOLM-13 cells was determined using CyQUANT direct dye. Cells were suspended at 1.5 $\times 10^5$ cells/ml and plated in 96 well with doxorubicin treatments at 5, 2.5 and 1 μM (4 wells per treatments) and incubated at 37^{O}C for 72 h. After incubation CyQUANT direct dye was added 100 μl per well and incubated for 1-2 h at 37 ^{O}C. The plate was read at 508 excitation and 527 emission wavelengths in a BMG LABTECH FLUOstar Omega Plate Reader

For CyQUANT direct, treated cells were read immediately after incubation period while for CyQUANT GR treated cells are suspended in an Eppendorf tube, supernatant removed, and cell pellets are then frozen at -70^{O}C overnight to be read at any convenient time.

3.4.3 Determination of alpha mangostin (α- mangostin), gallic acid (GA), vitamin C (Vit C) and combination with Doxorubicin (Dox) on cell viability using CyQUANT GR

The effect of α-mangostin, gallic acid, vitamin C alone and in combination with doxorubicin the chemotherapy drug, was determined on MOLM-13. The CyQUANT GR dye was used. Cells were suspended at a density of 1.5×10^5 cells/ml and plated in 24 well plates, with treatment of different concentrations (from low to high concentrations); α- mangostin 2.5, 5, 10, 20, 30, 40, and 50 μM, GA 5, 10, 15, 20, 30 μM, vitamin C 10, 15, and 30 μM were studied. IC_{50} concentration (concentration inhibiting 50% of cells) for each compound including Dox was determined using GraphPad prism software which creates a graph with % inhibition (y-axis) against concentration of the drug (x-axis). Concentration with 50% inhibition can be seen on the line plot of the graph

indicating high to low inhibition with corresponding drug concentration shown on the x-axis. These concentrations were used for combination with Dox. Combinations (α- mangostin 20 + Dox 1, Vit C 15 + Dox 1, and GA 15 + Dox 1 μM) were used to treat cells and incubated at 37^{O}C for 72 hours. Total volume in 24 well plate was 1000 μl, containing 1 in 20 dilutions of treatment. Negative control (cells without treatment) and positive control (Dox only) were included. The CyQUANT GR detects viable cells centrifuged in an Eppendorf tube after treatment period (48 or 72 h) and the pellet is frozen at -70^{O}C overnight. Cells were thawed at room temperature, suspended in the volume (1000 μL) used during treatment with CyQUANT GR buffer/dye and briefly vortexed. This suspension was plated in 96 well plate at 100 μL per well and allowed to incubate for 2-5 minutes protected from light, read in a Plate Reader with fluorescence wavelength of 480 nm excitation and 520 nm emission in the Microplate Reader. In this study the effect of doxorubicin singly and in combination with α-mangostin, gallic acid and vitamin c in AML, were determined to evaluate possible mechanisms of action. The apoptosis assays were conducted after the cell viability study.

3.5 Apoptosis Detection Assays

3.5.1 Apoptosis assay using Flow Cytometry

Induction of apoptosis was determined on MOLM-13 cells, seeded at a density 1.0×10^6 cells/ml and treated with α-mangostin 20 μM, GA 15 μM and Vit C 15 μM singly and in combination with Dox 1 μM incubated for 72 h. Alexa Flour 488 annexin V and Propidium Iodide (PI) for Flow Cytometry dead cell apoptosis kit was used for the apoptosis assay. Annexin V conjugated with the fluorophore Alexa Fluor 488 dye provides maximum sensitivity to identify apoptotic cells by binding to phosphatidyl serine translocated from the inner to the outer plasma membrane of apoptotic cells. Phosphatidyl serine on the outer surface of an apoptotic cell marks the cell for phagocytosis by macrophages. PI is a nucleic acid dye that bind tightly to the nucleic acid of dead cells due to loss of membrane integrity. PI dose not stain live cells and apoptotic cells. Annexin V and PI in binding buffer shows population of apoptotic cells with green fluorescence, dead cells with green and red fluorescence and live cells with little or no fluorescence using flow cytometer.

Annexin V/PI dye apoptotic method using flow cytometry is the most common method for studying apoptosis. Annexin V measuring PS is the most attractive method for apoptosis because it detects PS without penetrating the cells, not toxic in cellular research and. because it captures the changes in cell membrane (Demchenko, 2013). Annexin V/PI double labeling ideal for distinguishing apoptotic cells from dead cells.

Cells were harvested after incubation period and washed in phosphate buffer saline (PBS). Supernatant was discarded, and the cells were resuspended in 1X Annexin V binding buffer 100 μl per assay. Cells were stained with 5 μl Annexin V and 10 μl propidium iodide (PI) (100 μg/ml). Incubated at room temperature for 15 minutes in the dark, 400 μl binding buffer was added after incubation and kept on ice for analysis. Induction of apoptosis for quantification of cells (viable cells, early and late apoptotic cells, and necrotic cells) was determined using the flow cytometer (Becton- Dickinson (BD) fluorescence activated cell sorting (FACS) Calibur with Annexin V-FITC and propidium iodide (PI) double staining, detected with FL1 and FL3 channels, respectively.

3.5.2 Apoptosis assay using the Fluorescence Microscope

Fluorometric terminal deoxynucleotidyl transferase UTP nick end labelling TUNEL system which measures apoptotic cell's fragmented DNA by incorporation of fluorescein-12-dUTP at 3'-OH DNA ends using recombinant enzyme terminal deoxynucleotidyl transferase (rTdT) was used to detect DNA breakage in late stage apoptosis. The TUNEL assay is one of the most commonly used approach for studying apoptosis and because the hallmark of apoptosis is DNA fragmentation this approach has a high tendency in identifying apoptotic cells due to DNA break, and overlooking nonapoptotic DNA break (Stubenhaus & Pellettieri, 2018). MOLM-13 cells were seeded at 1.0×10^6 cells/ml in 24 well plate with α-mangostin 20 μM, GA 15 μM and Vit C 15 μM singly and in combination with Dox 1 μM incubated for 48 h. Cells were collected by centrifugation and layered on a slide coated with poly-L-lysine for adhesion of cells to the slide. Cells on slides were fixed with 4% paraformaldehyde by immersing the slides for 25 mins in a jar containing the fresh fixative. Slides were washed twice by immersion for 5 mins in PBS at room temperature. After washing in PBS, slides could be stored for 2 weeks in PBS at 4^OC or in 70% ethanol at -20^OC. The slides with cells were then permeabilised with 0.1% Triton X- 100 (10 μg/ml) or in 70% ethanol in deionized water for 5 mins. Slides were rinsed twice for 5 mins in fresh PBS and covered with 100 μl equilibrium buffer for 5-10 mins at room temperature. The surrounding of the slides was blotted around the equilibrate area to remove excess equilibrium buffer and 50 μl of recombinant enzyme terminal deoxynucleotidyl transferase (rTdT) incubation buffer was added. Slides were covered with plastic coverslips for even distribution and Incubated at 37^OC for 60 mins in a humidified chamber with soaked paper towel in water at the bottom of the chamber. This allows the tailing reaction to occur. Slides were cover with foil to prevent direct light on the slides. After incubation, the plastic cover slip was removed, the reaction was terminated by immersing slides in 2X SSC reagent jar for 15 mins at room temperature. Wash the slides 2-3 times by immersing in fresh PBS to remove unbind fluorescein-12-dUTP. Slides were stained and mounted in VECTASHIELD + DAPI to stain the nuclei and cover slip was added. Slides were analysed under a fluorescence microscope using standard fluorescein filter. Blue DAPI was viewed at 460nm. Slides can be stored at 4^OC overnight in the dark. Analysis was done using the fluorescence laser scanning inverted confocal microscope system, Leica TCS Sp5.

3.6 Mechanism of Action by Phytochemicals on Acute Myeloid Leukaemic Cell Line

3.6.1 Cell cycle assay

Cell cycle analysis was determined on MOLM-13 cells seeded at a density of 1×10^6 cells/ml in a 24 well plate treated with α-mangostin 20 μM, GA 15 μM and vitamin C 15 μM singly and in combination with Dox 1 μM incubated for 48 h. After incubation, cells were washed in PBS twice, pellets were fixed in 70% ethanol for at least 30 mins on ice or kept at 4^OC. The cells were centrifuged and resuspended with Propidium iodide (PI) 400 µl (50 µg/ml) and 50 µl of RNase A for 30 mins in the dark at 37^OC. Cells were analysed using Cell cycle analysis was carried out using flow cytometer (Becton- Dickinson (BD) fluorescence activated cell sorting (FACS) Calibur flow cytometer) and detected using FL2 channel. The flow cytometer detects the percentage of cells arrested in G_o/G_1, S or G_2M phases. The cell cycle assay detects and quantify DNA content present in the cells by staining with DNA binding dye such as PI.

3.6.2 Western blot

3.6.2.1 Expression of proteins using Western blot

MOLM-13 cells were treated for 48 h, separated from RPMI and washed three times in cold phosphate buffer saline (PBS) and kept on ice. Cells were suspended in halt protease inhibitor cocktail (100 µl) and then lysed in Radioimmunoprecipitate assay buffer (RIPA) buffer (400 µl) for 20 mins on ice, briefly vortex every 5 mins. Lysate were centrifuged at 14,000rpm for 15 mins at 4^OC and supernatant was transferred to a separate tube on ice for protein determination. Protein concentration was determined using Bradford assay and 30 µg of proteins were loaded, separated on 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% BSA 0.1% PBS Tween-20 for 1h and incubated overnight at 4^OC with any of the primary antibodies rabbit monoclonal to active caspase 3 (E83-77), rabbit monoclonal (EPR-362) to p21, rabbit monoclonal to caspase 9 (E23), rabbit monoclonal to p16 (EPR1473) 1:1000 in 5% BSA 0.1% PBS-Tween at 4^OC with gentle shaking overnight. The membrane after incubation was washed on a shaker three times for 5 mins each in 0.1% PBS Tween 20, the membrane was incubated with secondary antirabbit antibody1:3000 with 5% BSA in 0.1% PBS-Tween for 1 h. The blot/membrane after secondary incubation is view using enhanced chemiluminescent (ECL) horseradish peroxidase (HRP) substrate reagents in 1:1 ratio. Chemiluminescence detection is the

most common choice of protein detection that is convenient and has the greatest sensitivity. Chemiluminescence substrates are two components for horseradish peroxidase that consist of enhanced luminol solution and stable peroxidase solution. The image is captured using the Licor imaging instrument set with chemiluminescent wavelength at 2 mins and 700 gates at 0.5 mins. Western blot analysis allows the specific detection of a protein of interest in a mixture of proteins in lysate, therefore suitable for detection of proteins in this study.

3.6.2.2 Kinase inhibition using Western blot

FLT3 ligand (FL) was reconstituted in sterile PBS containing 0.1% endotoxin free human serum albumin by resuspending 10µg powder in 1ml and then prepare 100ng/ML. Wash WT-FLT3 cells (OCI-AML) twice in serum free media (RPMI) and incubate in 100ng/mL of FL for 15 mins at 37^{O}C. Then wash cells twice after incubation in cold PBS. Both MOLM-13 and OCI-AML after washing in cold PBS twice were lysed in Radioimmunoprecipitation assay buffer (RIPA) buffer for 20 mins on ice briefly vortex every 5 mins. Lysate were centrifuged in 14,000rpm for 15 mins at 4^{O}C. Protein concentration was determined using Bradford assay read in a plate reader at 595nm absorbance and 30 µg of proteins were loaded into the gels, transferred and probed with primary antibody. For FLT3 expression, block blot in 5% milk in 0.1% TBS-Tween, incubate in primary antibody Phospho-FLT3 Tyr591 1:1000 in 5% BSA 0.1% TBS-Tween at 4^{O}C with gentle shaking overnight and secondary antibody 1:3000 with 5% milk in 0.1% TBS-Tween. PBS-Tween was not used for detection of FLT3 expression. Tyrosine phosphorylation proteins were detected using antiphosphotyrosine antibody pY20 1:3000 in 5% BSA 0.1% PBS-Tween at 4^{O}C with gentle shaking overnight.

3.6.3 Kinase inhibition with Enzyme linked Immunosorbent Assay (ELISA)- Using Pathscan phospho-FLT3 (PanTyr) Sandwich Elisa

Micro well plate coated with FLT3 antigen was used for the study. PathfScan Phospho-FLT3 (panTyr) Sandwich ELISA kit #7761C was used to determine FLT3 expression. Micro well strip was broken off and kept at room temperature for use. Unused well strip was sealed immediately and kept at 4^{O}C. Cell lysate was added into appropriate micro well (100 µl) and sealed with tape, pressed firmly and incubated for 2 h at 37^{O}C or overnight at 4^{O}C. Gently, the tape was removed, supernatant discarded and washed 4 X with 200 µl of 1 X wash buffer. Each was done by striking

the plate on fresh towel hard enough to remove residual solution but not allowed to dry out. The underside of all wells was cleaned with a limit with free tissue. Reconstituted detection antibody in the kit was added (100 µl) and this was sealed with the tape and incubated at 37^{O}C for 1 h. After incubation content was discarded and wells was washed 4X with 200 µl of 1X wash buffer striking on fresh towel hard enough but not completely dried out. Reconstituted HRP linked secondary antibody was added to each well sealed with tape and incubated for 30 min at 37^{O}C. Micro wells was washed 4X in 1X wash buffer and TMB substrate (100 µl) was added after washing and incubated for 10 mins at 37^{O}C or 30 mins at 25^{O}C. Stop solution was added to each well (100 µl) and the plate is read within 30 mins after adding STOP solution. Underside of the wells was wiped and read at 450 nm absorbance wavelength. ELISA assay provides an alternative method to Western blot analysis for the detection and quantification of FLT3 protein.

3.7 Statistical Analysis

Data presented after analysis was expressed as % cell control for the cell viability, cell death, cell arrest and expression of proteins studied. Normality test (test for normality of data with probability plot to show if data was normally distributed ($p > 0.05$) or not normally distributed ($p<0.05$) (Appendix 1) and test for equal variance (test for equality of variance on Appendix 2) were done using Minitab software version 19 to show that data was normally distributed, and variance is equal. One-way ANOVA with Tukey's post hoc analysis to compare different results obtained and student t test to compare and show results with significant difference. P value < 0.05 was considered statistically significant. Minitab software version 19 was used for data analysis. IC_{50} was determined using the GraphPad.

The extent or nature of interaction of two drugs can be evaluated using combination index (CI) as a universal standard and most prevalent method for analysis of synergism, additive and antagonism. CI was determined using median effect analysis derived by Chuo (Chou, 2018). The median effect evaluates drug combinations and correlates drug dose and corresponding effect using the equation: CI= d1/D1+ d2/D2 where D1 & D2 are concentration of drug 1 and drug 2 while d1 & d2 is combined concentrations of both drugs.

Alternatively, the CompuSyn software by Ting Chao Chou can be used to evaluate the CI. The computer software is used for analyzing dose effect of single drugs with median effect equation,

and drug combinations with both median effect and combination index equation (Chou, 2006). Drug 1 (% inhibition of various concentrations of the first drug), Drug 2- (% inhibition of various concentration of the second drug) and Drug 3- (% inhibition of the combination of drug 1& 2) were entered the software analysis. A CompuSyn report was generated that consist of combination index plot with inhibition (Fa) and combination index (CI) values. A CI value of 0.1-0.90 indicates synergism, 0.90-1.10 indicates additive while 1.10-10 indicates antagonistic.

The CI value provides a quantitative and mathematical representation of the pharmacological interplay when drugs are combined.

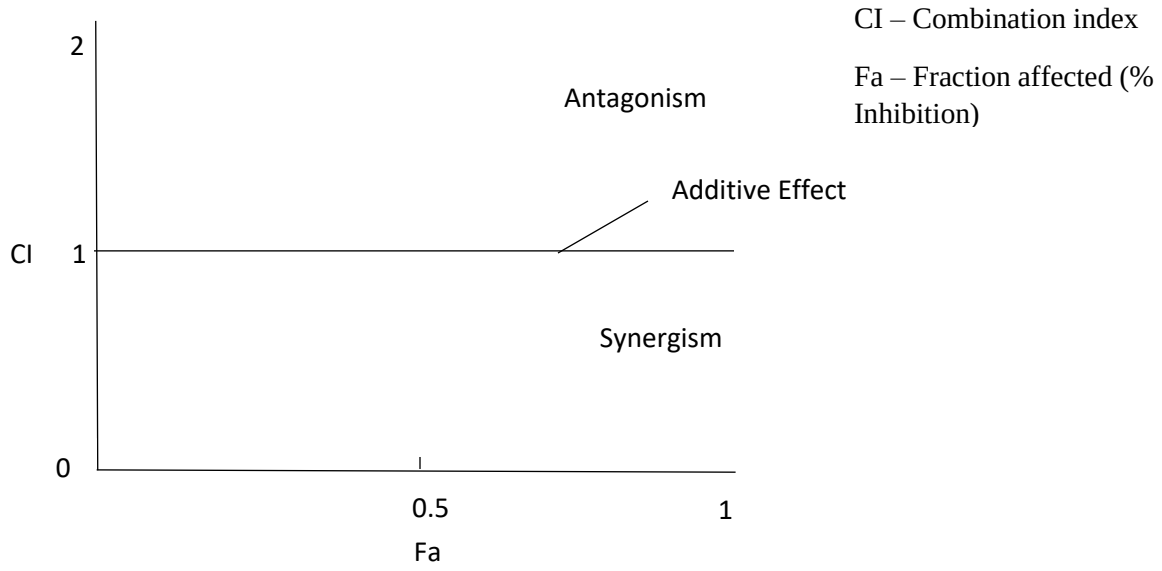


Figure 3.1: Combination Index Plot indicating effect of drug combination generated from the CompuSyn software. Synergism (<1), antagonism (>1) and additive (1).

CHAPTER FOUR

4. α-Mangostin and doxorubicin synergistically inhibited cell growth and induced cell apoptosis via the regulation of Bak protein and the inhibition of FLT3-ITD kinase in AML

4.1 Background

AML is a group of heterogeneous diseases characterised by immature haematopoietic precursors with uncontrolled proliferation and reduced or no differentiation in the bone marrow and blood stream (Chen *et al*., 2017). Standard therapy for decades in AML treatment include combination of anthracycline antibiotic such as doxorubicin (Dox) and cytarabine, with Dox associated with dose-dependent cardiotoxicity (Dombret & Gardin, 2016). AML is associated with numerous mutations and one gene of prognostic significance is the activating mutation in the Feline McDonough Sarcoma (FMS) like tyrosine kinase 3 (FLT3) that is associated with poor prognosis and outcome (Stockard *et al*., 2018). Mutations in FLT3, are the internal tandem duplication (FLT3-ITD) that occurs in the juxta- membrane and the tyrosine kinase domain (FLT3-TKD) that occurs in the kinase domain. The most common FLT3 mutation is the FLT3-ITD, one of the most common molecular genetic abnormalities that occurs in 20-30% of AML patients and predominant in cytogenetically normal AML patients (Stockard *et al*., 2018). The FLT3-ITD receptor tyrosine kinase mutation are associated with poor prognosis, confers resistance to treatment and high relapse risk. Unfortunately, this mutation could be the underlying mechanism of leukaemia in AML patients that could go unnoticed at the time of diagnosis, then become detected when resistance or relapse occurs. This is because FLT3 kinase is important for the regulation of haematopoiesis and therefore, a key driver of AML (Saultz & Garzon, 2016). The FLT3 receptor kinase activates downstream STAT, P13K/PKB, RAS/MAPK, ERK pathways, regulating early stem cell survival, myeloid differentiation and apoptosis, and are expressed in normal stem cells and progenitor cells (Larrosa-Garcia & Baer, 2017). Although midostaurin has been approved and other tyrosine kinase inhibitors have been developed for FLT3-ITD positive patients, drug resistance still occurs (Perl, 2017) (Fakih *et al*., 2018), which is a constant challenge in successful treatment, therefore, further understanding of the complexity of FLT3-ITD and specific targeting agents will improve clinical outcome (Stockard *et al*., 2018).

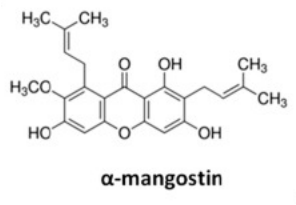
α−Mangostin, a major xanthone from the tropical fruit mangosteen, has been reported to have a wide range of health promoting benefits and biological activities such as anti-tumour, anti - inflammatory, anti-bacteria, cardioprotective, anti-diabetic and anti-oxidant properties(Sampath & Vijayaraghavan, 2007; Gutierrez-Orozco *et al*., 2013). Its effect on different types of cancers has been reported (Table 4.1). When studied in leukaemic cell lines, α-mangostin exerted the highest cytotoxicity to leukaemic cells compared to other xanthones found in the mangosteen fruit, showing complete growth inhibition through the induction of apoptosis (Matsumoto *et al*., 2003). Combination of α-mangostin with established chemotherapy drugs such as 5-flourouracil (5-FU) (Nakagawa *et al*., 2007) and cisplatin (Pérez-Rojas *et al*., 2016) has shown promising results. However, its combination with Dox in AML cell lines is yet unknown. Furthermore, it has been reported that α-mangostin acts on certain signaling pathways activated by FLT3 such as P13K/PKB pathway (Li *et al*., 2014) and MAPK, ERK1/2 (Lee *et al*., 2016 ; Kritsanawong *et al*., 2016). However, its effect, either singly or in combination with Dox in AML cells with FLT3-ITD mutation is yet to be determined. Although the development of liposomes such as Doxil (liposomal doxorubicin) has been reported to reduce the dose-dependent cardiotoxicity associated with Dox (Bulbake *et al*., 2017), approved liposome (Vyxeos - liposomal daunorubicin and cytarabine) for only certain types of AML (t-AML and AML with MDS changes) still has cardiotoxicity side effects (Perl, 2017; Crain, 2018). Daunorubicin which is in the same anthracycline family as Dox has similar, but milder side effects and is currently used in AML treatment (Alloghbi *et al*., 2017). The major limitation of Dox in clinical use is its dose related cardiotoxicity that may be irreversible (Bruynzeel *et al*., 2007). Therefore, there is a need to study the anticancer effect of Dox in reduced concentration and in combination with phytochemical for either enhanced anticancer effect with minimized toxicity to normal cells.

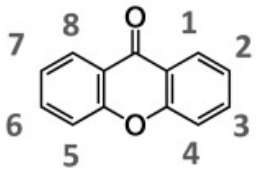
Table 4.1: *In vitro* anticancer effect of α-mangostin on cancer cells

Biological Properties

Anti-inflammatory
Neuroprotective
Cardioprotective
Antioxidant
Anti-fungal
Anti-parasitic

α-Mangostin: A Xanthone from Mangosteen fruit





1, 3, 6, Trihydroxy-7-methoxy-2, 8, bis (3-methyl-2-butenyl)-9H-xanthen-9-one. Mwt 410.46

Cancer/Cell line	Concentrations Studied	Anticancer Effect	Human & Animal Studies	References
Prostate Cancer (LN cap, 22RV1, DU145 and PC3	IC_{50} 5.9, 6.9, 22.5, 12.7 µM, respectively 2.5 & 10 µM 7.5 & 15 µM	Inhibit cell growth after 48 h Inhibition of colony formation at 2.5 µM in 22RV1 & PC-3 cells and 10 µM after 12-14 days G_1 cell arrest after 14 h at 7.5 & 15 µM ↑ P27 ^{Kip1} & cdk4, ↓ Cyclin D1 & D3, ↓ Cyclin E, ↓ Rb proteins. cdk4 inhibitor inhibit cell viability at 5 µM while cdk4/6 inhibit cell viability at 20 µM. ↓ Kinase activity in JNK3 and CyclinD1/cdk4 at 10 µM	Reduction of tumour growth by 65% after 34 days in Athymic nude mice implanted with 22Rv1 cells when treated with 100mg/kg of α-mangostin	(Johnson *et al*., 2012)
Gastric Adenocarcinoma cell lines BGC-823 and SGC 7901	0, 7, 12, 17, 24 µM optimal concentration at 17 µM	Inhibit cell growth after 6, 12, 18, 24 and 48 h Induce apoptosis with 12 µM after 6, 18 and 24 h ↓STAT3,↓ Bcl-2, ↓ MCL-1 expressions		(Shan *et al*., 2014)

Table 4.1: *In vitro* anticancer effect of α-mangostin on cancer cells

Leukaemia K562 cells	10 µM, no effect observed at 5 µM	Inhibit cell growth, induced apoptosis after 24 h and increase scavenging activity of OH^-, O^-_2, H_2O_2 and lipid peroxide. DNA damage in control observed		(Sun *et al*., 2009)
	0, 5, 10, 15, 20 µM	Inhibit cell growth after 24, 48 and 72 h with IC_{50} 16, 13, 8 µM, respectively. ↑ autophagy marker LC-311 expression G_0/G_1 phase arrest after 24 h with 20 µM of α-mangostin		(Chen *et al*., 2014)
B- Cell (CLL) cell lines EHEB & ESKOL	2.4 & 24 µM	Dose-dependent decrease in ESKOL after 48 h and EHEB after 72 h. B-CLL cells showed effect at 24 µM after 48 h. Induced DNA breakdown, Apoptosis, increase cytoplasmic nucleosome after 24 h. Caspase 3 was not stimulated	α-Mangostin was sensitive to normal peripheral blood mononuclear cell (PBMC) and purified B lymphocytes at 12 and 24 µM	(Menasria *et al*., 2008)
Head and Neck squamous cell carcinoma (HNSCC) cell lines HN-20, HN-30, HN-31	7 µM	Inhibit cell growth after 48 h ↑ Bax and p53, ↓ Bcl-2 after 24 and 48 h		(Kaomongkolgi *et al*., 2011)

Table 4.1: *In vitro* anticancer effect of α-mangostin on cancer cells

Lung cancer (Non-small cell lung cancer cells A549, human lung fibroblast cells WI-38 and human peripheral blood mononuclear cells (hPBMC)	2.5, 5, 10, 25 and 50 µM	Inhibit cell growth in dose-dependent manner after 24 h IC_{50} 10 µM and negligible cytotoxic effect on normal cells WI-38 and (hPBMC) after 24 h with IC_{50} 50 µM, respectively. ↑ Bax, ↓ Bcl-2, ↓ cellular migration, ↑ Apoptosis. ↑ catalase, glutathione peroxidase (GPx) and glutathione (GSH) levels at 1 and 3 µM and ↓ catalase, GPx and GSH at 5 and 10 µM		(Zhang *et al*., 2017)
Pancreatic Cancer cell lines BXPC-3 & Panc-1	0, 2, 4, 6, 8, 16, 32 µM	Inhibited cell growth after 12, 24 and 48 h. 8 µM significantly inhibited cell viability and 32 µM resulted in more than 80% loss of cell viability ↑ G_0G_1 arrest, ↓ Cyclin D1, ↓ migration and invasion, ↓ P13K/PKB expression	Nude mice implanted with BXPC-3 cells treated with 50 or 100 mg/kg showed reduced tumour growth	(Xu *et al*., 2014)
Colon Cancer cells DLD-1	20 µM	Inhibited cell growth after 24, 48, 72 and 96 h. G_0G_1 phase arrest was observed ↑P27 a multifunctional cdk inhibitor, ↓ Cyclin A, B1, D1, E1, cdc2, phosphor cdc2.		(Matsumoto *et al*., 2005)

Table 4.1: *In vitro* anticancer effect of α-mangostin on cancer cells

	20 µM 49 µM	Inhibited cell growth after 48 h ↓ NO Caspase 3 expression indicates caspase independent cell death, ↑ Bax ↓ bcl2, ↑ endonuclease-G (Endo-G) after 6 and 24 h, ↑ MAP, Erk1/2 after 24-48 h In colo 205 cells ↑ p53 and Bax, caspase 3, 8, and 9, tBid and Fas		(Nakagawa *et al*., 2007) (Watanapokasin *et al*., 2011)
Human Melanoma SK-MEL-28 Cancer cells	18 µM	Inihibit cell growth after 48 h IC_{50} 14 µM, ↑ sub G_1 peak, Caspase 3 and mitochondria potential after 48 h with 18 µM	Normal Skin Fibroblast CCD-1064Sk showed less cytotoxicity with IC_{50} 17.7 µM	(Wang *et al*., 2011)
	12 & 18 µM	↑ Caspase 8 after 48 h, ↑ Caspase 9 with 18 µM after 48 h, ↑ Cyclin D1, ↑ Cytochrome C, ↓ PKB1, phosphor-PKB & NFkB		(Wang *et al*., 2013)
Canine Osteosarcoma cell line D-17 Colorectal carcinoma cells HCT-116	37 µM	Inhibited cell growth after 24 h at IC_{50} 37 µM, ↑ GoG1 after 3 & 6 h, loss of mitochondria potential after 3 h Inhibit cell growth after 48 h with 12 µM, less effect observed in CCD-18Co normal cells at 27 µM ↑ caspase 3/7 ↑ Caspase 9, ↓ Caspase 8 at 24 and 48 µM, ↑ DNA fragmentation, ↓ mitochondria potential, ↑ chromatin condensation and nuclear fragmentation with 48 µM	Tumour established nude mice showed reduced tumour size after 15 and 20 days with no significant animal body weight loss	(Krajarng *et al*., 2012) (Aisha *et* al., 2011)

Table 4.1: *In vitro* anticancer effect of α-mangostin on cancer cells

		↑ MAPK/ERK, Myc/Max, p53, ↓ NFkB. No effect observed on Wnt, Notch, MAPK/JNK and TGFB signaling pathways at 18 µM		
Oral Squamous cancinoma cells (OSCC)	8 µM	↑ Bak, cleaved caspase 3 and PARP, G1 cell arrest with increase expression of p21 and p27		(Kwak *et al*., 2016)
Breast cancer cell (MCF-7 and MDA-MB-231		↑ PARP, Bax ↓ BCL-2 after 24 and 48 h. ↑ MAPK/ERK1/2 ↓ P13/PKB.		
MDA-MB-231	0-4 µM	↑G_0G_1, p21, Caspase 3, 8 and 9 but not caspase 4, ↓P13K/PKB		(Li *et al*., 2014)
	≥ 12 µM, 20 µM ideal for *in vitro* study	↓ cell growth and colony formation		(Kurose *et al*., 2012)
T47D	0, 15 and 30 µM	Loss of mitochondria membrane permeability (MMP), ↑ Bax, cytochrome c, caspase 9, caspase 3,		(Kritsanawong *et al*., 2016)
Cervical Cancer (Hela and SiHa)	0-30 µM	↓ Bcl-2 and PARP		(Lee *et al*., 2017)

Anticancer effect of α-mangostin in different cancers and specifically in leukaemia. Effect on apoptotic proteins (Bax, Bak and Bcl-2), caspase 3, 8 and 9, cell arrest (G_1 arrest), cell cycle arrest proteins (p53 and p21) and senescence protein (p16) were observed in different cancers.

4.1.1 Aim and Objectives

The primary aim of this study was to evaluate the potential of α-mangostin to sensitize AML cells (MOLM-13 cell lines containing a FLT3-ITD mutation) to the cytotoxic effect of doxorubicin.

Objectives

α-mangostin and doxorubicin, singly or in combination were tested on AML cell lines to determine:

- the cytotoxicity of the drugs
- their potential to induce apoptotic cell death
- their ability to change the expression of apoptotic proteins (Bax and Bak) and the anti-apoptotic protein (Bcl-2)
- if they induce apoptosis via the intrinsic or the extrinsic apoptotic pathways through their effect on caspase 3, 8 and 9 proteins
- their effect on the cell cycle and to evaluate their ability to induce cell arrest through the expression of p53, p21 and Cdc25 phosphatases
- their effect on senescence through the regulation of p16 protein expression
- their potential as FLT3 kinase inhibitors

RESULTS

As part of the method development, the doubling time of AML, MOLM-13 cell line was determined (Figure 4.2). In addition, a range of concentrations of Dimethyl Sulphoxide (DMSO) on cells and suitable for solubilizing the test compounds were determined using a cell viability assay (Method Section: 3.4.1). This was important to determine the cytotoxic effect of DMSO that could distort the effect of the test compounds used in this study. In this study the effect of doxorubicin singly and in combination with α-mangostin in AML was determined, to evaluate possible mechanisms of action on cellular processes such as cell growth, apoptosis, cell cycle arrest and senescence. Moreover, α-mangostin has been reported to inhibit the cell survival P13K/PKB pathway (Li *et al*., 2014), and the deregulation of this pathway could be as a result of FLT3-ITD mutation (Meshinchi & Appelbaum, 2009). Therefore, α-mangostin was further studied as potential FLT3-ITD kinase inhibitor, singly and in combination with doxorubicin on AML cell line MOLM-13 with FLT3-ITD mutation.

4.2 Evaluation of the doubling time on MOLM-13

To investigate the doubling time of cells, MOLM-13 cells were counted over a period (0-54 h) using the Trypan Blue exclusion dye assay (Method Section: 3.3), which showed the cell growth curve. The growth curve (Figure 4.1) showed steady increase in cell numbers over time. MOLM-13 cells increased in number with doubling effect observed at 8 h, which continued up to the 54 h incubation time point. This experiment was used to determine that cells were at log phase (proliferating) before assays were done.

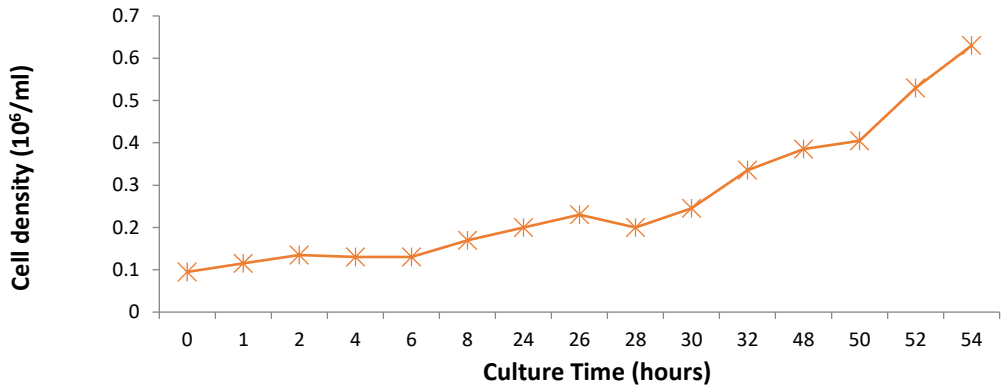


Figure 4.2: Cell counting of MOLM-13. Equal volumes of cell suspension and Trypan Blue were mixed and 10 µl of the mixture was loaded into a chamber of the haemocytometer. Manual cell counts were done using a phase contrast microscope (100× magnification).

4.3 Cytotoxic Effects of DMSO on Acute Myeloid leukaemic cells (MOLM-13) and Normal Monocytic cells

To determine the effect of DMSO on cells, a cell viability assay was performed with a range of DMSO concentrations (1-20% with final concentrations 0.05-1%) on MOLM-13 and control cells (normal macrophages) using CyQUANT GR after 72 h incubation (Method Section: 3.4.1). Descriptive statistics (Appendix 3) and ANOVA were performed to determine differences between treatments (Appendix 4). Inhibition of cell growth was less than 10% in higher concentrations, except at 0.8% (16%) concentration with inhibition of 13%. However, 20% inhibition of cell growth at low concentration 0.05% ($p<0.05$) in normal macrophage cells was observed This effect was different than what was expected. (Figure 4.3).

In MOLM-13, DMSO inhibited cells at 0.8% and 1% concentrations with 11% and 12% inhibition, respectively. DMSO showed no significant effect at 0.25% ($p>0.05$) and 0.05% ($p>0.05$) concentrations in MOLM-13. DMSO at 0.25% was observed suitable for preparation all concentrations of α- mangostin used in this study.

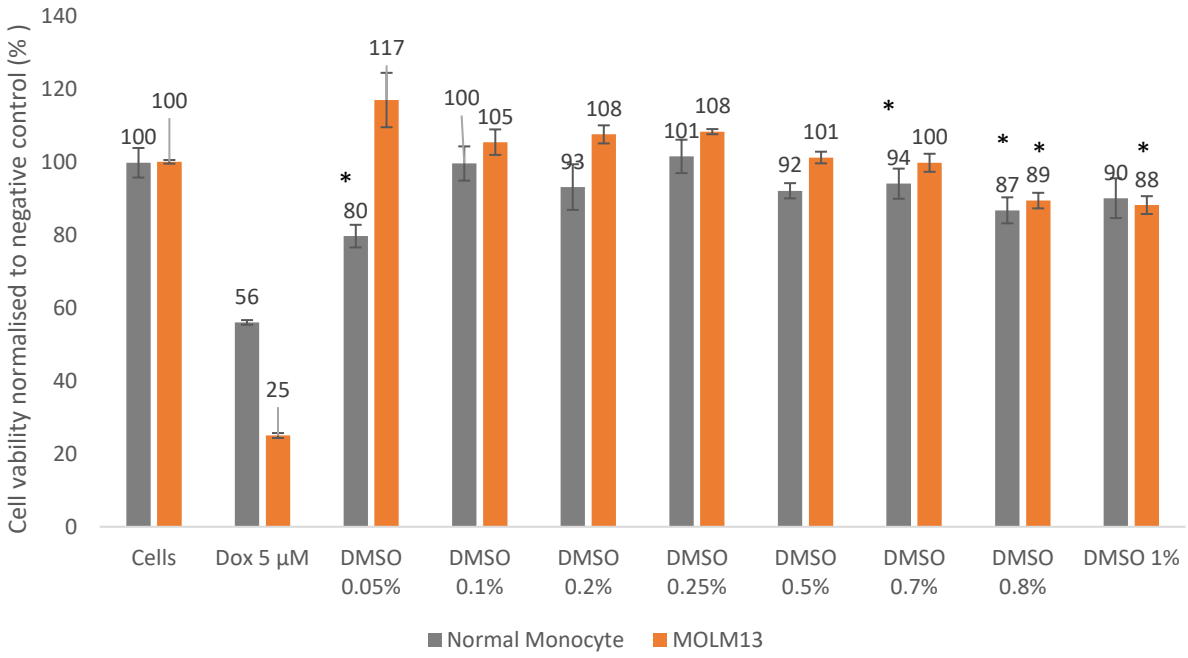


Figure 4.3: The effect of DMSO on cell viability after 72 hours of incubation. Cells (1.5×10^5 cells/ml) were treated with a range of DMSO concentrations for 72 h and the viability was determined using CyQUANT GR assay. Results were expressed as % control relative to negative control. Data represent three independent experiments (n=3) with each experiment carried out with four replicates (n=4 replicates). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *$p<0.05$ compared to negative control.

4.3.1 Effect of doxorubicin on Acute Myeloid Leukaemic cell line after 72 h of incubation

To determine the effect of doxorubicin on cell viability, MOLM-13 cells were treated with 1, 2.5 and 5 µM of Dox at 72 h. There was inhibition of cell growth by doxorubicin. Dox at 2.5 and 1 µM inhibited viable cells by 46% and 44%, respectively after 72 h treatment when compared to negative control 0.25% DMSO (cells without treatment). In this study, IC_{50} of Dox was 1 µM and this concentration was used for further studies and in combination with α-mangostin, gallic acid or vitamin C.

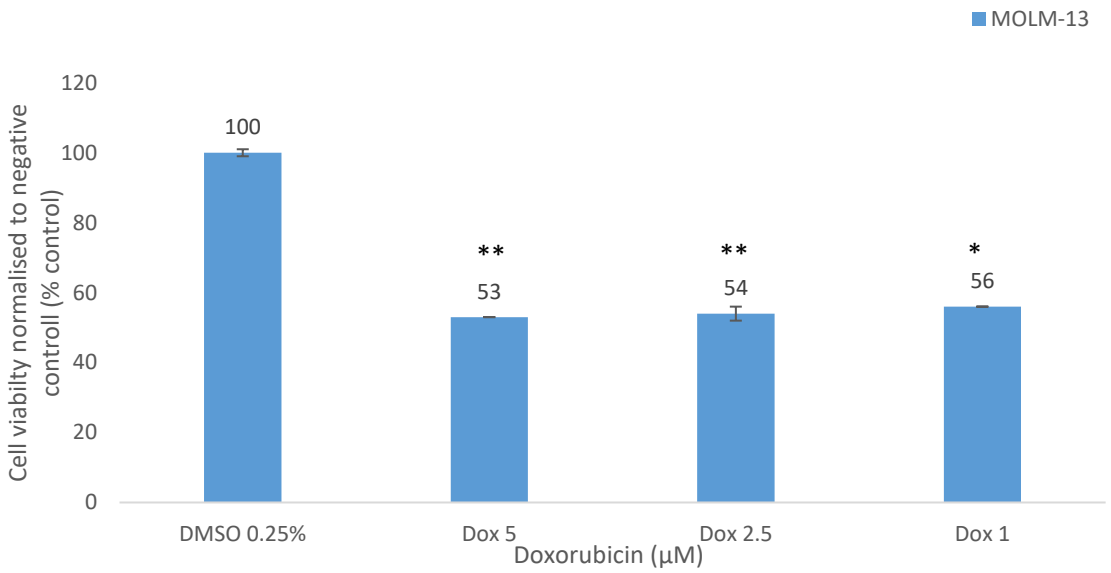


Figure 4.3.1: The effect of doxorubicin on MOLM-13 cells after 72 h of incubation. MOLM13 cells (1.5×10^5 cell/ml) were treated with various concentrations of Dox for 72 h. Cell viability was determined using CyQUANT GR. Results were expressed as % control relative to 0.25% DMSO control. Data represent three independent experiments (n=3) with three replicates (n=3 replicates) within an experiment. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis*$p<0.05$, **$p<0.01$ compared to negative control, IC_{50} of Dox was 1 µM

4.4 Alpha mangostin inhibits the proliferation of Acute Myeloid Leukaemic cell line in a dose-dependent manner

The cell viability following exposure to α- mangostin in MOLM-13 and normal monocytes after 72 h was examined using a cell viability assay (Method Section: 3.4.3). The effect of α-mangostin on both acute (MOLM-13) and chronic (K562) myeloid leukaemic cell lines were determined during method development. The viability assay, with a cell density of 1.5×10^5 cells/ml, was performed using CyQUANT GR after treatment with different concentrations (5, 10, 20, 30, 40, 50 µM) of α-mangostin. Inhibition of MOLM-13 cells was dose-dependent (Figure 4.4). Treatment

of MOLM-13 with α-mangostin at 50, 40, 30, 20 and 10 µM showed 87%, 85%, 82%, 81%, 56% inhibition, respectively when compared to no treatment cells. Thus indicating decreasing inhibition with decreasing concentration. Cell viability at 5 µM was 96% after 72 h, therefore, was not statistically significant ($p>0.05$). In normal monocytes, no cytotoxic effect with α-mangostin at 5 and 10 µM were observed. At 20 and 30 µM inhibition (20% and 26%, respectively $p<0.05$). At high concentrations, α-mangostin at 50 and 40 µM inhibited 50% and 52% of cells, respectively ($p<0.01$). In this study, α-mangostin at 20 µM was considered for combination studies in both MOLM-13 and K562 for the purpose of direct comparison, however combination and further studies was done only on MOLM-13 and not on K562 in the interest of time.

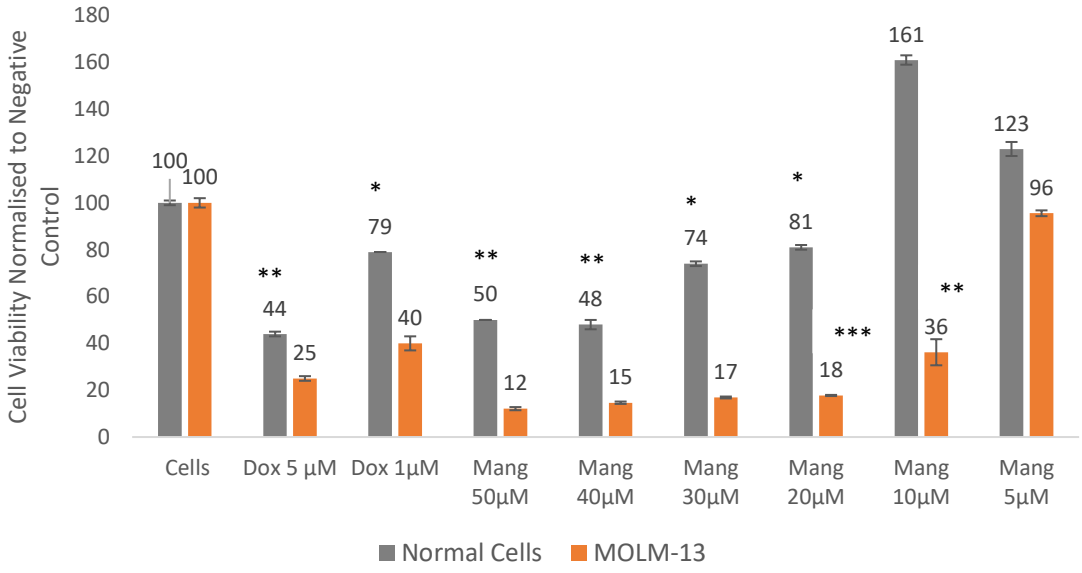


Figure 4.4: The effect of α-mangostin on normal monocytes and MOLM-13 cells after 72 h of incubation. MOLM-13 and normal monocytes were treated with various concentrations of α-Mangostin at a density of 1.5×10^5 cell/ml for 72 h. Cell viability was determined using CyQUANT GR. Results were expressed as % control relative to 0.25% DMSO control. Data represent three independent experiments (n=3) with four replicates (n= 4 replicates) within an experiment. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to negative control.

4.4.1 Effect of combination of α-mangostin and doxorubicin on cell viability

The combination of α-mangostin and the chemotherapy drug, doxorubicin was investigated in MOLM-13 to determine its effect on cells. α- Mangostin at 20 µM was combined with Dox at 1 µm to determine cell viability using CyQUANT GR after 48 h. MOLM-13 cells treated with α-mangostin and Dox combined showed significant inhibition of cell growth when compared to Dox 1 µM only ($p<0.01$) (Figure 4.4.1). Combination index (CI) analysis was done to determine synergistic, antagonistic and additive effect when the drugs were combined. CI value (0.85) was observed, showing possible synergism.

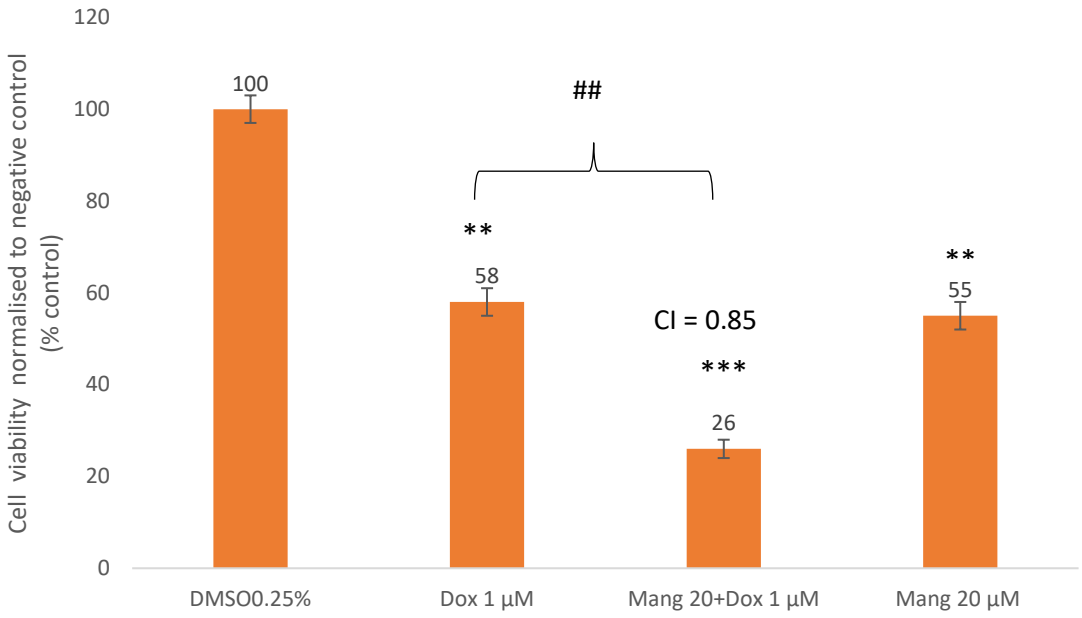


Figure 4.4.1: Effect of combination of α-mangostin and doxorubicin on cell viability after 48 h of incubation. MOLM-13 cells (1.5×10^5 cell/ml) was treated with Dox 1 µM singly and in combination with α-Mangostin 20 µM. Cell viability was determined using CyQUANT GR. Results were expressed as % control relative to 0.25% DMSO control. Data represent an independent experiment (n=1) with four replicates (n=4 replicates). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis **p<0.01, ***p<0.001 compared to negative control. $^{\#\#}$p<0.01 compared to Dox only.

4.4.1.1 Drug interaction of doxorubicin and α-mangostin

The type of interaction between Dox and α-mangostin was determine using the CompuSyn software with median effect analysis which derives combination index (CI) value of two drugs combined. CI value of 0.85 indicates that combined drugs showed synergism (Method Section: Figure 3.1).

Table 4.4.1.1: Combination index (CI) of data for combination effect of doxorubicin and α-Mangostin in MOLM-13 cells.

Doxorubicin (µM)	α-Mangostin (µM)	Effect (inhibition %)	CI Value	Result
1	20	0.74	0.85	synergism

CI value 0.1-0.90 indicates synergism, 0.90-1.10 indicates additive while 1.10-10 indicates antagonistic.

4.4.2 Effect of combination of doxorubicin and α-mangostin on cell Apoptosis

Apoptosis was determined using annexin V/propidium iodide dye (PI) in MOLM-13 cells. (Method Section: 3.5.1). Dox at 1 µM induced necrosis with 5.94% necrotic cells compared to those in early (0.69%) and late apoptosis (0.83%) after 72 h (Figure 4.4.2).

Apoptotic cell death with combination of α-mangostin and Dox increased compared to single drugs. Combination of α-mangostin (20 µM) and Dox (1 µM) induced 15.7% apoptotic cell death (both early and late apoptosis combined) while the single treatments, Dox (1µM) and α-mangostin (20 µM), inducing only 1.52% and 0.95% apoptotic cell death, respectively. In addition, percentage of cells going into necrosis was reduced with the drug combination (2.68%) compared to 5.94 with Dox 1 µM and 4.95% with α-mangostin single treatments. However, necrosis induction by the combined drug compared to Dox only was not statistically significant Therefore, more cell death by necrosis than apoptosis was observed with Dox only but more apoptosis than necrosis when combined with α-mangostin. Early apoptotic cell death by combined drug was statistically significant when compared to Dox only and cells without treatment ($p<0.001$) (Figure 4.2.2).

To verify if cell death was via the induction of apoptosis, the morphology of MOLM-13 cells were studied. Cell shrinkage, chromatin condensation and fragmentation are morphological characteristics of apoptosis observed using the TUNEL assay. DAPI staining showed some cells stained with the apoptotic dye and some fragmented with α-mangostin 20 µm single treatment indicating early signs of apoptosis. Shrinkage of apoptotic cells was observed in all treatments (Figure 4.4.2.1). Cells treated with α-mangostin (20 µM) only showed less intensity of apoptotic staining and more cell fragmentation while those in combination with Dox (1 µM) showed more apoptotic bodies and less fragmentation.

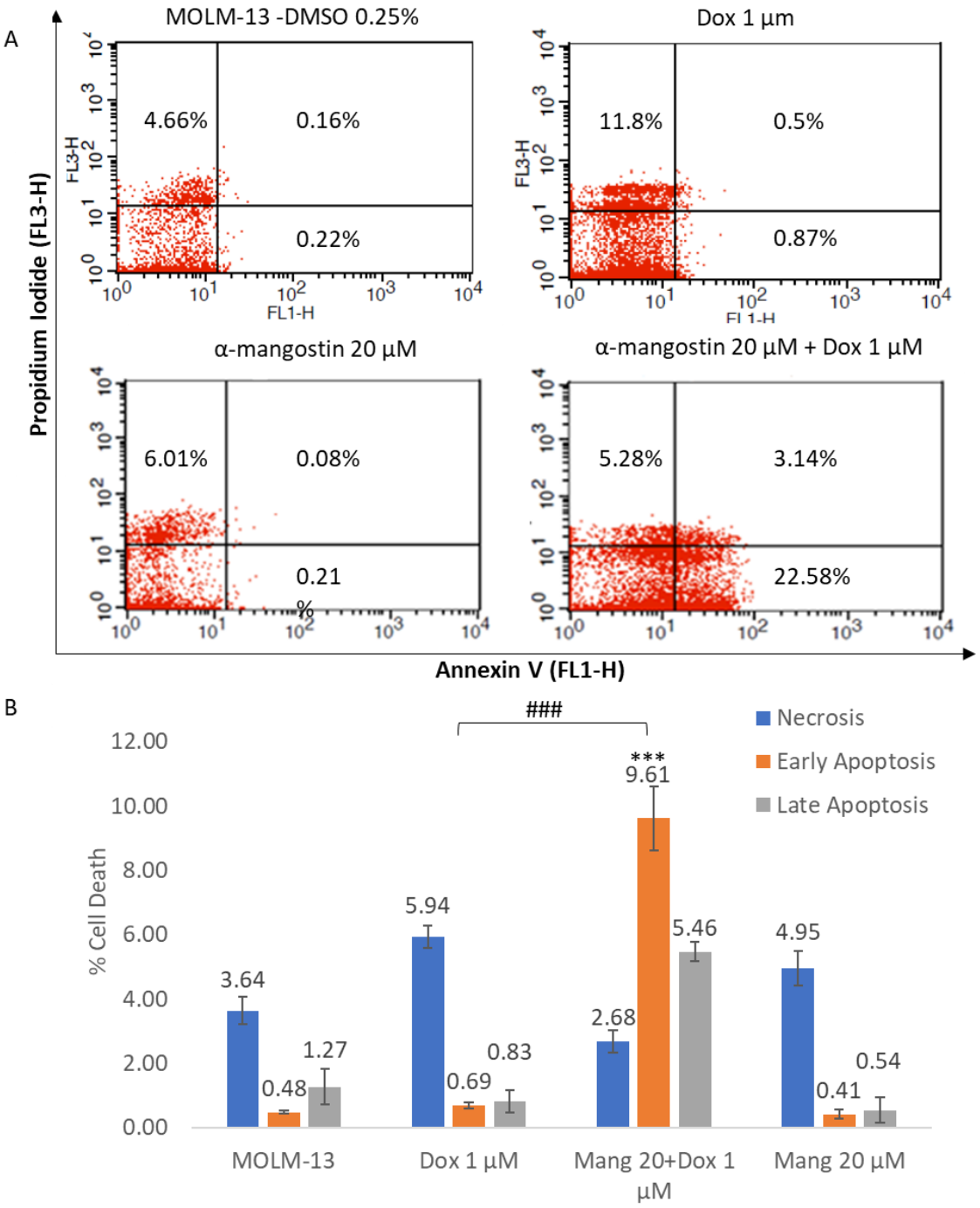


Figure 4.4.2: The Effect of doxorubicin and combination with α-mangostin on Induction of Apoptosis after 72 h on MOLM-13. **A.** Fluorescence activated cell sorting (FACS) illustrating distribution of % population undergoing apoptosis and necrosis of one independent experiment **B. G**raphical representation of % necrosis and apoptosis (early and late) of data from three independent experiments (n=3) with one replicate each (n=3 replicates). MOLM-13 cells were treated with α-mangostin at 20 µM and doxorubicin at 1µM for 72 h, cell apoptosis was determined using annexin V and propidium iodide. Representation of 10,000 cells staining profile. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis ***$p<0.001$ compared to negative control. $^{###}p<0.001$ compared to Dox only.

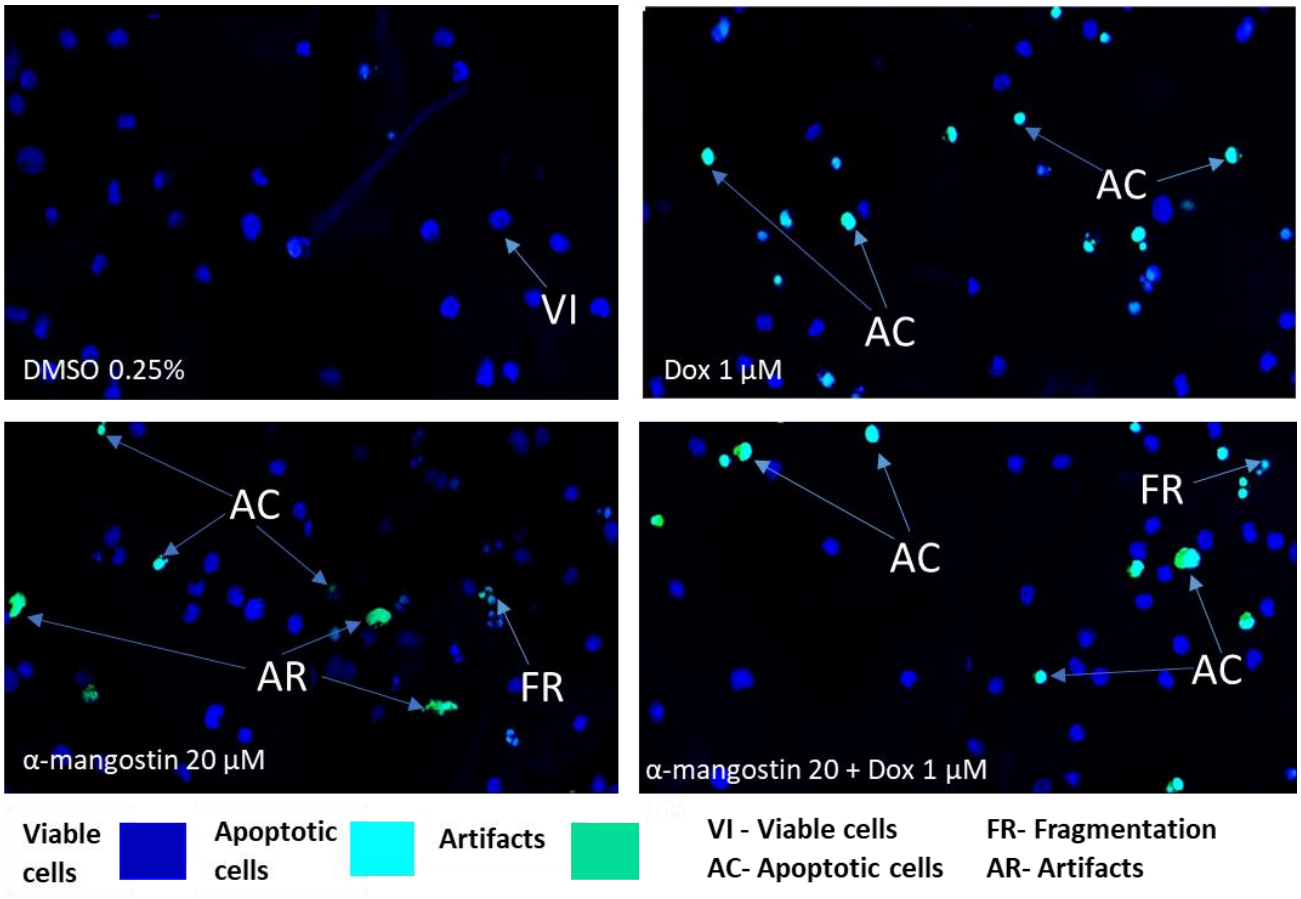


Figure 4.4.2.1: Apoptotic Effect of Doxorubicin and combination with α-Mangostin on MOLM-13 cells after 48 h with TUNEL assay. MOLM-13 cells were treated with α-mangostin at 20 µM and doxorubicin at 1µM for 48 h, morphology of apoptotic cells was observed. Image is representative of two independent experiments (n=2) with two replicates within an experiment. **AC**: Apoptotic cells, **FR**-Fragmentation. Cells were observed using the Fluorescence microscope

Mechanisms of apoptosis can be regulated by tumour suppressor protein p53. Expression of tumour suppressor protein p53 and other proteins it regulates, such as the cell cycle ($p21^{Cip1}$ and cdc25s phosphatases), Bcl-2 family (pro-apoptotic (Bak and Bax), anti-apoptotic (Bcl-2) and senescence ($p16^{ink4a}$ and $p21^{Cip1}$) proteins were studied using immunoblotting after 48 h cell treatment. In addition, expression of caspases (3, 8 and 9) that induce either intrinsic (caspase 3 and 9) or extrinsic (caspase 3 and 8) apoptotic pathways were also determined using immunoblotting.

4.4.3 Effect of combination of doxorubicin and α-mangostin on expression of Bcl-2 family of proteins

The Bcl-2 family protein regulates apoptotic cell death and the expression of these proteins were determined using Western blot analysis (Method Section: 3.6.2.1) to elucidate mechanisms involved in the cell death observed. More expression of pro-apoptotic proteins Bax and Bak, and less expression of anti-apoptotic protein Bcl-2 was observed with Dox and α-mangostin combination compared to single treatments (Figure 4.4.3). However only Bak was statistically significant ($p<0.05$) compared to Dox only**.**

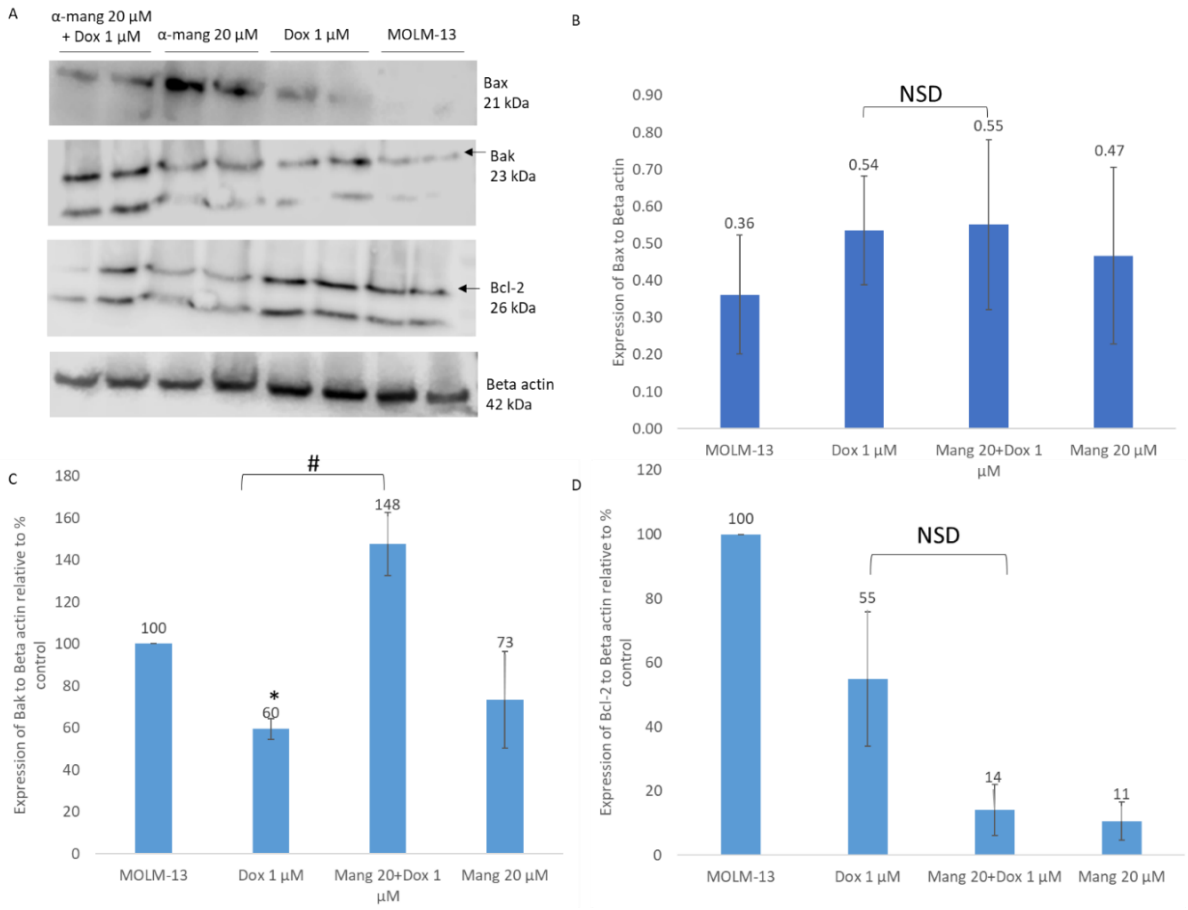


Figure 4.4.3: ***The expression of Bax, Bak, Bcl-2 after 48 h treatment with α-mangostin singly and in combination with doxorubicin in MOLM-13 cells using Western blotting.*** **A**. Immunoblotting results of apoptotic proteins (Bax, Bak and Bcl-2) and internal control (housekeeping gene Beta actin) after treatment with Dox singly and in combination with α-mangostin in MOLM-13 cells. Cell lysates were subjected to SDS-Page gel for immunoblotting analysis. **B**. Graphical presentation of Bax expression **C.** Graphical presentation of Bak expression **D**. Graphical presentation of Bcl-2 expression**.** Data represent two experiments (n=2) with three replicates (n=3 replicates) (two replicates within an experiment and one replicate from an independent experiment.) for Bax, Bak and Bcl-2. Results were presented as % control ± SD. Due to non quantifiable amount of protein in the control for Bax data was presented as protein expression relative to beta actin. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis ****p<0.05, **p<0.01 compared to negative control, #p<0.05 compared to Dox only as indicated, NSD- No Significant Difference as indicated.***

4.4.4 Effect of combination of doxorubicin and α-mangostin on expression of caspases (Caspase 3, 8 and 9) on MOLM-13

The expression of cleaved or activated caspase 3, 8 and 9 were determined using Western blot analysis. There was increased expression of caspase 3 and more expression of caspase 8 when Dox and α-mangostin were combined compared to Dox single treatment. Thus, caspase 3 mediated apoptosis was observed after α-mangostin and Dox combined treatment which may be involved with the activation of extrinsic pathway (via caspase 8). However, caspase 3 and 8 were not statistically significant when compared to Dox only. There was reduced expression of caspase 8 and 9 with single treatments compared to negative control. While 3-fold increase of caspase 3 by Dox only and 4-fold increase by α-mangostin single treatment was not statistically significant when compared to negative control.

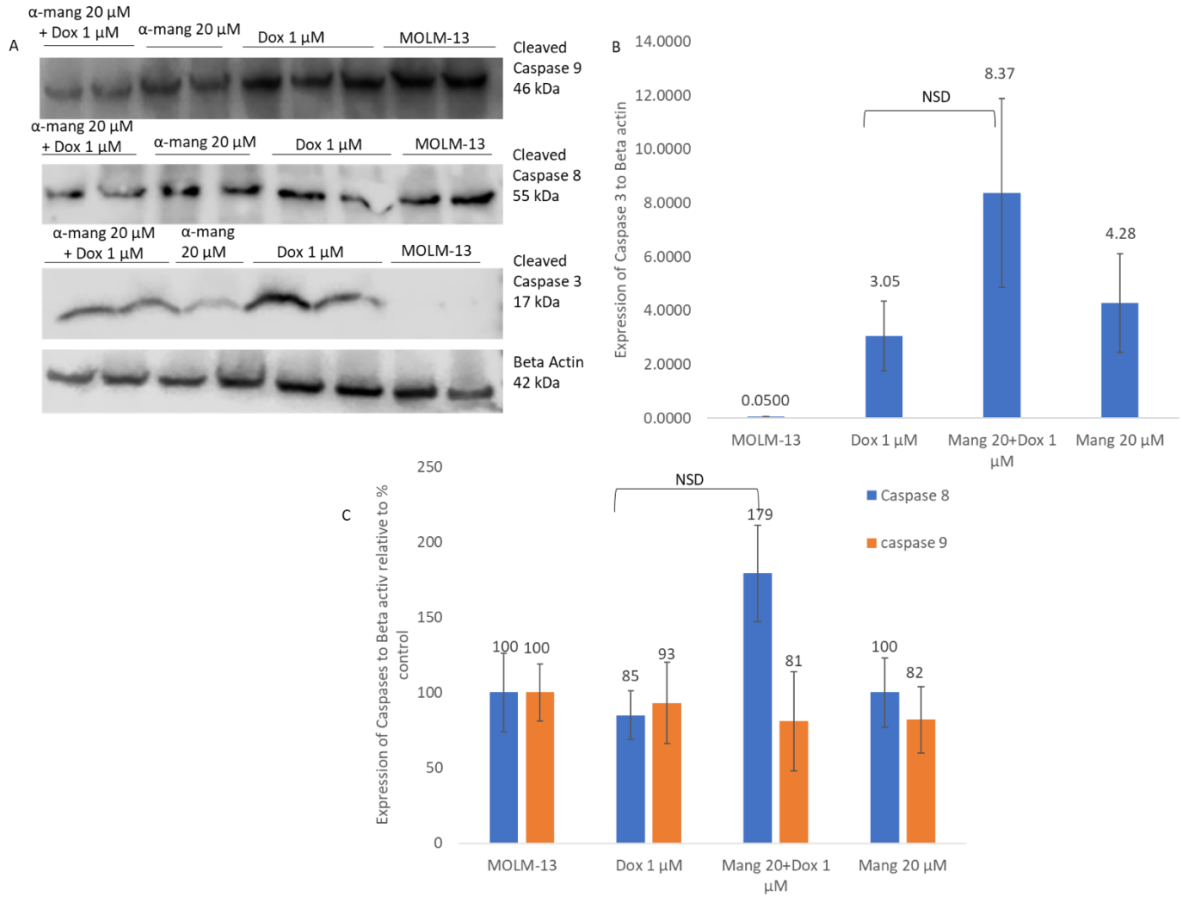


Figure 4.4.4: ***The expression of caspase 3, 8 and 9 after 48 h treatment with α- mangostin singly and in combination with doxorubicin on MOLM-13 cells after 48h using Western blotting.*** **A**. Immunoblotting results of caspase proteins (caspase 3, 8 and 9) after treatment with Dox singly and in combination with α-mangostin in MOLM-13 cells. Cells lysates was subjected to SDS-Page for immunoblotting analysis. **B**. Data represent two separate experiments (n=2) with two replicates within an experiment and one replicate from an independent experiment (n=3 replicates) for caspase 3. α-Mang data represent three independent experiments with one replicate each, n=3 replicates for caspase 3 relative to beta actin due to non quantifiable amount of protein in the control. **C**. Data represent two experiments (n= 2) with two replicates each (n=4 replicates) for caspase 8 and 9 (two replicates within an experiment). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *NSD- No Significant Difference as indicated*

4.4.5 Effect of combination of α-mangostin and doxorubicin on the cell cycle Effect on cell cycle was analysed using the Flow cytometer after staining with propidium iodide (Method Section: 3.6.1). MOLM-13 cells treated with Dox 1 μM showed S and G_2/M phase arrest (Figure 4.4.5). G_0/G_1 phase arrest of cells was observed after treatment with α-mangostin (20 μM) when compared to negative control. Combination of α-mangostin 20 μM and Dox 1 μM showed more cells were arrested at G_2/M phase compared to the single treatments of the drug.

There was significant G_2/M arrest ($p<0.001$) after treatment with Dox and α-mangostin combined compared to cells without treatment (negative control). However, more G_2/M phase arrest observed with Dox and α-mangostin combination (31%) was not statistically significant ($p>0.05$) when compared to Dox 1μM alone (24%.

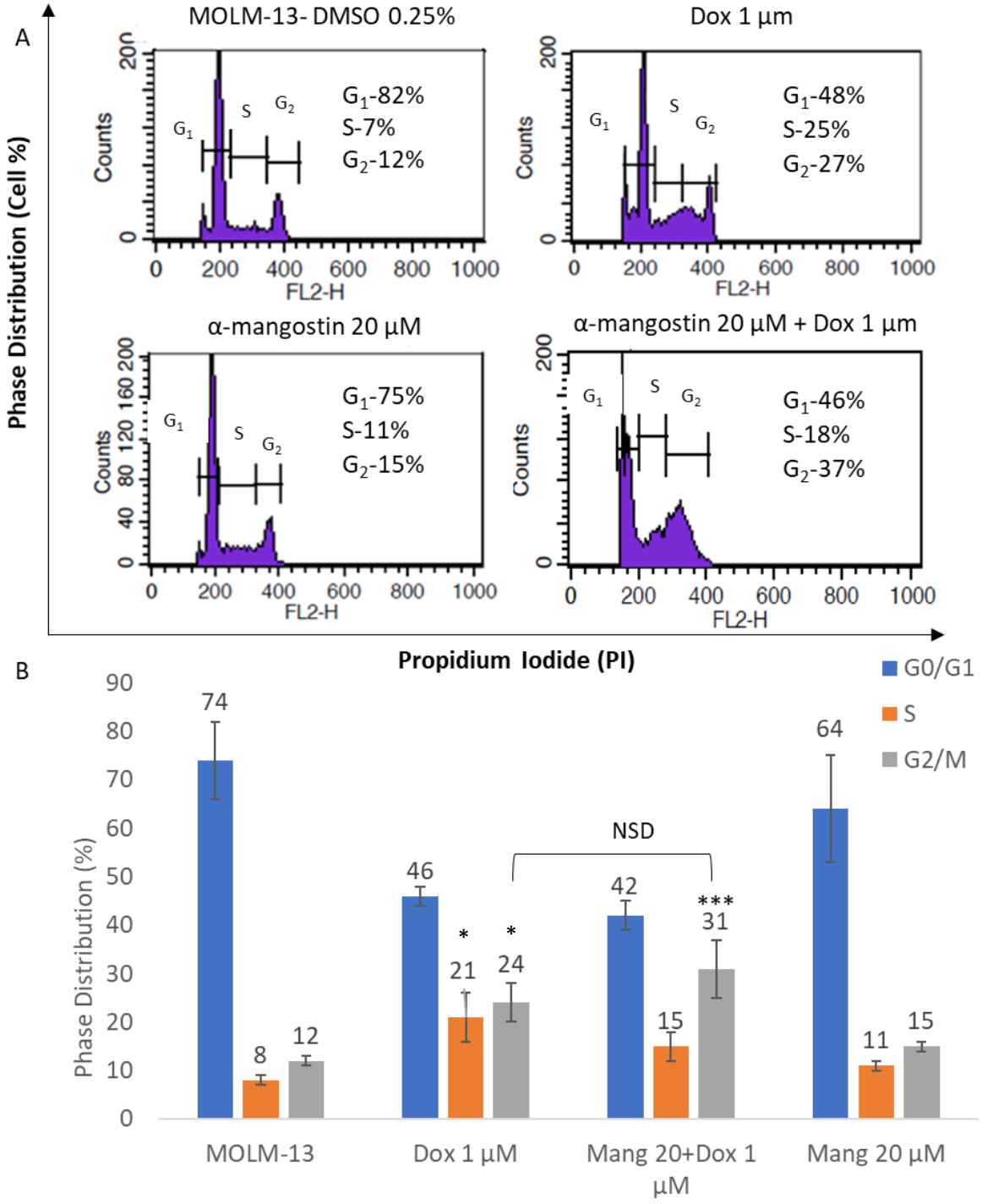


Figure 4.4.5: The effect of combination of α-mangostin and doxorubicin on Cell cycle after 48.

A.DNA histogram from the flow cytometer showing cell cycle arrest of MOLM-13 cells after treatment with α-mangostin 20 µM, Dox 1 µM and combination of Dox and α-mangostin for 48 h. Cells were harvested, stained with propidium iodide and analysed using flow cytometer. **B**. Data represent three independent experiments (n=3) with one replicate each (n=3 replicates). Cell cycle distribution was determined as % cell arrest in G_1, S and G_2/M phase Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis ***p<0.001 compared to control, NSD- No Significant Difference as indicated.

4.4.6 Effect of combination of doxorubicin and α-mangostin on expression of p53 in MOLM-13

There was expression of p53 after treatments. p53 regulates cell cycle, senescence and apoptosis when activated. There was expression of p53 after treatment with α-mangostin in combination with doxorubicin which was 2.0-fold increase compared to Dox only (Figure 4.4.6). Statistically significant results ($p<0.01$) was obtain when combined drug was compared to negative control but compared to Dox it was not statistically significant ($p>0.05$). α-Mangostin induced more expression of p53 (500%) compared to Dox only (323%). However, when compared to control it was not statistically significant.

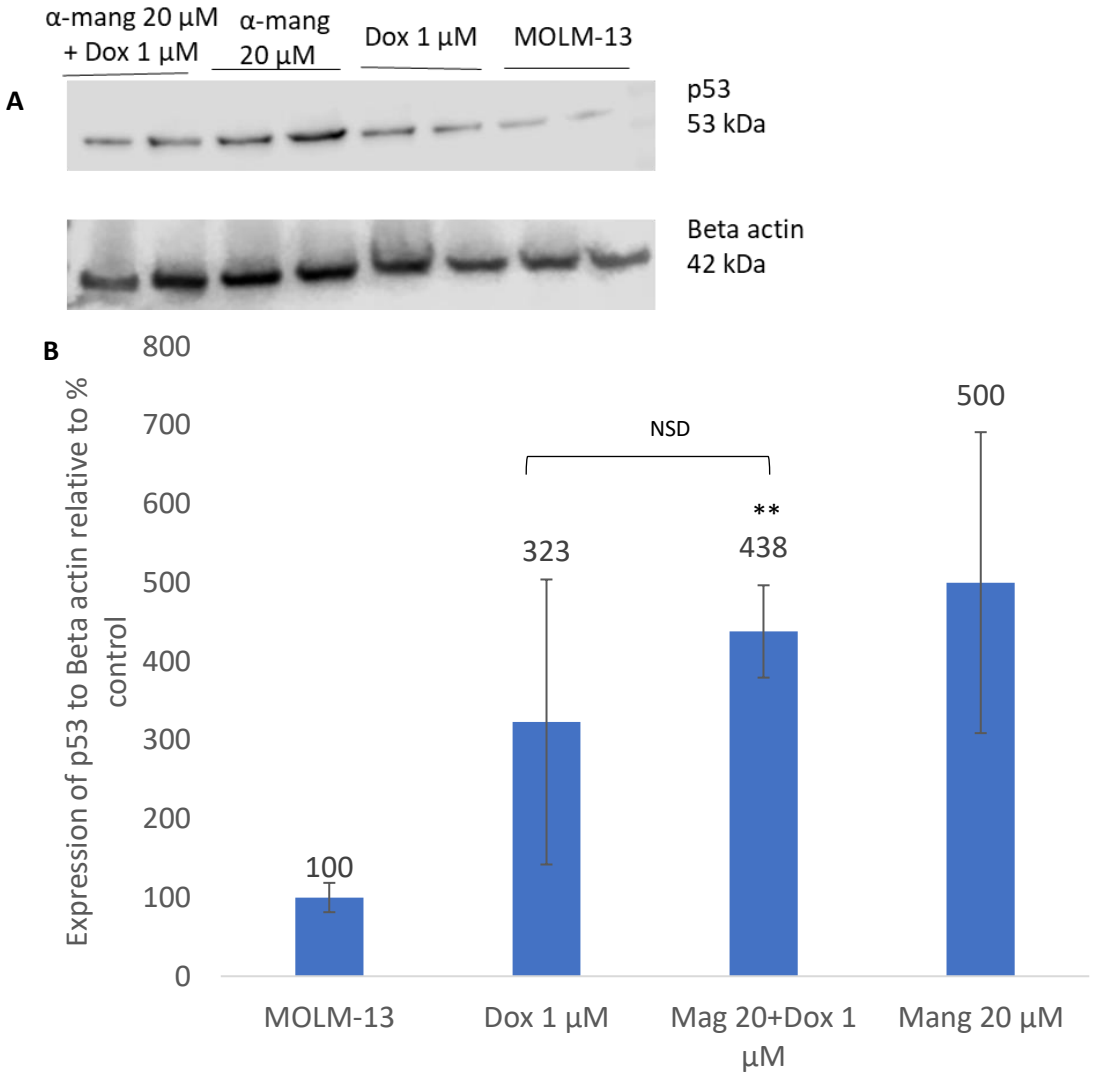


Figure 4.4.6: The expression of p53 after 48 h treatment with α-mangostin singly and in combination with doxorubicin in MOLM-13 cells using Western blotting. **A**. Immunoblotting results of p53 expression after treatment with Dox singly and in combination with α-mangostin in MOLM-13 cells. Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis **B**. Data represent two experiments (n=2) with three replicates (n=3 replicates) (Two replicates within an experiment and one replicate from an independent experiment). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *p<0.05, **p<0.01, ***p<0.001 compared to control, NSD- No Significant Difference as indicated

4.4.7 Effect of combination of doxorubicin and α-mangostin on expression of cell cycle arrest protein p21, Cdc25 phosphatases and senescence protein p16 on MOLM-13

Tumour suppressor protein p53 regulates the cell cycle via cell cycle complex inhibitor p21. Therefore, activation/ increase expression of p21 due to induction of cell cycle arrest was observed in this study. In addition, inhibition of cdc25 phosphatases that indicates induction of cell arrest was observed.

To evaluate the effect of α-mangostin singly and in combination with doxorubicin on the expression of $p21^{Cip1}$ and phosphatases cdc25A, cdc25B and cdc25C, Western blot analysis after 48 h treatment was performed. MOLM-13 cells treated with combination of α-mangostin and Dox showed increased expression of p21 (1.6-fold increase compared to Dox). However, combination was not statistically significant when compared to Dox only. Single treatments with either Dox or α-mangostin showed less than 1-fold increase (Table 4.4.9) expression of p21 which was not statistically significant when compared to control.

After combined treatment with Dox and α-mangostin, MOLM-13 cells showed reduced expression of cdc25 A and B compared to treatment with single drugs. Reduced expression of cdc25 A, B and C after treatment with Dox and α-mangostin was significant when compared to Dox only. Fold-decrease of cdc25A, B, C in cells treated with combination was 6.7; $p<0.05$, 1.7; $p \leq 0.05$; 1.3; $p \leq 0.05$, respectively, when compared to Dox only treated cells (Table 4.4.9).

$p21^{Cip1}$ has been reported to inhibit cell cycle and induce cellular senescence protein p16. To examine whether α-mangostin and doxorubicin induces cellular senescence after treatment, p16 expression was determined after 48 h using Western blotting. Expression of p16 was similar with combination and Dox only (Figure 4.4.7) suggesting senescence. α-Mangostin did not expressed p16.

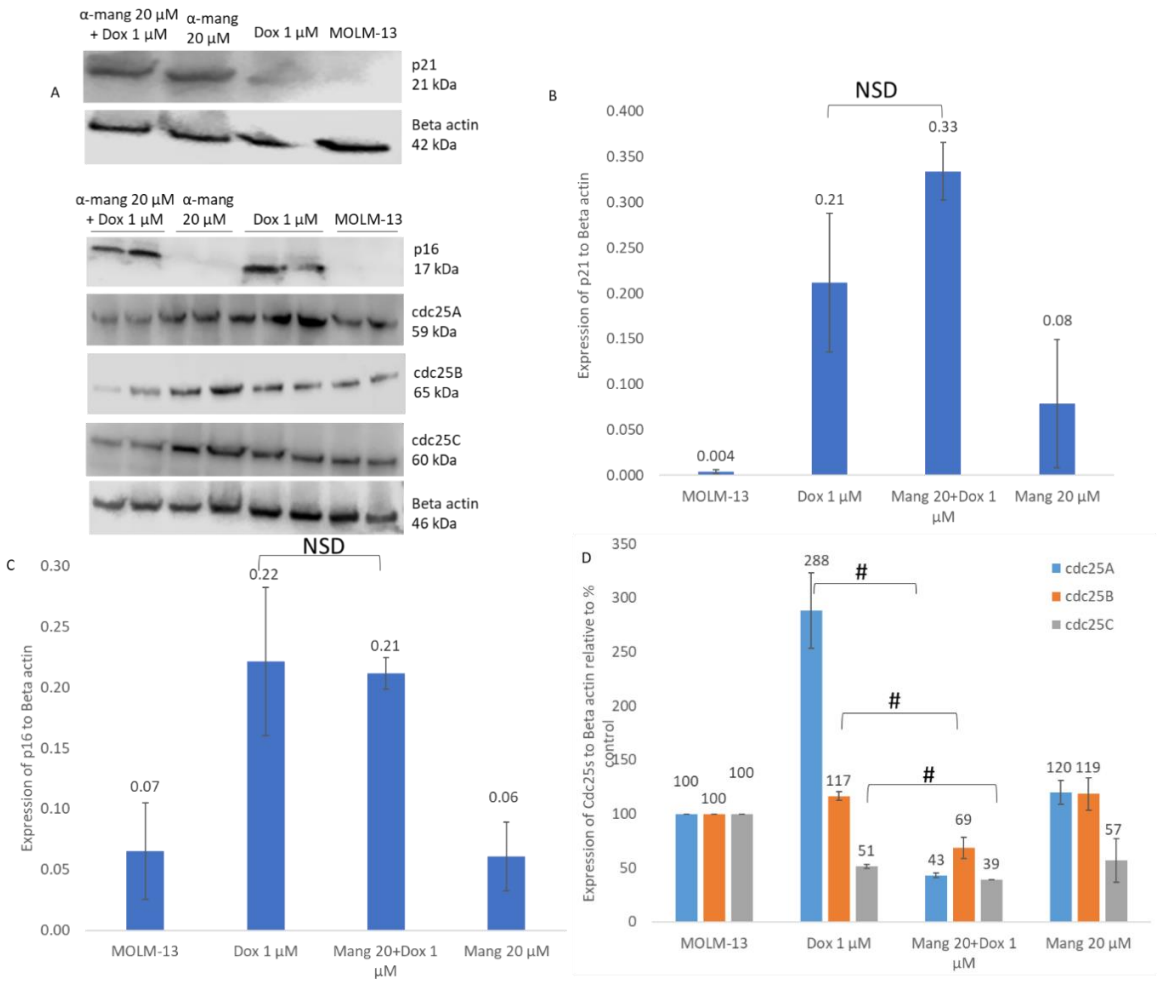


Figure 4.4.7: The expression of p21 Cdc25s and p16 after 48 h treatment with doxorubicin singly and in combination with α-mangostin on MOLM 13 cells using Western blotting. **A**. Immunoblotting results of p21, cdc25s and p16 expression after treatment with Dox singly and in combination with α-mangostin in MOLM-13 cells. Cells was subjected to SDS-Page for immunoblotting analysis **B.** Graphical presentation of p21 expression **C**. Graphical presentation of p16 expression, **D.** Graphical presentation of cdc25s expression. Results were presented as % control ± SD of data from two (n=2) experiments with three replicates (n=3) for cdc25A and cdc25B. Two replicates (n=2) within an experiment (n=1) for cdc25C. Three replicates (n=3) for p21 and four replicates (n=4) for p16 expressions relative to beta actin due to non quantifiable protein in control. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *p<0.05, **p<0.01 compared to negative control. #p<0.05 compared to Dox only. NSD- No Significant Difference as indicated.

4.4.8 α-Mangostin and Dox combination inhibits kinase activity of FLT3-ITD in MOLM-13

To detect effect in FLT3 phosphorylation and expression, both MOLM-13 (which expresses FLT3-ITD mutant) and OCI-AML (the negative control which expresses wild type FLT3) cell lines were used. In OCI-AML cells (FLT3-WT), there was addition of FLT3 ligand (FL) to stimulate FLT3 phosphorylation for detection since FLT3 WT receptor requires the binding of FL for phosphorylation. Thus, to reflect physiological situation, OCI-AML cells were incubated for 15 min with FL (100 ng/mL) at 37^{O}C. In MOLM-13 cells exogenous ligand stimulation is not required due to ITD mutation that result in active autophosphorylation of FLT3 without ligand binding the FLT3 receptor. Thus, the MOLM-13 cells were not exposed to FL.

Lysates (prepared from MOLM-13 cells after 2h treatments and OCI-AML cells without treatment) were probed with anti-phosphotyrosine (anti-P-Tyr) to detect phosphorylated tyrosine proteins and anti-FLT3 (Phospho-FLT3 (Tyr591) antibodies. FLT3-ITD expression was not significantly expressed (32% ± 0.11; $p>0.05$) in OCI-AML cells (despite stimulated with FL), compared to untreated MOLM-13 cells indicating the absence of the mutant gene. OCI-AML cells expressed phosphorylated tyrosine proteins while less expression was observed with Dox and α-mangostin combination. However, almost similar levels of phospho-FLT3 was detected with both OCI-AML (32 %) and combination treatment (23 %) in this study. In MOLM-13, FLT3 was phosphorylated in the absence of FL, consistent with expression of FLT3-ITD after a 2 h treatment (Fig. 4.4.8). Compared to negative control, α-mangostin stimulated more expression of phospho-FLT3-ITD (637% ± 0.01; $p<0.05$) compared to Dox (205% ± 0.25; $p>0.05$) in MOLM-13 cells. However, when combined there was a marked reduction of FLT3-ITD expression (23%±0.10 $p>0.05$ compared to Dox alone) by immunoblotting.

The inhibition of FLT3-ITD phosphorylation after treatment with α-mangostin in combination with Dox was confirmed with ELISA (Figure 4.4.8, B). Phospho FLT3 was inhibited with combination treatment by 77% ± 0.063 ($p>0.05$) and 78% ± 0.004 ($p<0.01$) using Western blotting and ELISA detection, respectively, compared to negative control, indicating the results are reproducible. α-Mangostin (20 µM) induced more expression of phospho FLT3 compared to Dox single treatment, similar to expression detected with Western blotting. In addition, more phospho FLT3 expression after stimulation with FL in OCI-AML cells was detected with ELISA (44% ±0.019; $p<0.05$) than with Western blotting (23% ±0.11; $p>0.05$) compared to negative control. Detection of more expression of phospho FLT3 in ELISA could be due to increased sensitivity of the ELISA technique. However, the reduced phospho FLT3 expression in OCI-AML cells with ELISA was significant compared to MOLM-13 control cells, indicating low level of expression and activity in OCI-AML FLT3-WT than in MOLM-13 FLT3-ITD. Interestingly, there was no significant difference between expression of phospho-FLT3 in OCI-AML-WT cells and treatment with combination in MOLM-13 cells expressing mutant FLT3-ITD ($p>0.05$). Thus, suggesting that treatment with combination could reduce the level and activity of phospho FLT3 to similar expression and activity in FLT3-WT cells (OCI-AML). More importantly, treatment with Dox single treatment in ELISA showed 99% expression of phospho FLT3 while expression when

combined with α-mangostin showed 22% expression and this was statistically significant when compared ($p<0.05$).

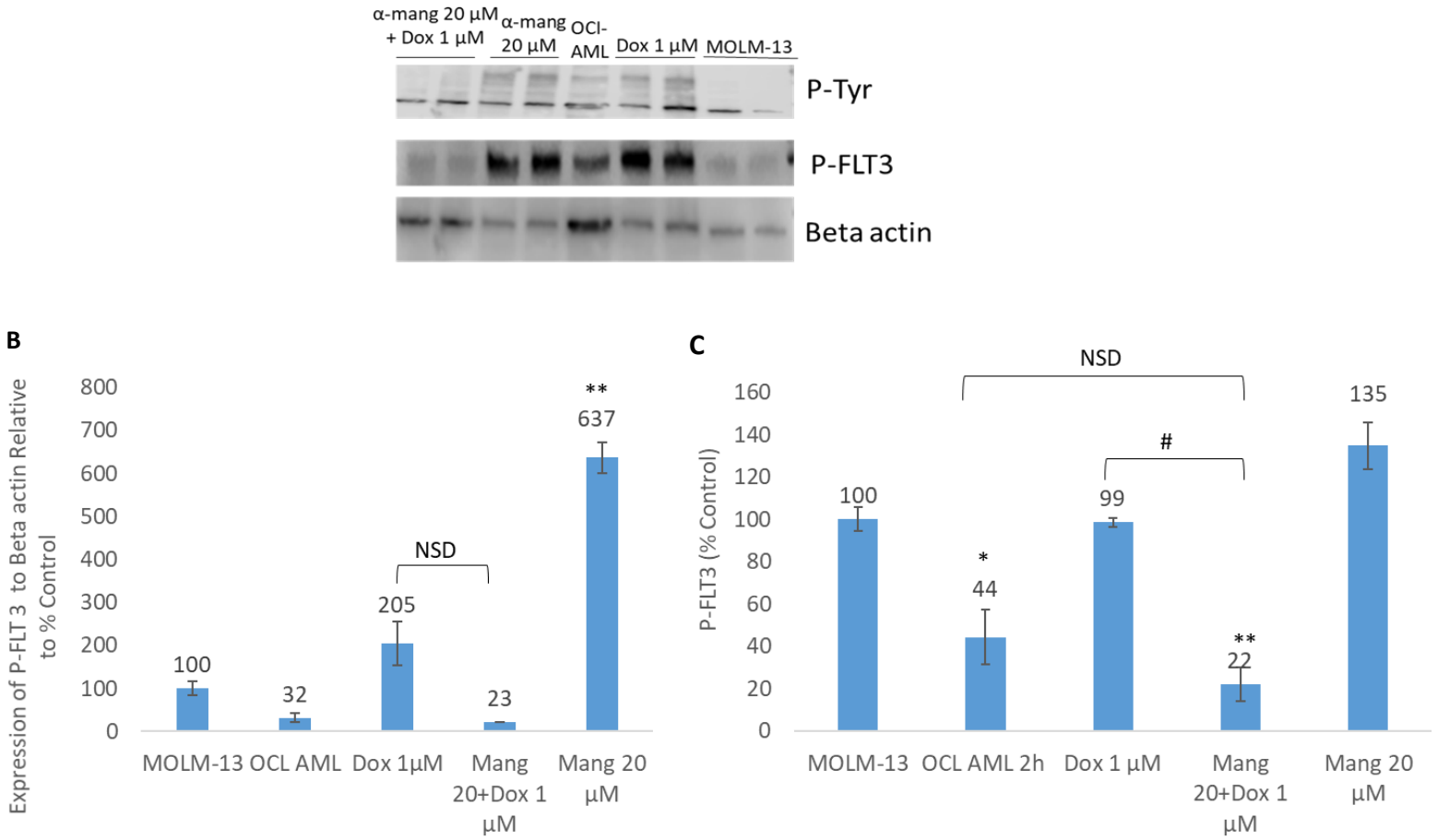


Figure 4.4.8: The inhibition of FLT3-ITD phosphorylation by α-mangostin and doxorubicin combination after 2 h treatment. MOLM-13 cells with FLT3-ITD mutation were treated with α-Mang 20 µM singly and in combination with Dox 1 µM, OCI-AML were stimulated with FL for 15 min at 37^{O}C. Both MOLM-13 and OCI-AML cells were lysed with RPMI and phosphatase inhibitor. (**A**) Immunoblot expression of phosphotyrosine (p-Tyr) and phospho-FLT3. (**B**) % control FLT3- ITD ± SD. Data represent two experiments (n=2) with three replicates (n=3 replicates) for Western blot (**C**) % control FLT3-ITD ± SD of three replicates (n=3) within an experiment(n=1) for ELISA. NSD -Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis *$p<0.05$, **$p<0.01$ compared to negative control, $^{\#}p<0.05$ compared to Dox only. NSD- No significant difference as indicated.

4.4.9: Summary of Data

Table 4.4.9 contains a summary of the results for the α-Mangostin/Dox study**.** Combined Dox and α-mangostin strongly and synergistically reduced the viability of MOLM-13 cells when compared to Dox only. Cell death was mainly via irreversible early apoptosis. The mechanism of action of the combined drug was through the induction of apoptosis that is associated with Bak. Although there was about 2-fold increase in caspase 3 and caspase 8 to Dox alone, the change was not statistically significant. There was statistically significant difference in all three cdc25 phosphatases cdc25A, cdc25B and cdc25C with Dox and α-mangostin combined compared to Dox only. Synergistic inhibition of phosphorylated FLT3 (p-FLT3) was also observed with combination treatment. Compared to control, combination significantly inhibited cell growth, showed early apoptotic cell death, G_2M cell arrest, increased expression of p53 and inhibited phosphorylated FLT3 (p-FLT3).

Dox single treatment compared to control, significantly reduced cell growth and expression of pro-apoptotic protein Bak. Treatment with α-mangostin alone significantly reduced cell viability and increased phosphorylated FLT3 (p-FLT3).

Table 4.4.9: Summary of results showing fold change with significance indicated against Dox only and negative control

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + Mang (20 μM)	Mang (20 μM)	Dox (1 μM) + Mang (20 μM)
CyQuant cell viability assay	0.58↓ (p<0.01)	0.26↓ (p<0.001)	0.55↓ (p<0.01)	0.45↓ (p<0.01)
Cell death assay				
Necrosis	1.63↑	0.74↓	1.36↑	0.45↓
Late apoptosis	0.65↓	4.30↑	0.42↓	6.58↑
Early apoptosis	1.44↑	20.02↑ (p<0.001)	0.85↓	13.93↑ (p<0.001)
Cell Cycle				
G0/G1	0.62↓	0.57↓	0.86↓	1.09↓
S	2.63↑(p<0.05)	1.88↑	1.38↑	1.4↓
G2/M	2.00↑(p<0.05)	2.58↑	1.25↑	1.29↑

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + Mang (20 μM)	Mang (20 μM)	Dox (1 μM) + Mang (20 μM)
Pro-apoptotic proteins Positive results are indicated by an **increase** in protein expressions compared to control				
Caspase 3 Extrinsic and intrinsic apoptotic pathways	3.05↑	8.37↑	4.28↑	2.75↑ (NSD)
Caspase 8 Extrinsic apoptotic pathway	0.85↓	1.79↑	1.00↑	2.11↑ (NSD)
Caspase 9 Intrinsic apoptotic pathway	0.93↓	0.81↓	0.82↓	1.15↓ (NSD)
Bak Intrinsic apoptotic pathway	0.60↓ ($p<0.05$)	1.48↑	0.73↓	2.47↑ ($p<0.05$)
Bax Intrinsic apoptotic pathway	1.50↑	1.53↑	1.31↑	No difference
Anti-apoptotic protein involved in the intrinsic apoptotic pathway Positive results are indicated by a **decrease** in protein expressions compared to control				
Bcl-2 Intrinsic apoptotic pathway	0.55↓	0.14↓	0.11↓	3.9↓ (NSD)
Tumour suppressor protein Positive results are indicated by an **increase** in protein expressions compared to control				
p53	3.23↑	4.38↑ ($p<0.01$)	5.00↑	1.36↑ (NSD)
Cell Cycle inhibitor & senescence promoter Positive results are indicated by an **increase** in protein expressions compared to control				
p21	0.21↑	0.33↑	0.08↑	1.57↑ (NSD)
Senescence stabiliser Positive results are indicated by an **increase** in protein expressions compared to control				
p16	3.14↑	3.00↑	0.86↓	1.05↓(NSD)
Cell Division Cycle (CDC) Positive results are indicated by a **decrease** in protein expressions compared to control				
cdc25A Control G_1/S & G_2/M	2.88↑	0.43↓	1.20↑	6.7↓ ($p<0.05$)
cdc25B Phosphorylation allows re-entry to mitosis	1.17↑	0.69↓	1.19↑	1.7↓ ($p<0.05$)
cdc25C Control G_2/M	0.51↓	0.39↓	0.57↓	1.31↓ ($p<0.05$)
Kinase Activity of FLT-3 Positive results are indicated by a **decrease** in protein expressions compared to control				
p-FLT3 Western blot assay	2.05↑	0.23↓	6.37↑ ($p<0.01$)	8.9↓ (NSD)
p-FLT3 ELISA assay	0.99	0.22↓ ($p<0.01$)	1.35↑	4.5↓ ($p<0.05$)

4.5 DISCUSSION

4.5.1 The potential of α-Mangostin and doxorubicin, singly or in combination, as anticancer agents

Resistance to chemotherapeutic agents in AML is a major challenge in successful treatment (Song *et al*., 2016). Standard therapy, with or without stem cell transplant has limited efficacy with 30% to 40% cure rate (Larrosa-Garcia & Baer, 2017) and 40%-60% relapse rate (Song *et al*., 2016). Intensive chemotherapy inducing side effects results in low tolerance and poorer outcome in elderly patients (Aziz *et al*., 2017). The genetic heterogeneity of AML accounts for its resistance to therapy and this has been demonstrated through molecular and cytogenetic studies to identify molecular mutations, establish prognostic factors and treatment selection in AML (Larrosa-Garcia & Baer, 2017). In addition, AML consist of aberrant regulation of signal transduction pathways such as P13K/PKB/mTOR, RAS/RAF/MEK/ERK/RSK and STAT/Pim-1/CREB that promote proliferation, cell survival and inhibit lineage development (Polak & Buitenhuis, 2012). In AML, treatment with anthracyclines such as doxorubicin (Dox) has been reported to induce dose-dependent cardiotoxicity (Tacara *et al*., 2013) and chemoresistance (Li *et al*., 2015), therefore becoming dose limiting. Some studies have reported that combination of drugs show greater cytotoxicity in cancer cells compared to single drug treatments, as they could target different pathways, mechanisms or cellular processes for potential synergistic or additive effect (Chuang *et al*., 2013; Adina *et al*., 2014). Phytochemicals combined with chemotherapy drugs may lead to increased efficacy, reduce toxicity on normal cells and prevent chemoresistance (Zhang *et al*., 2017). α−Mangostin is a phytochemical from the fruit mangosteen and has been reported to reduce central nervous system toxicity (neurotoxicity) induced by Dox (Tangpong *et al*., 2011), induce DNA damage and inhibit signaling pathways (P13k/PKB/MEK) (Aisha *et al*., 2012), with aberrant regulated signal transduction in AML. However, the anticancer effect of Dox combined with α-mangostin is yet to be explored in AML

α-Mangostin has been reported to inhibit the proliferation of several cancer cell lines such as T47D (breast cancer) (Kritsanawong *et al*., 2016), Bxpc-3, panc-1 (pancreatic cancer) (Xu *et al*., 2014), COLO 205, MIP-101(colon cancer) (Watanapokasin *et al*., 2011), as well as leukaemia (HL-60 and K562) (Novilla *et al*., 2016) cells. Dox is an anthracycline approved as standard chemotherapy drug for AML (Wunderlich *et al*., 2013) and several other cancers such as breast cancer (Chuang

et al., 2013), cervical cancer (Potter & Rabinovitch, 2005), lung cancer (Xiao *et al*., 2003) and gastric cancer (Yu *et al*., 2008).

In this study the anti-leukaemic effect of Dox, singly and in combination with α-mangostin was determined in AML cell line (MOLM-13) from a relapsed patient. The cytotoxic effect of the drugs was determined and combination with 20 µM of α-mangostin and 1 µM of Dox showed enhanced inhibition of cell growth ($p<0.001$) and indicated synergism (combination index = 0.85) (Figure 4.4.1). A synergistic effect is a combination effect that shows more numerical inhibition than individual drugs (Pérez-Rojas *et al*., 2016). The mechanism of action of Dox includes DNA damage as a topoisomerase II inhibitor, mitochondria targeting and generation of free radicals. In this study, Dox induced dose-dependent growth inhibition and cell apoptosis was more potent when combined with α-mangostin than the effect of the single drugs. α-Mangostin has been reported to diminish topoisomerase I and II to inhibit DNA synthesis and chromosomal segregation proteins (Mizushina *et al*., 2013) . Therefore, the suppression of cell growth by the combined drug leading to apoptosis may in part be due to the inhibition of topoisomerase enzyme. A previous study has reported synergistic effect of α-mangostin (10 µM) when combined with cisplatin (2 µM) to induce cytotoxic effect in cervical cancer cells (Pérez-Rojas *et al*., 2016). In addition, α-mangostin (15 and 20 µM) has been reported to sensitize 5 fluorouracil 5-FU resistant cells to cell death (Lee *et al*., 2016). This is the first reported study of the synergistic inhibition of MOLM-13 cells by the combination of Dox and α-mangostin. A low, but clinically relevant (McHowat *et al*., 2001) concentration of Dox (1 µM) was used in this study, and this could have the potential to reduce systemic toxicity and increase therapeutic index of Dox.

Cytotoxic effect of α-mangostin was reported in normal human cell line (human dermal fibroblast) and its effect was similar to effect observed with human cancer cells (Mizushina *et al*., 2013). In this study cytotoxic effect was observed in both normal monocyte cell line and AML MOLM-13 cells. However, more cytotoxic effect was observed in MOLM-13 cells than in normal monocyte, with 20 µM of α-mangostin inhibiting 82% of viable MOLM-13 cells and 19% of normal monocytes after 72 h (Figure 4.4).

Viable cells showing reduced growth may not indicate that cell death was caused by apoptosis only, as alternative cell death pathways such as necrosis may be involved. Some cells may go into cell arrest for repair due to DNA defects detected by the cell cycle inhibitor proteins.

In this study, more apoptosis and less necrotic cell death was observed with the combined drug cell treatment compared to Dox single treatment which showed more of necrosis and less apoptotic cells. This demonstrates that toxicity of Dox may enhance necrosis which involves inflammation that can affect normal cells. The morphology of MOLM-13 treated cells showed signs of apoptosis which includes reduction of cell size (shrunken cells) and fragmentation. Taken together, combination of Dox and α-mangostin induced apoptotic cell death on MOLM-13 cells and reduced necrosis compared to cell treatment with Dox only. Early apoptosis with combined drug was significant when compared to Dox only and cells without treatment ($p<0.001$). However, reduced necrosis was not significant when combined drug was compared to Dox only ($p>0.05$). This could also suggest that signaling pathways in AML that promote cell survival and growth were suppressed more with combination than single treatments.

Further analysis to determine mechanism of induction of apoptosis showed increased expression of pro-apoptotic proteins (Bak and Bax) in cells treated with combined drug treatment than single treatment with Dox and α-mangostin alone. However, only the expression of Bak protein was statistically significant when compared to Dox only ($p<0.05$). Low expression of the anti-apoptotic protein Bcl-2 was observed with the combined drug. However, compared to Dox only it was not statistically significant ($p>0.05$). More expression of cleaved caspase 3 with combination drug suggests more effect of the effector caspase and more expression of cleaved caspase 8 (179 %) than cleaved caspase 9 (81 %) suggest that increased expression of cleaved caspase 3 could be mediated by the extrinsic pathway of apoptosis which is cell death via binding of death ligands (Fas ligand) to trigger the death receptor pathway and activate caspase 8.

Dox has been reported to target the mitochondria via apoptotic proteins, induce the extrinsic pathway of apoptosis via Fas (Massart *et al*., 2004), inhibit apoptosis via Bcl-2 protein (Pilco-Ferreto & Calaf, 2016) and AMPK signaling (Gratia *et al*., 2012). Similarly, α-mangostin has been reported to induce apoptosis through the intrinsic and extrinsic apoptotic pathways by the activation of caspase 9, 8 and 3 (Zhang *et al*., 2017), induction of Bax protein and inhibition of Bcl-2, Mcl-1proteins (Shan *et al*., 2014) and modulate STAT3 (Shan *et al*., 2014) and P13/PKB signaling (Xu *et al*., 2014). However, caspase-independent apoptosis has also been reported with α-mangostin with the release of endonuclease-G, an apoptogenic factor from the mitochondria which involved miR-143/ERK5/c-Myc pathway. Therefore, in this study, although individual

drugs suggest expression of Bax and caspase 3, combination suggest expression of caspase 3 and caspase 8 than caspase 9, and expression of Bax and Bak proteins. Although this may suggest that extrinsic pathway (via caspase 8) was probably activated for effective cell growth inhibition, only Bak expression was significant when compared to Dox only treatment. Dox only significantly inhibited expression of Bak ($p<0.05$) compared to control, but when combined with α-mangostin Bak was expressed significantly ($p<0.05$). Activation of apoptotic protein Bak by Dox and α-mangostin combined can be explored for more understanding of mechanism of the combination treatment on apoptosis. Expression of caspase 3, caspase 8 and inhibition of Bcl-2 protein by combined drug was not significant when compared to Dox only ($p>0.05$), however expression of Bak protein was statistically significant ($p<0.05$).

Resistance of cells to apoptosis may lead to treatment failures as observed in patients with refractory or relapsed AML following initial response to chemotherapy (Li *et al*., 2015). Combination therapy in AML has been reported as a strategy to optimize the efficacy of treatments and prevent drug resistance through simultaneous targeting of proteins involved in cancer cell survival (Polak & Buitenhuis, 2012). This strategy can inhibit mutated signaling pathways such as PI3K/PKB which are highly deregulated in AML (Polak & Buitenhuis, 2012).

4.5.2 Modulation of the cell cycle by α-mangostin and doxorubicin in AML cells

Cancer cells are resistant to the regulation of the cell cycle, hence the extent to which anticancer drugs exert their effect on cancer cell cycle progression could determine their impact on cell growth and survival (Lee *et al*., 2016). p53 is an attractive drug target because when inactive it coorelates with disease progression in cancer cells and poor outcome (Zheng *et al*., 1999). In AML, the incidence of p53 mutation is rare (< 5%) therefore, p53 is considered inactive rather than mutated (Zheng *et al*., 1999).

Activation of p53 results in the regulation of cell cycle, apoptosis and senesencce pathways for induction of cell death in cancers. Interestingly, α-mangostin has been reported to induce expression of the cell cycle inhibitors, p21 and p27, with a subsequent G_0/G_1 phase arrest in a p53-dependent and p53-independent manner (Zhang *et al*., 2017). In a p53 independent action, G_0/G_1 arrest was induced by another tumour suppressor p16 which is not activated in cancer cells and activation of p16 is through p38 MAPK pathway which degrades Bim-1 protein that inhibit p16

and therefore, p16 can induce cell arrest (Korm *et al*., 2015). Therefore, α-mangostin can induce G_0/G_1 arrest via p53/p21 pathway or p38 MAPK/p16 pathway. In this study, cell arrest by α-mangostin was at G_oG_1 phase and Dox arrested cells at S and G_2M phase. α-Mangostin has been reported to induce G_0/G_1 arrest in many cancers such as melanoma (Wang *et al*., 2013), prostate cancer (Johnson *et al*., 2012), pancreatic cancer (Xu *et al*., 2014), breast cancer (Kurose *et al*., 2012) (Ibrahim *et al*., 2014), colon cancer (Matsumoto *et al*., 2005) and leukaemia (Chen *et al*., 2014) via inhibition of cdk4 activity (Johnson *et al*., 2012).

α-Mangostin has also been reported to induce G_2/M (colon cancer) (Mizushina *et al*., 2013), arrest and induction of cell arrest depends on the cell line, concentration of the drug and incubation time (Pérez-Rojas *et al*., 2016). G_0/G_1 arrest by α-mangostin (20 µM) after 24 h cell treatment of leukaemic cells (Chen *et al*., 2014) was observed in this study after 48 h. G_1/S transition is deregulated in most cancers (Zhang *et al*., 2017) and effective arrest of cells by α-mangostin at G_0/G_1 phase indicates the early detection of DNA defect in the cell cycle. The combined drug in this study showed more cell arrest at G_2M phase compared to Dox single treatment. G_2/M phase arrest has also been reported when α-mangostin was combined with cisplatin in cervical cancer model (Pérez-Rojas *et al*., 2016). G_2M phase arrest suggest irreversible cell arrest, with the tendency of cells going into apoptosis and not DNA repair. G_2/M phase arrest is also considered an early stage of senescence. α-Mangostin and Dox has individually been reported to act on topoisomerase activity, which is needed for the DNA synthesis, hence the halt at G_2/M phase. However, arrest at G_2/M with the combined drug was not significant when compared to the sole treatment with Dox ($p>0.05$) but significant when compared to untreated control ($p<0.01$).

Induction of apoptosis and cell cycle arrest involves the activation of the tumour suppressor protein p53. p53 regulates cellular processes through activation of proteins involved in the induction of cell cycle arrest (p21), apoptosis (Bax) and senescence (p21 and p16) (Blanpain *et al*., 2011), after its separation from its inhibitor (MDM2) and activation by either ATR/Chk1 or ATM/Chk2 kinases following the detection of DNA defect (Sun *et al*., 2017). Targeting ATR/Chk1 pathway has been reported to overcome resistance in AML (Davida *et al*., 2017). While the degradation of p53 due to phosphorylation of MDM2 by PKB signaling has been reported to promote chemoresistance in gastric cancer (Yu *et al*., 2008). Markers of senescence include senescence associated β- galactosidase (SA-βgal), increased p53, p21, p16 expression, and other cyclin

dependent kinase inhibitors (cdkIs) such as p15 and p27 (Yingjuan & Xinbin, 2013; Rufini *et al*., 2013) and trimethylation of H3k9 (Milanovic *et al*., 2017). In the interest of time, p21 and p16 were studied as senecence proteins. p21 has been reported to often initiate senescence (Gire & Dulic, 2015), while p16 establish senescence as the gaudian and stabiliser of senesencence state (Maya-Mendoza *et al*., 2014). Further studies for confirmation will include more markers such as SA-βgal and trimethylation of H3k9.

In this study, cells treated with the combination Dox and α-mangostin showed more expression of p53, p21 and Bak when compared to Dox single treatment. However, only expression of Bak was statistically significant ($p<0.05$). p53 was significant compared to cell without treatment ($p<0.01$). This may suggest that the tumour suppressor protein, p53, may regulates cell cycle arrest and apoptosis with the combined treatment. In addition, p53 regulates induction of senescence through p21 and this was stabilized with the expression of p16 protein. Although protein expression of p21, p16, Bax and Bak may suggest the activity of cell arrest (p21, 0.33%), senescence (p21, 0.33%, p16; 0.21%) and apoptosis (Bax, 0.55%, Bak, 148%) with combination treatment. However, only Bak was significant ($p<0.05$) compared to Dox, indicating only apoptosis and not cell cycle arrest and senescence activity suggested. In this study, cells treated with Dox showed expression of cell cycle inhibitor (p21, 0.21%) and senescence (p16, 0.22%) proteins levels compared to cells without treatment. However, p21 and p16 were not statistically significant compared cells without treatment. When Dox was combined with α-mangostin the expression was enhanced for p21, 0.33%) while p16 expression was similar to Dox only. However, the enhanced expression of p21 was not statistically significant ($p>0.05$) when compared to Dox only. DNA damaging agents are known to induce senescence. Hence, the expression of the p16 and p21 proteins may be due to DNA damaging effects of the drugs.

4.5.3 Combined Doxorubicin and α-Mangostin as potential FLT3-ITD kinase inhibitors

The kinase inhibitor imatinib has been reported to revolutionise the treatment of chronic myeloid leukaemia (CML) due to its effect on BCR-ABL, a non-receptor tyrosine kinase (Sacha, 2014). There has been development of several kinase inhibitors (AC220, Sunitinib and crenolanib) for the treatment of AML and their effect is only beginning to have an impact or improve overall survival outcome in AML patients. The absence of complete remission with kinase inhibitors in

AML treatment could be due to different kinase mutations observed in AML, including FLT3-ITD, FLT3-TKD, NPMI, DNMT3. While only one mutation (BCR-ABL) is targeted in CML resulting in long term remission (provided the kinase inhibitor in continuously taken) (Hunter, 2014), treatment regime in AML is tailored to suit the specific mutation observed (Perl, 2017), thereby the 'oncogenic signature' is determined prior to treatment of each patient (Zhou & Chng, 2018). Thus, treatment with kinase inhibitors is more complicated in AML than in CML and there are constant developments of more resistant mutations in AML even after treatment with kinase inhibitors and after allogenic transplant (Fakih *et al*., 2018). The RATIFY study recently reported promising results in AML, with significantly improved survival rate with the FLT3 tyrosine kinase inhibitor, midostaurin in combination with standard chemotherapy in younger patients (18-59 years) (Gallogly *et al*., 2017). Midostaurin became the first approved drug for targeted FLT3 inhibition since the discovery of FLT3 mutations in the year 2000, with more than 50% reduction in bone marrow and circulating blast cells (Levis, 2017). However, acquisition of point mutation due to drug resistance (Fakih *et al*., 2018), and minor rashes among patients treated with midostaurin have been reported (Perl, 2017).

In this study, a novel combination α-mangostin and Dox (DNA damaging agents) was studied to determine their effect on FLT3-ITD mutation *in vitro*. α-Mangostin has been reported to show growth inhibitory effects on the leukaemic cell lines, HL-60 and K562 cell lines via apoptosis (Sun *et al*., 2009), however, to the author's knowledge no effect has been reported for MOLM-13 or any other cell lines which has the FLT3-ITD mutation. This study compares cell treatments with Dox alone to co-treatment with α-mangostin in MOLM-13 cells to determine possible inhibition of phosphorylation caused by FLT3-ITD mutation.

α-Mangostin has been reported to inhibit MAPK/ERK1/2 (Kritsanawong *et al*., 2016) and PKB (Xia *et al*., 2016) signaling pathways, including cyclin dependent kinase (cdks) through the induction of cdk inhibitors (CKIs) that suppress cdk activity in the cell cycle (Korm *et al*., 2015). Dox has also been reported to induce cytotoxicity through ERK pathway and the transcriptional regulator of its cytotoxicity is pivoted by the activating transcription factor 3 (ATF3) (Park *et al*., 2012). ATF3 is a common stress responsive transcription factor that regulates gene expression and has been reported to interact with p53 in DNA damage (Zhao *et al*., 2016). MAPK/ERK and P13K/PKB are secondary mediators associated with FLT3 regulated cell growth (Genevra *et al*.,

2015). Ligand mediated FLT3 receptor activation is crucial for normal haematopoiesis, whereas in AML, activation is ligand independent due to mutations (Parcells *et al*., 2006). MAPK is a signaling component required for controlling cellular proliferation, survival, differentiation and apoptosis (Kritsanawong *et al*., 2016).

To the author's knowledge this is the first reported study on the effect of α-mangostin on FLT3 kinase. In this study, the expression of FLT3-ITD after 2 h treatment showed no effect on phosphorylated tyrosine and phosphorylated FLT3 after treatment with α-mangostin. However, α-mangostin induced inhibition of one of the isoforms of cell division cycle 25 (cdc25) phosphatases (cdc25C) compared to untreated cells $p<0.05$. FLT3 mutation has been reported to promote abnormal expression of cdc25 phosphatases, which are crucial cell cycle regulators involved in human malignancies specifically myeloid leukaemia (Perner *et al*., 2016). Their overexpression correlate with the prognosis of clinical outcome in cancers (Kristjansdottir & Rudolph, 2004). Reduced expression of any of the three homologues of cdc25 (cdc25A, cdc25B and cdc25C) may indicate inhibition of phosphorylation caused by FLT3-ITD in AML. Cdc25 can be regulated by transcriptional control, phosphorylation, degradation of cdc25 itself and use of the opposing kinase, Wee1 (Bouldin & Kimelman, 2014). In this study, α-mangostin induced G_0/G_1 cell cycle arrest and showed reduced protein levels cdc25C (57%) but with an increased cdc25 A and B (120% and 119%, respectively) compared to untreated cells. Indicating more expression of cdc25A and cdc25B but not cdc25C. This may result in cells re-entering the cell cycle because more expression of cdc25B suggest shorter resumption time of the cell cycle after arrest and more cdc25A expression may suggest reactivation of the cell cycle, irrespective of the phase of cell cycle arrest. Despite reports of inhibiting kinases (MAPK & PKB) that promote survival pathways in FLT3-ITD (Kritsanawong *et al*., 2016), α-mangostin did not inhibit FLT3-ITD phosphorylation in this study. The result suggests that α-mangostin can induce cytostatic effect on MOLM-13 cells but have no effect on the main driver of AML, which is FLT3.

Similar to the effect of α-mangostin, Dox treated cells, did not show inhibitory effect on the activity of FLT3-ITD in MOLM-13 cells. Increased expression (205% and 99%; $p>0.05$) by immunoblotting and ELISA, respectively, compared to untreated cells) of phospho FLT3-ITD with Dox treatment indicates that it is not a FLT3 kinase inhibitor. In addition, expression of cdc25 phosphatases was similar to those observed with α-mangostin single treatment (increased cdc25A

and B, but reduced cdc25C). Cell cycle arrest at G_2/M phase by Dox treatment correlated well with the over-expression of cdc25A (288%) and cdc25B (117%) while reduced levels of cdc25C (51%) proteins were observed. Dox has been reported to induce resistance in melanoma cell line due to ERK1/2 mutation (Goetz *et al*., 2014), and ABCB1 overexpression in K562, and this was implicated in resistance to kinase inhibitors in CML (Eadie *et al*., 2016). This suggests that Dox could support kinase mutation to induce chemoresistance.

In this study, the negative control OCI-AML cells (with wild type FLT3) did not express phospho FLT3-ITD and the reduced expression was significant compared to untreated control MOLM-13 ($p = 0.02$; ELISA). The sole treatment of MOLM-13 with Dox or α-mangostin showed stimulatory rather than inhibitory effect on FLT3-ITD phosphorylation, therefore, kinase inhibition was not observed. However, when combined, Dox and α-mangostin treatment showed inhibitory effect on protein express of phosphotyrosine and phospho FLT3. This is the first report of the potential of a novel combination of Dox and α-mangostin as potential FLT3-ITD inhibitors. The combination resulted in inhibition of FLT3-ITD (23% & 22%, $p<0.01$ by immunoblotting and ELISA, respectively) and significant when compared to Dox single treatment ($p<0.05$) for the ELISA result. To further understand the mechanism that may be associated with the inhibition of phospho FLT3-ITD, the expression of cdc25 homologues was studied. Cdc25A was significantly reduced (43%; $p = 0.02$); showing a 6.7-fold decrease when compared to Dox only treatment. Cdc25A has been reported as an early transducer of the oncogenic signaling of FLT3-ITD during cell cycle, a promising target for inhibiting cell proliferation and re-induces monocytic differentiation (Bertoli *et al*., 2015). Therefore, reduced expression of cdc25A indicates inhibition of phospho FLT3-ITD, resulting in reduced abnormal signaling. Most importantly, reduced cdc25A re-induce monocytic differentiation of AML FLT3-ITD cells, thereby re-introducing differentiation. This effect is important as the main defect in AML blast cells is lack of terminal differentiation and excessive immature cells.

Combination of α-mangostin and Dox also showed reduced cdc25B (69%) which was significant when compared to Dox only ($p<0.05$). Reduction of cdc25B indicates that the cells would less likely to return to the cell cycle. In addition, the combination treatment showed even more reduced cdc25C protein expression (39%) compared to cdc25B (69%; $p>0.05$) and less compared to Dox treatment alone cdc25C (51%; $p<0.05$). However, only two replicates ($n=2$) were analysed for

cdc25C. More number of replicates (n>2) may show more interesting results. Cdc25C is reported to contribute to the protection of the genome during cytotoxic stress by avoiding mitotic entry and localizing strictly in the cytoplasm (Perner *et al*., 2016). However, in AML, its protective function is lost due to constant phosphorylation by FLT3-ITD. Cdc25 inhibitors has been reported to target FLT3-ITD and not wild type FLT3 (normal FLT3) (Bertoli *et al*., 2015). Inhibition of FLT3-ITD and subsequent reduction of cdc25C expression allows the cell cycle regulators to gain control of the cell cycle and inhibit progression of AML cells from one phase to the other in the cell cycle. In this study the drug combination (Dox and α-mangostin) induced more cells at G_2/M phase arrest. Furthermore, with reduced expression of cdc25B and cdc25C, possibly hindering mitotic entry of AML cells. Reduced expression of cdc25A indicates that reactivation of cell cycle after arrest is likely not going to occur until the cell cycle regulators have acted following inhibition of FLT3-ITD phosphorylation. It is therefore, tempting to state that targeted therapies for FLT3 mutation should focus also on cdc25 phosphatases especially since these phosphatases have been reported to have major effect on cancer cells specifically AML cells but not on normal cells (Perner *et al*., 2016).

In this study, inhibition of MOLM-13 by Dox and α-mangostin combined is associated with apoptosis, expression of Bak and cell arrest via cdc25s phosphatases, leading subsequently to the inhibition of FLT3-ITD phosphorylation. In addition, induction of apoptosis and cell cycle arrest are reported in this study to suppress growth of MOLM-13 more potently with the combined drug formulation than sole treatment with α-mangostin or Dox only.

Combination of kinase inhibitors has been reported to be promising in AML treatment than single inhibitors (Takahashi *et al*., 2006). Combination of a FLT3 inhibitor (midostaurin) and a cell survival pathway inhibitor (mTOR inhibitor - RAD001) has also been proposed for AML treatment and currently at phase 1 in clinical trial (NCT00819546) (Fakih *et al*., 2018). This present study showed that the combined drug but not the single agents inhibited FLT3-ITD phosphorylation. This suggest that the reported survival pathway inhibitor (α-mangostin) and DNA damaging agent (Dox) could possess another promising mechanism when combined, that is as an inhibitor of FLT3 phosphorylation. It is noteworthy that kinase inhibition which has been effective on CML may not be of the same worth with AML treatment due the presence of different kinase mutations in AML cells, several of these which have not yet been identified.

Pointing to the need to study other mechanisms involved in AML, another tyrosine kinase involved with AML has been identified. Burton's tyrosine kinase (BTK) has been reported to promote AML blast proliferation, migration and adhesion to stromal cells (Genevra *et al*., 2015). Its pharmacological inhibition and genetic knockdown impaired AML blast cell growth suggesting significance to AML cell proliferation (Wu *et al*., 2016). Ibrutinib, a BTK kinase inhibitor that was used to validate BTK kinase in B-Cell related malignancy has been reported to selectively target FLT3-ITD mutation in FLT3 positive AML cell lines (Wu *et al*., 2016). This suggests that targeting FLT3-ITD and BTK kinase could be promising in AML treatment and it may not be necessary a multi-kinase inhibitor. In addition, development of resistance after treatment with FLT3 inhibitors or after transplant could be overcome by targeting Axl. Axl is a receptor tyrosine kinase that is upregulated after treatment and mediates survival and proliferation of AML cells, which in turn expresses AXL ligand growth arrest specific gene 6 (GAP6) and further amplifies growth and therapy resistance (Janning *et al*., 2015; Ben-Batalla, *et al*., 2013). Studies targeting FLT3 and AXL in refractory/relapse FLT3-ITD AML cells are ongoing using gilteritinibib (Fakih *et al*., 2018). In addition, hypomethylating agents in combination with FLT3 inhibitor for the elderly is yet to be defined (Fakih *et al*., 2018). Therefore, targeting survival pathways together with FLT3 could improve treatment outcome. Survival pathways that could be targeted alongside FLT3 inhibition include mTOR, P13K, MAPK-ERK1/2, STAT5 (Fakih *et al*., 2018) and warrants further studies.

4.5.4 Concluding Comments

Overcoming chemoresistant with cancer therapy has been reported to improve survival outcome and reduce relapse or refractory diseases. The rigidity of focus on overall survival outcome has been reported to be the main reason why it has taken so long to find novel and effective treatment for AML. The first major breakthrough occurred in 2017 with the RATIFY study since the discovery of FLT3 mutation in AML cells in 1996 (Stone *et al*., 2018; Levis, 2017). Understanding of the complex genetic heterogeneity of each subset of AML may be more important than using overall survival of the disease as the only endpoint or focus, thereby limiting the good an making a perfect enemy of what could be a novel breakthrough in each subsets of AML (Levis, 2017), after a thorough understanding of mechanism involved.

In this study, combination of Dox and α-mangostin showed synergistic inhibition of the growth of AML in a relapsed cell line, MOLM-13. Inhibition of cell cycle promoters (cdc25s) by combination treatments suggests regulation of the cell cycle and detection of aberrant cell signaling hence the reactivation of DNA damage mechanism pathway through the activity of the guardian of the genome p53. The induction of senescence which is reported to suppress tumorigenesis may suggest reactivation of senescence pathway that results in cells that should not undergo repair in the cell cycle (due to p16 as the guardian and stabilizer of senescence) and will not divide even in the presence of mitotic factors. However, in this study increase expression of p16 to suggest senescence by combination and Dox only was not statistically significant and more assays aside from p16 are required to confirm senescence.

α-Mangostin and Dox sole treatments induced, rather than inhibited FLT3 kinase activity, but had significant inhibitory effect in FLT3 kinase activity when combined. Further work is warranted to explore the mechanism by which the drug combination affects FLT3 phosphorylation. Other mechanisms such as proteasome inhibition, mTOR inhibition, Heat Shock Protein 90 (HSP90) inhibition or MEK inhibition which are involved in AML can be investigated (Prada-Arismendy *et al.*, 2017). Further studies can investigate if the combined Dox and α-mangostin drug are involved in the regulation of antiapoptotic signaling (Bcl-2, Bcl-xl, Bad) due to overexpression, down regulation of downstream signaling (PKB, MAPK, STAT5), decreased expression of surviving genes (survivin) and Pim kinase via STAT5 pathway to inhibit its cytoprotective function (Zhou & Chng, 2018).

The limitation of this study is that a known FLT-ITD inhibitor was not included as a control drug. In addition, FLT3-ITD activates potently STAT5 pathway in contrast to FLT-WT and therefore, protein markers for the STAT5 pathway would have been informative.

Novel drug combinations with multiple mechanism of action including inhibition of kinases involved in the activation of cancer cells proliferation and survival may be a good strategy for drug development. Such combinations could have reduced toxicity on normal cells and adverse effect while inducing long-term cytotoxicity on cancer cells.

In conclusion, AML cells treated with a combination of α-mangostin and Dox promotes the induction of apoptosis is associated with Bak expression and inhibition of FLT3-ITD that could

be effective to overcome resistance in AML and therefore, require further study to understand it mechanism as a targeted FLT3-ITD therapy.

CHAPTER FIVE

5. Gallic acid and doxorubicin combination induces cellular processes (apoptosis, cell arrest and senescence) as DNA damage agents in AML

5.1 Background

Gallic acid (GA) is a phenolic compound that has been reported in numerous studies to inhibit growth and induce antiproliferative effect in human cancer cell lines by induction of cell cycle arrest and apoptosis (Table 5.1). Regulation of cell cycle arrest and apoptosis can be mediated by tumour suppressor protein p53 (Blanpain *et al*., 2011). p53 as a guardian of the genome activate cell cycle arrest, induces apoptosis and senescence pathway as a DNA damage response when DNA defect is detected (Blanpain *et al*., 2011).

GA has been reported to be a stronger antioxidant than popular antioxidants such as ascorbic acid, uric acid and Trolex (analogue of water-soluble vitamin E) (Schlesier *et al*., 2002). GA exhibit scavenging effects against oxidative radicals such as DPPH (43.9%), H_2O_2 (60%), $HO\cdot$ and HOCL (at low concentration) but no scavenging activity on superoxide ($O_2^{\cdot -}$) (Badhani *et al*., 2015). In addition, GA has been reported to restore reduced levels of endogenous antioxidants such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione reductase (GRx) which were inhibited by excessive free radicals, resulting in oxidative stress (Priscilla & Prince, 2009). For instance, reduced activities of SOD and CAT due to superoxide radicals has been reported, including inactivation of GRx due to excessive GSSG (glutathione disulfide) (Priscilla & Prince, 2009).

GA antioxidant activity can be influence by metal chelation (presence of iron), GA concentration in the Fenton type systems and peroxidase (presence of H_2O_2). Thereby exhibiting dual function due to presence or absence of these factors. GA has also been reported to promote production of hydroxyl radical (HO) in the presence of iron (Fe^{3+}) and at low concentration (pro-oxidative activity) but act as scavenger of HO in the absence of iron and at high concentration (anti-oxidative activity) (Badhani *et al*., 2015). Strong reducing ability and weak metal chelating effect is ascribed to GA pro-oxidative action at low concentration. With increasing concentration of GA, the reducing power increases thereby inhibiting oxidation process by hydroxyl radical scavenging (Yen *et al*., 2002). In the presence of H_2O_2, formation of oxidative species is inhibited

(antioxidative effect) while in the absence of H_2O_2, GA exhibits pro-oxidative effect (formation of oxidative radicals) (Serrano *et al*., 2010).

Although low concentration of gallic acid has been reported as good antioxidants and high concentration inducing cellular damage as prooxidant (Fan & Lou, 2004; Setayesh *et al*., 2017). There has been reports of low concentration acting as prooxidant and inducing DNA damage as apoptotic agents (Yen *et al*., 2002; Badhani *et al*., 2015). Therefore, indicating contradictory effect with GA and considerable attention should be paid to its contrary actions as the protective effect of phenols *in vitro* may not have the same effect *in vivo* due to the problem of phenol metabolism (Fan & Lou, 2004).

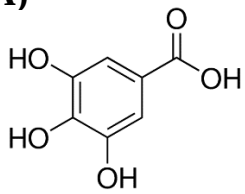
GA antioxidant activity has been reported to induce apoptosis that is dependent on formation of reactive oxygen species (ROS), calcium ion involved and activation of caspase 3 in HL-60 leukaemia cell line (Yeh *et al*., 2011). It inhibits superoxide dismutase in HL-60 cell line to induce apoptosis (Zhang *et al*., 2002). Other anti-leukaemic effects includes mitochondria caspase dependent cell death, inhibition of the growth of FLT3-ITD cells more than non FLT3-ITD cells, inhibition of PKB/mTOR pathway and enhances AML standard chemotherapeutic agents (cytarabine and daunorubicin) when combined (Gu *et al*., 2018). Its inhibition of ABL/BCR kinase in CML (Chandramohan Reddy *et al*., 2012), has led to the study of its derivatives as ABL inhibitors (Raghi et al., 2018). In human colon cancer cell line (HCT116) it induces apoptosis through p53 and calcium pathways (Yang *et al*., 2018).

GA in low doses has been reported to reduce the effect of genomic instability and oxidative damage caused by obesity in mice (Setayesh *et al*., 2017). However, its exact mechanism of anticancer effects associated with p53 activation, regulating cell cycle arrest, apoptosis and senescence singly and in combination with chemotherapy drugs is still unclear. Chemotherapy drugs induce DNA damage to cancer cells and gallic acid has also been reported to induce DNA damage (Weng *et al*., 2015). Therefore, analysis of proteins in response to p53 activation and mechanism regulated will provide more information on anticancer effect and could be used as a predictive and /or prognostic tool. More understanding is important considering that gallic acid or phenols are abundant in most fruits, plants and could be combined alongside chemotherapy drugs in cancer treatment by patients. Herein, the anticancer effect of the chemotherapy drug, doxorubicin singly and in combination with gallic acid in AML cell line MOLM-13 was studied.

Table 5.1: *In vitro* anticancer effect of gallic acid in cancer cells

Fruits, plants and herbs with gallic acids- Mango peel (polymeric form), Nuts, wine, cocoa (hot chocolate), berries, grapes, Indian gooseberry, oak bark, sundew, golden root, white tea, clove, bearberry, oak, Chinese mahogany

Gallic acid (GA)



3, 4, 5 trihydroxy benzoic acid

Biological properties
Antioxidant
Anticarcinogenic
Antiviral
Antibacterial
Antiallergenic
Anti-inflammatory

Cancer/Cell line	Concentration studied	Anticancer Effect	Human/Animal studies	References
Human Hepatocellular carcinoma (HepG2 & SMMC-7721)	6.25, 12.5, 25.0, 50.0, 100.0 µg/ml at 24, 48 and 72 h	IC_{50} SMMC-7721 cell- 22.1 ± 1.4 µg/ml. IC_{50} HepG2 cells 28.5±1.6 µg/ml via apoptosis ↑ Caspase 3 and 9 ↓ Caspase 8 ↑ Bax and Cytochrome C (cytosol) ↓ Bcl-2 & Cytochrome C (mitochondria) after 48 h	Normal cells HL-7702- IC_{50} 80.9± 4.6µg/ml	(Sun *et al*., 2016)
Human Lung adenocarcinoma	100, 200, 400 µM after 24 and 48 h	↑ Caspase 3 ↓ Caspase 8, ↑ Ros and DNA fragmentation		(Maurya *et al*., 2011)
Lymphoblastic leukaemia cell line (Jarkat)	0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µM after 24 and 48 and 72 h	20 µM showed 93%, 87%, 65% after 24, 48 and 72 h, respectively. 100 µM showed 13%, 8%, and 5% after 24, 48 and 72 h. IC_{50} 24 h - 60 µM, IC_{50} 48h- 50 µM, IC_{50} 72 h-30 µM Inhibited imatinib resistant and sensitive leukaemic cell lines (IR-K562 and K562, respectively). When combined with Imatinib synergistic effect was observe.		(Sourani *et al*., 2015)

Table 5.1: *In vitro* anticancer effect of gallic acid in cancer cells

	10, 25, 50, and 75 µM after 24 h	↑ p27, p21 ↓ cyclin D1/2/3 and cyclin E Induced apoptosis after 24 h G0/G1 arrest, ↑ DNA damage, ↑ mitochondria membrane potential (MMP), ↑Cytochrome c, ↑ poly ADP ribose polymerase (PARP), ↑caspase 3, 8 and Bax. ↓ Bcl-2 (GA triggered caspase cascade and mitochondria dependent pathways).		(Chandramohan Reddy *et al*., 2012)
	50 µM after 6, 12 and 24 h	↓ BCR-ABL kinase activity, ↓Nf-kB, ↓Cox-2		
Human promyelocytic leukaemia (HL-60)	25 and 50 µM after 6, 12 and 24 h	Inhibited cell growth, G0/G1 arrest after 24 h. ↑ p21, p27, ↓ cyclin D1/2/3 & cyclin E. No ROS stimulation after 0.5, 1, 3, 6 and 12h at 50 µM. ↑ Fas, FasL, cyto c, Caspase 3, 8, & 9. ↑ AIF, Endo G, BID, Bax, Bcl-2 and caspase 4 at 50 µM after 6, 12 and 24 h. (caspase cascade and mitochondria-dependent pathways activated)		(Yeh *et al*., 2011)
Breast cancer cell line MDA-MB-231 & HS578T (Triple negative breast Cancer	10, 25, 50, 75 and 100 µM after 6, 12 and 24 h	Inhibited cell growth induced G0/G1 arrest, ↑ PARP, caspase 3, & 9. ↓ Cyclin D, cdk4, cyclin E, cdk2 after 24h at 5, 25 and 50 µM. ↑ p38 MARK that influences p21 and p27 increase Inhibited cell proliferation and induced apoptosis.	Normal cells MCF-10F at 24 h no effect but after 48 h significant effect observed with 50 µM	(Lee *et al*., 2017)

Table 5.1: *In vitro* anticancer effect of gallic acid in different cancer cell lines

Breast cancer cell line MCF-7	5, 25 and 50 µM after 24 and 48 h	Induce both extrinsic (FAS, FASL) and intrinsic pathways and increase cross link between both pathways		
Human glioblastoma (brain cancer) cell line U251, U87, mouse brain, epithelia cells		U251 cell line inhibited more than U87 at low concentration. 30 and 40 µg/ml inhibit cell migration. 40 µg/ml inhibit invasion in U87 after 24 h. ↓ ADAM17, p-ErK, P-PKB after 24h		(Wang *et al*., 2014)
Human Oral squamous cell carcinoma (HOSCC) UMI	10, 20, 30 and 40 µg/ml for 24 h	↑ proapoptotic proteins (TNF-α, TP53βP2, GADD45A); ↓ antiapoptotic proteins (survivin & CIAP1)	Selective dose-dependent cytotoxicity (95, 91 and 89%) in brain epithelia cells	(Yong *et al*., 2010)
Human non-small cell lung cancer cell line (NCI-H460)	50-500 µM for 24 & 48 h	G_2/M phase arrest, DNA damage and apoptosis. ↑ Caspase 3, 8 and 9, Bax and Bad. ↓ cytochrome C, MMP, Bcl-2, BCL-XL		(Chia *et al*., 2010)
Human colorectal adenocarcinoma cell line HCT-15	200 µmol/L after 72h, IC_{50} 740 µmol/L,	Sub G1 phase arrest after 72h. ↓ MMP, ↑ Ros at 24, 48 and 72 h	Inhibited growth *in vivo* in Nude mice bearing NCI-H460 xenograft tumors	(Ji *et al*., 1999)
Human bladder transitional carcinoma cell line (TSGH-8301)	40 µM	Induces G_2/M phase arrest, ↑ 14-3-3β ↓ cdk1, cyclin B, cdc25C		(Subramanian *et al*., 2016)
Postate cancer cell line (DU145)	IC_{50} 15.6 µg/ml	G_2/M phase arrest after 24 and 48 h. Induce DNA breakage at 100 µg/ml after 1,4, 8, 12, and 24 h. ↓ Bcl-Xl, MMP. ↑ cleaved caspase 3, Ros, cytochrome C (Mitochondria pathway), Synergistic combination of GA and Dox observed		(Ou *et al*., 2010)
				(Chen *et al*., 2009)

5.1.1 Aim and Objectives

The aim of this study was to determine the anticancer effect of gallic acid and doxorubicin, singly or in combination as DNA damage agents on cellular process such as apoptosis, cell cycle and senescence

Objectives

The effect of gallic acid and doxorubicin, singly and in combination in AML cell line MOLM-13 was investigated to determine:

- the cytotoxic effect of the drugs
- their potential to induce apoptotic cell death
- their ability to change the expression of apoptotic proteins (Bax and Bak) and the anti-apoptotic protein (Bcl-2)
- their effect on caspase 3, 8 and 9 proteins to induce intrinsic, extrinsic apoptosis or both
- their effect on cell cycle and to evaluate their ability to induce cell arrest through the expression of p53, p21 and Cdc25 phosphatases
- their effect on senescence through the regulation of p16 expression

RESULTS

In this study, the inhibitory effect of GA and doxorubicin were determined in MOLM-13 and normal cells. Combination of IC_{50} concentration of gallic and doxorubicin was determined on cell viability as well as its effect on apoptosis and cell cycle arrest, singly and when combined with Dox. Further studies on the effect of tumour suppressor protein p53 which regulates apoptosis, cell cycle arrest and senescence was determined. Therefore, effect on apoptotic proteins (Bax, Bak, and Bcl-2), caspase 3, 8 and 9, cell cycle arrest proteins (p21, cdc25) and senescence protein (p16) were determined using Western blot with single and combination treatments.

5.2 Effect of gallic acid on cell viability after 72 h of incubation

Inhibitory effect of gallic acid on MOLM-13 was determined using a range of concentrations (1-30 µM) (Method Section:3.43). Dose-dependent inhibition was observed. At 15, 20 and 30 µM gallic acid showed strong evidence of inhibition ($p < 0.01$). In normal macrophage cells, gallic acid showed inhibition at 20 and 15 µM (16% and 20%, respectively) (Figure 5.2). IC_{50} of gallic acid was 15 µM on MOLM-13 and showed less effect on normal cells compared to MOLM-13, therefore 15 µM was used with Dox 1 µM for combination and further study. Descriptive statistics (Appendix 5) and ANOVA (Appendix 6) were done. $p<0.05$ was considered significant.

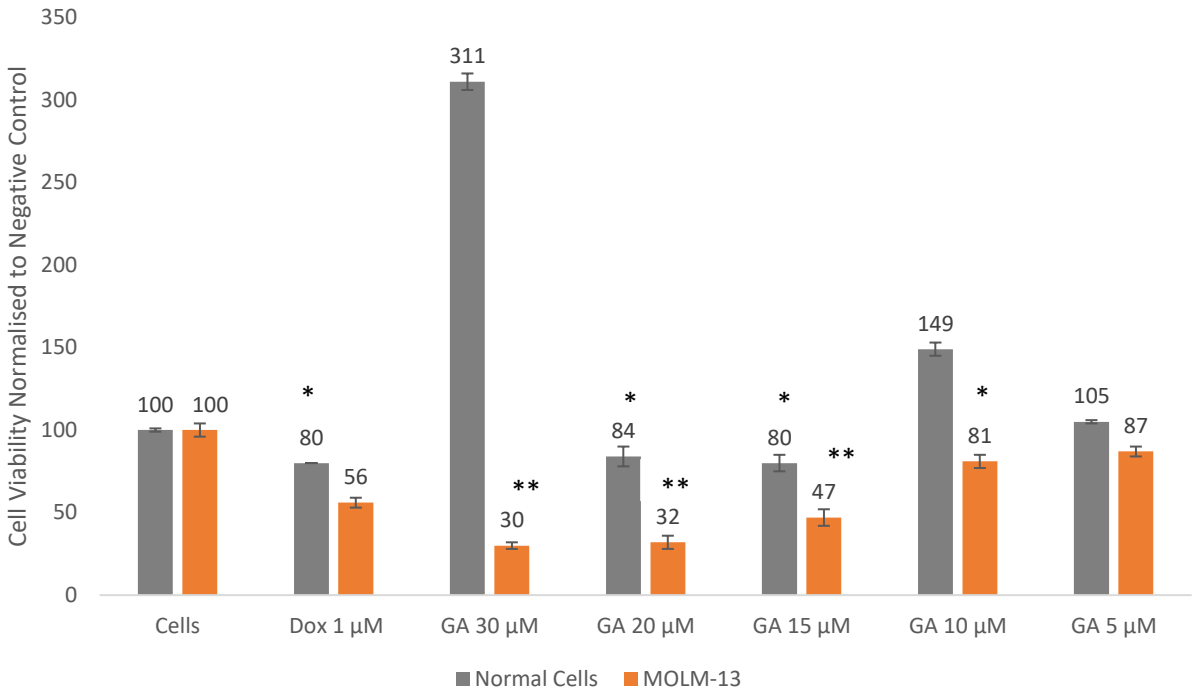


Figure 5.2: The effect of gallic acid on cell viability (normal monocytes and MOLM-13 cells) after 72 h of incubation. MOLM-13 and normal monocyte cells were treated with different concentrations of gallic acid and the viability was determined using CyQUANT GR. Result was expressed as % control relative to cells only. Data represent two independent experiments (n=2) with four replicates each. (n=4 replicates). IC_{50} MOLM-13 72 h was 15 µM, Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis **p<0.01, *p<0.05.

5.2.1 Combination of gallic acid and doxorubicin on cell Viability

Combination of gallic acid and chemotherapy drug Dox was conducted and effect was determined using the cell viability assay. Combination of Gallic acid at 15 µM and Dox at 1 µM induced inhibition of 84% and was statistically significant ($p < 0.05$) when compared to positive control drug Dox. Gallic acid single treatment also inhibited cell growth which was significant when compared to control. However, more inhibition was observed with combined drug than single treatment with gallic acid.

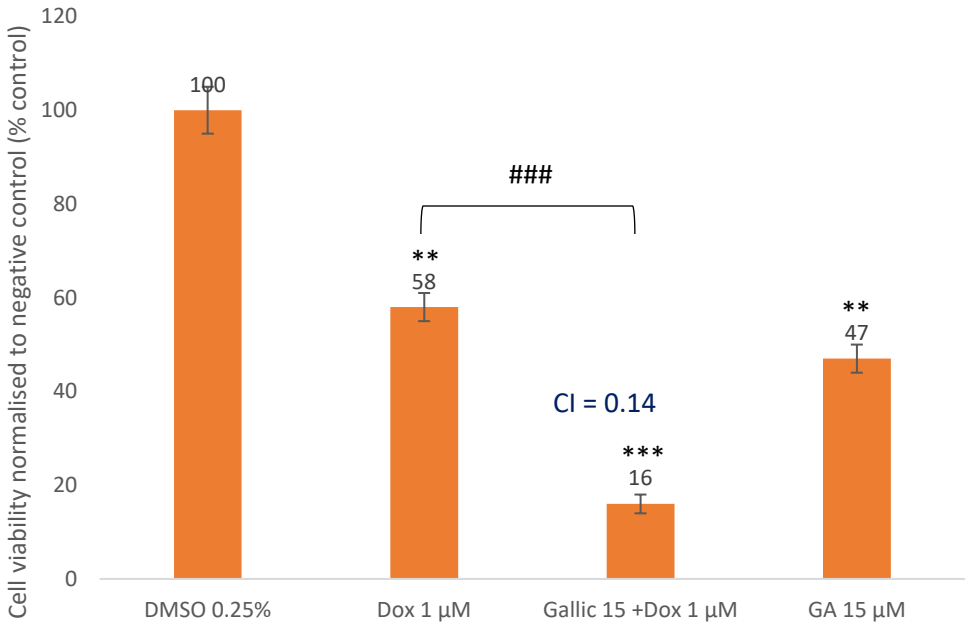


Figure 5.2.1: Effect of combination of gallic acid and doxorubicin on Cell viability. MOLM-13 was treated with Dox 1 µM singly and in combination with Gallic acid 15 µM on MOLM-13 at a cell density of 1.5 × 10^5 cell/ml for 72 h. Cell viability was determined using CyQUANT GR. Result was expressed as % control relative to 0.25% DMSO control. Data represent an independent experiment (n=1) with four replicates (n=4). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis **p<0.01, ***p<0.001 compared to control. ###p<0.001 compared to Dox only. IC_{50} for Gallic acid in MOLM-13 was 15 µM, IC_{50} of Dox was 1 µM.

5.2.1.1 Drug interaction of doxorubicin and gallic acid

Quantitation of the type of interaction between Dox and gallic acid was done using median effect analysis with the CompuSyn software which derives combination index (CI) value between two or more drugs. CI value of 0.14 indicates that combined drugs showed synergism (Figure 3.1).

Table 5.2.1.1: Combination index (CI) data for combination effect of doxorubicin and gallic acid in MOLM-13 cells.

Doxorubicin (µM)	Gallic acid (µM)	Effect (inhibition %)	CI Value	Result
1	15	0.85	0.14	synergism

Combination index (CI) value of 0.1-0.90 indicates synergism, 0.90-1.10 indicates additive effect and 1.10-10 indicates antagonistic effect.

5.3 Combination of gallic acid and doxorubicin on cell apoptosis

Gallic acid at 15 µM combined with Dox 1 µM showed more apoptosis when compared to Dox 1 µM single treatment. Gallic acid 15 µM treatment showed less necrosis (2.99%) when compared to combination with Dox 1µM (5.11%), while Dox single treatment induced 5.94%. However, necrotic cell death with combined drug was not statistically significant when compared to Dox only. Apoptotic cell death was 6.65% with gallic acid single drug treatment and when compared to apoptosis induced by combined Dox and gallic acid, more apoptotic cell death (11.54% both early and late apoptosis) was observed (Figure 5.3). The result demonstrated that combination has induced more cells to go into apoptosis and this was statistically significant when compared to Dox only and cells without treatment ($p<0.001$)..

Cell morphology was studied for characteristic apoptotic cell changes using the TUNEL assay and staining with DAPI (Method Section: 3.5.2). Treatment with GA 15 µM showed some apoptotic cells stained with DAPI and reduced in size. Combination with Dox 1 µM showed more cells undergoing apoptosis and some showing cell fragmentation (Figure 5.3.1)

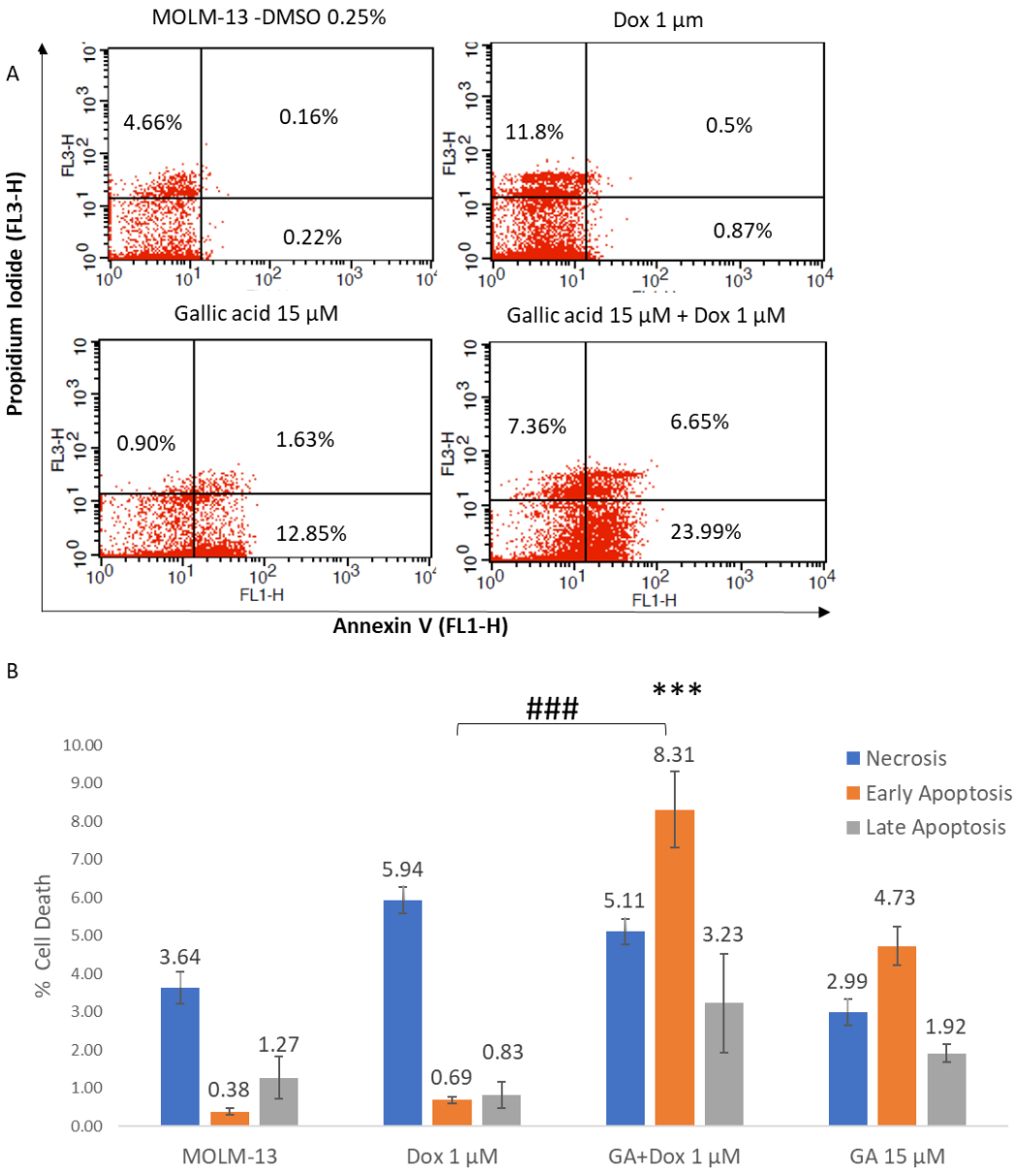


Figure 5.3: The Effect of doxorubicin and combination with gallic acid on induction of apoptosis after 72 h on MOLM-13. **A.** The lower right of fluorescence activated cell sorting (FACS) indicates early apoptosis (annexin V staining only). The upper right of FACS indicates late apoptosis (annexin V/PI staining). The upper left of (FACS) indicates necrosis (PI staining only). **B**. Graphical representation of % Necrosis and apoptosis (early and late). Data represent three independent experiments (n=3) with one replicate each (n=3 replicates). MOLM-13 cells were treated with GA at 15 µM and Dox at 1µM for 72 h, cell apoptosis was determined using annexin V and propidium iodide. Annexin/ PI staining of apoptotic cells using Flow cytometer illustrating distribution of % population undergoing apoptosis and necrosis. Representation of 10,000 cells staining profile. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis ***p<0.001. compared to negative control,

p<0.001 compared to Dox only.

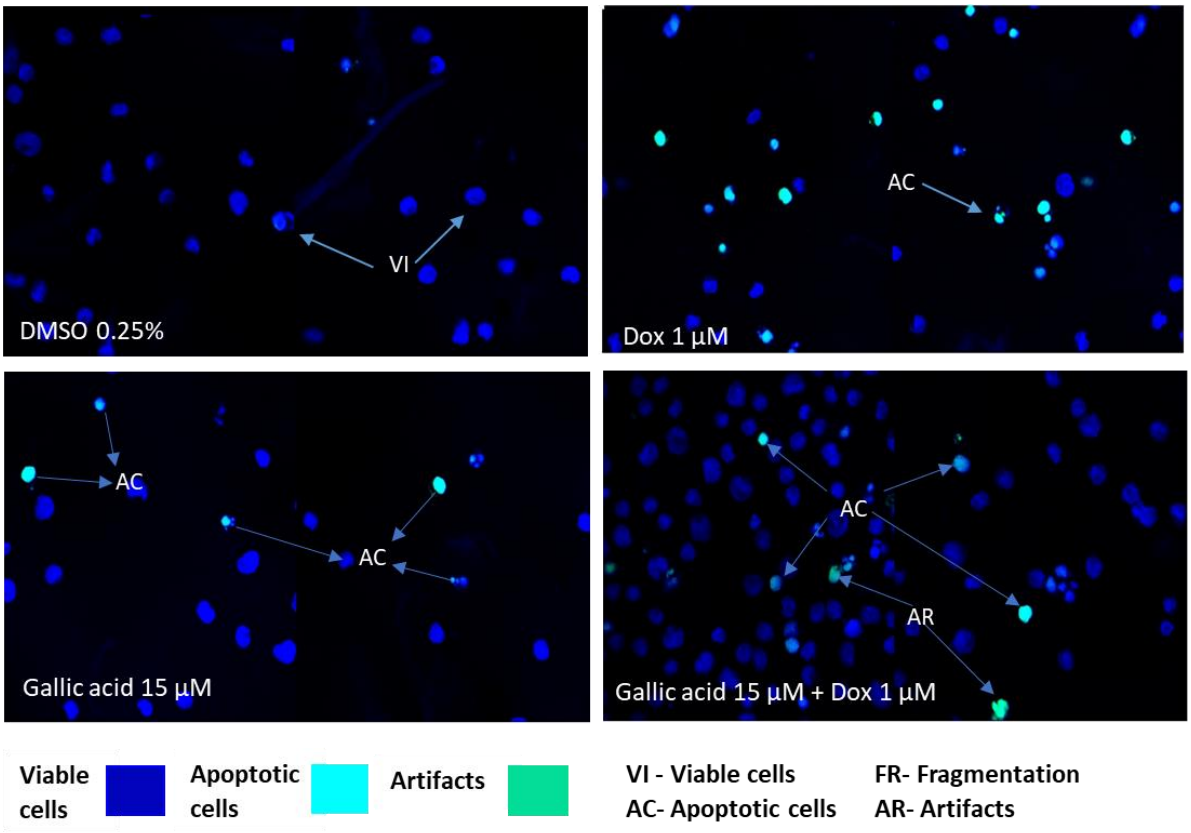


Figure 5.3.1: Apoptotic effect of doxorubicin and combination with gallic acid in MOLM-13 cells after 48 h using TUNEL assay. MOLM-13 cells were treated with GA at 15 µM and Dox at 1µM for 48 h, morphology of apoptotic cells was observed using the Fluorescence microscope. Image represent data from two independent experiments (n=2) with two replicates within an experiment

Mechanism of action was determined through expression of proteins after treatments with Dox singly and in combination with gallic acid. Gallic acid and Dox are DNA damaging agents and their effect on expression of proteins involved in apoptosis, cell cycle arrest and senescence regulated by p53 was observed. GA treatment singly and in combination with Dox after 48 h was studied on MOLM-13 cells using immunoblotting.

5.4 Effect of gallic acid and doxorubicin on expression of apoptotic proteins in MOLM-13

The expression of pro-apoptotic proteins (Bak and Bax) differs following singly and combined drug. Single treatment with Dox or GA showed reduced pro-apoptotic protein (Bak) expression. Dox combined with GA treatment increased Bak expression significantly when compared to no treatment control cells ($p<0.001$) and when compared to Dox only treatment ($p<0.01$). However, cells treated with Dox only was observed to express Bax more (217 %) than when combined with GA (106%). There was increased expression of anti-apoptotic protein Bcl-2 when Dox was combined with GA. Gallic acid single treatment induced increased expression of Bcl-2 on cells

compared to Dox only treatment. Thus, suggesting GA enhanced expression of Bcl-2 when combined with Dox.

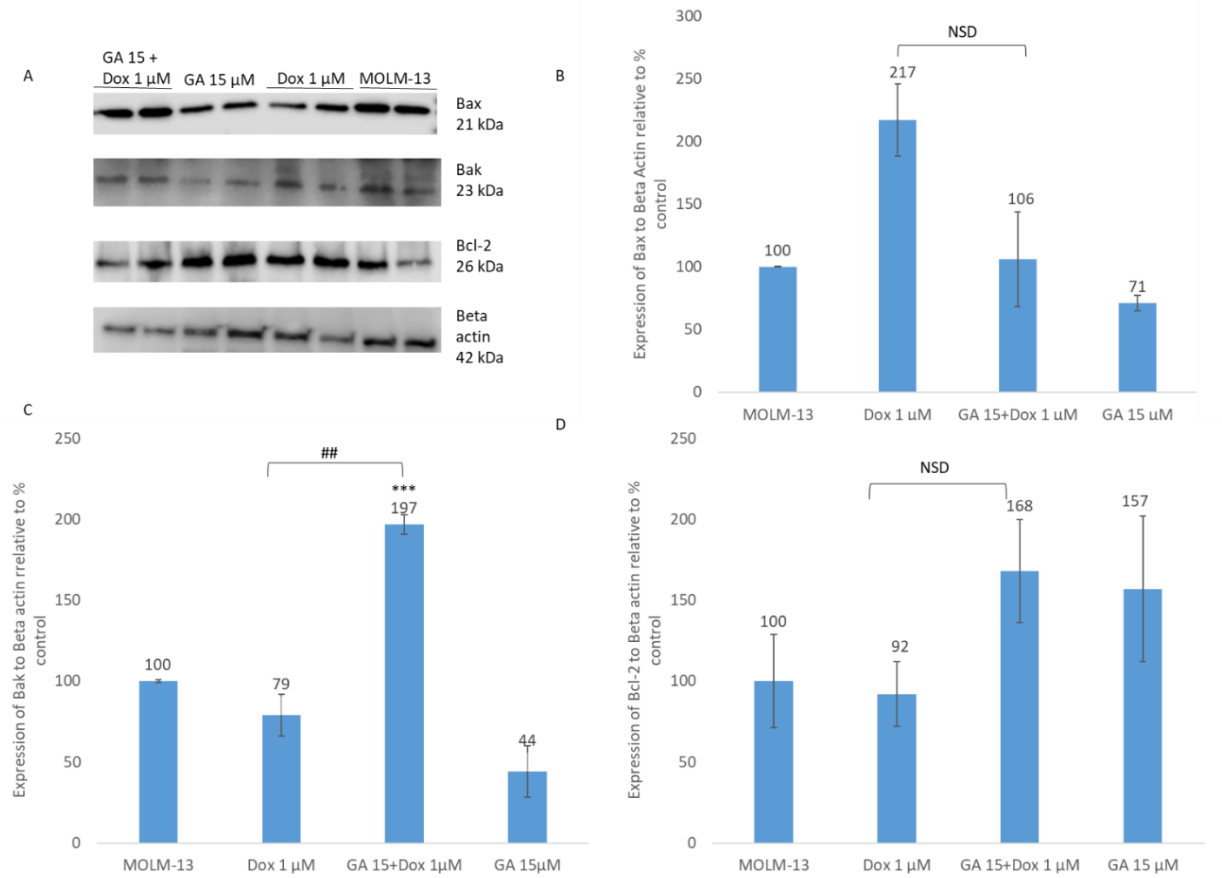


Figure 5.4: The expression of Bax, Bak and Bcl-2 after 48 h treatment with gallic acid singly and in combination with doxorubicin in MOLM-13 cells using Western blotting. A. Immunoblotting results expressing apoptotic proteins after treatment with Dox single and in combination with GA in MOLM-13 cells. Cell lysate was subjected to SDS-Page for immunoblotting analysis. (**B**) Expression of Bax (**C**) Expression of Bak (**D**) Expression of Bcl-2. Results were presented as % control ± SD of Data representing an experiment (n=1) with two replicates for Bax (n=2 replicates). Two experiments with four replicates (n=4) for Bak and Bcl-2 (two replicates within each experiments). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis ******p<0.001 compared to negative control*. *\#\#p<0.01 compared to Dox only as indicated. NSD- No Significant Difference as indicated***

5.4.1 Effect of gallic acid and doxorubicin on the expression of caspases in MOLM-13

The activation of caspase 3, 8 and 9 were determined after 48 h treatment. Treatment with single drugs (Dox or GA) showed intrinsic pathway of apoptosis via increased caspase 9, but only slight increase in caspase 8. However, increase expression of caspase 9 by single drugs was not statistically significant when compared to control. Caspase 3 was not expressed by single treatments. Combined drug showed reduced expression of all caspases and were not statistically significant when compared to Dox only in all three caspases. A 2-fold decrease in caspase 3 and 1-fold decrease in caspase 8 and 9 was observed when combined drug was compared to Dox only (Table 5.4.5).

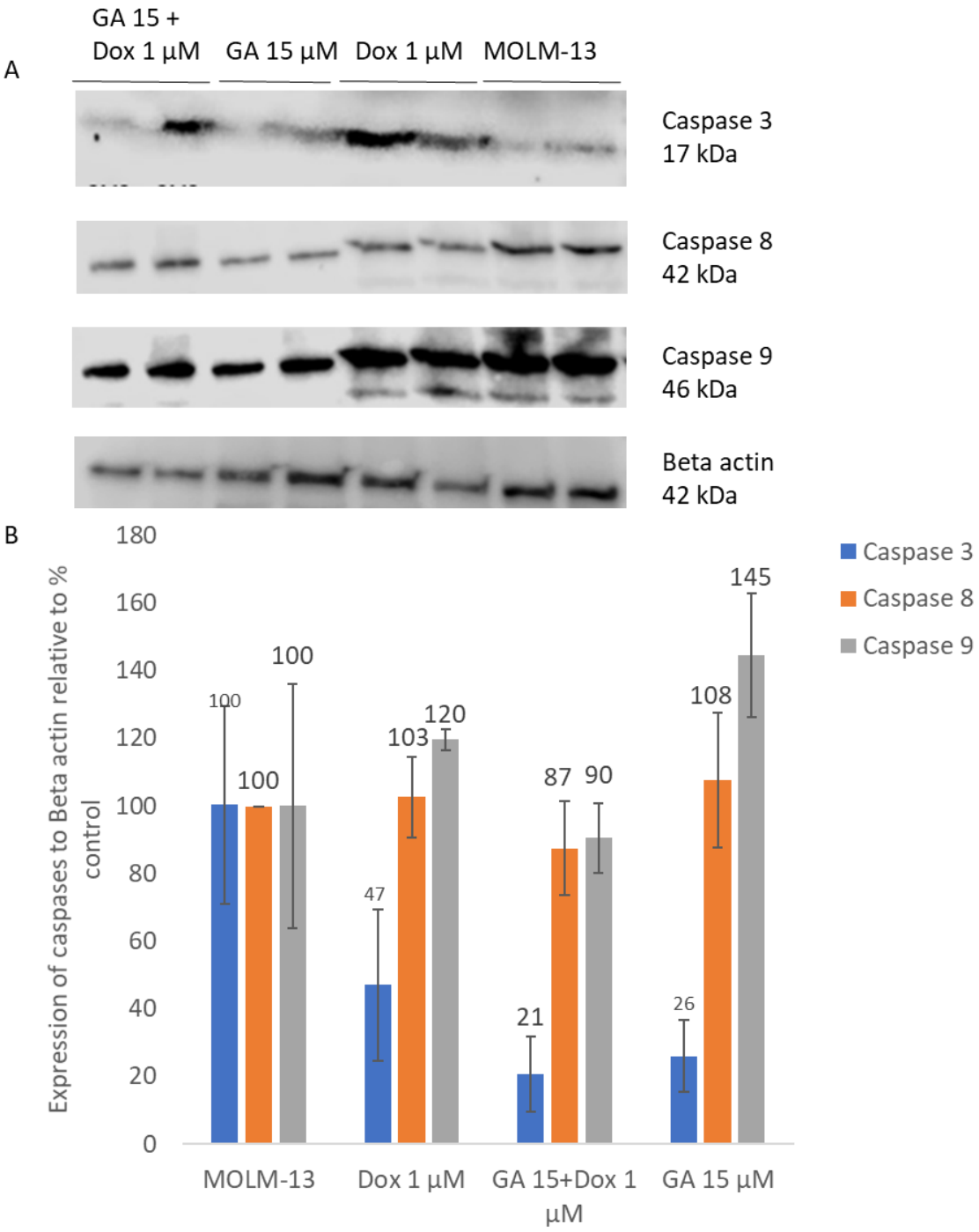


Figure 5.4.1: Effect of gallic acid and doxorubicin on expression of caspase 3, 8 and 9 in MOLM-13 after 48 h using Immunoblotting. **A**. Immunoblotting results of caspase proteins after treatment with Dox single and in combination with GA in MOLM-13 cells. Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis. **B**. Results of caspase 3, 8 and 9 expression were presented as % control± SD. Data represent two experiments (n=2) with three replicates (n=3 replicates) (two replicates within an experiment and one replicate from an independent experiment) for caspase 3 and 9. Two replicates (n=2) within an experiment for caspase 8. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. ***p<0.01 compared to negative control.*

5.4.2 Combination of gallic acid and doxorubicin on the cell cycle

In this study, 64%, 10% and 21% cell populations of MOLM-13 cells at G_0/G_1, S and G_2/M phases of cell cycle were observed following 15 µM GA treatment. Compared to the negative control, more cells were seen in G_2/M phase following GA or Dox treatment singly, (21% with GA 15 µM, 24% with Dox 1µM and 12% with control) but this increase was not significant ($p>0.05$; 0.26).

Combination of GA15 and Dox 1 µM showed G_0/G_1 population of cells with 42%, S phase with 17% and G_2/M phase with 30% indicating G_2/M arrest when compared to negative control with 12% and more G_2/M arrest when compared to the positive control drug Dox 1 µM with 24%. This suggests that combination of GA and Dox stimulated more G_2/M arrest since individually treatment showed less arrest (24% Dox 1 µM and 21% GA 15µM) while Dox and GA combined induced 30%. Statistically, Dox and GA combined treatment arrest at G_2/M phase was significant ($p < 0.01$; 0.001) when compared to the negative control. However, this G_2/M phase arrest observed with Dox and GA combined treatment was not significant ($p > 0.05$) when compared to both Dox 1 µM single treatment and gallic acid 15 µM single treatment after 48 h.

Cell arrest at S phase was not significant with the Dox and GA combined treatment showing 17%, while negative control showed 8%. Comparing Dox and GA combined treatment, GA 15 µM and Dox 1 µM individual treatment at S phase, Dox 1 µM showed 21%, GA 15 µM showed 10% and combined Dox and GA showed 17% indicating more arrest at S phase was with Dox single treatment and this was statistically significant.

In summary, although the combination of GA and Dox 1 µM induced more G_2/M arrest of the cell cycle than GA singly used. This combination format has not reached a statistically significant difference in the effect of inducing G_2/M arrest when compared to Dox 1 µM single treatment and Gallic 15 µM single treatment.

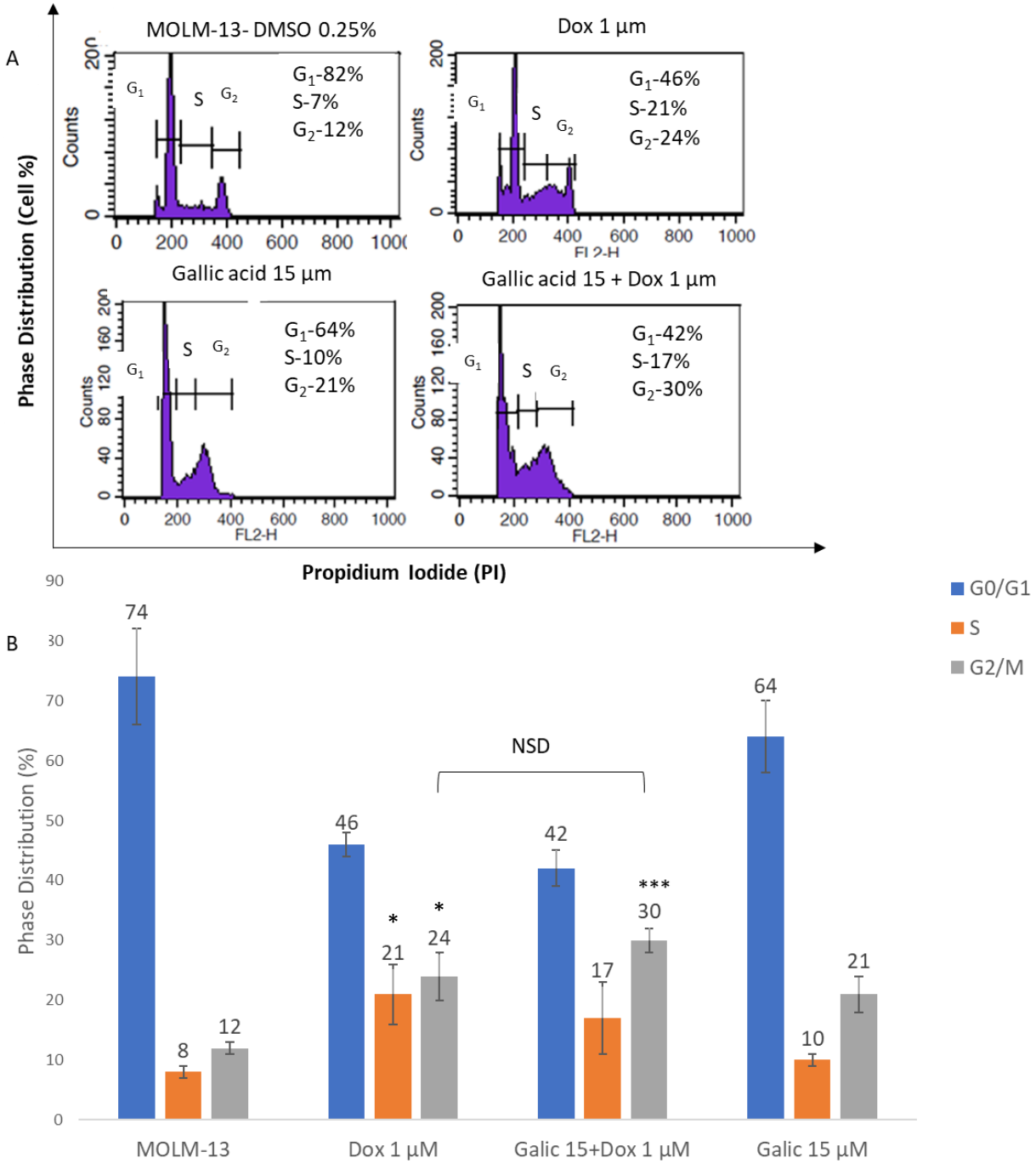


Figure 5.4.2: The Effect of doxorubicin and combination with gallic acid on induction of cell cycle arrest after 48 h. A. DNA histogram showing cell cycle distribution of MOLM-13 cells after treatment with GA 15 µM, Dox 1 µM and combination of Dox and GA for 48 h ***B.*** Data represent three independent experiments (n=3) with one replicate each (n=3 replicates). Cells were harvested, stained with propidium iodide and RNase, then analysed using flow cytometer. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. ***p<0.001 compared to negative control as indicated, NSD- No Significant Difference as indicated.

5.4.3 Effect of gallic acid and doxorubicin on expression of p53 in MOLM-13

Activation of p53 in response to DNA damage can lead to cell cycle arrest, apoptosis and senescence. To determine the activation of p53 with gallic acid 15 µM singly and in combination with Dox 1 µM on MOLM-13, expression of p53 was determined using western blotting after 48 h treatment. There was suppressed expression of p53 with combination compared to Dox and gallic acid single treatments (Figure 5.4.3). Dox expressed p53 while GA showed reduced p53 expression.

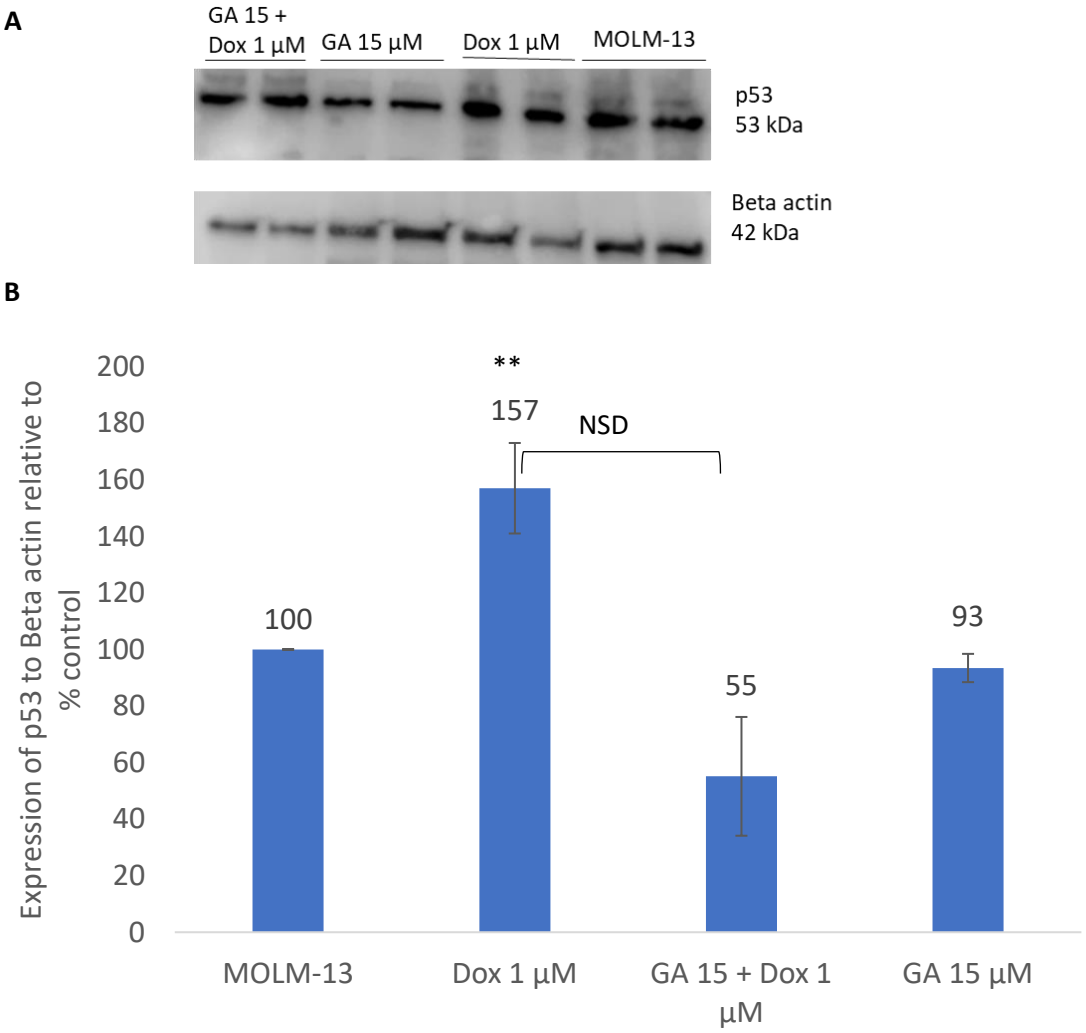


Figure 5.4.3: The expression of p53 after 48 h treatment with gallic acid singly and in combination with doxorubicin on MOLM-13 cells using Western blotting. **A**. Immunoblotting results of p53 expression after treatment with Dox single and in combination with GA in MOLM-13 cells. Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis. **B**. Graphical presentation of p53 expression. Results was presented as % control ± SD of two experiments (n=2) with three replicates (n=3) (Two replicate within an experiment and one replicate from an independent experiment. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis, *NSD- No significant difference as indicate*

5.4.4 Effect of gallic acid and doxorubicin on expression of cell cycle arrest protein p21, Cdc25 phosphatase and senescence protein p16 in MOLM-13

To investigate the role of activated p53 on cell cycle arrest, expression of cdk interacting protein (cell cycle inhibitor) $p21^{Cip1}$ and cdc25 phosphatases in relation to cell cycle arrest was determined after 48 h with gallic acid (15 µM) singly and in combination with Dox 1 µM

Gallic acid was observed to show increased expression of p21 (340 %) compared to Dox only (298 %) and no drug treated control although no statistically significant significance was found. Combination of gallic acid and Dox induced less expression (308%) of p21 compared to Gallic acid single treatment but more expression when compared to Dox only. Examining cdc25 activity showed that Dox and GA combined treatment reduced the expressions of both cdc25A (54%) and cdc25C (79%) indicating a reduced dephosphorylation of cdk by cdc25A and C thereby preventing cell cycle progression and allowing cell cycle arrest as indicated by increased p21 expression. However, combined Dox and GA treatment induced more expression of cdc25B (196%) was not statistically significant when compared to Dox only ($p>0.05$)) and no drug treated control sample ($p>0.05$) despite reduced expression by individual treatment, with Dox showing 88% and GA with 68%.

Dox single treatment and GA single treatment showed reduced expression of cdc25B and C, but when combined enhanced inhibition of cdc25A expression was observed followed by cdc25C. Cdc25B was increased when combined. This indicated that expression of cdc25s that has been linked to abnormal phosphorylation of FLT3-ITD mutation could be regulated by combined DNA damaging agents (Dox and GA) on MOLM-13 cells. In addition, familial platelet disorder that has been linked to mutation of cdc25C could be regulated with Dox and GA combination. However, reduced expression of cdc25A and cdc25C by combination treatment was not statistically significant when compared to Dox only and negative control.

To determine the effect of increased expression of $p21^{Cip1}$ on senescence pathway, the expression of p16 that is activated to suggest stabilized cellular senescence was determined. Dox 1 µM single treatment induced increase expression of $p16^{Ink4a}$ (297 %) while combination of Dox and GA induced less expression (122%) compared to Dox only. However, combined drug p16 expression was not significant compared to Dox only but significant compared to negative control Thus, indicating expression of senescence stabilizing protein either with Dox itself or when combined with GA after 48 h. Most DNA damaging agents induce expression of senescence and this was determined using p16 protein which suggest senescence. In this study both agents showed increased expression of p21 and p16. When combined compared to single treatment, although increased p16 expression was observed with combination, more was expressed with single treatments. Therefore, p16 expression by combination compared to Dox only was not significant ($p>0.05$) but compared to control it was significant ($p>0.01$). Therefore, p16 also known as senescence stabilizer was expressed by either combination or single treatments, but combination seems to inhibit p16 expression.

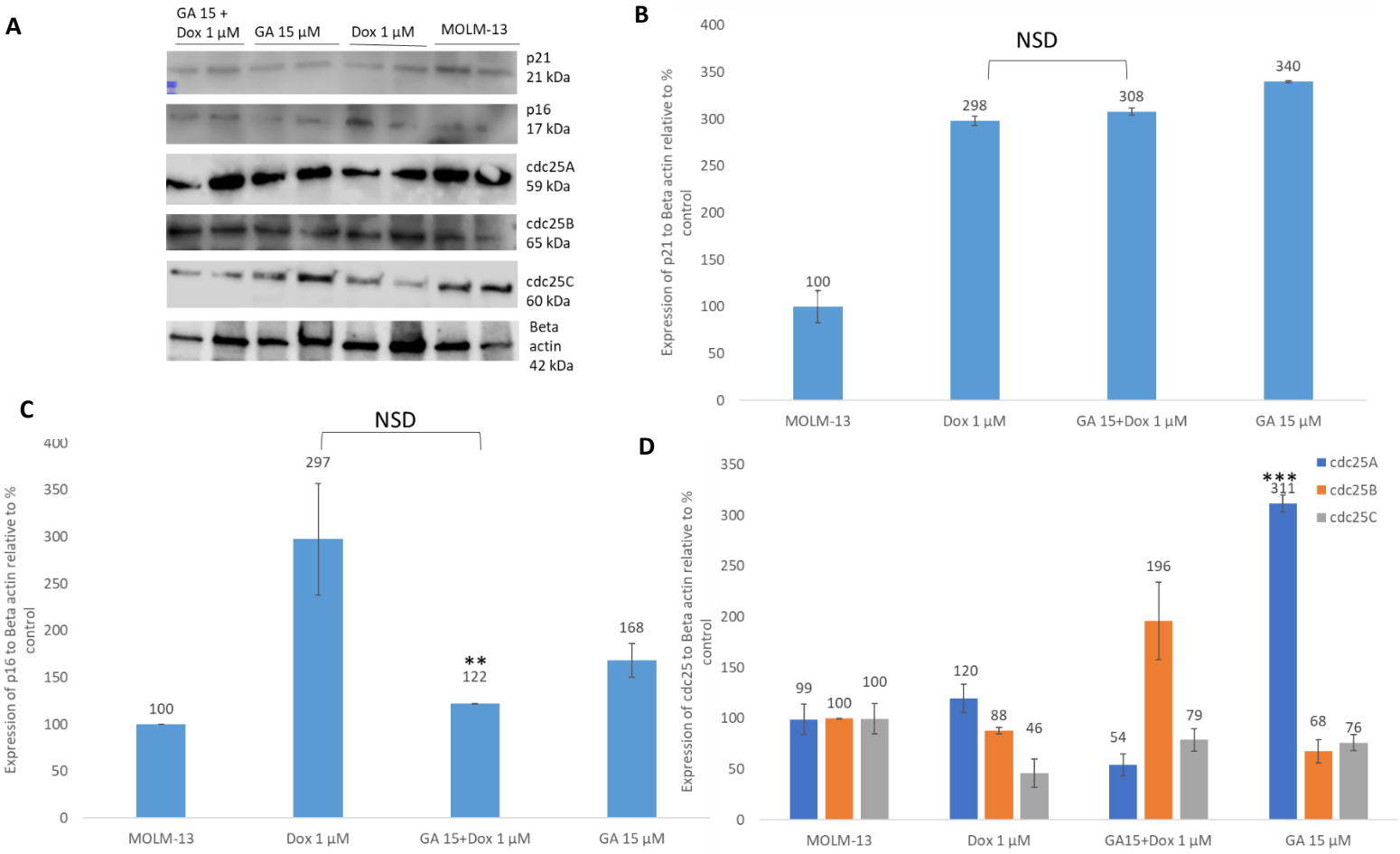


Figure 5.4.4: The expression of p21, Cdc25s and p16 after 48 h treatment with gallic acid singly and in combination with doxorubicin in MOLM-13 cells using Western blotting. A. Immunoblotting results of p21, cdc25s and p16 expression after treatment with Dox single and in combination with GA in MOLM-13 cells. Cells lysate was subjected to SDS-Page for immunoblotting analysis **B**. Graphical expression of p21 **C**. Graphical expression of p16 **D**. Graphical expression of cdc25s. Results were presented as % control ± SD. Data represent two replicates (n=2 replicates) within an experiment (n=1) for p21, p16, cdc24A and cdc25C. Data represent two experiments(n=2) with three replicates (n=3) (two replicates within an experiment and one replicates from an independent experiment) for cdc25B. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. ***p<0.01 ***p<0.001 compared to negative control, ### p<0.001 compared to Dox only as indicated. NSD- No Significant Difference as indicated.*

5.4.5 Summary of Data

Table 5.4.5 contains summary of results gallic acid/Dox study. Combination of gallic acid and Dox showed significant inhibition of cell growth compared to Dox only ($p<0.001$) (Table 5.4.5). In addition, early apoptotic cell death was observed with Dox and gallic acid combined treatment compared to Dox alone. Induction of apoptosis was associated with pro-apoptotic protein Bak. Although there was 2-fold decrease in cdc25A and cdc25B, and 1-fold decrease in cdc25C with combination treatment, they were not statistically significant when compared to Dox only. Gallic acid and Dox combined synergistically inhibited cell growth, showed early apoptotic cell death and G_2/M cell arrest compared to negative control. Moreover, significant p16 protein expression was observed with Dox and gallic acid combined treatment when compared to negative control. Despite p21 referred to as initiator of senescence and p16 referred to as the stabiliser of senescence which suggest senescence, biomarkers for senescence will be recommended to confirm senescence.

Gallic acid treatment significantly inhibited cell growth when compared to negative control. There was significant increase in cdc25A expression with gallic acid treatment when compared to negative control. Although there was 3-fold increase in p21 expression with gallic acid treatment when compared to control, it was not statistically significant. Dox only treatment inhibited cell growth with increase expression of tumour suppressor protein p53, which was significant ($p<0.01$) when compared to negative control.

Table 5.4.5: Summary of results showing fold change and indicating where single or combined drugs were significant against control and against Dox.

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + GA (15 μM)	GA (15 μM)	Dox (1 μM) + GA (15 μM)
CyQuant cell viability assay	0.58↓ (p<0.01)	0.16↓ (p<0.001)	0.47↓ (p<0.01)	0.28↓ (p<0.001)
Cell death assay				
Necrosis	1.63↑	1.40↓	0.82↓	0.86↓
Early apoptosis	1.82↑	21.87↑ (p<0.001)	12.45↑	12.04↑ (p<0.001)
late apoptosis	0.65↓	2.54↑	2.31↑	0.59↑
Cell Cycle				
G0/G1	0.63↓	0.56↓	0.87↓	1.09↑
S	2.6↑(p<0.05)	2.13↑	1.25↑	1.24↑
G2/M	2.0↑ (p<0.05)	2.50 ↑(p<0.001)	1.75↑	1.25↑

Table 5.4.5: Cont'd

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + GA (15 μM)	GA (15 μM)	Dox (1 μM) + GA (15 μM)
Pro-apoptotic proteins Positive results are indicated by an **increase** in protein expressions compared to control				
Caspase 3 Extrinsic and intrinsic apoptotic pathways	0.47 ↓	0.21↓	0.26↓	2.24 ↓ (NSD)
Caspase 8 Extrinsic apoptotic pathway	1.03↓	0.87↑	1.08↑	1.18↓ (NSD)
Caspase 9 Intrinsic apoptotic pathway	1.20↓	0.90↓	1.45↑	1.33↓ (NSD)
Bak Intrinsic apoptotic pathway	0.79↓	1.97↑	0.44↓	2.49↑ ($p<0.01$)
Bax Intrinsic apoptotic pathway	2.17↑	1.06↑	0.71↓	2.05 ↓ (NSD)
Anti-apoptotic protein involved in the intrinsic apoptotic pathway Positive results are indicated by a **decrease** in protein expressions compared to control				
Bcl-2 Intrinsic apoptotic pathway	0.92↓	1.68↓	1.57↓	1.82↑ (NSD)
Tumour suppressor protein Positive results are indicated by an **increase** in protein expressions compared to control				
p53	1.57 ↑ ($p<0.01$)	0.55 ↓	0.93↓	2.86↓ (NSD)
Cell Cycle inhibitor & senescence promoter Positive results are indicated by an **increase** in protein expressions compared to control				
p21	2.98↑	3.08↑	3.40↑	1.03↑ (NSD)
Senescence stabiliser Positive results are indicated by an **increase** in protein expressions compared to control				
p16	2.97↑	1.22↑ ($p<0.01$)	1.68↓	2.43 ↓ (NSD)
Cell Division Cycle (CDC) Positive results are indicated by a **decrease** in protein expressions compared to control				
cdc25A Control G_1/S & G_2/M	1.20↑	0.54↓	3.11↑($p<0.001$)	2.22↓ (NSD)
cdc25B Phosphorylation allows re-entry to mitosis	0.88↑	1.96↓	0.68↑	2.23↓ (NSD)
cdc25C Control G_2/M	0.46↓	0.79↓	0.76↓	1.72↓ (NSD)

5.5 DISCUSSION

5.5.1 Gallic acid in combination with doxorubicin induces G_2M cell cycle arrest and apoptosis in MOLM-13 cells

DNA damage response with anticancer drugs, singly and in combination may be of benefit to AML patients. In this study, GA showed dose-dependent growth inhibition of MOLM-13 after 72 h treatment. Comparing with the effect observed from treated normal monocytes cells, more cytotoxic effects were observed with MOLM-13 cells. It was reported that one cup of green tea and black tea contains 2.8 mg and 57 mg of GA, respectively (Henning *et al*., 2013). 3 mg of GA was reported to yield 88 µmol L^{-1} in 200 mL volume (Yang *et al*., 2018), therefore an IC_{50} value of GA treatment (15 µM) in this study suggest that the concentration is within the biological available range, since GA can be found in urine and serum after consumption and showed less cytotoxic effects against normal monocytes (Figure 5.2). Sourani *et al*., (2016), studied the effect of GA on lymphoblastic leukaemia cell line and showed dose dependent inhibition which is consistent with our study and reported IC_{50} of 30 µM after 72 h (Sourani *et al*., 2016). This suggests that the effect of GA concentrations could differ in various cells and GA seems to have greater effect and be more sensitive to AML MOLM-13 cells than ALL due to reduced IC_{50} value (15 µM) in this study.

GA induction of apoptosis has been associated with oxidative stress, increased intracellular Ca^{2+} levels and mitochondria dysfunction (Yeh *et al*., 2011). GA treatment revealed apoptotic cell death and morphology showed positive cells from TUNEL study. Furthermore, combination of GA 15 µM and Dox at IC_{50} 1 µM in this study showed significant ($p<0.001$) inhibition of cell growth with CI value (0.14) indicating synergism. Doxorubicin is known to induce dose-dependent cardiotoxicity and when it was combined with GA in this study, more of apoptotic cell death and less necrosis was observed, in addition to more TUNEL positive cell. Which suggest increased apoptosis was induced by the apoptotic pathway and toxicity associated with Dox was reduced. This was similar to one earlier report of GA reducing the toxicity of the chemotherapy drug methotrexate that can cause chronic liver damage in rats (Safaei *et al*., 2018) and reported protective effect of GA against toxicity induced by cyclophosphamide in male Wister rats (Oyagbemi *et al*., 2016). Gallic acid at 80 µM was reported to induce 80% apoptosis after 48 h in lymphoid leukaemia cell line (Jurkat cells) (Sourani *et al*., 2016). However, in promyelocytic

leukaemia cells (HL-60), 39% of apoptosis was observed at 80 µM after 48 h (Madlenera *et al*., 2007). In this study (AML -MOLM-13), 15 µM after 72 h induced 7% (early and late) apoptosis, indicating that induction of apoptosis by GA differs depending on concentration, incubation time and cell origin. Combination of GA and Dox induced more apoptotic cell death (12%) and early apoptosis cell death was statistically significant when compared to Dox only ($p<0.001$) (Figure 5.2.1). More early apoptotic cell populations were observed than late apoptotic cells from both singly GA treatment and when combined with doxorubicin. GA has been reported to induce early apoptosis in some cell lines of oral squamous carcinoma and late apoptosis in other cells lines of the same cancer origin as well (Chia *et al*., 2010). Thus, it may suggest that a difference in sensitivity to induction of apoptosis by gallic acid occurs in different human cancer cell lines.

The mechanism of apoptosis is regulated by different proteins that result in self programmed cell death. Activation of caspases that act as effectors of apoptosis is a common feature, and other proteins such as p53, Bcl-2 and Bax also regulates apoptosis. Apoptosis is a cell death mechanism programmed to induce non-inflammatory cell death which is characterised by chromatin condensation, cell shrinkage and fragmentation.

GA did not induce expression of caspase 8 either singly or in combination with Dox in this study. Dox treated cells showed reduced Bcl-2 expression (92%). However, when cells were treated by Dox combined with GA, more expression of Bcl-2 was observed (168%), while GA single treatment showed (157%), indicating GA enhancing increased expression observed when combined. However, expression of Bcl-2 by combined drug was not statistically significant when compared to Dox only ($p>0.05$). Expression of Bax in this study was analysed using two replicates ($n=2$). Further study will include more replicates for more statistical information on Bax. GA has been reported to increase expression in Bcl-2, Bax, apoptosis inducing factor (AIF), endonuclease G (Endo G) and Bid proteins in HL-60 cell line (Yeh *et al*., 2011), and inhibit the expression of Bcl-2 on K562 and Jurkat cells but showed an increase expression of Bax and AIF (Maioral *et al*., 2016). Bcl-2 has been reported to prevent the release of AIF form the mitochondria. AIF proapoptotic activities has provided more understanding of apoptosis regulation and mitochondria dysfunction. Bax and Bcl-2 regulates mitochondria potential and membrane permeability. Increased expression of Bcl-2 in this study suggests that the induction of apoptosis may involve other proteins, although increased expression of Bak was observed when Dox and GA were

combined ($p<0.01$). Therefore, increased early apoptosis (Fig. 5.3) observed with Dox and GA combined was associated with Bak expression which was statistically significant when compared to Dox only ($p<0.01$) and negative control ($p<0.001$). Dox single treatment significantly ($p<0.01$) inhibited the expression of Bak in this study when compared to combination and gallic acid only treatment did not express Bak. Expressions of caspase 8 and Bak did not increase following the treatment of Dox but the increase expressions of caspase 9 and Bax may suggest the intrinsic pathway of apoptosis by Dox only treatment. However, increased expression of caspase 9 and Bax by Dox only was not significant when compared to untreated cells and combination treatment. In this study, GA showed increase expression of Bcl-2 (157%) and caspase 9 (145%). However, expression was not significant compared to control and Dox only.

Further analysis on combination of gallic acid and doxorubicin was done to determine the effect on cell cycle. GA has been reported to induce apoptosis, cell cycle arrest, delay DNA repair, inhibit expression of DNA repair genes and p53 messenger mRNA (Weng *et al*., 2015; Sourani *et al*., 2016). Cell cycle plays an important role in regulating cell proliferation and division, therefore, agents resulting in DNA damage which could induce cell arrest in the cell cycle and inhibit proliferation would be an effective strategy to control growth of cancer cells. GA in this study inhibited cell cycle progression in MOLM-13 at G_2/M phase. In another study carried out by Yeh and co-workers, they reported that that GA induces G_0/G_1 arrest rather than G_2/M in promyelocytic leukaemia HL-60 cell line (Yeh *et al*., 2011). Which was different from what we have found from MOLM-13 cell line. Therefore, the mechanism of GA in inducing cell cycle arrest may vary depending on cell line used. To relate the varying results to humans especially in AML with different subtypes due to its diverse prognostic factors and molecular heterogenicity, the cell line similar to the subtype of AML should be studied, and effective concentration of GA determined for maximum effect. Doxorubicin, a topoisomerase II inhibitor inhibited cell cycle progression at S and G_2/M phase in this study. Interestingly, its combination with GA showed more cells arrested at G_2/M phase. However, the increase was not statistically significant when compared to Dox only. Combination of doxorubicin and polyphenols which is a class of GA has been reported to accumulate cells at S and /or G_2/M phase, which was associated with DNA damage in leukaemic cell lines (Mahbub *et al*., 2015). G_2/M arrest observed in both single treatments and more arrest when combined has been considered as the first step to irreversible permanent cell arrest and known as the onset of senescence which indicates no more cell cycling (Krenning *et al*., 2014).

Cell cycle regulation involves DNA replication, chromosomal segregation and detects DNA defects following DNA damage. The p53 protein regulates cell cycle arrest that ultimately inhibits cancer progression when defects are detected. This study further investigated the expression of p53, a critical tumour suppressor associated with the induction of cell cycle arrest, apoptosis and senescence (Blanpain *et al*., 2011). p53 induces cell cycle arrest and caspase dependent apoptosis, therefore cell cycle arrest protein was also investigated. Senescence is a permanent cell cycle arrest mechanism that promotes inhibition of cancer growth.

Doxorubicin induced expression of p53 in this study, but when combined with GA it was associated with reduced p53. Dox induced apoptosis in tumor cells was reported to be dependent on p53, showing increase p53 expression and correlating with our study (Wang *et al*., 2004). While in normal cells it is independent of p53, occurring through H_2O_2 mediated mechanism (Wang *et al*., 2004). This suggests that Dox induced apoptosis via transcriptional activation of p53 may be crucial in tumour cells than in normal cells. The use of p53 inhibitor, suppressed p53 activation by Dox but did not prevent apoptosis in cardiomyocytes and normal cells (Wang *et al*., 2004). Other mechanisms of Dox such as DNA damage, necrosis, cell cycle arrest and oxidative stress have been reported to be p53 independent (Shin *et al*., 2015). Dox was shown to induce necrosis which is mediated by p53 independent poly- (ADP –ribose) polymerase 1(PARP1), a necrotic signaling pathway (Shin *et al*., 2015). In this study, there was reduced expression of p53 by combined drug when compared to Dox only. In addition, Dox only treatment showed significant expression of p53 ($p<0.01$) when compared to negative control.

The activation of p53 is through ATM/chk2 or ART/chk1 pathways which in turn activate the cell cycle inhibitor p21 that act on the cell cycle to induce arrest. In this study, both Dox single treatment and its combination with GA induced G_2/M arrest. However, more cell arrest observed at G_2/M phase with combined Dox and GA treatment was not statistically significant when compared to Dox only. p21 is a member of the kinase inhibitor protein (cip/kip family-$p21^{cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) and it inhibits cdk/cyclin complex when formed. In this study increased levels of p21 following GA single treatment (340%) was observed compared to Dox only (298%). However, more p21 protein (308%) was expressed after combined treatment of Dox and GA compared to Dox only, but the level was less than GA single treated sample, suggesting GA enhancing p21 expression and cell arrest with gallic acid and Dox combined treatment. Although

p21 expression with gallic acid single treatment showed 3-fold increase (Table 5.4.5) compared to control, it was not statistically significant. In this study, combined drug expressing p21 (308%) was not statistically significant when compared to Dox only (298%). More number of replicates may show significant effect since number of analysed in this study was only two (n=2).

Taken together, this suggests that increase expression of cell cycle inhibitor p21 disrupt cdk1/cyclin B complex associated with G_2/M phase transition and therefore, cell arrest in this phase was observed. Recently, GA has been reported to increase expression of both cell cycle inhibitors, p21 and p27 at G_1 phase arrest in breast cancer cell line MDA-MD-231 (triple negative) (Lee *et al*., 2017); and leukaemia cell line HL-60 (Yeh *et al*., 2011). However, at G_2/M phase arrest in breast cancer cell line both p21 and p27 were expressed (Hsu *et al*., 2011). However, p27 not p21 was responsible for more arrest at G_2/M phase after gene knockdown of p21 which did not affect cell arrest, while knockdown of p27 did (Hsu *et al*., 2011). This suggest further studies on cell cycle inhibitor p27 on GA induced G_2/M phase arrest in MOLM-13. On the other hand, Dox has been reported to induce S and G_2/M arrest in Hela cells and in an interesting twist, it was reported that those cells that were not arrested undergo apoptosis after mitosis while those arrested at S and G_2/M phase indeed survived and did not undergo apoptosis (Miwa *et al*., 2015). Therefore, demonstrated a heterogeneous population of chemoresistant cells with the arrested cells that survived (Miwa *et al*., 2015). In that study, cell cycle was used as fate monitoring of drug treated heterogeneous cancer cell populations to distinguish chemoresistant from chemo-sensitive cancer cells (Miwa *et al*., 2015). Dox was reported to induce apoptosis after mitosis (Miwa *et al*., 2015). Miwa *et al*., (2015), demonstrated that arrested cancer cells contain both population of chemo-sensitive and chemoresistant cells and that future novel drugs and combinations should target arrested cancer cells for apoptosis. In this study, combination of Dox and GA arrested at G_2/M phase with more arrest at G_2/M than single treatments. This could suggest targeting both populations (chemo-sensitive and chemoresistant) of cancer cells due to increased number of cell arrested with Dox and GA combined treatment. Dox already reported effective at inducing apoptosis on cells that escape arrest (Miwa *et al*., 2015), its combination with GA could suggest enhanced anticancer effect in MOLM-13 as indicated with increased expression of p21 and Bak, when combined. However, only increased expression of Bak by combined drug was significant when compared to Dox only ($p<0.01$).

Since the cell cycle inhibitors prevent cells from progressing in the cell cycle, there are certain phosphatases that promote progression of cells from one phase to the other and they are the cdc25s. The cdc25s are of three homologs; cdc25A, cdc25B and cdc25C. Cdc25A is implicated in G1 and S phase arrest, cdc25C is implicated in G_2/M phase while cdc25B is involved in S phase arrest. Inhibition of cdc25s is through phosphorylation binding of 4-3-3 protein as mediated by chk1 or chk2 (Gardinoa & Yaffe, 2011). Cdc25s activates cdk which binds to cyclin to promote progress of cells in the cell cycle and the inhibition of cdc25 protein expression suggest the signaling of arrest at each phase of the cell cycle. There was reduced expression of cdc25C in all treatments, cdc25A was reduced only in combination of GA and Dox, and cdc25B was increased in GA and Dox combined and GA single treatments. This suggests that G_2/M arrest observed in all treatments was associated with reduced cdc25C level. GA has been reported to arrest cells at G_2/M phase by down regulating cdc25C activity (Chen *et al*., 2009). Cdc25C protein promote entry from G2 to mitosis by removal of inhibitory phosphate loop phosphorylation on cdk1 (Gardinoa & Yaffe, 2011). Cdc25A was reported to not only regulates early phases of the cell cycle but also involved in G_2/M transition and in this study reduced expression was observed in combination treatment suggesting that Dox and GA combined treatment regulated both cdc25A and cdc25C to induce G_2/M arrest. In this study, reduced expression of cdc25 A and C with combined drug was not statistically significant when compared to Dox only. However, only two replicates (n=2) were analysed for cdc25A and cdc25C. More number of replicates (n>2) may show more interesting results.

Another mechanism regulated by p53 when activated is the permanent cell arrest which is known as cellular senescence also mediated by cell cycle inhibitors p21 and p16. However, more biomarkers are required to confirm senescence. The cell cycle inhibitor p16 belongs to a family of the inhibitor of cdk4 (INK4) which are (INK4 family) $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. The p21 initiates senescence while the p16 stabilises it. P16 is reported silenced or deleted in most cancers, its mutation in AML is rare (Keegan *et al*., 2014). Senescence plays a role in aging and tumour suppression. Therefore, regulated expressions of p53, p21 and p16 were studied and as reported by Yang *et al*., (2012), these proteins (p53, p21 and p16) suggest senescence (Yang *et al*., 2012). Other senescence biomarkers that have also been reported includes senescence associated beta galactosidase (SA-β galactosidase), retinoblastoma protein (RB), and trimethylation of H3K9. In this study, Dox treatment and Dox combination with GA showed the increase expression of p16

protein (297% and 122%, respectively). However, GA single treatment showing expression of 158% indicates expression observed with combination was reduced. This result showed that combined treatment of Dox and GA although significantly increased p16 ($p<0.01$) when compared to untreated cells, expression was reduced compared to single treatments.

Dox p16 expression was inhibited when combined with gallic acid. Clearly, both gallic acid and Dox individual drugs expressed p16 than when combined. Dox only treatment and gallic acid alone in this study could be suggested as a senescence marker, therefore indicating decrease p16 treatment and possible senescence with gallic acid and Dox combination treatment. However, number of replicates analysed for p21 and p16 expression were two (n=2). Therefore, more replicates may provide more significant effect.

Dox has been reported to induce senescence in cancers such as leukaemia and breast cancers. At 50 nM Dox was reported to induce senescence in the absence of p53 and p16 through initiation of autophagy and miR-375, which has anti-proliferative function and involved in regulatory pathways (Yang *et al*., 2012). In that study, autophagy a form of cell death was reported to be initiated by Dox after it was observed that Dox induced senescence on cells. To the best of our knowledge, the effect of GA induced senescence on cancer cell lines has not been reported. This study showed that both Dox and GA expressed senescence protein (p16) in MOLM-13 singly and significant expression ($p<0.01$) was observed in cells when the two drugs was combined, compared to the cells without treatments. However, compared to Dox only, p16 expression was not statistically significant ($p>0.05$). Although combination treatment expression was reduced when compared to single treatments, it was significant compared to negative control suggesting senescence activation.

Senescence has been reported as tumour suppressor mechanism, however, it has been considered to be involved in chemo-resistance. Senescence is a state of permanent cell cycle arrest even in the presence of mitogens or growth factors hence suppressing the growth of cancer cells. Therefore, combination of Dox and GA in treated MOLM-13 cells indicates regulated expression of p16 protein ($p<0.01$ compared to control) and may aid to prevent chemo-resistant and suppress tumour.

In conclusion, this study investigated the effect of GA treatment singly and in combination with Dox in MOLM-13 and demonstrated that the combination treatment inhibited cell growth by inducing G_2/M arrest to stop proliferation. There was upregulation of apoptotic protein Bak

compared to cells without treatment and/or Dox treated cells. Therefore, early apoptotic cell death was enhanced when combined in this study was associated with the expression of Bak. These findings revealed mechanism of GA combined with Dox on cellular processes (cell arrest, apoptosis and senescence) as potential anticancer agents in MOLM-13 cells. Therefore, further study is recommended to determine the effect of combined treatment as DNA damaging agents

CHAPTER SIX

6. Vitamin C and doxorubicin combination may exert anticancer effect through the regulation of apoptosis, cell arrest and senescence in acute leukaemic cells

6.1 Background

The role of vitamin C (ascorbate, L-ascorbate) in the development, growth and regulation of cancer has been a subject of debate for decades (Vissers & Das, 2018). There are reports of anticancer effects of vitamin C in both *in vitro* and *in vivo* studies (Table 6.1). However, human clinical studies has been inconsistent (Nauman *et al*., 2018), while aiming to determine pharmacological doses of vitamin C that is toleratable for cancer patients with advanced cancers (Stephenson *et al*., 2013; Vissers & Das, 2018). Some studies have reported clinical benefits of high doses of vitamin C for pancreatic cancer patients (Drisko *et al*., 2018) and advanced cancers (Stephenson *et al*., 2013), however these studies were done on a small number of patients. Vitamin C has been reported to be effective in colorectal cancer (Jihye *et al*., 2015; Pires *et al*., 2018), improved outcome in breast cancer patients has been linked to vitamin C supplements (Greenlee *et al*., 2012; Sant *et al*., 2018) and in clinical study with intravenous administration (Vollbracht *et al*., 2011). In leukaemia, it is reported to restore TET2 gene that reverses self renewal of abnormal haematopoietic stem and progenitor cell either singly (Cimmino *et al*., 2017) and in combination with chemotherapy drug (Zhao *et al*., 2018).

Anticancer activity of vitamin C reflects its redox, enzyme co-factor or prooxidant activities (Vissers & Das, 2018). Vitamin C is the oldest, commonly used and most studied anticancer therapy due to its historical background. It is reported to be a reducing agent and exhibit antioxidant function in physiological condition and pro-oxidant role in pathological condition indicating a switch over role (Chakraborthy *et al*., 2014). Hence physiological concentrations (low concentrations 0.1 mM) and pharmacological concentrations (high concentrations 0.3-20 mM or above) were determined (Chen *et al*., 2005). However, regardless of its concentration and condition, it anticancer activity is associated with production of hydrogen peroxide (H_2O_2) that is selective to cancer cells. Hence the conflict in vitamin C cancer therapy is due to its role as antioxidant or pro-oxidant.

In many studies vitamin C refelects H_2O_2 generation as a pro-oxidant due to oxidative stress that specificly target cancer cells from 1mM concentration and above (Chen *et al*., 2005; Du *et al*., 2010; Parrow *et al*., 2013). Anticancer effect below 1 mM, or 100 µM or even as low as 1 µM has been reported to enhance cancer cells susceptibility to doxorubicin, etoposide or cisplatin (Kurbacher *et al*., 1996), and the mechanism although unclear involves cell survival pathways relating to p53. However, at high concentrations (18 mM & 8.5 mM) antagonistic effect was observed with chemotherapy drugs (Heaney *et al*., 2008). Multiple mechanisms of vitamin C reported in diferent cancers includes caspase dependent and independent apoptosis (Lin *et al*., 2006), autophagy (Du *et al*., 2010), ATP depletion (Lv *et al*., 2018), cell cycle arrest (Herst *et al*., 2012) and DNA damage (Lv *et al*., 2018).

Although there are clinical concerns of vitamin C impeding the action of chemotherapy drug (Heaney *et al*., 2008; Ludke *et al*., 2017; Perrone *et al*., 2009), phase I trial combination treatment of gemcitabine and vitamin C did not show any adverse events (Monti *et al*., 2012), along side other studies of synergistic effect of the combination of vitamin C and chemotherapy drugs (Espey *et al*., 2011; Martinotti *et al*., 2011 Kassouf *et al*., 2006; Cieslak & Cullen, 2015).

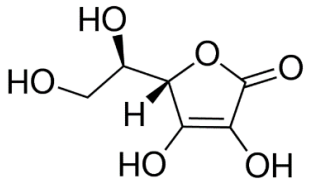
Doxorubicin is an effective antitumour drug that induces DNA damage by DNA intercalation and ROS on cancer cells to activate DNA damage response (DDR) and inhibit cell growth. Adverse effect such as cardiotoxicity associated with doxorubicin was found to be as a result of lipid peroxidation and the use of antioxidant such as vitamin C could minimize adverse effects caused by oxidative stress and promote recovery (MVOB *et al*., 2016). Vitamin C has been reported to enhance anticancer effect of chemotherapy drugs including doxorubicin (Bober *et al*., 2017).

There is the need for clear understanding of the mechanism of combination of drugs which is still poorly understood as their effect with chemotherapy is still in doubt (MVOB *et al*., 2016), (Vissers & Das, 2018).

Table 6.1: Anticancer effect of vitamin C on different cancer cell

Most fruits contain Vitamin C and they include- Lemon, Orange, Rose Hips, Chilli peper, wild strawberry, potatoe, Broccoli, Cranberry, Spinach, Cayanne peper, strawberry, tomatoes,

Vitamin C



$C_6H_8O_6$

Biological properties
Antioxidant
Anticarcinogenic
Immune function
Hormonal regulation
Gene transcription
Epigenetic regulation
Barrier integrity

Cancer/Cell line	Concentration studied	Anticancer Effect	Human/Animal studies	References
Human leukaemic cell lines HL- 60, NBA, and NBA-R1	100, 250, 500, 750 and 1000 µM	Inhibited cell growth at 24 h H_2O_2 induced apoptosis ↑ Bax, ↓ Bcl-2 ↑ cytosol cytochrome C ↓ Mitochondria cytochrome C ↑ Caspase 3 and 9, cleavage of PARP, ↓ caspase 8 G1/S phase arrest	Inhibit cell growth of AML cells from patients in dose-dependent manner (0, 0.1, 0.25, 0.5, 1 and 2mM). ↑ H_2O_2. Five out of eight patients showed beneficial effects with 100g per day without significant toxicity	(Park *et a*l., 2004)
Human ovarian cancer cell lines SK-OV-3, OVCAR-3, 2774	100, 250, 500, 750 and 1000 µM	No significant inhibition observed due to lower uptake efficiency No apoptosis observed		(Park *et al*., 2004)
Colon cancer cells HCT1 16	100 µg/ml	Inhibited cell growth after 24 h, enhanced inhibition, cell damage and DNA fragmentation when combined with cisplatin ↑ p53		(An *et al*., 2011)
Prostate cancer cell lines DU-145 and LNCap Prostate cancer cell lines DU-145 cells treated with 1mM ascorbate after 24 h	0-10 M 0.1 and 0.3 mM	Inhibited cell growth in dose-dependent manner. Inhibited cell growth, ↑caspase 3, G_0/G_1 cell arrest was induced with 1mM after 24 h		(Maramag *et al*., 1997) (Fromberg *et al*., 2011)

Table 6.1: Anticancer effect of vitamin C on different cancer cell lines

Murine melanoma cells B16F10 were treated with of Vitamin C.	0.05, 0.1, 0.15, and 0.2mM	Inhibited cell growth, G1 phase arrest was induced with 0.2mM, ↑ p53, ↑ p21, ↓ Cdk2.		(Hahm *et al*., 2007)
Breast cancer cell line MDA-(MB231 and MCF7)	1.5mM and 1mM	Dose dependent inhibition, G0/G1 arrest and apoptosis was induced and moderate PI3k pathway activation		(Guerriero *et a*l., 2014)
Human lung adenocarcinoma Spc-A-1 cells	36.62 μM/L for 48 h	Ascorbic acid induced G0/G1 cell arrest		(Li *et al*., 2010)
Hepatocellular carcinoma cell line HLE and Huh7 cell lines	1mM for 48 h	Vitamin C arrested cells at G2 phase in HLE and G1 phase in Huh7 cell lines, ↑ p21. In HLE cell line, ↓ Cyclin B		(Sajadian *et al*., 2016).

Anticancer effect of vitamin C in different cancers including leukaemia. Effect on apoptotic proteins (Bax, Bak and Bcl-2), caspase 3, 8 and 9, cell arrest (G_1 arrest), cell cycle arrest proteins (p53 and p21) and senescence protein (p16) were also observed in different cancers.

6.1.1 Aim and Objectives

The aim of this study was to investigate the anticancer effect of vitamin C and doxorubicin, singly and in combination to establish anticancer mechanism in the regulation of apoptosis, cell cycle and senescence in AML cell line.

Objectives

The aim of the research was to achieve the following objectives which includes;

- to determine the cytotoxic effect of the drugs
- to evaluate their potential to induce apoptotic cell death
- to determine their ability to change the expression of apoptotic proteins (Bax and Bak) and the anti-apoptotic protein (Bcl-2)
- to determine effect on caspase 3, 8 and 9 proteins
- to study their effect on cell cycle and to evaluate their ability to induce cell arrest through the expression of p53, p21 and Cdc25 phosphatases
- to determine effect on senescence through the regulation of p16 expression

RESULTS

Pro-apoptotic effects of doxorubicin singly and in combination with vitamin C were studied in MOLM-13 cell line. This study determined effect on cell viability, apoptosis, cell cycle and determined the expression of tumour suppressor protein p53 which regulates cell cycle arrest, apoptosis and senescence. Therefore, provides more understanding of the mechanism of doxorubicin (Dox) and vitamin C combined drug for possible synergistic and enhanced anticancer effect.

6.2 Effect of vitamin C on cell viability (MOLM-13 cells) after 72 h of incubation

The cytotoxic effect of vitamin C in MOLM-13 was determined in this study after 72 h incubation using the cell viability assay (Section: 3.4.3). Vitamin C at 15 and 30 µM inhibited cell growth by 35% and 38%, respectively. Statistically, inhibition at 15 and 30 µM were significant ($p<0.001$) indicating very strong evidence of inhibition when compared to no treatment cells. Imatinib a chemotherapy drug (acted as positive control) at 2.5 µM inhibited cell growth and was statistically significant ($p<0.05$) when compared with untreated cells. There was no statistical difference between inhibition at 15 µM and 30 µM ($p>0.05$). Vitamin C at 10 µM had stimulatory effect on MOLM-13 cells (Figure 6.2). Descriptive statistics and ANOVA of MOLM-13 in appendix (Appendix 7 & 8, respectively). In normal macrophage cells, which are normal cells used in this study, no inhibition was observed with all concentrations (10-30 µM) studied. IC_{50} at 15 µM was used for combination and further study with MOLM-13.

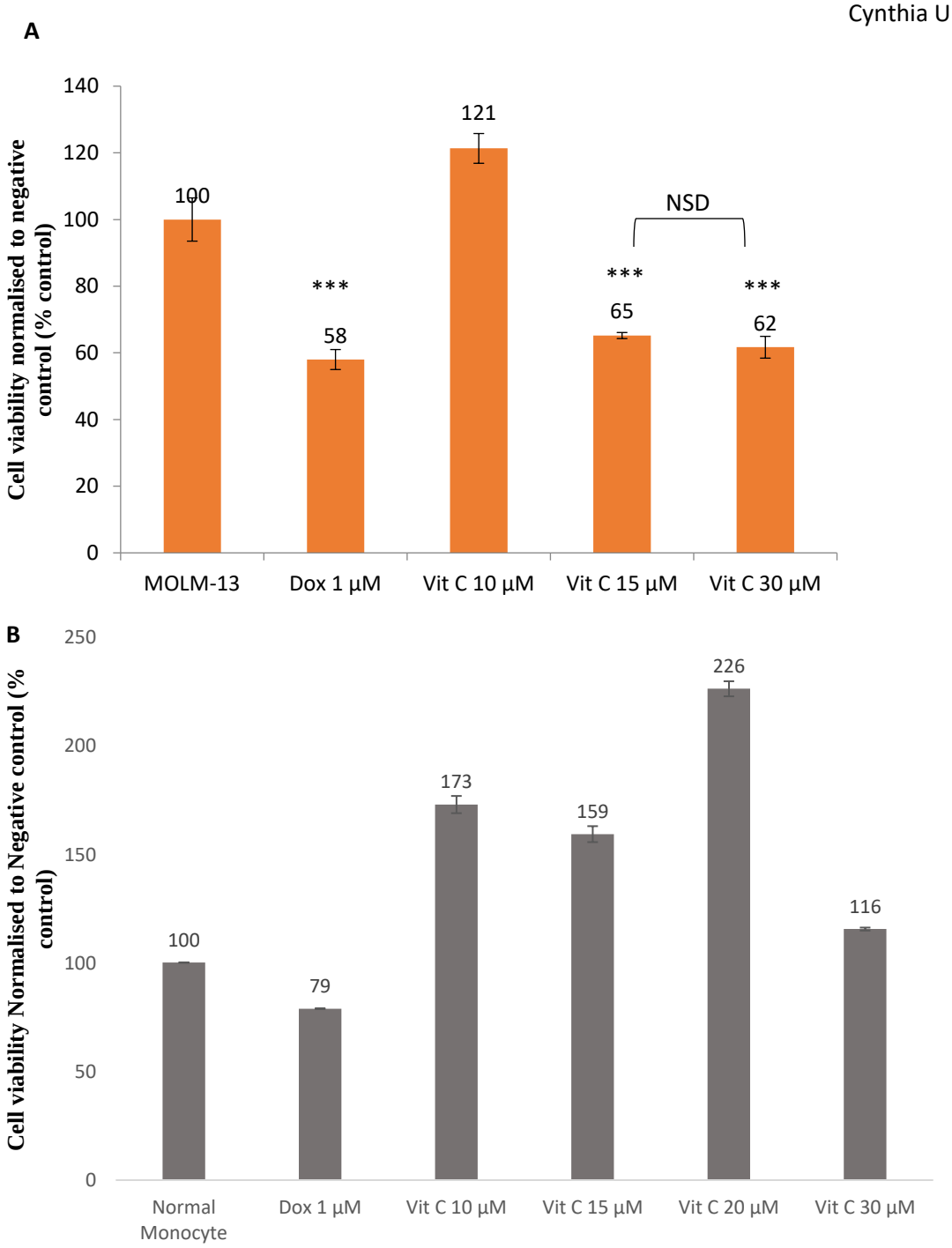
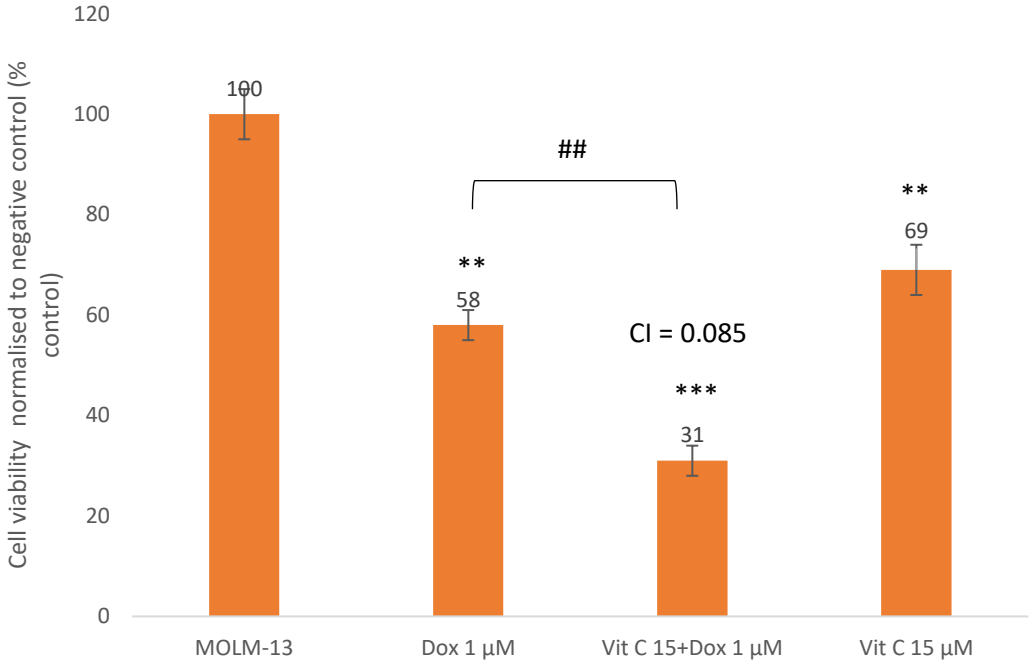


Figure 6.2: The effect of vitamin C on cell viability (A. MOLM-13 B. Normal monocytes cells) after 72 h of incubation. MOLM-13 and normal monocytes were treated with various concentrations of vitamin C for 72hrs and viability determined using CyQUANT GR. Results were expressed as % control compared to cells without treatment. Data represent two independent experiments (n=2) with four replicates (n=4 replicates) within an experiment. IC_{50} MOLM-13 was 15 µM. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis***p< 0.001. NSD- No significant difference as indicated.

6.2.1 Combination of vitamin C and doxorubicin on cell viability

There could be promising effect with combination of vitamin C and Dox. This was conducted in this study and effect was determined using CyQUANT GR. Combination of vitamin C at 15 µM and Dox at 1 µM induced inhibition of 69% and statistically significant ($p < 0.01$) when compared to positive control drug Dox. Suggesting enhanced anticancer effect when combined (Figure 6.2.1). Quantitation of the type of interaction between Dox and vitamin C was done using median effect analysis which derives combination index (CI) value. CI value 0.09 indicates synergism (0.1-0.90) with 0.70% inhibition.



6.2.1.: Effect of combination of vitamin C and doxorubicin on Cell viability. MOLM-13 was treated with Doxorubicin 1 µM singly and in combination with Vitamin C 15 µM on MOLM-13 at a cell density of 1.5×10^5 cell/ml for 72 h. Cell viability was determined using CyQUANT GR. Combination index (CI) value 0.085 indicates synergism. Results were expressed as % control compared to cells without treatment. Data represent an independent experiment (n=1) with three replicates (n=3 replicates). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 compared to negative control, $^{\#\#}$p<0.01 compared to Dox only as indicated. NSD- No Significant Difference as indicated.

6.2.1.1 Drug interaction of doxorubicin and Vitamin C

Determination of the type of interaction between Dox and vitamin C was done using median effect analysis with the CompuSyn software, which derives combination index (CI) value between two or more drugs. CI value of 0.085 indicates that combined drugs showed synergism (Figure 3.1).

Table 6.2.1.1: Combination index (CI) of data for combination effect of doxorubicin and vitamin C in MOLM-13 cells.

Doxorubicin (µM)	Vitamin C (µM)	Effect (inhibition %)	CI Value	Result
1	15	0.70	0.085	synergism

CI value 0.1-0.90 indicates synergism, 0.90-1.10 indicates additive while 1.10-10 indicates antagonism

6.3 Combination of vitamin C and doxorubicin on apoptosis

The effect of vitamin C on apoptosis of MOLM-13 was determined after 72 h incubation using apoptosis assay (Section: 3.5.1). Vitamin C single treatment induced 3.33% apoptosis and 4.63% necrotic cell death while Dox 1 µM induced more necrotic cell death of 5.94% and less apoptotic cell death 1.72%. However, combination of vitamin C at 15 µM and Dox 1 µM resulted in a dramatic increase in cell death with more cells going into apoptosis with 11.29% (both early and late apoptosis) and less necrosis (3.37%) when compared to Dox 1 µM and vitamin C 15 µM individual treatments. Apoptosis with combined drug was statistically significant when compared to Dox only ($p<0.001$). However, reduced necrosis cell death 3.73% with combination was not statistically significant when compared to Dox 1 µM only (5.94 %) ($p>0.05$).

The morphological changes of cells undergoing apoptosis were studied using Tunel assay and stained with DAPI (Section - 3.5.2). MOLM-13 cells treated with vitamin C 15 µM showed some apoptotic cells which were reduced in size and stained with apoptotic dye indicating apoptosis. Combination of vitamin C and Dox 1 µM showed more apoptotic cells compared to single treatments (Figure 6.3.1).

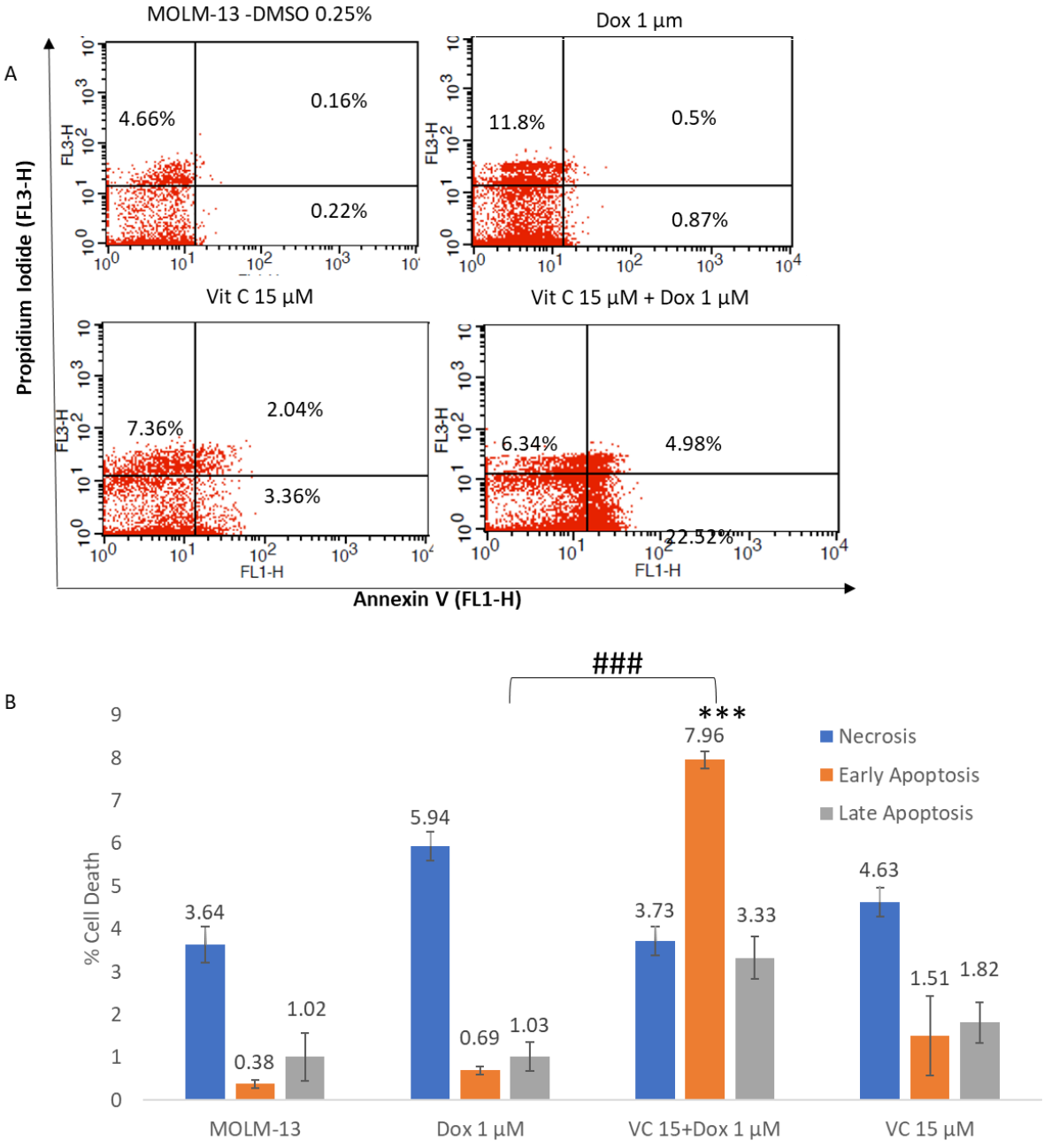


Figure 6.3: The effect of doxorubicin and combination with vitamin C on induction of apoptosis after 72 h on MOLM-13. **A**. Annexin/ PI staining of apoptotic cells using Flow cytometer illustrating distribution of % population undergoing apoptosis and necrosis. The upper right of FACS indicates late apoptosis (annexin V/PI staining). The upper left of (FACS) indicates necrosis (PI staining only). Representation of 10,000 cells staining profile. **B**. Graphical representation of % Necrosis and Apoptosis (Early and Late). Data represent three independent experiments (n=3) with one replicate each (n=3 replicates). MOLM-13 cells were treated with vitamin C 15 µM and doxorubicin at 1µM for 72 h, cell apoptosis was determined using annexin V and propidium iodide. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. ***p<0.001 compared to negative control, ### p<0.001 compared to Dox only.

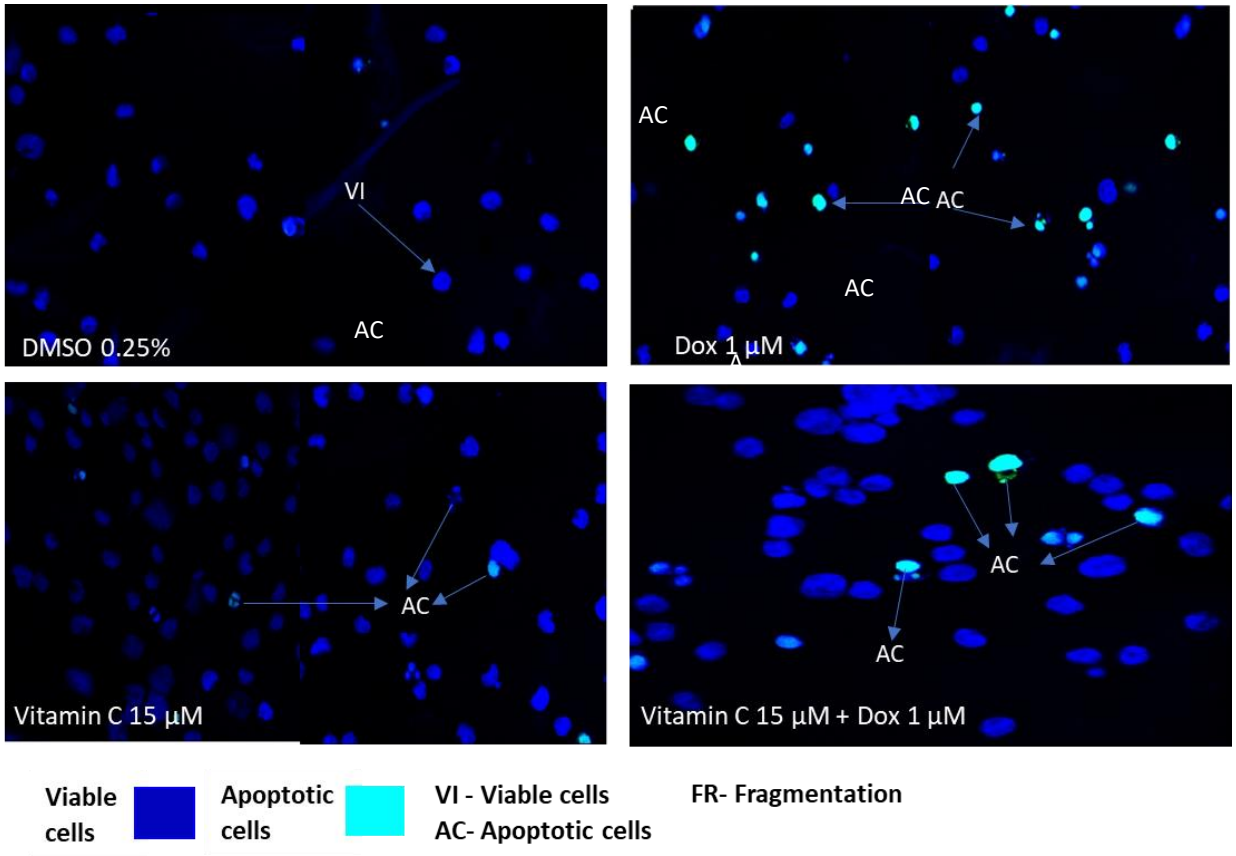


Figure 6.3.1: Apoptotic effect of doxorubicin and combination with vitamin C on MOLM-13 cells after 48 h using TUNEL assay. MOLM-13 cells were treated with vitamin C at 15 µM and doxorubicin at 1µM for 48 h, morphology of apoptotic cells was observed using the Fluorescence microscope. Image represent data from two independent experiments (n=2) with two replicates within an experiment

6.4 Effect of doxorubicin and vitamin C on expression of Apoptotic proteins in MOLM-13

To understand the induction of apoptosis, proapoptotic proteins (Bax and Bak) that promote mitochondria membrane permeability and antiapoptotic protein (Bcl-2) were studied using Western blot (Method Section: 3.6.2.1). Apoptosis regulator (Bax) (71%) was expressed more than Bcl2 antagonist/killer (Bak) (14%; $p<0.01$) after treatment with vitamin C.

Cells treated with Dox only expressed 60% of Bak and 158% of Bax. However, Bax level increased after treatment with vitamin C and Dox combined than Bak compared to Dox only. Combination showed enhanced expression of Bax (233%1-fold increase) compared to 158% of Dox only treatment. However, increased expression of Bax after treatment with combined drug was not statistically significant when compared to Dox only. Anti-apoptotic protein (Bcl-2) was expressed more when combined than single treatments (Figure 6.4).

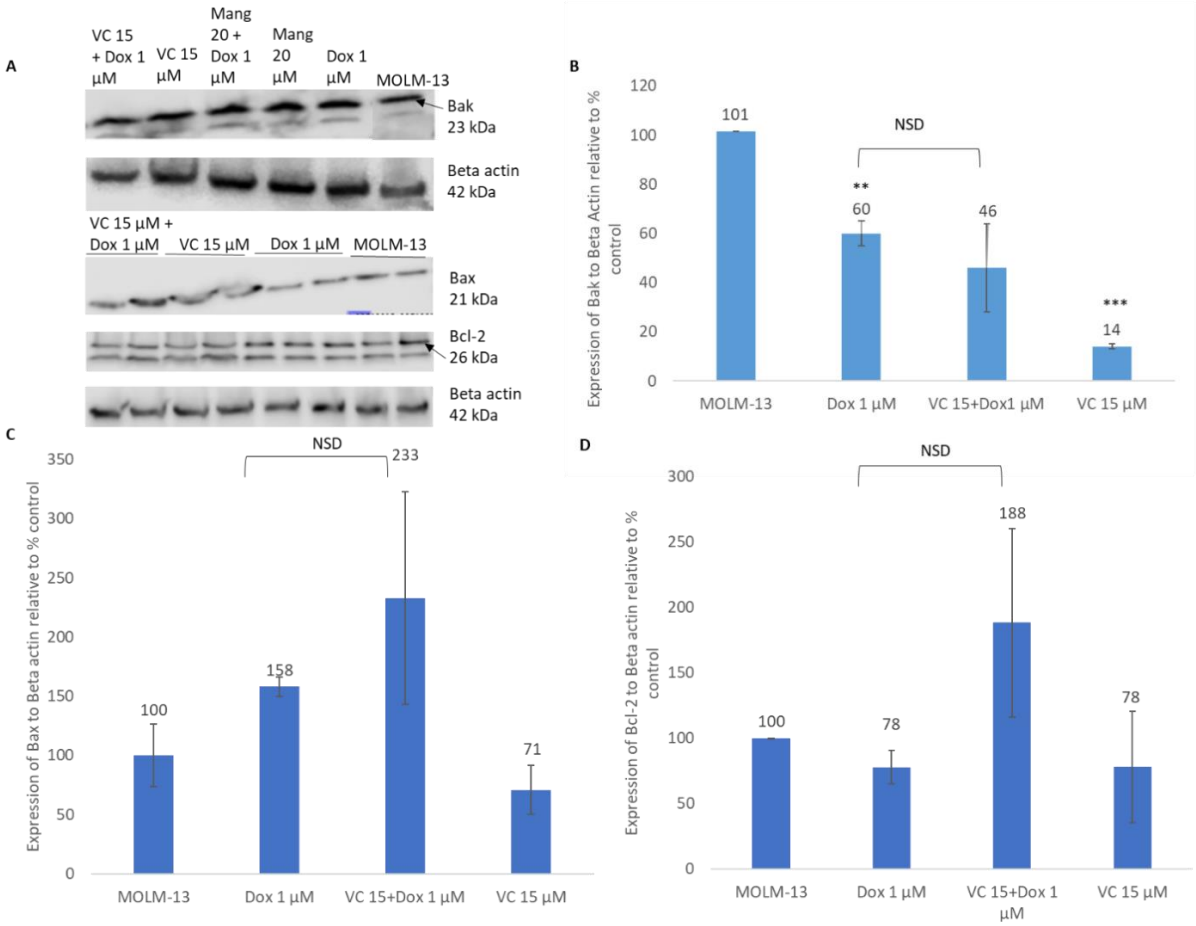


Figure 6.4: The expression of Bax, Bak and Bcl-2 after 48 h treatment with vitamin C singly and in combination with doxorubicin in MOLM-13 cells using Western blotting. **A**. Immunoblotting results of apoptotic proteins after treatment with Dox single and in combination with vitamin C in MOLM-13 cells. Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis. **B**. Expression of Bax **C**. Expression of Bak **D**. Expression of Bcl-2. Results were presented as % control ± SD. Data represent two experiments (n=2) with three replicates (n=3) for Bak, Bax,and Bcl-2 expressions (Two replicates within and experiment and one replicates from an independent experiment). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. ***p<0.01, ***p<0.001 compared to negative control, NSD- No Significant Difference as indicated.*

6.4.1 Effect of doxorubicin and vitamin C on expression of caspases

To investigate whether vitamin C singly and in combination with Dox could affect the activation of caspases, expression of cleaved caspase 3, 8, 9 were studied after 48 h using Western blot**.** Vitamin C 15 µM single treatment induced increased expression of caspase 9 (173%) than caspase 8 (113). Reduced effect was observed with Dox 1 µM single treatment showing more caspase 9 expression (90%) than caspase 8 (79%).

It was observed that cells treated with vitamin C and Dox single treatment showed reduced expression of the common pathway protein (caspase 3) with 43% for Dox only and 11% for vitamin C treatment. Interestingly, similar trend was observed when vitamin C and Dox were combined with expression of caspase 9 (213%) more than caspase 8 (166%) compared to cells without treatment. In addition, reduced caspase 3 (37%) was observed.

This suggests that vitamin C individual treatment may activate the intrinsic and extrinsic pathways of apoptosis and when combined with Dox, enhanced expression was observed with caspase 9 showing 2.4-fold increase and caspase 8 showing 2.1-fold increase when both compared to Dox only. However, enhanced expression of caspase 8 and 9 by combined drug were not statistically significant compared to Dox only and negative control. Vitamin C in this study was observed to enhance the activity of Dox in activation of caspase 8 and 9 when combined. Reduced expression of caspase 3 by vitamin C single treatment (11 %) and Dox single treatment (43%) was maintained after treatment with combination of Dox and vitamin C (37%).

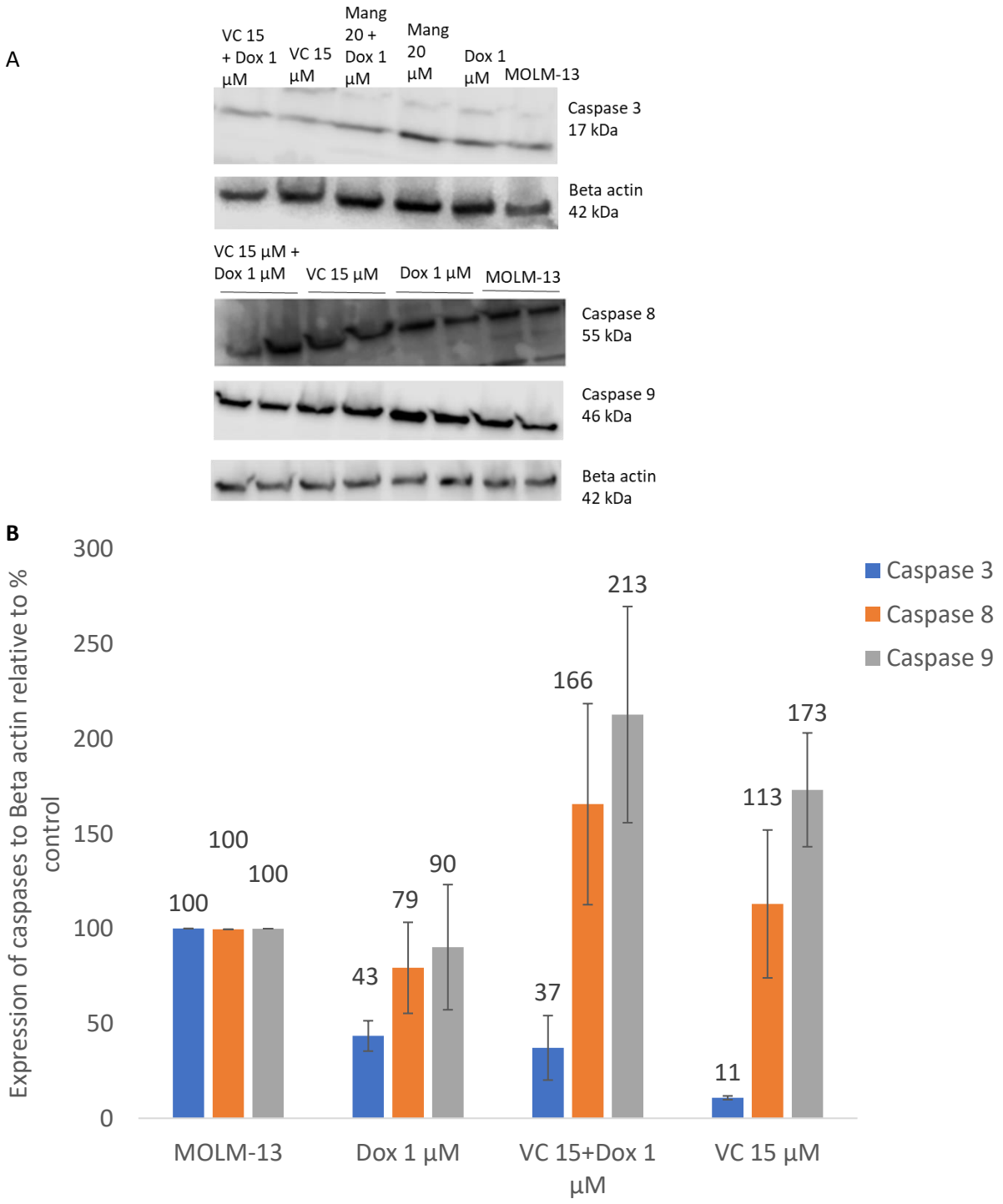


Figure 6.4.1: The expression of caspase 3, 8 and 9 after 48 h treatment with vitamin C singly and in combination with doxorubicin on MOLM-13 cells using Western blotting. **A**. Immunoblotting results of caspase proteins after treatment with Dox single and in combination with vitamin C in MOLM-13 cells. Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis. **B**. Graphical expression of caspase 3, 8 and 9. Results were presented as % control ± SD. Data represent two experiments (n=2) with three replicates (n=3 replicates) (two replicates within an experiment and one replicate from independent experiment) for caspase 3, 8 and 9. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis.

6.4.2 Combination of doxorubicin and vitamin C on the cell cycle

Induction of cell cycle arrest is prior to induction of apoptosis and certain phase of the cell cycle is sensitive to apoptosis (Smith *et al*., 2000; Chen *et al*., 2009). Cell cycle arrest was determined after 48 h (Section: 3.6.1). Cells treated with vitamin C at 15 µM show arrested at G_0/G_1 phase (70%). Vitamin C at 15 µM showed no significant arrest when compared to DMSO control at S phase (12%) ($p>0.05$) and G_2/M (12%) ($p > 0.05$) phase. This indicates G_0/G_1 arrest when compared to untreated cells.

Treatment with combination of vitamin C 15 µM and Dox 1 µM showed cells were arrested at G_2/M (33%). Importantly, more G_2/M phase arrest was observed when vitamin C at 15 µM was combined with Dox 1 showing 33% cell arrest while Dox 1 µM treatment showed 24%. However, this increase was not statistically significant ($p>0.05$) but was significant when compared to cells without treatment ($p<0.001$). Therefore, very strong evidence of significant arrest was observed ($p<0.001$) when compared to untreated cells.

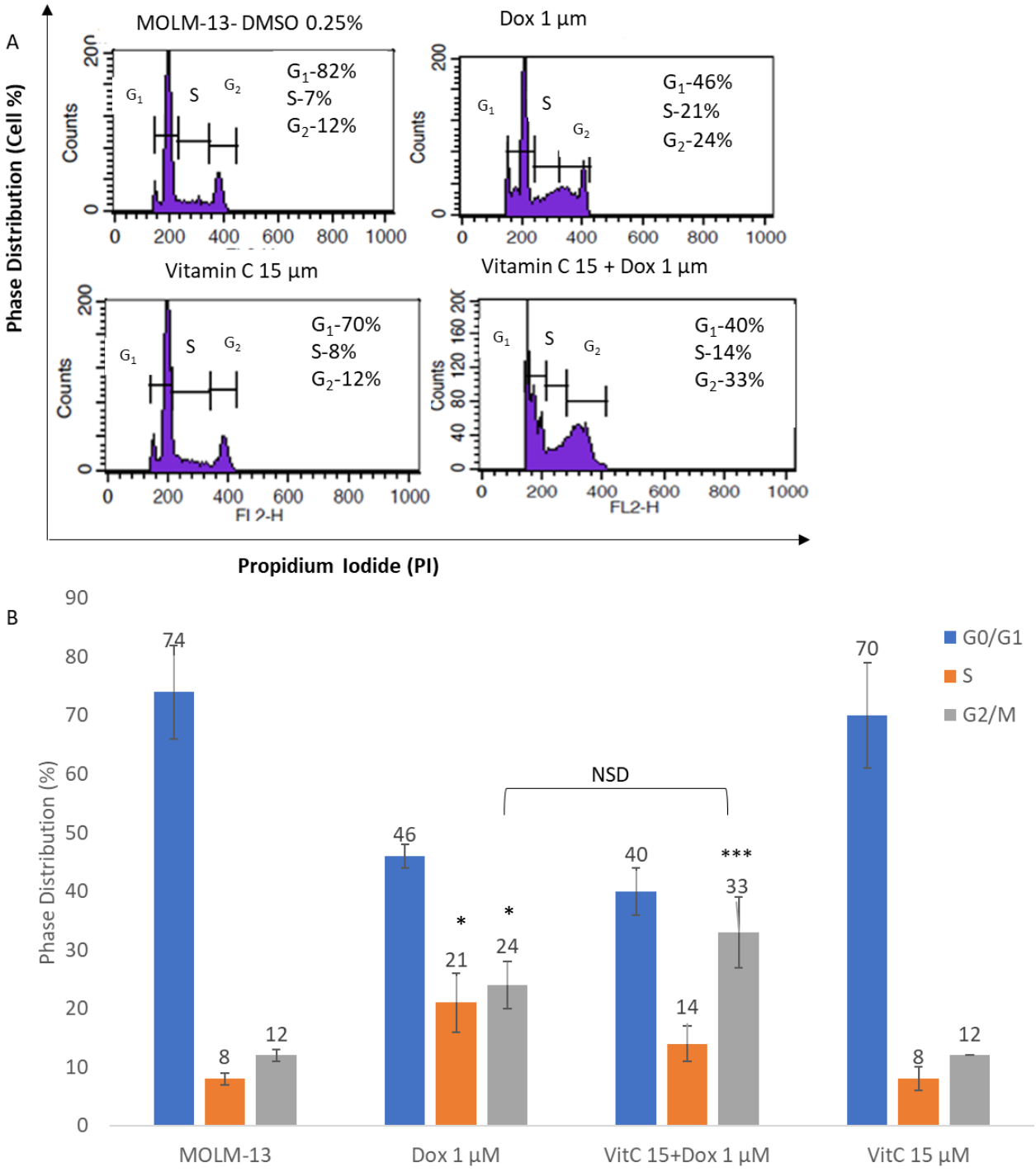


Figure 6.4.2: The effect of combination of vitamin c acid and doxorubicin on the cell cycle after 48 h incubation. A. DNA histogram showing cell cycle distribution of MOLM-13 cells treated with vitamin C 15 µM, Dox 1 µM and combination of Dox and vitamin C for 48 h***. B.*** Data represent three independent experiments(n=3) with one replicate each (n=3 replicates). Cells were harvested, stained with propidium iodide, RNase added and then analysed using flow cytometer. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 compared to control. NSD- No Significant Difference as indicate

6.4.3 Effect of doxorubicin and vitamin C on expression of p53 in MOLM-13

The most observed biological outcome when p53 is activated is the induction of the cell cycle arrest and apoptosis (Chen, 2016). Cell cycle arrest and apoptosis induction by p53 can be exploited due to its significant antitumour potential (Chen, 2016). To investigate whether vitamin C could play a role in activating the tumour suppressor protein p53. MOLM-13 cells was treated with vitamin C 15 µM singly and in combination with Dox 1 µM and expression of activated p53 determined using Western blot after 48 h. Results showed reduced p53 expression (50 %) with vitamin C 15 µM treatment compared to increased expression (235 %) after treatment with Dox 1 µM. Combination of vitamin C 15 µM and Dox 1 µM showed reduced p53 expression (84 %) which when compared to Dox 1 treatment it was 2.8 fold decrease in expression, thus indicating that combination did not show more expression of p53 and also vitamin C reduced the activity of Dox on expression of p53.

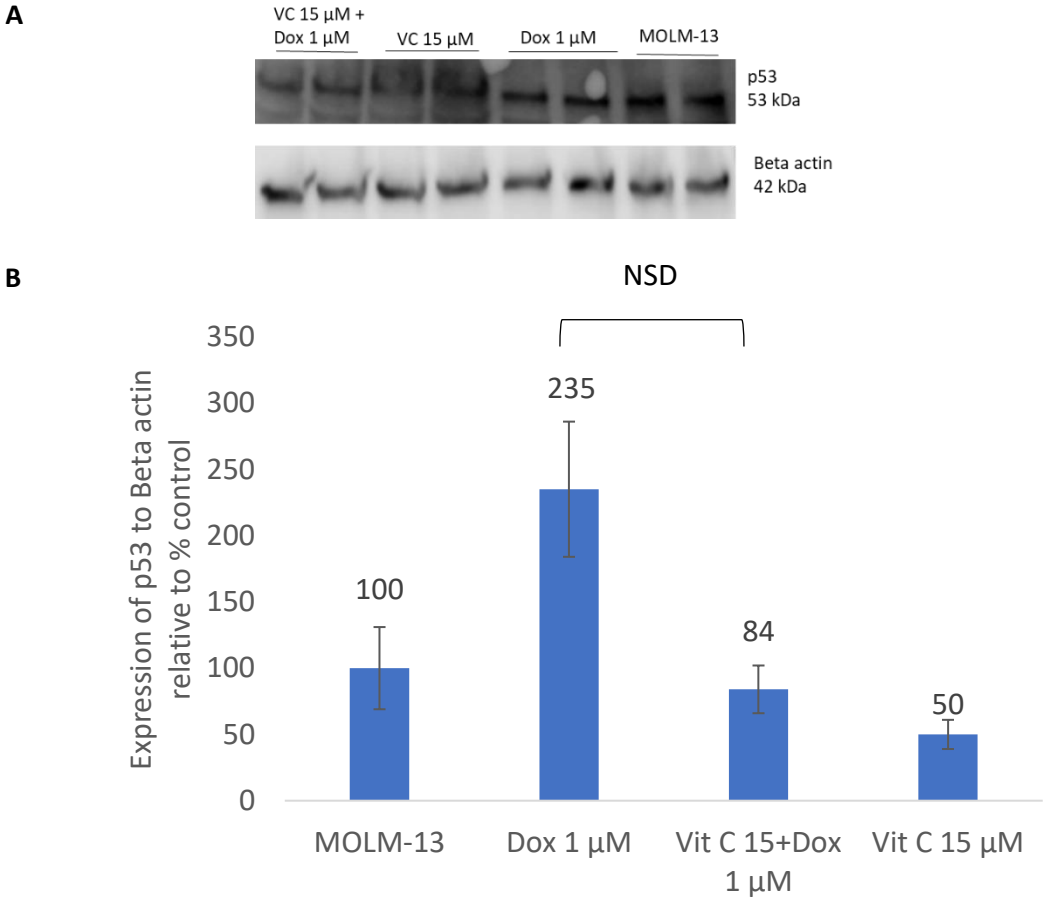


Figure 6.4.3: The expression of p53 after 48 h treatment with vitamin C singly and in combination with doxorubicin on MOLM-13 cells using Western blotting. **A**. Immunoblotting results of p53 expression after treatment with Dox single and in combination with vitamin C in MOLM-13 cells. **B**. Results were presented as % control ± SD. Data represent two experiments (n=2) with three replicates (n=3 replicates) (two replicates within an experiment and one replicate from independent experiment). Cells were harvested, lysate was prepared and subjected to SDS-Page for immunoblotting analysis. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. *NSD- No significant difference as indicated*

6.4.4 Effect of Vitamin c on expression of cell cycle arrest protein p21^{cip1} and senescence protein p16 in MOLM-13

Expression of cell cycle regulatory protein p21^{Cip1} which induce cell cycle arrest was determined after treatment with vitamin C singly and in combination with Dox after 48 h using Western blotting. It has been observed that vitamin C and Dox single treatments showed stimulated expression of p21. Dox 1 µM showed 190 % while vitamin C 15 µM showed even more expression (276%). Expression by both agents individually was strongly enhanced when vitamin C and Dox were combined (455%) showing 2.4-fold increase compared to Dox only (Table 6.4.5). vitamin C single treatment showing more expression of p21 than Dox, enhanced the activity of Dox when combined in the expression of cell cycle inhibitor p21 in MOLM-13 cells. However, increased expression of p21 by combination was not statistically significant when compared to Dox only.

To determine whether senescence stabilizing protein p16 was expressed, p16^{Ink4a} expression was determined. There was similar expression of p16 (0.26%) after treatment with vitamin C compared to Dox single treatment (0.27%). DNA damaging agents that induce DNA breaks has been reported to induce senescence in cancer cells (Zeng *et al*., 2018). Hence, the increased expression observed in this study

Cells treated with vitamin C and Dox combined showed more expression of p16, with 1.6-fold increase (0.44%) when compared to Dox only treatment and vitamin C only. Therefore, showing that the senescence protein stabilizer was expressed after treatment with both vitamin C and Dox individually and when combined more expression was observed compared to Dox only. Expression observed when vitamin C and Dox were combined was not statistically significant when compared to Dox only ($p>0.05$). However, compared to cells without treatment, combination was significant ($p<0.05$).

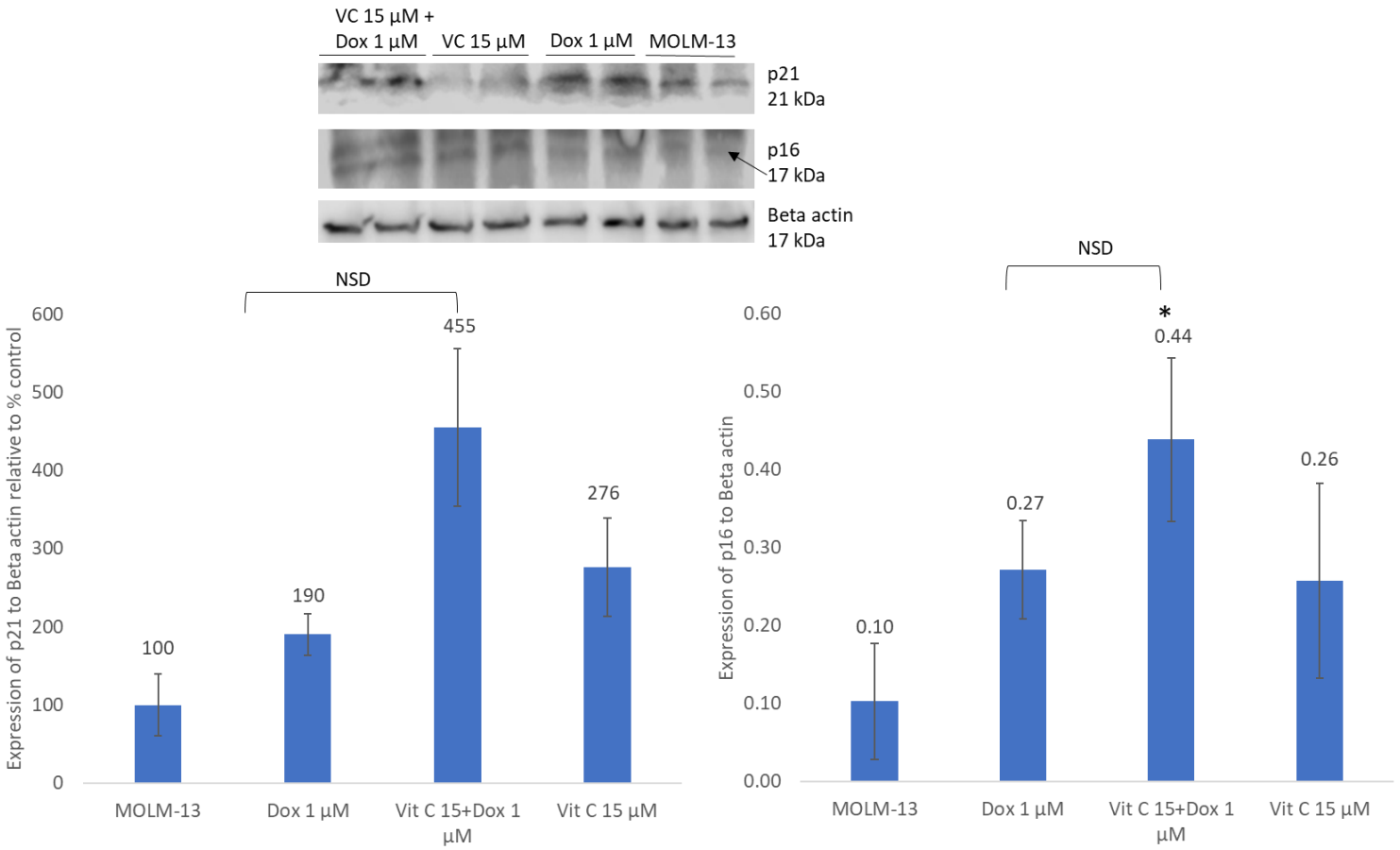


Figure 6.4.4: The expression of p21 and p16 after 48 h treatment with vitamin C singly and in combination with doxorubicin in MOLM-13 cells using Western blotting. **A**. Immunoblotting results of p21 and p16 expression after treatment with Dox single and in combination with vitamin C in MOLM-13 cells. Cell lysates was subjected to SDS-Page for immunoblotting analysis. **B**. Graphical presentation of p21 expression **C.** Graphical presentation of p16. Results were presented as % control ± SD. Data represent two experiments (n=2) with three replicates (n=3 replicates) for p21 (two replicates within an experiment and one replicate from independent experiment). Non quantifiable amount of protein in the control of p16 resulted in data presented as relative to beta actin of two experiments (n=2) with three replicates (n=3 replicates). Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc analysis. **p<0.05, **p<0.01 compared to negative control. ##p<0.01 compared to Dox as indicated, NSD- No Significant Difference as indicated*

6.4.5 Summary of Results

Table 6.4.5 contains summary of results for vitamin C/Dox study. Combination of vitamin C and Dox inhibited cell growth significantly and induced early apoptosis when compared to Dox only. Although there was 2-fold increase of caspase 8 and caspase 9 expression with combination of vitamin C and Dox treatment, compared to Dox only, they were not statistically significant. In addition, 1-fold increase in pro-apoptotic protein Bax with combination of vitamin C and Dox treatment was not significant when compared to Dox only. Furthermore, 2 -fold increase in p21 and 1-fold increase in p16 by combination treatment was not statistically significant when compared to Dox only treatment. When compared to negative control, combination of vitamin C and Dox inhibited cell growth, induced early apoptosis and arrested cells at G_2/M phase. There was 4-fold increase in p16 expression with combination of vitamin C and Dox compared to negative control which was statistically significant. p16 has been referred to as the stabiliser of senescence. However, biomarkers will be recommended to confirm senescence.

Dox single treatment significantly inhibited cell growth when compared to control. 2-fold increase in p53 and p16 expression with Dox single treatment was not statistically significant when compared to negative control. In addition, 1-fold increase in Bax and p21 with Dox only treatment was not statistically significant. Vitamin C single treatment inhibited cell growth and about 3-fold increase fin p21 and p16 expressions were not statistically significant when compared to negative control. p21 inhibit the cell cycle and reported to initiate senescence.

Table 6.4.5: Summary of results showing fold change when compared to control and Dox with significance indicated where observed

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + Vit C (15 μM)	Vit C (15 μM)	Dox (1 μM) + Vit C (15 μM)
CyQuant cell viability assay	0.58↓ (p<0.01)	0.31↓ (p<0.001)	0.69↓ (p<0.01)	0.61↓ (p<0.01)
Cell death assay				
Necrosis	1.63↑	1.02	1.27↑	0.63↓
Late apoptosis	1.01	3.26↑	1.78↑	3.23↑
Early apoptosis	1.81↑	20.95↑ (p<0.001)	3.97↑	11.54↑ (p<0.001)
Cell Cycle				
G0/G1	0.62↓	0.54↓	0.95	1.15↓
S	2.63↑(p<0.05)	1.75↑	1.00	1.5↓
G2/M	2.00↑ (p<0.05)	2.75↑ (p<0.001)	1.00	1.37↑ (NSD)

Table 6.4.5: Cont'd

	Fold change compared to negative control / p value			Fold change compared to Dox (1 μM) / p value
	Dox (1 μM)	Dox (1 μM) + Vit C (15 μM)	Vit C (15 μM)	Dox (1 μM) + Vit C (15 μM)
Pro-apoptotic proteins Positive results are indicated by an **increase** in protein expressions compared to control				
Caspase 3 Extrinsic and intrinsic apoptotic pathways	0.43↓	0.37↓	0.11↓	1.16↓
Caspase 8 Extrinsic apoptotic pathway	0.79↓	1.66↑	1.13↑	2.10↑
Caspase 9 Intrinsic apoptotic pathway	0.90↓	2.13↑	1.73↑	2.37↑
Bak Intrinsic apoptotic pathway	0.60↓ ($p<0.01$)	0.46↓	0.14↓ ($p<0.001$)	1.30↓
Bax Intrinsic apoptotic pathway	1.58↑	2.33↑	0.71↓	1.47↑
Anti-apoptotic protein involved in the intrinsic apoptotic pathway Positive results are indicated by a **decrease** in protein expressions compared to control				
Bcl-2 Intrinsic apoptotic pathway	0.78↓	1.88↑	0.78↓	2.41↑
Tumour suppressor protein Positive results are indicated by an **increase** in protein expressions compared to control				
p53	2.35↑	0.84↓	0.50↓	2.79↓
Cell Cycle inhibitor & senescence promoter Positive results are indicated by an **increase** in protein expressions compared to control				
p21	1.90↑	4.55↑	2.76↑	2.39↑ (NSD)
Senescence stabiliser Positive results are indicated by an **increase** in protein expressions compared to control				
p16	2.7↑	4.4↑ ($p<0.05$)	2.6↑	1.63↑ (NSD)

6.5 DISCUSSION

6.5.1 Vitamin C enhances the cytotoxicity of doxorubicin in AML MOLM-13 cell line

Vitamin C has been reported to inhibit the growth of leukaemic cells and induced G_0/G_1 elongation (cell cycle arrest) (Park *et al*., 2004), have selective H_2O_2 cytotoxicity (Chen *et al*., 2005) and reduce cardiotoxicity of doxorubicin (Ludke *et al*., 2017). However, the mechanism of combined Dox and vitamin C induced cytotoxicity with regards to apoptosis, cell arrest and senescence as regulated by tumour suppressor protein p53 on leukaemic cells is still unclear. Therefore, mechanism of low dose vitamin C (15 µM) and Dox (1 µM) combined in MOLM-13 was examined in this study.

In this study, inhibition of cell growth following vitamin C treatment was observed in a dose dependent manner with IC_{50} of 15 µM which is less than the physiological concentration (100 µM) and this concentration can be easily achievd in the plasma via supplement or diet (Sant *et al*., 2018). Vitamin C selectively inhibited MOLM-13 cells but no inhibitory effect was observed in normal cells in this study. Vitamin C has been reported not to significantly affect the proliferation of normal cells (Kim *et al*., 2008). It has been suggested that the presence of catalase (antioxidant) is responsible for the suceptible (cancer cells) and resistance (normal) of cells to the cytotoxicity of vitamin C (Parrow *et al*., 2013). This is because H_2O_2 has reduced effect on normal cells due to the precence of catalase, while in cancer cells absence of catalase results in their susceptibility to vitamin c in induction of cell death through H_2O_2 generation. Resistance to vitamin c in breast cancer cell line was reverse after knockdown of catalase resulting in suceptibility and cell death, and sensitivity of pancreatic cancer cells to vitamin C was reverse after pretreatment with catalase or an adenovirus vector containing catalase complementary DNA (cDNA) (Du *et al*., 2010; Klingelhoeffer *et al*., 2012). Catalase in addition to inhibiting vitamin C mediated cell death in normal cells, prevents depletion of cellular ATP levels, although ATP may not be a component of vitamin C induced cytotoxicity (Parrow *et al*., 2013). H_2O_2 and superoxide may play pivotal role in sensitivity to vitamin C (Parrow *et al*., 2013).

Doxorubicin inhibited MOLM-13 cells in a dose-dependent manner and IC_{50} 1 µM, which on normal cells showed less than 30% inhibition was used in this study for combination. Physiological concentration (100 µM and below) of vitamin C has been reported to be synergistic with

chemotherapy drugs (Kurbacher *et al*., 1996). In this study 1 µM of Dox combined with 15 µM vitamin C showed more effect in inhibiting cell growth (31%) ($p<0.001$) compared to single treatment with vitamin C (69%) or Dox. (58%). This result is in aggrement with a report showing that low dose vitamin C (5 µM) combined with chemotherapy drug (0.1 µM methotrexate) and vitamin C 5 µM combined with 10 µM methotrexate inhibiting growth of triple negative breast cancer cell lines (Wu *et al*., 2017).

Apoptosis is a cellular response to stresses (oxidative or genotoxic) and DNA damage. It is considered a therapeutic response (MVOB *et al*., 2016). In this study combination of Dox and vitamin C when compared to single treatment wit Dox modulated increased apoptosis from 1.72% to 11.29%, decreased necrosis from 5.94% to 3.73% and increased cell arrest at G_2M phase of the cell cycle from 24% to 33%. Although early apoptotic cell death with combined drug was statistically significant when compared to Dox ($p<0.001$). However, cell arrest at G_2M phase was not significant when compared Dox only treatment ($p>0.05$). Vitamin C has been reported to protect against cardiotoxicity induced by Dox as an adjuvant therapy that effectively counteract Dox induced cardiotoxicity (Ludke *et al*., 2017). Which is consistent with the result in our study indicating decreased necrosis observed when Dox was combined with vitamin C. Therefore, suggesting protective function of vitamin C against Dox adverse effect.

Combined apoptotic effect of vitamin C and chemotherapy drugs when combined has been suggested. Vitamin C has been reported in generating extracellular H_2O_2 that regulates apoptosis and redox signalling by the release of cytochrome c, an early event of apoptosis. While chemotherapy drugs induce apoptosis through response to stress and DNA damage, thus combined anticancer effect when combined with vitamin C (MVOB *et al*., 2016). Pharmacological doses of vitamin C (0.3 mM-20 mM or 100 mM) have been reported to inhibit the effect of methotrexate, doxorubicin and cisplatin when applied in combination with them (Heaney *et al*., 2008). However, in some studies, pharmacolgical doses have shown increased anticancer effect in ovarian cancer in combination with placlitaxel and carboplatin (Ma *et al*., 2014) and in pancreatic cancer in combination with gemcitabine (Espey *et al*., 2011). Thus suggesting that synergism depends on the type of chemotherapy drug combined and cancer studied.

Mechanism of induction of apoptosis after treatments in this study was observed to occur via mitochondria dependent pathway and extrinsic pathway. which was indicated by expression of

caspase 8 and 9 proteins after single treatment with vitamin C and more when combined with Dox. However, expression of capase 8 and caspase 9 was not significant ($p>0.05$) when combined drug was compared to Dox only treatment and vitamin C single treatment.

Expression of Bax (apoptosis regulator) which is activated by tumour suppressor protein p53 was observed after treatment with Dox and vitamin C combined. However, increased Bax expression by combined drug was not statistically significant when compared to Dox only. Vitamin C significantly ($p<0.01$) inhibited Bak in this study. Expression of p53 could play a role in the induction of apoptosis, cell cycle arrest and senecence which are DNA damage response pathways activated when p53 is active. Whether vitamin C activates p53 has been a subject of debate but in this study less expression of p53 protein was observed after single treatment with vitamin C.

Vitamin C has been reported to induce G_0/G_1 cell cycle arrest in leukaemia cells and it arrest is dependent on cell type and application doses (Parrow *et al*., 2013). In this study G_0/G_1 arrest was observed with vitamin C single treatment, S and G_2/M cell arrest with Dox single treatment and more G_2/M arrest with combined Dox and vitamin C treatment. However, more G2/M arrest observed with vitamin C and Dox combined was not significant compared to Dox only but was significant ($p<0.001$) when comapred to negative control. G_2/M arrest suggests irreversible cell arrest that could lead to apoptosis or senescence. Upon further analysis of cell arrest mechanism, it was observed that there was increased expression of cell cycle arrest protein p21, when Dox and vitamin C were combined compared to Dox single treatment. However, p21 expression with combined drug was not statistically significant when compared to Dox single treatment ($p>0.05$). p21 acts on cdk/cyclin complex to inhibit progrssion from one phase of the cell cycle to the other and its expression suggest its activation by p53 through either ATM/CHK2 or ATR/CHK1 pathways. Vitamin C has been reported to induce G1 phase arrest in B16F10 melanoma cells and during G1 arrest there were increased p53-$p21^{wafi/cip1}$ levels leading to the induction of cell arrest (Hahm *et al.*, 2007). cdk2 necessary for G1 progression was reported to be inhibited when $p21^{wafi/Cip1}$ increased (Hahm *et al*., 2007). There has been no reports of the effect on cell cycle by combination of vitamin C and Dox in AML cell line. This study is therefore the first to report of the effect induced by a combination treatment on MOLM-13, through the induction of G_2/M arrest. Simialr observation was reported with vitamin C inducing mild G_2/M arrest when combined with the chemotherapy drug Mitoxantrone in breast cancer cell lines compared to G_0/G_1 arrest

observed with vitamin C single treatment (Guerriero *et al*., 2014). However, G_2/M arrest with vitamin C and Dox treatment in this study was not significant when compared to Dox only.

Senescence proteins to suggest senescence were determined and it was observed that cells treated with vitamin C and Dox single treatments induced p21 and p16 expression, with vitamin C expressing more p21 than Dox single treatment. When Dox was combined with vitamin C, the expression of p21 and p16 increased suggesting vitamin C enhanced the activity of Dox in affecting the expression of p21, while p16 protein expression was similar for single treatments, which are crucial proteins in the activation of irreversible senescence, a cancer inhibiting mechanism. However, expression of p21 and p16 with combined drug was not significant when compared to Dox only ($p>0.05$). p21 via p53 initiate the mechanism of senescence, while p16, the guardian of sensencence stabilise senescence. Therefore in this study, vitamin C and Dox combined may suggest some activity of senescence via p16 which was significant when compared to negative control.

Although vitamin C inducing p53 activation has been a subject of debate, there has been report of vitamin C inhibiting p53 expression in bladder cancer cells (Kim *et al*., 2008). However, vitamin C has also been reported to increase p53 expression when combined with cisplatin after 24 h in colon cancer cell line HCT116 compared to single treatment with vitamin C (An *et al*., 2011). In addition it has been reported to increase expression of p53 in melanoma cells (Hahm *et al*., 2007). In this study, vitamin C inhibited the expression of p53 and when combined with Dox it suppressed the expression (84%) when compared to Dox single treatment (235%) although combination was higher than vitamin C single treatment (50%).

Vitamin C has been reported in the cell line B16F10 to induce G1 arrest in melanoma via increase in p21 and p53 expression (Hahm *et al*., 2007). In this study, vitamin C induced G1 arrest via expression of p21 without signifcant effect on p53. In addition, it was observed that vitamin C induced increased expression and activity of p21 and p16. This effect was stronger when combined with Dox compared to Dox only treatment. Suggesting vitamin C positive effect on combination treatment. However, p21 and p16 expression was not statistically signifiantly when compared to Dox only. Vitamin C has been reported to inhibit p53 induced senescence through inhibition of ROS and p38 MAPK in bladder cancer cells (Kim *et al*., 2008). It has been reported to suppress

expression of p53 which was independent of its inhibitor Mdm2 and then inhibition of senescence (Kim *et al*., 2008).

Taken together, vitamin C could be observe to exert different anticancer mechanisms with regards to p53, p21 and p16, while inducing G1 arrest in most cancer cells such as melanoma cells (Hahm *et al*., 2007), prostate (Fromberg *et al*., 2011), breast (Guerriero *et al*., 2014), lung (Li *et al*., 2010) and liver (Sajadian *et al*., 2016). It inhibited the expression of p53 in bladder cancer via reduction of ROS and p38MAPK, colon cancer and in this study with leukaemic cells while increase expression was observed in melanoma cells via checkpoint kinase 2.

To the best of the author's knowledge, this is the first time that a study of the effect of vitamin C on both p21 and p16 proteins either singly and with Dox was conducted. Although there was reduced p53 expression and senescence features reported in bladder cancer, reduced p53 expression in this study did not affect or reduced the expression of p21 and p16 either singly or in combination with Dox. However, vitamin C has been reported to induce expression of both p53 and p21 in melanoma cells (Hahm *et al*., 2007). Similar to our study in leukaemia, vitamin C induced more of p53 when combined with cisplatin than single treatment with vitamin C in colon cancer (An *et al*., 2011). Therefore, expression of p53, 21 and p16 could differ in different cancer cells depending on the mechanism involved, which suggest further study of each cellular mechanism in each cancers cells to be established.

In conclusion, physiological dose (low concentrations) rather than pharmacological dose, enhanced the actvity of Dox in this study with MOLM-13 cells. Vitamin C and Dox combined treatment induced synergistic inhibition of cell growth, induced apotosis via both intrinsic (caspase 9) and extrinsic (caspase 8) pathways. However, both caspase 8 and caspase 9 were not significant when compared to Dox only and negative control. G_2/M cell arrest observed when combined was via increased expression of p21, and the mechanism of induced senescence was stabilized by expression of p16. However, although combined drug showed increased G_2/M arrest, p16 and p21 expression, they were not statistically significant when compared Dox single treatment. This study reveals that vitamin C combined with Dox inhibit cell growtth and induced more early apoptosis compared to Dox only in AML MOLM-13 cells.

CHAPTER SEVEN

7. General Discussion and Concluding Comment

The potential of treatments as FLT3 kinase inhibitors to overcome abnormal phosphorylation of ITD mutation in AML cell line was evaluated in this study**.** FLT3-ITD is a driver mutation (initiation and development of AML) that confers poor prognosis for reduced overall survival (Ma *et al*., 2015). Treatment with FLT3 inhibitors is limited by incomplete response and acquired resistance (Guo *et al*., 2012), resulting in high relapse rate after treatment (Green *et al.*, 2015). Therefore, a promising therapeutic target for AML. Treatment of viable cells could undergo apoptosis, cell cycle arrest or senescence due to activation of tumour suppressor protein p53 which is reported inactive in over 50% of cancer cells. p53 could be inactive due to mutation or overexpression of p53 inhibitors (MDM2 and MDM4). However, induction of DNA damage or oxidative stress by anticancer drugs could trigger the release of p53 bound to its inhibitor resulting in its activation and induction of cellular outcome (Figure 7.1).

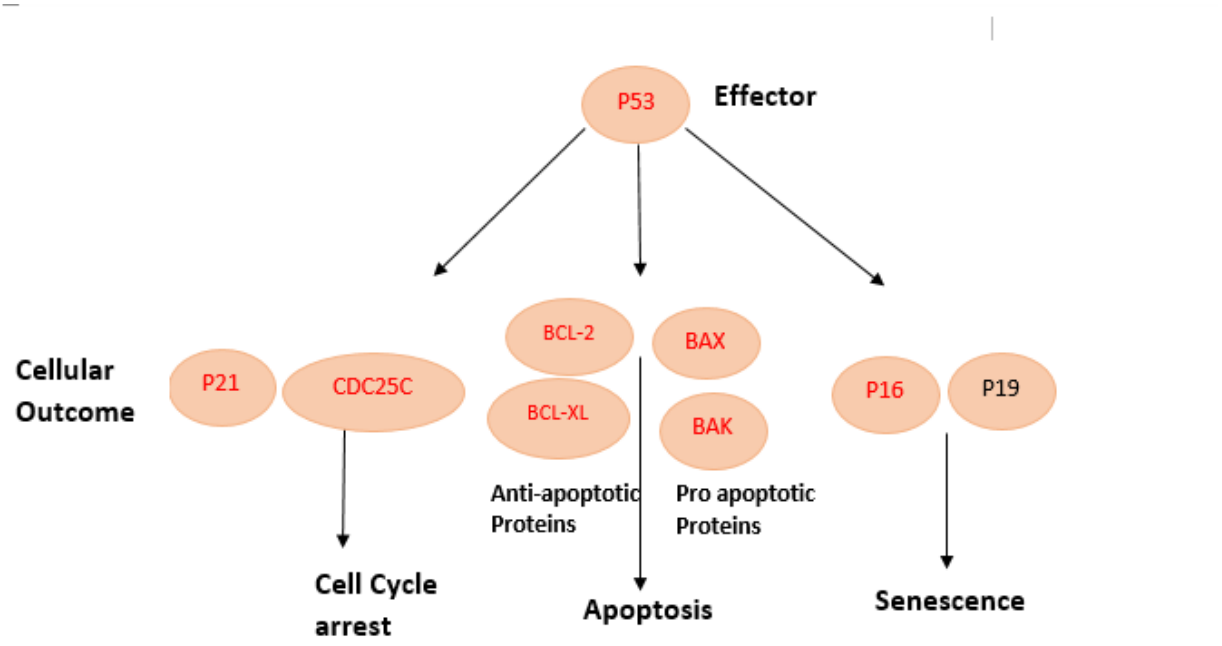


Figure 7.1: DNA damage response pathway (DDR) involving p53 and cellular outcomes (cell cycle, apoptosis and senescence). Adapted from (Blanpain *et al*., 2011).This study was designed to determine the therapeutic potential of three phytochemicals (α-mangostin, gallic acid and vitamin C) to sensitise the AML cell line, MOLM-13 to the cytotoxic effect of doxorubicin (Dox). Dox is already known to induce cell death in cancer cells through the inhibition of topoisomerase II inhibitor and the above-stated phytochemicals have been reported to induce apoptosis and cell cycle arrest in different cancer cells, including leukaemia cells *in vitro* (Table 4.1, Table 5.1 and Table 6.1). Other cellular

processes including senescence, in addition to cell apoptosis and cell cycle arrest were investigated to determine the possible mechanism of action of the drugs. In addition, the potential of the drugs as FLT3 kinase inhibitors to overcome abnormal phosphorylation of ITD mutation in AML cell line was investigated.

Doxorubicin (Dox) has been reported to have a synergistic effect when it was combined with vitamin C to enhance cytotoxicity on breast cancer cell lines (Kurbacher *et al*., 1996; Bober *et al*., 2017). However, currently there are no studies reported on the combined treatment on acute myeloid leukaemia. In another study, Dox neurotoxicity was suppressed by xanthone supplement (containing α-mangostin), indicating possible protective effect of α-mangostin (Tangpong *et al*., 2011). In addition, the cardiotoxicity induced by Dox was shown to be prevented by gallic acid (Kulkarni & Viswanatha, 2015) and a synergistic inhibition of cell growth in prostate cancer cell line when combined with gallic acid was also observed (Chen *et al*., 2009). This suggests that these phytochemicals could be protective of normal cells and could synergies with doxorubicin to induce significant anticancer effects in AML cell line. The aim of this study was to evaluate the anticancer effect of the established chemotherapy drug doxorubicin both singly or in combination with the phytochemicals α-mangostin, gallic acid and vitamin C, in a relapsed acute myeloid leukaemic cell line MOLM-13.

Interestingly, all combinations studied (Dox and α-mangostin; Dox and gallic acid; Dox and vitamin C) showed synergistic inhibition of cell growth compared to single treatments. This result is consistent with previous report on gallic acid demonstrating synergistic interaction with doxorubicin in prostate cancer cell line (DU145 cells), inhibiting proliferation of cells (Chen *et al*., 2009).

Induction of programmed cell death can be activated via intrinsic/mitochondria or extrinsic pathways. Proapoptotic proteins (Bax and Bak) in response to apoptotic signals form pores on outer mitochondria membrane and release cytochrome C, which forms apoptosome with Apoptosis Activating Factor 1 (Apaf-1) in the presence of ATP. Resulting in the activation of caspase 9 and then caspase 3 due to caspase cascade as classical intrinsic pathway of apoptosis. In this study, treatment of the acute leukaemic cell line, MOLM-13, with Dox only, showed an increased expression of the proapoptotic protein Bax but not Bak, and reduced anti-apoptotic protein, Bcl-2 compared to cells without treatment. However, statistically only Bak inhibition was significant

($p<0.05$) when compared to control. Cell viability studies indicated that combined treatments of Dox with either α-mangostin, gallic acid or vitamin C could enhance the cytotoxicity. Therefore, when Dox was combined with α-mangostin, induction of apoptosis was associated with the expression o Bak and inhibition of antiapoptotic protein Bcl-2 were observed. In addition, it was observed that combination treatment of Dox and α-mangostin expressed some significant level of Bak ($p<0.05$ compared to Dox), while Dox when combined with gallic acid induced more significant level of Bak ($p<0.01$) compared to Dox only. However, although treatment with Dox combined with vitamin C showed enhanced Bax expression, compared to Dox only it was not statistically significant ($p>0.05$). This suggests that more apoptotic effect observed with all combinations compared to Dox only treatment was associated with Bak expression except vitamin C combination. There was increased expression of antiapoptotic protein Bcl-2 after treatment with Dox combined with either gallic acid or vitamin while reduced levels was observed with Dox and α-mangostin combination. However, reduced or increased expression of Bcl-2 observed with the combinations was not statistically significant when compared to Dox only treatment. Bcl-2 or other antiapoptotic proteins such as Bcl-xl or MCL-1 inhibits the effect of Bak and Bax by preventing their pore forming activity on outer mitochondria membrane (Galluzzi *et al*., 2018). Gallic acid has been reported to increase expression of Bcl-2 but induced apoptosis via Apoptotic inducing factor (AIF) and increased expression of AIF suggest its effect on GA induced apoptosis (Maioral *et al*., 2016). Therefore, Dox stimulated Bax but not Bak and inhibited Bcl-2 in MOLM-13 cells. Bax expression increased when α-mangostin and vitamin C were combined with Dox but not with gallic acid. However, increased expression of Bax with α-mangostin and vitamin C combinations were not significant when compared to Dox only treatment. Although Bak level was reduced following Dox treatment, it was increased significantly when α-mangostin was combined ($p<0.05$) and when gallic acid was combined ($p<0.01$). Vitamin C inhibited expression of Bak when combined with Dox.

Formation of apoptosome after the release of cytochrome C activate caspase 9 which joins the apoptosome complex to activate effector caspase 3 leading to cell death via intrinsic or mitochondria pathway. Activation of caspase 8 is via binding of stress ligand to death receptor which activate formation of death inducing signaling complex (DISC) that converts procaspase 8 to active caspase 8 which initiates the extrinsic pathway. Caspase 8 activated, could either activate caspase 3 directly or indirectly via Bid which is translocate to the mitochondria to activate Bak

and Bax to trigger permeabilisation of the mitochondria. It was observed that MOLM-13 cells treated with Dox only did not suggest expression of any caspase, while α-mangostin treated cells stimulated expression of caspase 3 compared to untreated cells. However, statistically it was not significant. Although combination of Dox and α-mangostin suggests only expression of caspase 3 and 9, combination of Dox and gallic acid did not express any caspases compared to Dox only treatment. Moreover, when doxorubicin was combined with vitamin C enhanced expression of caspase 8 and 9 were shown in comparison to negative control. However, the enhanced expression of caspase 8 and 9 were not significant when compared to Dox only and negative control. Initiation caspases (caspase 9 and caspase 8 for intrinsic and extrinsic pathway, respectively) and effector caspase (caspase 3 for both pathways) amplify apoptotic signaling pathways that result in rapid cell death. Activation of caspases indicates an irreversible commitment to cell death (Elmore, 2007). Thus, both pathways (intrinsic or mitochondria and extrinsic pathway) of apoptosis might be involved with vitamin C and Dox combined treatment. Enhanced synergistic expression Bak following doxorubicin combined with α-mangostin treatment could be involved in releasing cytochrome C from the mitochondria during apoptosis.

Taken together, doxorubicin by itself did not stimulate expression of caspase 3 in this study. However, when combined with α-mangostin expression of caspase 3 was observed compared to control. When combined with gallic acid reduced expression was observed and with vitamin C, it failed to enhance the ability of Dox to stimulate caspase 3. α-Mangostin has been reported not to stimulate expression of caspase 3 in B-cell (CLL) leukaemia cell line (Menasria *et al*., 2008). The result is consistent with findings from this study. However, although not statistically significant, combination of α-mangostin with Dox in this study was observed to enhance caspase 3 expression than single treatment with Dox only. Caspase 8 level reduced after treatment with Dox, and when combined with α-mangostin caspase 8 was stimulated. When combined with vitamin C expression of caspase 8 was observed. In addition, Dox did not induce caspase 9 expression and when combined with α-mangostin it was inhibited. Interestingly, caspase 9 level increased when vitamin C was combined with Dox. However, compared to Dox only increased expression of caspase 9 was not statistically significant. Therefore, 2-fold increase in caspase 8 and 9 (Table 6.4.5) with vitamin C and Dox combination treatment was not statistically significant when compared to Dox only.

Induction of apoptosis following cell cycle arrest could be due to lack of DNA repair resulting in tumour suppression. Cell cycle deregulation underlies uncontrolled cell proliferation in cancer (Williams & Stoeber, 2012). Both apoptosis and cell cycle arrest limit the propagation of mutations following induction of cellular stress such as DNA damage. The cell cycle consists of G_0/G_1, S and G_2/M phases, which are regulated by specific cyclin/cdk complexes that allow migration of cells from one phase to the other till the cell cycle is completed. In addition, there are cell cycle inhibitors such as p21 that inhibits cell progression in the cell cycle when DNA defect is detected. The mechanism of cell cycle arrest was studied and combination effects on arrest of the cell cycle was determined. Doxorubicin induced S and G_2/M phase arrest. When combined with α-mangostin, gallic acid or vitamin C more G_2/M arrest was observed. However, combinations showing more G_2/M phase arrest was not statistically significant when compared to Dox only ($p>0.05$), but significant when compared to untreated cells ($p<0.001$) Thus, synergistic effects were observed with the induction of more G_2/M arrest in all combinations. In addition, G_2/M arrest observed in all combinations and Dox only treatment has been reported to indicate irreversible cell arrest, leading to induction of apoptosis or senescence, as G_2/M arrest is also referred to as the onset of senescence which is an anti-tumour mechanism (Feringa *et al*., 2018).

Furthermore, to determine molecular mechanism of cell arrest and apoptosis due to DNA damage response, p53 expression was studied, which has been reported inactive in leukaemia (Zheng *et al*., 1999). p53 activate p21 (cdk/cyclin complex inhibitor) which induces cell arrest and was also determined in this study. Doxorubicin stimulated the expression of p53, which was also shown for α-mangostin single treatment. However, vitamin C and gallic acid single treatments did not induce the expressions of p53. Combination of Dox and α-mangostin induced enhanced expression of p53 compared to Dox only treatment (although not statistically significant $p>0.05$) and compared to negative control. Surprisingly, the expression of p53 after single treatment with α-mangostin was high when compared to combination with Dox and untreated cells. For the first time to the best of our knowledge, α-mangostin was reported to induce p53 expression more than its combination treatment with Dox in a leukaemic cell line. MOLM-13 cells treated with combination of Dox and gallic acid showed reduction of p53 expression (55%) compared to Dox treatment and reduced p53 expression (84%) compared to control was observed with vitamin C combined with Dox treated cells. Indicating that Dox expressed p53 more compared to when combined with vitamin C and gallic acid. Therefore, Dox combined with α-mangostin suggest more activity of p53 to

compared Dox only. However, despite increased expression of p53 with α-mangostin combination, compared to Dox only, it was not statistically significant.

Doxorubicin as well as all three studied phytochemicals showed increased p21 levels. Cells treated with all combinations in this study expressed more of p21 compared to Dox single treatment. While vitamin C and gallic acid enhanced Dox when combined, Dox enhanced α-mangostin in expression of p21. Therefore, this suggests that irrespective of the level of p53 expressed, the cell cycle inhibitor p21 was activated either singly or in combination with doxorubicin. However, in all combinations p21 expression were not statistically significant when compared to Dox only ($p>0.05$). Rise in p21 levels at G2 arrest observed with all combinations could occur due to enhanced ATR dependent signaling via p53 that drives cells to permanent cell cycle exit (Feringa, et al., 2018). In addition, DNA damage detected at G_2/M result in irreversible cell cycle exist within a few hours while there is recovery from DNA damage when arrest occurs at any other phase of the cell cycle (Krenning, et al., 2014).

Induction of senescence could involve activation of p21, p16, pRB and p53 proteins (Kuilman *et al*., 2010) and most DNA damage agents induce senescence. Senescence is an anticancer mechanism that inhibit tumorigenesis (Milanovic *et al*., 2017). In this study, senescence stabilising protein p16 was studied to suggest effect on senescence pathway. Doxorubicin induced expression of p16 as well as p21 and p53. This study showed that vitamin C and gallic acid single treatment induced p16 expression while α-mangostin treated cells did not express p16. However, all the phytochemicals combined with doxorubicin may induce cellular expression of p16. Therefore, Dox enhanced α-mangostin in expression of p16 while vitamin C enhanced Dox when compared to Dox only treatment. Although gallic acid and Dox single treatments induced p16 expression, when combined there was significant expression of p16 ($p<0.01$) compared to untreated cells but the expression was reduced when compared to Dox only treatment (Figure 5.4.4). These results suggest that senescence may be activated with combined treatment of Dox and the phytochemicals after G_2/M arrest. However, increase expression of p16 with combinations compared to Dox only treatment were not statistically significant.

Cells treated with α-mangostin and vitamin C when combined with doxorubicin showed dramatic increase in expression of p16. There was 4-fold increase ($p<0.05$) with vitamin C combination and 3-fold ($p>0.05$) increase with α-mangostin combination, while gallic acid combination showed 1-

fold ($p<0.01$) increase in p16 when compared to control. α-Mangostin and gallic acid when combined with Dox induced increased p21 level although more expression of (p53 and p21 was observed with α-mangostin and doxorubicin combination. However, statistically, p21, p16 and p53 expressions with combination treatments were not significant when compared to Dox only.

Therefore, doxorubicin combined with α-mangostin could induce apoptosis was associated with Bak expression, gallic and dox combined also induced apoptosis was associated with Bak while vitamin C combined with Dox may suggest induction of apoptosis via caspase 8 and caspase 9. However, the increased expressed expression of caspase 8 and 9 were not statistically significant when compared to Dox only in this study. Further studies on anticancer effect observed with Dox and α-mangostin combined on FLT3 mutation in AML was determined. FLT3 gene found on chromosome 13q12 auto phosphorylate and dimerises upon binding of its ligand. Resulting in exposure of the intracellular tyrosine kinase domain that leads to phosphorylation of downstream molecules and activation of signaling cascades that promotes gene transcription that regulates survival, proliferation and differentiation of cells (Larrosa-Garcia & Baer, 2017). FLT3-ITD is a mutation in AML that is not only known to be common, but involved the most in AML, have the worse prognosis and treatment outcome. It is known as the driver of AML disease due to its important role in haematopoiesis that includes cell growth and survival and are therefore, found in haematopoietic stem cells and progenitor cells. FLT3-ITD result in loss of autoinhibition which leads to a constitutively active tyrosine kinase. Hence, FLT3 mutation has become an attractive target in AML.

In this study, doxorubicin singly and in combination with α-mangostin was further studied to determine effect on FLT3-ITD phosphorylation that is reported to promote aberrant signaling and proliferation of AML cells. Interestingly, doxorubicin and α-mangostin single treatments did not inhibit the expression of FLT3-ITD phosphorylation, however, when combined synergistic response was observed that resulted in significant inhibition when compared to Dox only treatment ($p<0.05$) and negative control ($p<0.01$).

FLT3-ITD mutation involving the cell cycle regulation has been reported and the inhibition of FLT3 resulted in reduced cdc25s phosphorylation (Perner *et al*., 2016). Aberrant signaling of FLT3-ITD increased cdc25 expression and this was dependent on STAT5 (Bertoli *et al*., 2015). It has been reported that inhibition of cdc25s triggered cell arrest and cell death in FLT3-ITD and

resistant cells but not WT-FLT3 (normal FLT3 cells) (Bertoli *et al*., 2015). In this study, expression of cdc25A, cdc25B and cdc25C were determined. Combination of Dox and α-mangostin showed reduced expression of all three cdc25s (cdc25A-43%, cdc25B-69%, cdc25C-39%) after treatment and the reduction was statistically significant when compared to Dox only treatment ($p<0.05$). This result is in good agreement with inhibition of FLT3-ITD phosphorylation observed with Dox and α-mangostin combined treatment. Hence, inhibition of FLT3-ITD receptor could prevent constant aberrant phosphorylation of cdc25s, which promotes cell arrest and cell death in AML cells with FLT3-ITD mutation.

Cdc25A and cdc25C has been reported in oncogenic FLT3-ITD signaling as an early target and an important player in proliferation of AML cells and differentiation arrest (Bertoli *et al*., 2015) (Perner *et al*., 2016). Therefore, Dox and α-mangostin combined is reported in this study to inhibit early target (cdc25 inhibitor) of FLT3-ITD signaling and promising FLT3-ITD inhibitor in AML therapy. To the best of the author's knowledge this is the first report on doxorubicin combined with α-mangostin on FLT3-ITD phosphorylation in AML MOLM-13 cells. Therefore, further research is warranted to explore possible mechanism of these significant clinical findings with doxorubicin when combined with α-mangostin.

What is already known?

ATR and ATM are kinases that activate chk1 and chk2 in response to single strand and double strand break, respectively. Chk1 and Chk2 respond to genotoxic stress. Chk1 is always active, further activated by DNA damage, strictly restricted to S and G_2/M phase hence activated during S and G_2/M arrest irrespective of double and single DNA strand break. p53 is not mutated in AML but inactive. P21 is a two-faced protein due to exhibition of different activity or function that is dependent on p53 status. Anticancer activity of p21 is when activated by p53 while oncogenic effect is when not activated by p53. In AML p21 displays anticancer effect and is independent of p53. P16 is a stabilizer and guardian of senescence which is an anticancer mechanism. Removal of senescence cells reduces aging by 25%.

G_2M arrest is maintained by p53 and p21, induced by DNA damage agents and seen as the first step of irreversible permanent cell arrest and termed onset of senescence. Hence all combinations in this study showing more G_2/M arrest indicates irreversible cell arrest. P21 is not involved in p53 induced cell death but involved in p53 induced senescence. p53 inactivation, tumour suppressor

protein levels, strength of oncogenic signal level and absence of p16 can result in re-entering of cells into the cell cycle due to premature senescence. α-Mangostin belongs to class of compounds called xanthone, it is an antioxidant, it inhibits topoisomerase I and II, induce apoptosis and cell arrest on cancer cells and has effect on normal cells.

What is not known? (New findings/Key points)

In this study, synergism of doxorubicin when combined with α-mangostin, gallic acid and vitamin C in inhibition of cell growth in AML cell line MOLM-13 was observed. Combination of Dox and α-mangostin induced the most apoptotic effect with increased p53, Bax, Bak, caspase 8 and caspase 3 compared to other combinations studied in MOLM-13. However, compared to Dox treatment, only Bak protein was significant. α-Mangostin induced more expression of p53 than Dox, gallic acid and vitamin C in MOLM-13. For the first time to the best of our knowledge, α-mangostin is reported to induce p53 expression more than its combination treatment with Dox in a leukaemic cell line MOLM-13. Gallic acid and Dox combined induced cell cycle arrest as DNA damaging agents by arresting cells at G_2/M phase in MOLM-13. All combinations studied showed G_2/M phase arrest. However, G_2/M cell arrest by combination was not significantly different from cell arrest by Dox only.

Vitamin C and α-Mangostin when combined with doxorubicin dramatically induced increased expression of p21 in MOLM-13. Gallic acid for the first time is reported to suggest induction of senescence. α-Mangostin in combination with Dox suggest more expression of p53 protein compared to other combinations studied. Doxorubicin in combination with α-mangostin induced synergistic inhibition of FLT3-ITD phosphorylation and subsequent inhibition of cdc25s.

7.2 Concluding Comments

The future of cancer treatment is in the combination of anticancer agents to target multiple pathways deregulated in cancers. Other advantages of combination include targeting more critical molecular process, delivering low dose with lower toxicity, increase tolerance and reduce resistance (Chen *et al*., 2009). In AML, tailored treatment is deemed highly necessary for each patient due to the heterogeneity of the disease and presence of different mutations. The current

study showed that doxorubicin can be enhanced when combined with α-mangostin, gallic acid or vitamin C. Dox in this study, was observed to inhibit cell growth via expression of Bak and p53 which was statistically significant when compared to negative control. However, expression of p16 was not significant when compared to negative control.

α-mangostin treatment alone in this study significantly reduced cell viability, increased phosphorylated FLT3 (p-FLT3) and although 5-fold increase in p53 expression was observed with α-mangostin treatment, compared to control it was not statistically significant.

Gallic acid treatment significantly inhibited cell growth and increased cdc25A expression when compared to negative control. However, 3-fold increase in p21 expression with gallic acid treatment was not statistically significant when compared to control.

Vitamin C single treatment inhibited cell growth and about 3-fold increase in p21 and p16 expressions were not statistically significant when compared to negative control in this study.

This study on combinational effects on apoptosis, demonstrated that, Dox combined with α-mangostin induced more apoptotic effect due to the expression of both proapoptotic proteins (Bax and Bak), effector caspase (Caspase 3) and inhibition of antiapoptotic protein Bcl-2. However, only Bak was significant when compared to Dox only. MOLM-13 cells treated with vitamin C combined with Dox did not express Bak but only Bax and showed expression of caspase 8, 9 and cell cycle inhibitor p21 and p16. However, compared to Dox only, all proteins expressed with vitamin C and Dox combination were not statistically significant. Gallic acid combination with Dox expressed Bak significantly compared to Dox only and negative control.

Therefore, combination of α-mangostin and doxorubicin compared to other combinations (Dox and gallic acid, Dox and vitamin C) could induce apoptosis, inhibit FLT3-ITD phosphorylation and further reduction of its early targets cdc25s phosphatases. Therefore, a promising therapeutic effect is expected after a combination treatment with FLT3-ITD for AML cells. Gallic acid has been reported to inhibit ABL kinase in CML and its derivatives are been studied as ABL inhibitors (Raghi *et al*., 2018). This study supports further research on doxorubicin and α-mangostin combination as potential FLT3-ITD inhibitor in trials and clinical studies. However, low cytotoxic effect of α-mangostin was observed in normal monocyte cells in this study.

7.3 Limitations of the Research

This study was done on only MOLM-13, more cell lines such as MV4;11 and MOLM-14 with FLT3-ITD mutation should be included for future work.

A known FLT3-ITD inhibitor was not included as positive control in this study. Therefore, future work should include a known FLT3 inhibitor not only cells without the FLT3 -ITD.In this study only p16 and p21 were studied for senescence. Therefore, future work on senescence should include more biomarkers for senescence such as senescence associated beta galactosidase (SA-β galactosidase) and trimethylation of H3K9 which were not studied in this research.

For expression of proteins where number of replicates were two (n=2), more replicates should be analysed.

7.4 Recommendation for Future Work

The use of more FLT3-ITD cell lines and a positive control drug such as midostaurin is recommended. Further study can include knock down of FLT3 in FLT3-ITD positive cells using ShRNA or SiRNA. This will distinguish the effects on FLT3 phosphorylation after combined treatment with Dox and α-mangostin in FLT3 knockdown cells and non-knockdown cells.

Further investigation of the mechanism of FLT3-ITD inhibition by Dox and α-mangostin combination drug, by studying signaling pathways such as STAT5, PKB, ERK, activated by FLT3 is recommended. This will determine the effect of Dox combined with α-mangostin on these signaling pathways as FLT3-ITD inhibitors.

Cell cycle inhibitor p27 that has been reported responsible for gallic acid in inducing G_2/M arrest in breast cancer, therefore it should be studied in addition to the effect of p21 observed in this study. This will determine if both p21 and p27 are involved in G_2/M arrest in MOLM-13 cells

DNA damage induced by Dox and gallic acid. Combination treatment can be measured by γH2AX foci formation, which is a marker of DNA damage expressed by only synergistic combinations as further confirmation of more DNA damage aside from arrest at G_2/M phase. In addition, normal cell line with DNA inhibited can be included in the study to compare the effect of Dox combined with gallic acid on DNA inhibited cells and MOLM-13.

Expression of 4-3-3 protein is recommended for further studies because it indicates strong G_2 checkpoint arrest. Inappropriate mitotic entry can occur when there is interference with 4-3-3: Wee1 binding or 4-3-3: cdc25 binding (Gardinoa & Yaffe, 2011). All combinations studied induced more G_2/M phase arrest than single treatment with Dox only.

More number of replicates (n=3 and more) will be included for future studies.

Further studies on senescence will include more assays such as senescence associated beta galactosidase (SA-β galactosidase), retinoblastoma protein (RB), trimethylation of H3K9, Markers of DNA damage such as yH2AX and morphological changes such as increase in cell area and Increase in size of nucleus.

Further studies on vitamin c will include analysis of catalase in cancer cells and normal cells.

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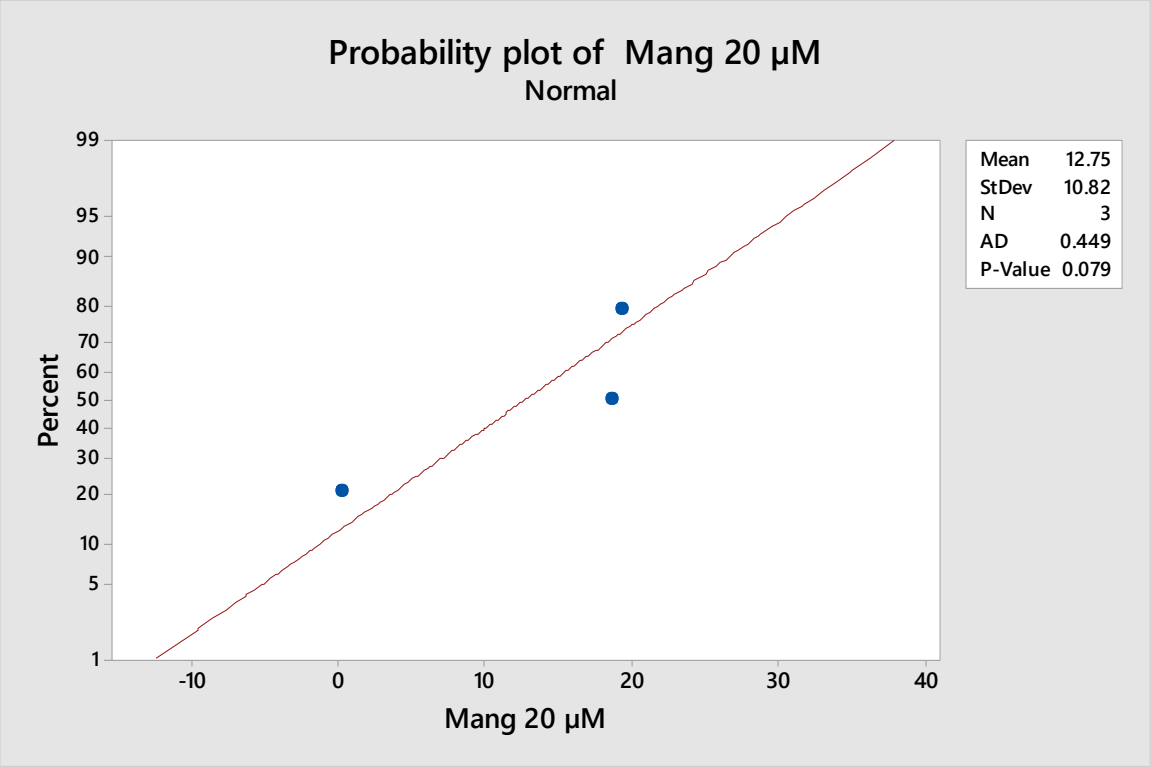
APPENDIX



Appendix 1: Normality graph of α-mangostin 20 µM after 72 h incubation.

Appendix 2: Test and CI for two variances: MOLM-13 and α-mangostin 5 µM.

Test

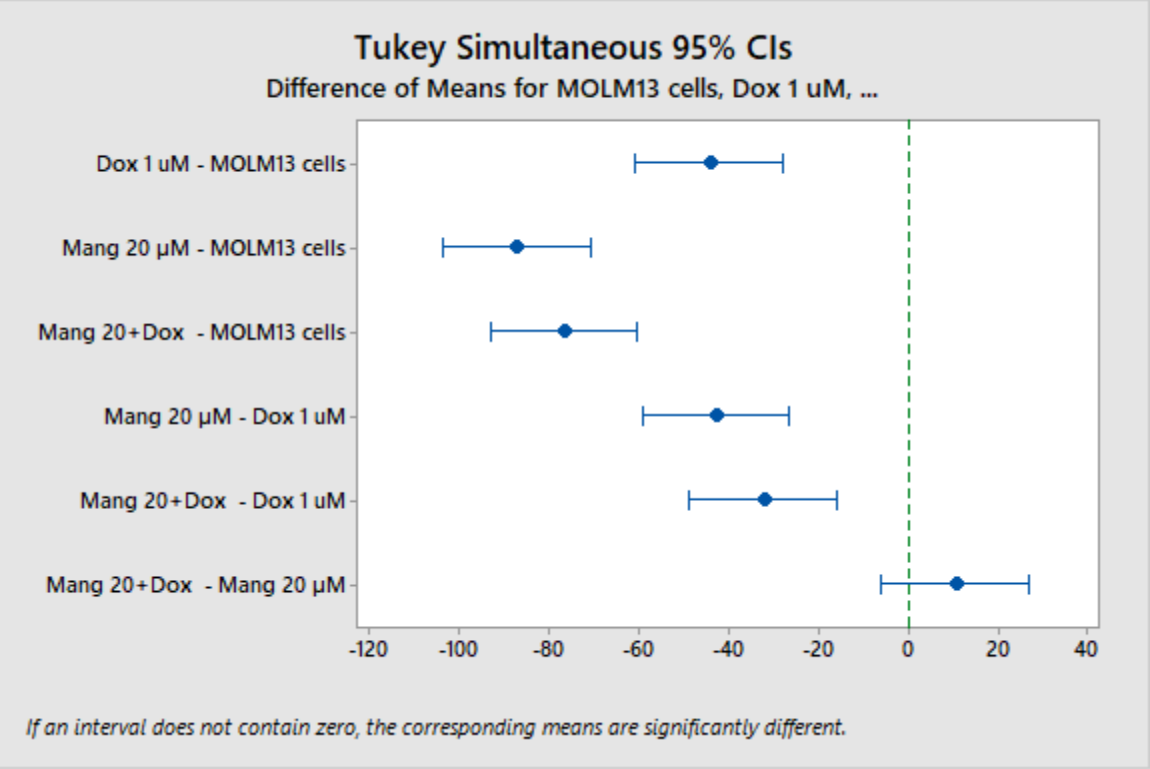
Null hypothesis	$H_0: \sigma_1 / \sigma_2 = 1$
Alternative hypothesis	$H_1: \sigma_1 / \sigma_2 \neq 1$
Significance level	$\alpha = 0.05$

Method	Test Statistic	DF1	DF2	P-Value
Bonett	0.16	1		0.687
Levene	0.02	1	4	0.894

Variance equal ($p>0.05$).

Appendix 3: Descriptive statistic of α-mangostin 72 h

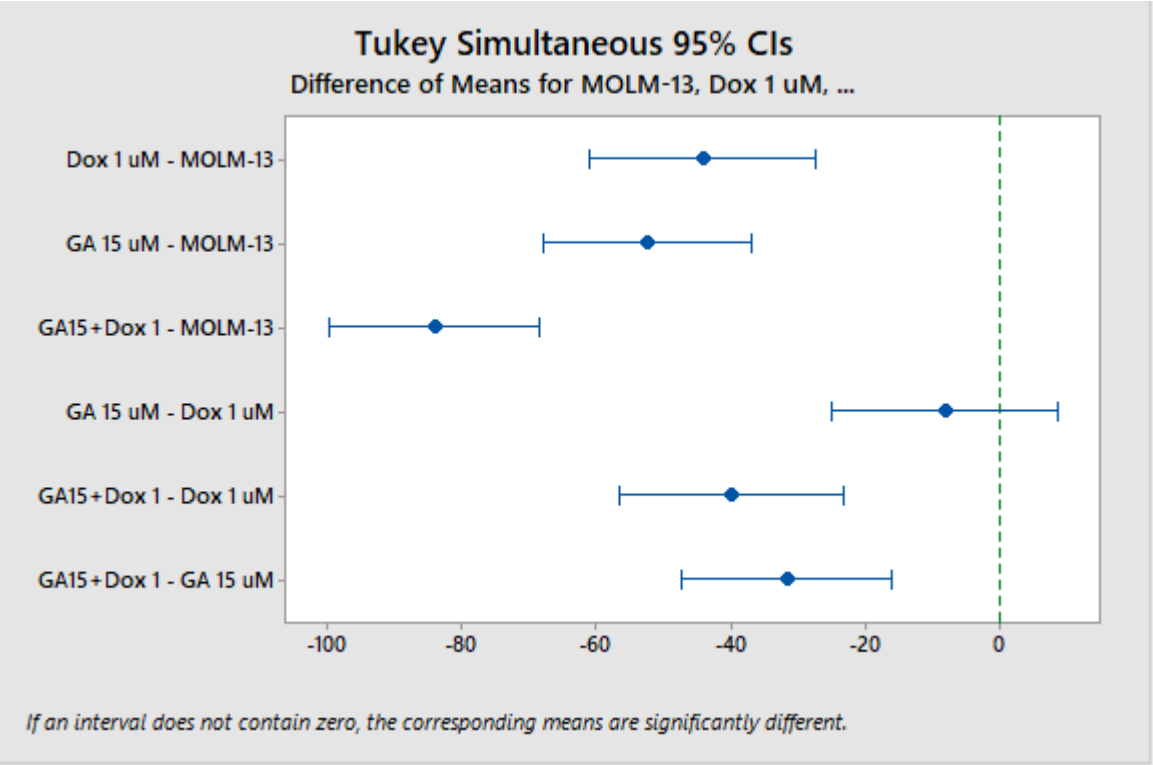
Variables	Total Count	Mean	SE Mean	StDev	Minimum	Maximum
MOLM-13	3	100.00	0.934	1.62	98.14	101.03
Dox 1 µM	3	55.62	3.47	6.02	51.29	62.49
α-Mang 50 µM	3	12.986	0.904	1.566	11.408	14.540
α-Mang 40 µM	3	15.313	0.487	0.844	14.650	16.263
α-Mang 30 µM	3	17.575	0.538	0.932	0.26	18.646
α-Mang 20 µM	3	12.75	6.25	10.82	0.26	19.39
α-Mang 10 µM	3	38	5.66	9.81	27.28	45.69
α-Mang 5 µM	3	100.3	0.725	1.25	98.68	101.17
α-Mang 2.5 µM	3	83.5	13.9	24.0	55.9	90.0
α-Mang 20 µM +Dox 1 µM	3	23.231	0.732	1.269	22.058	24.577



Appendix 4: α-Mangostin 72 h one way ANOVA

Appendix 5: Descriptive statistics of gallic acid 72 h

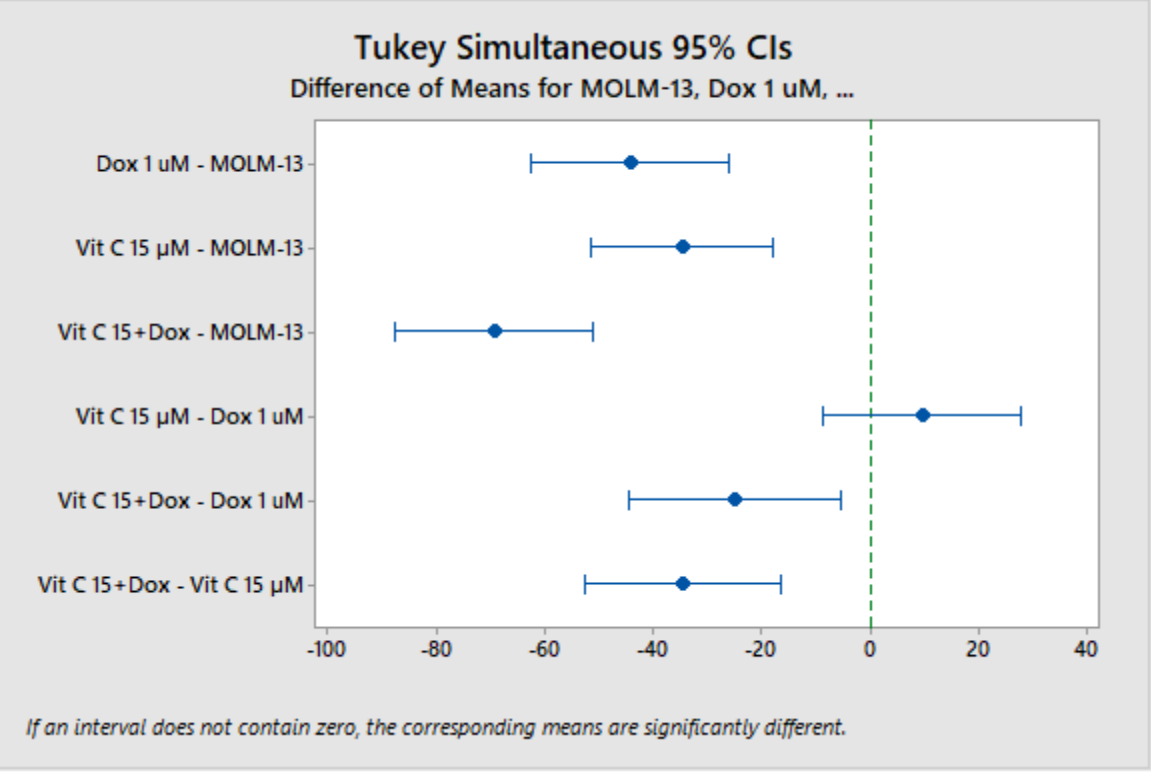
Variables	Total Count	Mean	SE Mean	StDev	Minimum	Maximum
MOLM-13	4	99.90	3.99	7.99	91.23	106.89
Dox 1 µM	3	55.62	3.47	6.02	51.29	62.49
GA 30 µM	4	29.91	1.73	3.58	26.10	34.45
GA 20 µM	4	31.58	3.58	7.15	26.51	42.17
GA 15 µM	4	47.39	4.93	9.86	37.16	58.04
GA 10 µM	4	80.64	4.00	8.00	72.23	88.94
GA 5 µM	4	87.47	2.77	5.54	83.09	94.99
GA 1 µM	2	89.14	6.47	9.15	82.67	95.62
GA 15 µM +Dox 1 µM	4	15.65	1.63	3.25	11.21	18.38



Appendix 6: Gallic acid 72 h one-way ANOVA

Appendix 7: Descriptive statistics of vitamin C 72 h

Variables	Total Count	Mean	SE Mean	StDev	Minimum	Maximum
MOLM-13	4	100.00	6.49	12.97	83.41	114.06
Ima 2.5 µM	3	55.646	0.541	1.082	51.027	53.239
Vit C 10 µM	4	121.33	4.46	8.92	111.69	132.23
Vit C 15 µM	4	65.205	0.904	1.808	63.665	67.457
Vit C 30 µM	4	61.69	3.27	6.53	55.61	68.25
Vit C 15+ Dox 1 µM	3	30.56	1.82	3.14	27.14	33.33
Dox 1 µM	3	55.62	3.47	6.02	51.29	62.49



Appendix 8: Vitamin C 72 h one-way ANOVA

Appendix 9: p16 expression relative to beta actin vitamin c and Dox treatments

	MOLM-13	Dox 1	Vit C 15+Dox 1	Vit C 15
p16	0.02	0.28	0.61	0.48
	0.25	0.38	0.25	0.24
	0.04	0.16	0.46	0.05
Average	**0.10**	**0.27**	**0.44**	**0.26**

p21 expression relative to % control with vitamin c and Dox treatments

3 replicates	MOLM-13	Dox 1 µM	Vit C 15+Dox 1 µM	Vit C 15 µM
	73	237	241	155
	40	151	492	267
	174	160	577	372
AVG	96	183	437	265
%Control	**100**	**190**	**455**	**276**

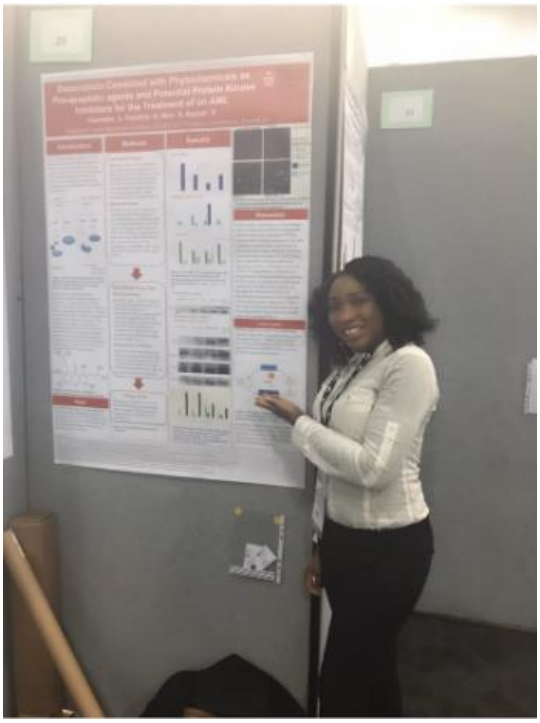
Bcl-2 expression relative to % control with gallic acid and Dox treatments.

	MOLM-13	**Dox 1**	**GA 15+Dox 1**	**GA 15**
	68	127	138	46
	87	88	119	49
	60	39	145	131
	185	114	269	402
AVG	**100**	**92**	**168**	**157**

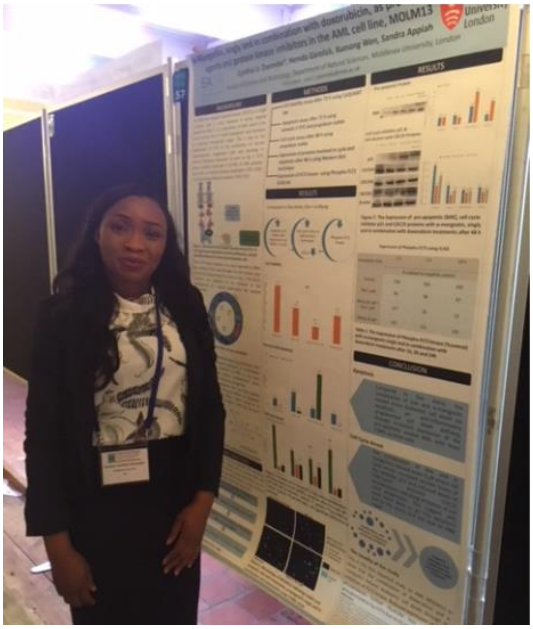
Appendix 10: **List of seminars and conferences attended**

- Tumour Microenvironment - Basic Science to Novel Therapies (Including 3D models' workshop) - 14th to 16th June 2017. Nottingham Conference center, Nottingham, UK.
- National conference research institute conference (NCRI): BACR delegate at BT convention center Liverpool UK, 5- 8th November 2017.

- BACR Development of Cancer Medicines event on the 21st November 2017 at the Royal Society of Medicines, 1 Wimpole Street, London.
- 4th EACR conference, a matter of Life or Death from basic cell death mechanism to Novel Cancer Treatments 1-3rd Feb 2018 Amsterdam, Netherlands.
- ESMO Symposium on Signalling Pathways in Cancer 2018: Cyclin-dependent kinases (CDKs) Organised by ESMO in partnership with the EACR 23 - 24 March 2018 || Vall d'Hebron Institute of Oncology, Barcelona, Spain.
- BACR student conference, Francis Crick institute. Nov 26th, 2018.



- National Conference Research Institute Conference (NCRI): BACR delegate at BT convention center Liverpool UK, 5- 8th November 2017.



- 4th EACR conference, a matter of Life or Death from basic cell death mechanism to Novel Cancer Treatments 1-3rd Feb 2018 Amsterdam, Netherlands.

- ESMO Symposium on Signalling Pathways in Cancer 2018: Cyclin-dependent kinases (CDKs) Organised by ESMO in partnership with the EACR 23 - 24 March 2018 || Vall d'Hebron Institute of Oncology, Barcelona, Spain.