Induction of death in myeloid leukaemia cells by doxorubicin and betulinic acid, singly and in combination was associated with the regulation of apoptosis, autophagy and the PI3K/Akt pathways

A thesis submitted to Middlesex University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Milan Vu*
M00389076

Director of Studies: Dr Sandra Appiah
Supervisors: Dr Nick Kassouf & Dr Celia Bell
*PhD Research Degree

Department of Natural Sciences, Faculty of Science and Technology
Middlesex University London

Table of contents

List of Tables and Figures

Tables

Figures

Chapter 4

Chapter 5

List of Abbreviations

ABL-1	Abelson murine leukemia viral oncogene homolog 1
ADP	Adenosine diphosphate
AKT	Protein kinase B
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Apaf-1	Apoptosis protease activating factor 1
Apo-1/CD95	Apoptosis antigen 1/ Cluster of Differentiation 95
ATCC	American Type Culture Collection
ATG	Autophagy-related genes
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BCR	Breakpoint Cluster Region (gene)
BCR-ABL1	Fusion (oncogene) gene of BCR and ABL1
BetA	Betulinic acid
BH	Bcl-2 homology domain
Bid/t-Bid	BH3 interacting-domain death agonist/ truncated Bid
BSA	Bovine serum albumin
CDS	Coding sequence
CFSE	5-(and -6) carboxyfluorescein diacetate succinimidyl ester
CI	Combination index
cIAP1/2	Cellular Inhibitor of APoptosis1/2
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myelogenous/ Myeloid Leukemia
CRISPR	Clustered regularly interspaced short palindromic repeats
CYLD	Cylindromatosis
dATP	Deoxyadenosine triphosphate
DCFDA	2',7'–dichlorofluorescin diacetate
DEPC	Diethyl Pyrocarbonate
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
ECL	Electrochemiluminescence/ Electrogenerated chemiluminescence
ED_{50}	Median effective dose
EDTA	Ethylenediaminetetraacetic acid
FAB	French–American–British classification
FACS	Florescence activated cell sorter
FADD	Fas-associated protein with death domain
Fas	Fas cell surface death receptor = Apo-1/CD95
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIP200	RB1 inducible coiled-coil 1 (RB1CC1)/ ATG17
FL-	Fluorescence channel

FLD	Flexible loop domain
FLICE	Alias symbol of caspase-8
$FLIP_L$	CASP8 and FADD like apoptosis regulator/ FLICE-Like inhibitory protein (Long)
FLT3	Fms like tyrosine kinase 3/ receptor-type tyrosine-protein kinase FLT3
FSC	Forward Scatter
Fwd	Forward primer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC_{50}	Half maximal inhibitory concentration
IDH1/IDH2	Isocitrate dehydrogenase 1/2
IgG	Immunoglobulin G
ITD	Internal tandem duplications
JNK	c-Jun amino (N)-terminal kinases
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LUBAC	Linear ubiquitin chain assembly complex
MAPK	Mitogen-activated protein kinas
Mcl-1	Induced myeloid leukemia cell differentiation protein Mcl-1
MFI	Mean fluorescent intensity
MLKL	Mixed lineage kinase domain-like protein
MLL	Mixed-lineage leukemia
MOMP	Mitochondrial outer membrane permeabilisation
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NPM	Nucleophosmin
NSD	No statistical difference ($p > 0.05$)
NTC	Non-template control
p53	Phosphoprotein 53
PARP	Poly-ADP-ribose polymerase
PBL	Peripheral blood lymphoblast
PBS	Phosphate buffer saline
PBS-T	Tween20 in PBS
PCR	Polymerase chain reaction
PDK-1	phosphoinositide-dependent protein kinase-1
Ph chromosome	Philadelphia chromosome
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinases
PKCδ	Protein kinase C delta type
PS	Phosphatidylserine
PTEN	Phosphatase and tensin
PUMA	p53 upregulated modulator of apoptosis
RAPTOR	Regulatory Associated Protein of mTOR
Rev	Reverse primer
RIPK	Receptor-Interacting serine/threonine-Protein Kinases
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription-PCR
SAP	Stress-activated protein kinase
SCD-1	Stearoyl-CoA desaturase-1

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspase
Sp	Specificity protein (transcription factor)
SSC	Side Scatter
TBHP	Tert-butyl hydrogen peroxide
TCR	T-Cell Receptors
TKI	Tyrosine kinase inhibitor
TLR	Toll-Like receptors
TM	Transmembrane region
TNF	Tumor necrosis factor
TNF-R1/R2	Tumor necrosis factor receptor 1 (CD120a)/ 2 (CD120b)
TRADD	TNFR (TNF receptor)-associated death domain
TRAF 2/5	TNFR (TNF receptor)-associated factor 2/ 5
TRAIL	TNF-related apoptosis-inducing ligand
ULK	Unc-51-like kinases (homologue of Atg1)
UTR	Untranslated region
UV	Ultraviolet
VP16	Etoposide chemotherapy drug
VPS15	Vacuolar protein sorting regulatory subunit of VPS34 (p150)
VPS34	Class III PI 3-kinase subgroup vacuolar protein sorting
WBCs	White blood cells
WHO	World Health Organization
XIAP	X-chromosome linked inhibitor of apoptosis
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide assay
z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone
ΔΨm	Mitochondrial membrane potential

Acknowledgements

I would like to express my gratitude to everyone who helped and supported me in completion of my PhD work. Special thanks go to Dr Sandra Appiah for being my Director of Studies and providing much needed mentorship throughout this journey. I would also like to thank the rest of my supervisory team, Dr Celia Bell and Dr Nick Kassouf for data collection, technical support in the lab and helping me throughout my write up. I am thankful for the invaluable constructive criticism provided during the research, which inspired me and motivated me to complete the work. I would like to express my gratitude to other researchers, including several Middlesex University staff. Expertise from Dr Torben Lund was greatly valued and his impact as a brilliant co-author in our collaborative paper was truly appreciated. Some outstanding help was provided by researchers at Middlesex University institute. Their opinions, guidance and technical support contributed to improvement of my research work. I am incredibly thankful and many apologies for not listing all the names.

Acknowledgement also goes to my fellow students and PhD candidates, mainly Rosemary Isioma Ofili, Noor Hasan, and John Nursiah as well as Dr Uzoma Cynthia Osemeke who also guided me prior taking on PhD studies. I am grateful for all the assistance in the lab and for supporting me in every possible way. It would not have been possible without their contribution and support. Many thanks to my Middlesex University co-workers and fellow GAAs/past GAAs, who always encouraged me. I am very glad to have the opportunity working with this wonderful team who had a positive impact on me and gave me more confidence. I would also like to thank my family, especial my parents, for their patience and emotional support in my studies. Lastly, I would like to apologise to anyone I have not mentioned by name in this statement but who helped me in achieving my goals. My genuine gratitude and thank you all.

I must acknowledge that there were some invaluable lessons through certain challenges and overcoming these have served to demonstrate my true strength. I would like to declare that no support was provided by my residence of origin; therefore, no claim of my achievements goes to them.

Abstract

Relapsed acute myeloid leukaemia (AML) cells are difficult to eradicate due to their more refractory nature. Deregulation of the B-cell lymphoma 2 (Bcl-2) family of proteins in these cells, which regulates cell death through apoptosis, autophagy and other cell processes, may contribute to this trend. Using chemical agents could modulate these signalling pathways. Drugs such as doxorubicin (Dox) are effective but displayed some clinical drawbacks, while compounds such as betulinic acid (BetA) have shown some interesting activity against cancerous cells. These drugs may complement each other in sensitising and eradicating aggressive AML cells. Therefore, in this study, cell death regulation and induction in AML cell line from a relapsed MLL-rearranged cell model (MOLM-13) was investigated with an established chemotherapy drug Dox and a bioactive compound BetA, singly and in combination.

The cytotoxic effect of the compounds was measured by CyQUANT Direct® (which estimates cell numbers by nuclear content of viable cells). Proliferation rate (using 5(6)-carboxyfluorescein diacetate succinimidyl ester) and cell death population (using Annexin V/propidium iodide) of the treatments were studied using flow cytometry. The contribution of reactive oxygen species (ROS) stimulation in MOLM-13 cell death mechanism was measured by 2',7'–dichlorofluorescin diacetate (DCFDA) staining. Protein and gene expressions of regulators involved in apoptotic, autophagy and cell-survival (PI3K-AKT) pathways were examined by Western blot and reverse transcription polymerase chain reaction (RT-PCR) analysis, respectively.

Dox showed non-discriminatory inhibition of cell viability ($p < 0.05$) in all tested cell lines including non-cancerous (HEK293T) cell control whilst BetA selectively inhibited viability ($p < 0.05$) of AML MOLM-13 and CML K562. The combination index (CI; which determines multiple drug effect interactions) of BetA (20 μM) and Dox (1 μM) showed that the inhibitory effect on AML cells was synergistic (CI < 1). Dox potently inhibited cell division of MOLM-13 cells over a three-day period, which was not negatively affected by co-treatment with BetA combination. Although Dox inhibited ($p <0.05$) PI3K-AKT markers in MOLM-13 cells, suggesting a decrease in cell survival, potential sign of drug resistance was observed possibly due to significant downregulation ($p <0.05$) of PTEN. BetA-Dox combined treatments increased ($p < 0.01$) ROS formation within MOLM-13 cells between 0.5-1.5 h but the single treatments did not significantly alter ROS levels. Additionally, more cells resided in irreversible late apoptotic stage after drug combination (BetA + Dox) in MOLM-13 co-treatment compared to the single treatments ($p < 0.05$). However, a contradicting effect was observed in leukaemic SC/U-937 monocytes, where combination treatments attenuated Dox-induced cell death. Here for the first time an isoform of anti-apoptotic Bcl-2 protein p15-20-Bcl-2 was detected in MOLM-13, alongside the usual p26-Bcl-2-α. This p15-20-Bcl-2 was sensitive to treatments and was reduced ($p < 0.05$) upon exposure to Dox and the drug combination, unlike p26-Bcl-2-α present in all tested cells. Dox and the drug combination reduction in Beclin 1 (autophagy inducer) expression could indicate a modulation of autophagy in MOLM-13 cells but it is not clear whether this confers a protective or cell-death inducing effect. The outcome of combination studies on Bcl-2 family and autophagy protein expressions was in parallel with the mRNA regulation; additionally, the transcript further postulated the protective nature of BetA combination in Dox-treated SC/U-937 cells. Further recommended studies should investigate the targeting potential and the role of the novel p15-20-Bcl-2 and define the underlying cell-dependent effect mechanism of the combination treatments in enhancing and alleviating cell death.

Chapter 1

Literature review

1 Literature review

1.1 Leukaemia

Leukaemia is the 11th most common type of cancer, as well as the cause of cancer death based on UK data from 2015-2016 (Cancer Research, 2019). Acute leukaemia types are aggressive and progress rapidly (Hoffbrand and Moss, 2011). Acute myeloid leukaemia (AML) has the highest mortality rate among the different types of leukaemia (Table 1.1) (Cancer Research, 2019). Chronic myeloid leukaemia (CML) is a rare disease but it affects over 100,000 patients worldwide per year. Compared to AML, CML has prolonged progression of the disease. Both AML and CML are predominant diseases of adults, accounting for approximately 25% and 20% of adult leukaemia, respectively (WHO, 2014; Estey and Döhner, 2006; Deschler and Lübbert, 2006). Therefore, both types of leukaemia represent a significant global health burden.

Table 1.1 Incidence and death rate of leukaemia in UK (2015 and 2016 data)

Types of leukaemia	Incidence (UK, 2015)		Mortality (UK, 2016)		
	% Leukaemia incidence	Cases by gender	% of all cancer deaths	% Leukaemia deaths	Cases by gender
AML	37.2%	M: 1753 F: 1373	2%	63.7%	M: 1481 F: 1120
ALL	9.9%	M: 460 F: 372	<1%	6.2%	M: 143 F: 110
CML	8.8%	M: 408 F: 334	<1%	5.4%	M: 119 F: 100
CLL	44.1	M: 2326 F: 1383	<1%	24.7	M: 624 F: 384

 Leukaemic progression can be either rapid (acute) or prolonged (chronic) and different haematopoietic progenitors, myelogenous or lymphocytic, may be affected. Therefore, four main leukaemia types exist; acute lymphoblastic leukaemia (ALL), chronic lymphoid leukaemia (CLL), acute myeloid leukaemia (AML), and chronic myeloid leukaemia (CML) (Cancer research UK 2019). M: male; F: female

1.1.1 Myeloid progenitor neoplasia

Uncontrolled proliferation of functionless or immature myeloid progenitor cells (myeloblasts) leads to leukaemia. White blood cells (WBCs) from the myeloid pathway such as neutrophil, monocytes, eosinophils and basophiles are affected by the disease. Abnormal WBCs and blast cells build up in the bone marrow, eventually replacing healthy normal cells and obstructing their roles (Hoffbrand and Moss, 2011). Besides the classification based on their progression rate, both AML and CML can be further grouped by their common genetic mutations. This grouping affect patients' response to chemotherapy and treatment success (Section 1.2). The classification and sub-classifications of leukaemia according to World Health Organisation are summarised in Figure 1.1.

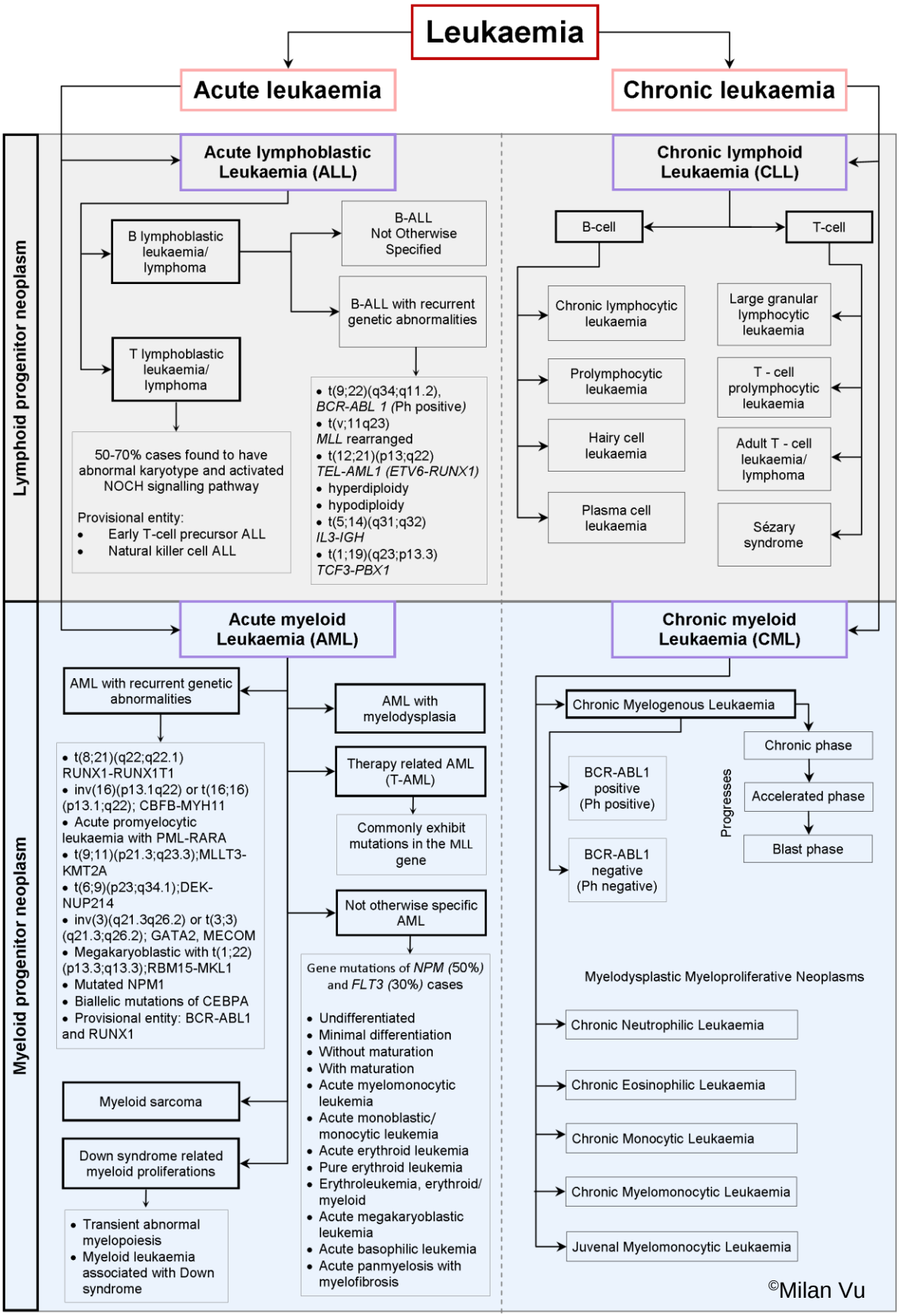


Figure 1.1 Classification and sub-classifications of leukaemia according to World Health Organisation

Sources of information: Arber *et al*., (2016); Campo *et al*., (2011); Vardiman *et al*., (2009)

1.1.2 Acute myeloid leukaemia (AML)

Acute myeloid leukaemia has a relatively high mortality rate due in part to its rapid progression. The disease also has a high relapse incidence, which leads to production of more refractory cells (Ding *et al.,* 2012). Although genetic mutation burden of AML at both diagnosis and relapse is not as common as in other cell types (Hassan *et al.,* 2017), there is still some potential association with the development of additional mutations or epigenetic shift at relapse that may cause drug-resistance leading to poor prognosis (Ding *et al.,* 2012). In human AML cells, anti-apoptotic proteins in the B-cell lymphoma-2 (Bcl-2) family are upregulated, and they play a prominent role in myeloid leukaemogenesis (Zinkel *et al.,* 2006; Bensi *et al.,* 1995).

Apoptosis is one of the main targets for inducing cancer cell death with chemotherapy drugs. However, cancer cells have evolved a mechanism to counter apoptotic cell death contributing to chemoresistance (Zahreddine and Borden, 2013; Hu and Xuan, 2007). Current standard treatments for AML are purported to be less effective in patients where the balance of the Bcl-2 family of proteins are deregulated; these patients tend to exhibit chemoresistance (Campos *et al.,* 1993).

1.1.3 Chronic myeloid leukaemia (CML)

About 95% of CML cases show a chromosomal abnormality known as Philadelphia (Ph) chromosome, which may incline to blast crisis (fatal stage) transformation (Theml, 2004). Ph chromosome is an aberration of chromosome 22, which contains fused gene BCR-ABL1, due to reciprocal translocation t(9;22) (q34;q11) between chromosome 9 and 22. An oncogene ABL1 from chromosome 9 moves and fuses with BCR gene on chromosome 22 while part of chromosome 22 fuses to chromosome 9 (Hoffbrand and Moss, 2011). The BCR-ABL1 is a chimeric gene that encodes for a fusion protein of a bigger size (210 kDa; p210) compared to normal ABL1 protein product (145 kDA), which results in excessive activity of tyrosine kinase (Jabbour *et al.,* 2015; Hoffbrand and Moss, 2011). Tyrosine kinase regulates many cellular responses in cytoplasmic and nuclear signal transduction pathways, affecting cell growth, division, differentiation and death. Oncogenic activation of tyrosine kinase causes disruption of these functions, leading to cancer pathogenesis by increasing cell proliferation and decreasing apoptosis (Kawthalkar, 2013; Paul and Mukhopadhyay, 2004). The prognosis of CML has rapidly improved with the development of tyrosine kinase inhibitors targeting the abnormal kinase produced by Ph chromosome, which was introduced in the 90s (Iqbal and Iqbal, 2014; Fleming, 2012). Although the CML mortality rate has reduced by a third over the last decade, the trend is not witnessed in its acute counterpart (Cancer Research, 2019).

1.2 Current treatment for myeloid leukaemia and their limitations

1.2.1 Treatment for AML

Cytotoxic chemotherapy drugs such as cytarabine (a drug of marine origin) and anthracyclines (daunorubicin and doxorubicin) are typically involved in the standard treatment for AML patients. These drugs are commonly taken intravenously as combined infusion, where cytarabine acts as a nucleoside analog and anthracyclines work as topoisomerase II inhibitors in combating the growth of cancer cells (Cools, 2012; Tallman *et al.,* 2005). Thanks to recent advances in sequencing techniques, genomic abnormalities such as FLT3 or IDH1/IDH2 mutations can be targeted by various kinase inhibitors, if applicable (Madanat *et al.,* 2019). Although the current chemotherapy regime has improved the remission rate in some AML patients, there are serious side-effects due to its aggressiveness (Whitaker and Green, 2014). For example, the administration of drugs such as gemtuzumab ozogamicin was discontinued at one point due to the high toxicity of the drugs, decreasing their effectiveness (Fleming, 2012). In addition, doxorubicin exerts toxic effects on healthy tissues and is known to produce cardiomyopathy, leading to congenital heart failure at doses exceeding 600 mg/m^2 in 36% of cases, thus hampering its clinical purpose (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004). The exact molecular mechanism leading to cardio toxicity is still unclear despite the extensive clinical use of doxorubicin (Yang *et al.,* 2014). Furthermore, development of multidrug resistance in leukemic cells treated by doxorubicin is on the rise (Tallman *et al.,* 2005; Minotti *et al.,* 2004). Despite the use of doxorubicin in AML therapy, the drug can markedly decrease blood cells in the bone marrow, which further increases the risk of developing leukaemia, especially in higher doses (Medline Plus, 2012) .

1.2.2 Treatment for CML

Standard anti-leukaemic drugs such as cytarabine and cyclophosphamide are Food and Drug Administration (FDA) approved to treat CML in combination with other more targeted drugs (National Cancer Institute, 2019). Currently, the gold standard first-line treatment for CML is imatinib mesylate, a tyrosine kinase inhibitor (TKI) that targets Ph chromosome through blocking adenosine triphosphate binding site of BCR-ABL tyrosine kinase (Druker, 2008). TKIs inhibit tyrosine phosphorylation which leads to suppression of downstream signalling pathway associated with CML cell survival (Marcucci *et al.,* 2003). The treatment stabilises hematopoietic cell numbers to consequently achieve a normal lifespan (Scheinberg and Jurcic, 2004). Combination of imatinib with anthracycline drugs such as doxorubicin or idarubicin is sometimes administered in treatment regime of CML blast crisis phase due to the resemblance to the acute type and having more unfavourable prognosis (American Cancer

Society, 2015; Hehlmann, 2012). However, utilising additional potent drug in the combination drug regime often presents with adverse cytotoxic effects on healthy tissues.

As about 95% of CML cases are positive for Ph chromosome (Scheinberg and Jurcic, 2004), TKI therapy has led to much lower mortality rate by CML since their introduction in the 2001. Imatinib-treated patients have been reported to have 89% survival at 5 years, with the disease not progressing to more serious phases in around 93% of patients (Iqbal and Iqbal, 2014; Druker, 2008). However, the acute myeloid type causes the highest mortality compared to the other major leukaemia types. Unlike CML, AML has more complicated sub-categorisation due to very diverse genetic abnormalities within the cells (Fig. 1.1) and most cases have no specific target like the Ph chromosome in CML. However, suppressing the Bcl-2 anti-apoptotic molecule, which are elevated in the AML cancer cells, may have some targeting potential. Bcl-2 is one of the anti-apoptotic proteins in the Bcl-2 family and it has been reported to be involved in different programmed death pathways.

1.3 Induction of programmed cell death to treat leukaemia

One of the hallmarks of cancer cells is their ability to evade cell death and continuously replicate (Hanahan and Weinberg, 2011). Normal cells undergo a series of cell cycle events during cellular division; when errors are detected, cells are signalled to die by apoptosis (Macdonald *et al.,* 2004). However, cancer cells have the capability to evade the killing process and continuously replicate (Panno, 2004). Modulation of the signalling pathways to induce cell death has been described as a potential means of targeting various cancers. There are different modes of programmed cell death including apoptosis, autophagy, necroptosis or other regulated cell death that may be employed by anti-cancer drugs to fight malignancies.

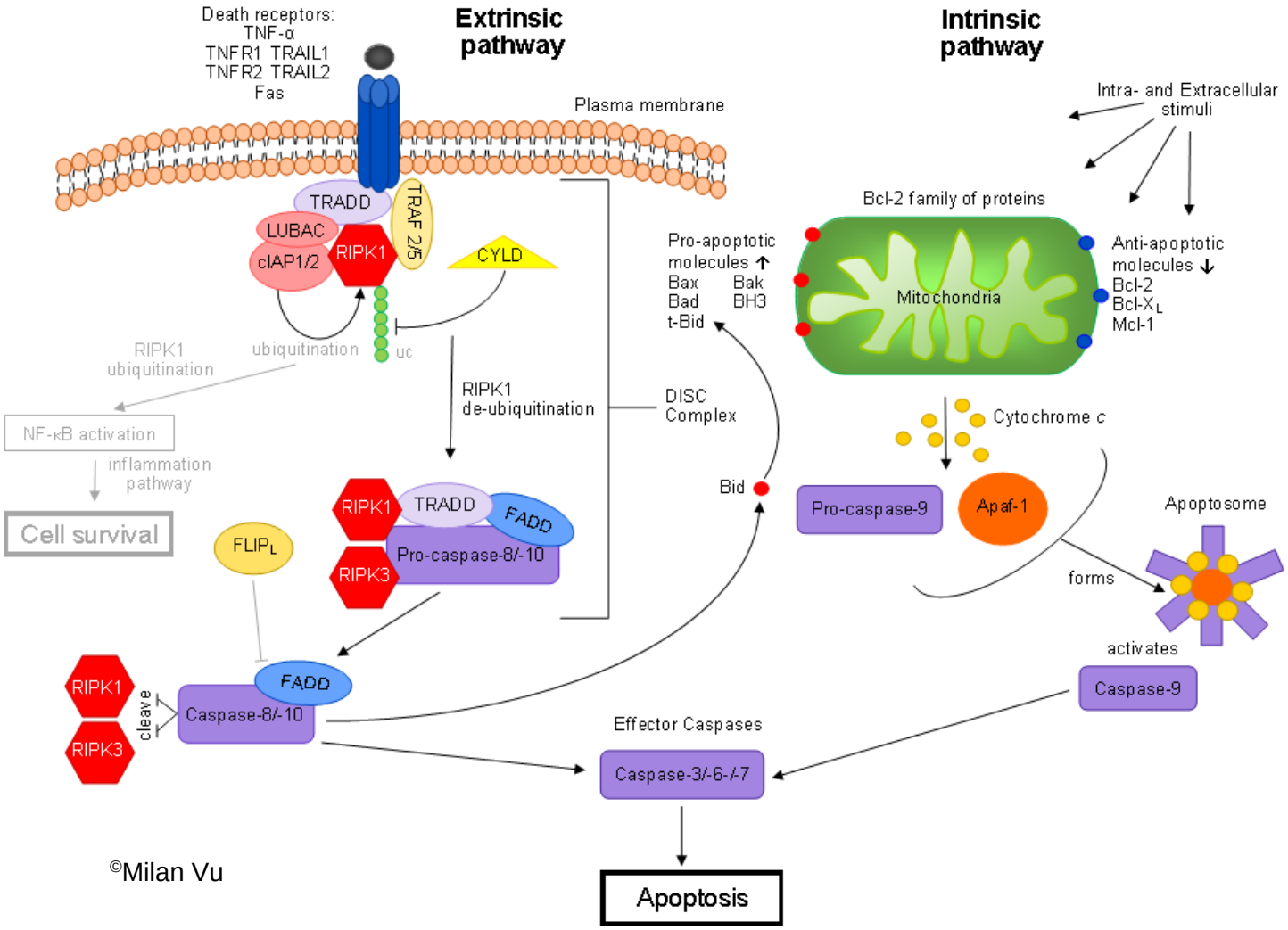
The resistance against apoptotic death induction in cancer cells is very common, especially in relapse or transformed cases of leukaemic cells. There are many factors and mechanisms contributing to this cell resistance, limiting the use of chemotherapy drugs. Several means of drug resistance could be developed by cells through loss of caspase regulation, increased upregulation of anti-apoptotic Bcl-2 family proteins, or by expressing caspase inhibitors (Horita *et al.,* 2008; Hu and Xuan, 2007). Since even the most vigorous resistance in cancer cells is often restricted to a particular signalling pathway (Hu and Xuan, 2007), targeting different programmed death pathways may hinder or slow down the ability of cancer to develop and sustain resistance. The interplay between the cellular processes that can induce cell death can further elucidate on the mechanism of action of drug, which could be eventually exploited as a strategy against cancer.

1.3.1 Programmed cell death I: Apoptosis

The primary cell death pathway targeted by most chemotherapy drugs is apoptosis, termed as programmed cell death I. Apoptosis is characterised morphologically by cell shrinkage, nuclear condensation and DNA fragmentation (Panno, 2004). It is a caspase-dependent process, regulated via intrinsic or extrinsic signalling pathways, which ultimately results in activation of a series of caspase proteins. Caspase enzymes are cysteine proteases that cleave proteins at aspartic acid residues and trigger a cascade of proteolytic events to execute cell death (Ashkenazi, 2008; Sellers and Fisher, 1999).

The intrinsic pathway (mitochondrial pathway; Fig. 1.2) is mediated by intra- and extracellular stimuli, resulting in mitochondrial perturbation and membrane degradation leading to the release of Cytochrome *c* (Ouyang *et al.,* 2012). The mitochondrial perturbation is induced via transcriptional and post-translational activation of the Bcl-2 family of proteins, consisting of pro-apoptotic molecules (Bax, Bak, Bad, BH3 and t-Bid) and anti-apoptotic molecules (Bcl-2, Bcl-X_L, and Mcl-1) (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). If the equilibrium between the pro- and anti-apoptotic molecules is imbalanced, the cell signals for either survival or death depending on the direction of the shift (Nikoletopoulou *et al.,* 2013; Adams and Cory, 2007). An apoptosome is formed after the release of Cytochrome *c*, which interacts with Apaf-1 and procaspase 9. This leads to the activation of initiator caspase-9, which can trigger cleavage of downstream executioner caspases-3, -6 and -7 resulting in apoptosis (Li *et al.,* 2016; Ouyang *et al.,* 2012).

The extrinsic apoptotic signalling pathway is triggered by the binding of death ligands to death receptors on cell surfaces (Fig. 1.2). Tumour necrosis factor (TNF) superfamily (TNF-R1, TNF-R2), TNF-related apoptosis-inducing ligands (TRAIL; TRAIL1, TRAIL 2) and Fas receptors (Apo-1/CD95) are some of the death receptors involved in this pathway (Li *et al.,* 2016). The ligation forms a death-inducing signalling complex (DISC) which consists of Fas-associated death domain-containing protein (FADD) and procaspases -8 and -10. Upon activation, the initiator caspases-8 and -10 activate by cleavage of the executioner caspases-3, -6, -7 (Li *et al.,* 2016; Sellers and Fisher, 1999). In addition, caspases-8 and -10 can stimulate Bid, the pro-apoptotic member of Bcl-2 family of proteins, which has an impact on the permeability of mitochondria (Li *et al.,* 2016).



©Milan Vu

Figure 1.2 Schematic representation of extrinsic and intrinsic apoptotic signalling pathways

Sources of information: Li *et al*., (2016); Nikoletopoulou *et al.,* (2013); Ouyang *et al.,* (2012)

1.3.2 Programmed cell death II: Autophagy

Autophagy is a cellular conservative process, using a catabolic 'self-cannibalising' mechanism, to either enhance cell survival or promote cell death (Nikoletopoulou *et al.,* 2013; Ouyang *et al.,* 2012). It recycles aged cellular content such as organelles and proteins to provide new building blocks and generate energy, thus maintains and protects cell under stress conditions (Feng *et al.,* 2015). Initially activation of autophagy was thought to be an adaptive response of malignant cells in cancer therapy, to develop resistance and promote survival (Radogna *et al.,* 2015). However, over-activation of autophagy may lead to non-apoptotic cell death, known as programmed cell death II (Ouyang *et al.,* 2012). Autophagy in different circumstances acts as a guardian or executioner of tumour cells, and promotes both chemoresistance and chemosensitivity (Nikoletopoulou *et al.,* 2013; Ouyang *et al.,* 2012). The link between autophagy and other cell death types has been elucidated (Goodall *et al.,* 2016; Radogna *et al.,* 2015) and in certain settings, autophagy inhibits apoptosis (Liang *et al.,* 2007), facilitates/cooperates with apoptosis (Ding *et al.,* 2007), or acts as an alternative cell death when apoptosis is blocked (Nikoletopoulou *et al.,* 2013). The role of autophagy can vary from cell to cell and thus needs to be confirmed in cancers; nevertheless, its modulation can be potentially valuable in cancer treatment.

Autophagy is triggered under conditions of metabolic stress such as nutrient starvation and growth factor or hormone deprivation; cellular stress such as hypoxia, reactive oxygen species, endoplasmic reticulum stress, or viral infection (Li *et al.,* 2016; Feng *et al.,* 2015; Nikoletopoulou *et al.,* 2013). Morphologically, autophagy forms an isolation membrane in the cytoplasm that engulfs cytosolic cargo, creating an autophagosome (Feng *et al.,* 2015; Tait *et al.,* 2014). Autophagy proceeds in several steps: initiation, membrane formation, nucleation and elongation of vesicle, autophagosome formation, and autolysosome formation. More than 30 autophagy-related genes (ATG) have been identified which encodes for intracellular (Atg) protein components regulating autophagosome formation, cargo collection and lysosome trafficking (Li *et al.,* 2016; Qiu *et al.,* 2015).

Initiation of autophagy complex is caused by mammalian target of rapamycin (mTOR) inhibition, which is due to activation of adenosine monophosphate-activated protein kinase (AMPK) (Qiu *et al.,* 2015; Radogna *et al.,* 2015) (Fig. 1.3). During metabolic stress, this protective regulator, AMPK, phosphorylates the binding partner of mTOR, Regulatory Associated Protein of mTOR (RAPTOR) thus reducing mTOR kinase activity (Feng *et al.,* 2015; Qiu *et al.,* 2015). mTOR kinase is a metabolic sensor of nutrients, growth factors, and energy, and signals downstream regulators of anabolic and catabolic processes, promoting cell growth and protein synthesis (Radogna *et al.,* 2015; Nikoletopoulou *et al.,* 2013).

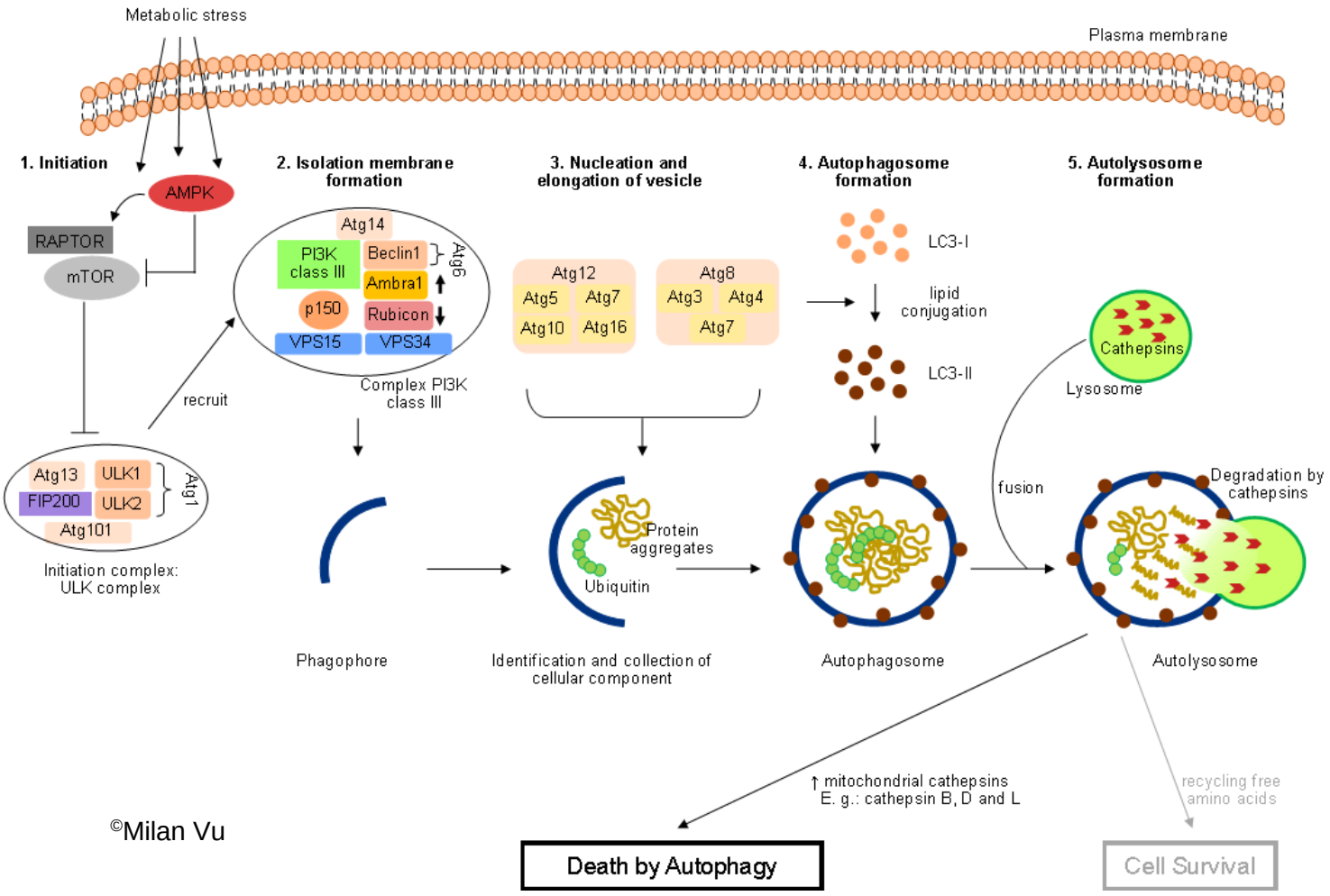


Figure 1.3 Schematic representation of autophagy signalling pathway leading to regulated cell death

Sources of information: Radogna *et al.,* (2015); Qiu *et al.,* (2015); Feng *et al.,* (2015); Tait *et al.,* (2014); Nikoletopoulou *et al.,* (2013); Ouyang *et al.,* (Ouyang *et al.,* 2012)

When mTOR is inhibited, Atg13 cannot be phosphorylated and will bind to a homologue of Atg1 (Unc-51-like kinases; ULK1 and ULK2), forming a ULK complex (pre-autophagosomal structure) with scaffold protein FIP200 and Atg101, leading to the recruitment of other Atg proteins (Ouyang *et al.,* 2012). Subsequently, vesicle nucleation is activated via complex phosphatidyl inositol-3 kinase (PI3K) class III, which is composed of class PI3K III (VPS34), p150 (VPS15), Atg 14 and Beclin 1 (mammalian orthologue of Atg6) proteins, regulated by Bcl-2 protein family, Rubicon and Ambran 1 (Nikoletopoulou *et al.,* 2013).

The process of ubiquitination affects the stability of Atg proteins. Conjugation systems of Atg12 (Atg5, Atg7, Atg10, and Atg16) and Atg8 (Atg3, Atg4, and Atg7) are involved in vesicle elongation (Feng *et al.,* 2015; Radogna *et al.,* 2015). These form phagosphore with misfolded proteins and the organelles aggregate (Feng *et al.,* 2015). The Atg conjugation process facilitate protein lipidation of Atg8 to LC3 (the mammalian homologue of Atg8; microtubule-associated protein 1 light chain 3), allowing autophagosome maturation by inducing the lipid

conjugation of phosphatidylethanolamine. This results in conversion of soluble LC3-I to an autophagy-vesicle-associated LC3-II form, able to act as a scaffold protein and bind on the surface membrane, forming an autophagosome (Li *et al.,* 2016; Radogna *et al.,* 2015).

The final step of autophagy is autolysosome formation where autophagosomes and lysosomes fuse together. The intracellular cargo of organelles are degraded by lysosomal cathepsins (enzyme proteases), freeing amino acids and permitting the energy recycle for the cells (Feng *et al.,* 2015; Radogna *et al.,* 2015). The recycling process by cathepsins is to maintain homeostasis. However, the elevated concentration of these proteases may result in cell death. For instance, cathepsins that affect caspases or mitochondria such as cathepsin B, D and L are associated with cellular demise (Radogna *et al.,* 2015).

1.3.3 Programmed cell death III: Necroptosis

Necroptosis is programmed cell death by necrosis, morphologically characterised by cell and organelle swelling, plasma membrane rupture, mitochondrial dysfunction and cellular collapse (Li *et al.,* 2016). Necrosis is considered a passive cell death, a response to cell demise by a trauma or pathogen, associated and often followed by inflammation processes (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). In contrast, necroptosis is regulated cell death that is caspase-independent and the key induction proteins are Receptor-Interacting serine/threonine-Protein Kinases (RIPK) (Nikoletopoulou *et al.,* 2013; Ouyang *et al.,* 2012). In recent years, necroptosis has gained more attention in targeting cancer cells. Necroptosis-induced death has been reported in apoptosis-resistant cancer cells as a back-up pathway mechanism when caspases are inactivated (Fulda, 2014; Ouyang *et al.,* 2012).

Necroptosis is stimulated by ligation of membrane death receptor such as tumour necrosis factor (TNF; TNFR1, TNFR2), Fas, and TNF related apoptosis inducing ligand (TRAIL1, TRAIL2) (Fig. 1.4), similarly to extrinsic apoptotic pathways (Nikoletopoulou *et al.,* 2013). Other death receptors such as T-Cell Receptors (TCR) and Toll-Like receptors (TLR) may also initiate necroptosis (Li *et al.,* 2016; Tait *et al.,* 2014). The ligation leads to formation of intracellular complexes triggering various responses regulating the cell death pathway (Li *et al.,* 2016). Complex I is comprised of TNFR-associated death domain (TRADD), TNFR-associated factor 2 and 5 (TRAF2, TRAF5), cellular Inhibitor of APoptosis1/2 (cIAP1/2), Linear Ubiquitin Chain Assembly Complex (LUBAC), and Receptor-Interacting Protein Kinase 1 (RIPK1) (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). cIAP1/2 ubiquitinates RIPK1 by conjugation with ubiquitin chain and propagates pro-survival by activation of Nuclear Factor κB (NF-κB) (Li *et al.,* 2016), demoting cell death. However de-ubiquitination of RIPK1 by cylindromatosis (CYLD), a de-ubiquitinating protein, leads to the formation of complex II (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013).

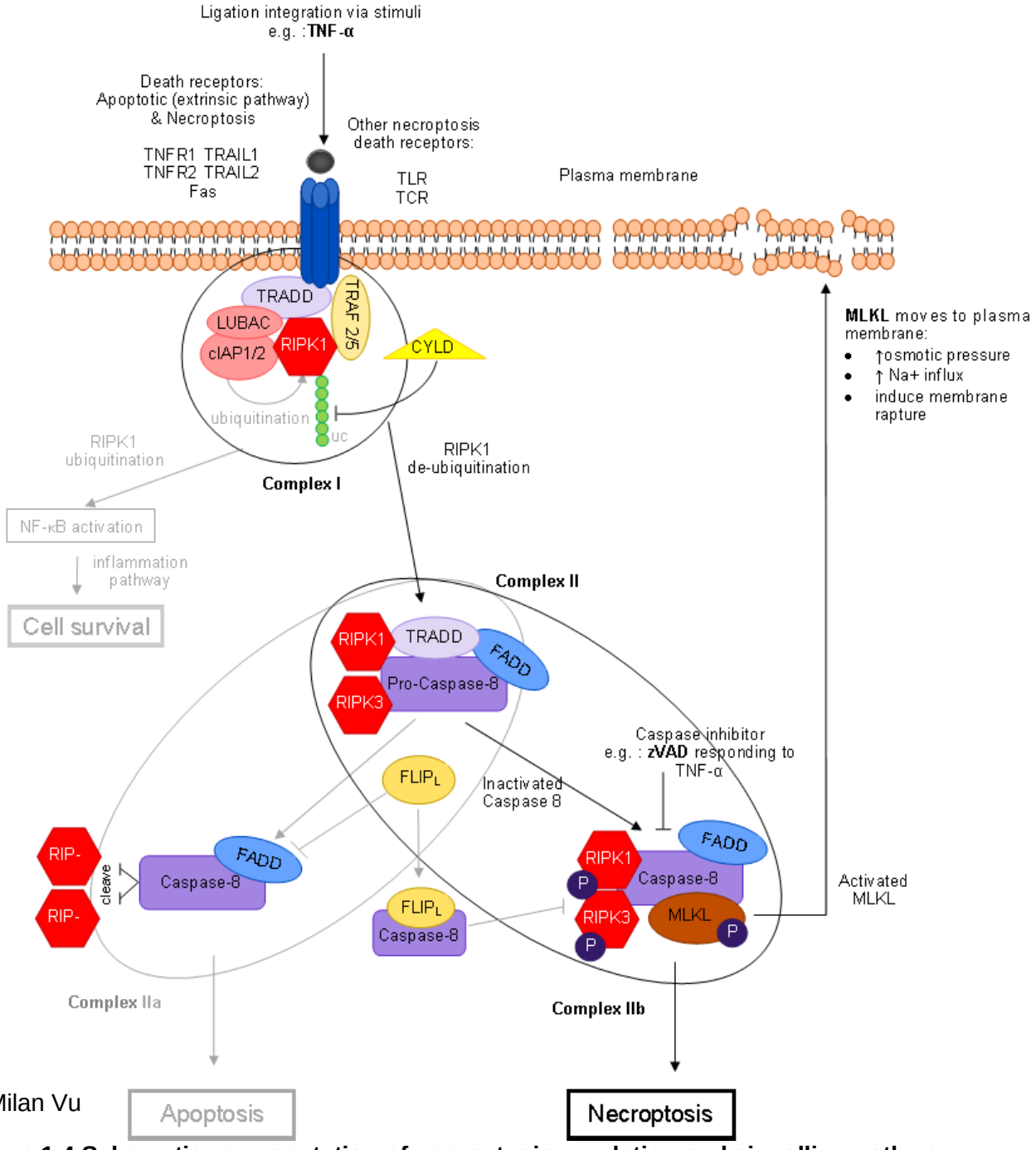


Figure 1.4 Schematic representation of necroptosis regulation and signalling pathway
Sources of information: Li *et al*., (2016); Fulda, (2014); Tait *et al.,* (2014); Nikoletopoulou *et al.,* (2013); Ouyang *et al.,* (2012)

Complex II, consisting of TRADD, Fas-associated dead domain (FADD), pro-caspase-8 and de-ubiquitinated RIPK1 and RIPK3, may propagate into one of the two sub-formations, IIa and IIb (Li *et al.,* 2016). This complex is regulated by $FLIP_L$, FLICE-like inhibitory protein, which can suppress both apoptosis mediation by preventing FADD assembly with caspase-8 homodimers and necroptosis through formation of caspase-8-$FLIP_L$ heterodimer (Tait *et al.,* 2014). Pro-caspase-8 activation will result in complex IIa (Tait *et al.,* 2014), where activated

caspase-8 cleaves RIPK1 and RIPK3, initiating caspase activation cascade and apoptotic cell death (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013).

Complex IIb starts to form once caspase-8 is inactivated (by caspase inhibitors such as z-VAD, a short peptide that inhibits caspases) which enables RIPK1 and RIPK3 to trans-phosphorylate and recruit mixed lineage kinase domain-like protein (MLKL), resulting in necroptosis (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). MLKL, a pro-necrosome protein, upon phosphorylation moves to the plasma membrane and forms channels that increase osmotic pressure, Na+ influx, and membrane rupture (Li *et al.,* 2016).

1.3.4 Cross-talk between the programmed cell death pathways

There is a complex cross-talk between autophagy and apoptosis. For instance, both pathways can be induced by similar stimuli such as metabolic stress (Tait *et al.,* 2014). Furthermore, over-activation of autophagy can induce non-apoptotic cell death (Ouyang *et al.,* 2012). Modulating this type of programmed cell death may enhance killing apoptosis-resistant cancer cells. Necroptosis, has gained more attention in recent years as another non-apoptotic programmed cell death (Fulda, 2014; Ouyang *et al.,* 2012). Similar to autophagy, there is cross-talk between apoptosis and necroptosis pathways (Tait *et al.,* 2014; Nikoletopoulou *et al.,* 2013). Necroptosis may act as a back-up mechanism that is triggered when apoptosis fails to execute cell death (Fulda, 2014). Therefore, death by necroptosis may trigger cellular demise in resistant cells where other death signalling pathways are inhibited.

Investigative work on the regulation of B-cell lymphoma 2 (Bcl-2) family of proteins in AML cells could provide some insight in drug targeting. Deregulation of Bcl-2 family and overexpression of the anti-apoptotic member Bcl-2 protein in AML may possibly contribute to drug chemoresistance (Zinkel *et al.,* 2006; Bensi *et al.,* 1995). In addition, the role of Bcl-2 protein is not limited to only apoptosis but can regulate other cellular processes, including some major types of cell death pathways (Reed, 2008). Comprehension of the mechanical interrelationship between various cell death modes such as apoptosis, autophagy and necroptosis may be advantageous in enhancing the execution of AML cell that gained drug resistance.

1.3.5 Mechanism and interactions between Bcl-2 family proteins

The Bcl-2 protein family regulates the loss of mitochondrial membrane permeability by interacting with each other, which may subsequently result in apoptotic death induction (Gross and Katz, 2017). Some of these proteins are composed of several hydrophobic and amphipathic α-helices but all contain one or more Bcl-2 homology (BH) domains, which are central to Bcl-2 protein family since BH regions enables the interaction between the family

members (Warren *et al*., 2019; Li *et al*., 2016; Shamas-Din *et al*., 2013). These members of the protein superfamily can be further categorised as functionally pro-survival or pro-apoptotic based on the presence of their conserved BH domains (Li *et al*., 2016).

There are three categories of the Bcl-2 family: (1) anti-apoptotic members (Bcl-2, Bcl-XL, Mcl-1, Bcl-w, Bcl-B, Bcl-2A1), also known as 'the guardians', traditionally contain up to four BH (BH1, BH2, BH3 and BH4) regions and can inhibit activity of some pro-apoptotic Bcl-2 members, thus prevent apoptosis (Warren *et al*., 2019; Vogler *et al*., 2017). The pro-apoptotic members are further subcategorised into (2) multi-domain and (3) BH3-only group (Vogler *et al*., 2017; Shamas-Din *et al*., 2013). The multi-domain members (Bax, Bak, Bok) are known as 'effectors/executioners' that contain several BH (BH1, BH2 and BH3) domains and upon activation (through formation of homodimers that oligomerize) form pores in the outer mitochondrial membrane to release Cytochrome *c* and induce apoptosis (Warren *et al*., 2019; Kale *et al*., 2017; Shamas-Din *et al*., 2013). The pro-apoptotic BH3-only protein members contain just one BH3 domain and act as either (3a) activators (BID, BIM) or (3b) sensitizers (BAD, BMF, HRK). Both regulate and facilitate apoptosis through binding to the other multi-domain BH members. The activators can promote the pro-apoptotic effectors/executioners while sensitizers inactivate the anti-apoptotic guardians through competitive binding to release the sequestered BH3-only activators (Warren *et al*., 2019; Gross and Katz, 2017; Shamas-Din *et al*., 2013). In addition, some BH3-only members such as PUMA and NOXA may act as both sensitizers and activators (Gross and Katz, 2017).

The interaction between the Bcl-2 family members is owing to the presence of BH3 domain, which is essential in folding of the hydrophobic pocket and forming of the binding groove, thus becoming a receptor for BH3 domains of the other family members (Warren *et al*., 2019; Kale *et al*., 2017). Some members such as Bcl-2, Bcl-XL, Mcl-1, BAK, BAK, Bim contain at the carboxyl-terminal helix a transmembrane (TM) region domain, which is vital for anchoring to cellular membranes, targeting the proteins to mitochondrial outer membrane (Warren *et al*., 2019; Vogler *et al*., 2017). The binding affinity and the localisation of these proteins can determine their dominance in the interaction. For instance, Bax has a higher affinity to the anti-apoptotic Bcl-2 or Bcl-XL, while Bak tends to form a binding partner with anti-apoptotic Mcl-1 or Bcl-XL (Kale *et al*., 2017; Shamas-Din *et al*., 2013). BH3-only protein activators have high affinity towards the multi-domain pro-apoptotic effectors Bax and Bak, while the BH3-only sensitizers bind to the anti-apoptotic guardians (Shamas-Din *et al*., 2013).

The interplay between the three Bcl-2 family member subgroups has been described by several mechanical models. The direct activation model suggests that BH3-only sensitizers have to free the BH3-only activators from the anti-apoptotic guardians, thus the activators can

prime and stimulate the multi-domain pro-apoptotic members by forming heterodimers together, thus the activation of effectors is through direct binding interaction. In the other model, indirect activation model, both BH3-only sensitizer and activator members have to engage first with anti-apoptotic members, which inhibit apoptosis by sequestering the pro-apoptotic effectors through a binding complex. Therefore, BH3-only proteins may not directly activate the effectors in this model (Ku *et al*., 2011). The complex interactions between the members of Bcl-2 family determine the cell signalling response. Therefore, investigating their regulation in cells that are undergoing death can provide some insight into the mechanism of the drug and cell death modulation.

1.4 Phytochemicals and the modulation of cell death in cancer

A possible strategy to overcome drug resistance associated with relapsed and refractory leukaemia is combination therapy, which may potentially target several signalling transduction pathways that are involved in multiple regulated cell death. Several novel compounds, most of which are phytochemicals, such as betulinic acid, are currently under investigation for their anti-cancer properties. Over 60% of employed chemotherapy drugs originate from natural sources (Shah *et al.,* 2013; Cragg *et al.,* 2005) with many of these compounds derived from plants. For instance, the alkaloid vincristine is an approved chemotherapy drug used for treating leukaemia (National Cancer Institute, 2019) and is a constituent of the plant species *Vinca rosea* L. (Apocynaceae). Vincristine inhibits microtubule assembly leading to the termination of the cell cycle and resulting in the induction of apoptosis (Safarzadeh *et al.,* 2014). Some of these phytochemicals such as curcumin (Guo, Yong *et al.,* 2015; Rao *et al.,* 2011; Wu *et al.,* 2011; Jia *et al.,* 2009) and resveratrol (Jiao *et al.,* 2013; Banerjee Mustafi *et al.,* 2010; Li *et al.,* 2010; Puissant *et al.,* 2010) have demonstrated the ability to induce both apoptotic and autophagic cell death in leukaemic cell lines. In addition, the phytochemical shikonin can induce apoptosis (Mao *et al.,* 2008; Yoon *et al.,* 1999), autophagy (Shi and Cao, 2014) as well as an additional death pathway through necroptosis in various cancer cell lines (Han *et al.,* 2007)

1.4.1 Chemotherapy drug, doxorubicin

Doxorubicin (Dox) is an anthracycline chemotherapy agent derived from a natural source, which was isolated from soil-based pigment producing bacterium (*Streptomyces peucetius*) (Yang *et al.,* 2014; Takemura and Fujiwara, 2007). Anthracylicnes such as Dox is a planar molecules consisting of aglycone (tetracyclic ring with adjacent quinone-hydroquinone groups, methoxy substituent, short side chain and carbonyl) and sugar moieties (3-amino-2, 3, 6-trideoxyl-L-fucasyl moiety) called daunosamine that is attached by a glycosidic bond (Yang *et al.,* 2014; Minotti *et al.,* 2004). Specifically in Dox, the side chain terminates with the primary alcohol unlike in other anthracylines (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004).

The mechanism of action for Dox has been described in early studies by (Fornari *et al.,* 1994) where the drug was shown to inhibit cell growth by blocking an enzyme topoisomerase II, which is essential for cell division. Later studies defined Dox-induced cell death to be apoptotic (Casares *et al.,* 2005; Wang *et al.,* 2004; Panaretakis *et al.,* 2002; Gamen *et al.,* 2000). A summary of selected studies reported the evaluation of Dox in cancer treatment is given in Table 1.2.

Although Dox is approved for treatment of haematological malignancies like acute lymphoblastic or myeloid leukaemia (Szwed and Jozwiak, 2014), it is also employed in other cancers of epithelial origin either as sole chemotherapy or as a poly-chemotherapy regime (National Cancer Institute, 2019). Despite its wide usage against various tumours, the incubation period and concentration of Dox must be carefully considered since its effect on cellular response varies in different conditions (Lüpertz *et al.,* 2010). There are some concerns regarding the small therapeutic window that shows non-discriminatory toxicity towards healthy cells (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004). Therefore, an approach that can utilise Dox anti-tumour activity and capability to induce cell death but also abate its many limitations is desired.

Table 1.2 Doxorubicin induced cell death signalling pathways in cancer cells

Doxorubicin

Concentration/ Incubation time	Cell line/Experimental model	Mechanical Pathway	Type of death	References
5 µM 0.05-0.5 µM	MCF-7 (breast tumour)	-DNA strand cleavage=> DNA damage 5 µM: Non-protein-associated => free radical mediated 0.05-0.5 µM: protein-associated => Topoisomerase II inhibitor		(Fornari *et al.,* 1994)
100 µM (Supraclinic concentrations)	Cell-free system	- Free radical formation (ROS) - DNA damage		(Gewirtz, 1999)
1 µM /16-24 h	JURKAT cells (acute T lymphocyte cell leukaemia)	-Caspase activation (2, 3, 4, 6, 7, 8, 9, and 1 [z-VAD sensitive]) -mitochondria membrane potential disruption	Apoptosis	(Gamen *et al.,* 2000)
0.018 µM 0.11 µM (clinically relevant) /24, 48, 72 h	U266 (multiple myeloma) Daudi (Burkitt's Lymphoma) MEF (WT deficient of Bax, Bak or Bid)	-mitochondrial depolarisation -Bak (precedes Cyt *c*) and Bax activation (BAK mainly) -BH3-only protein=> Bid cleaved to tBid (active form) -Cytochrome *c* release -Caspase activation -p38 SAPK (pathway in response to stress stimuli) =>inhibition decreased Bax, Bak activity -PI3K (counteract apoptotic response) => inhibition induced dox-apoptosis	Apoptosis	(Panaretakis *et al.,* 2002)
0.02 µM = IC_{50} 1 µM (experiments) / 0-48 h	KB-3 (derivative of HeLa) expresses a low level of wildtype p53	-PARP cleavage -↑ caspase-3 -Cytochrome *c* release -JNK activation, but no JNK protein expression change and no induction of c-Jun phosphorylation or AP-1 activation -↑ p53 and p21	Cell death (apoptosis)	(Brantley-Finley *et al.,* 2003)

Table 1.2 (cont'd)

Concentration/ Incubation time	Cell line/Experimental model	Mechanical Pathway	Type of death	References
0.01-0.5 µM /0-24 h	*Non-transformed cells:* BAECs (bovine aortic endothelial cells) ARCMs (adult rat cardiomyocytes) *Tumor cell lines:* PA-1 (human ovarian teratocarcinoma) MCF-7 (human breast adenocarcinoma)	-↑ caspase-3 (BAECs, PA-1) -p53 independent (BAECs, ARCMs), p53-dependent (PA-1, MCF-7) - no oxidative stress in tumour cell lines but ↑ oxidative stress in BAECs	Apoptosis	(Wang *et al.,* 2004)
0.09 µM (low dose) /0-12 days	Huh-7 (human hepatoma/ hepatocellular carcinoma)	*Low dose:* -Induced SA-β-gal (senescence marker) / 6 day - Senescence like phenotype morphology => cell volume enlargement, flattened, multinucleated, vacuolated/6 day -↑ mRNA of osteonectin, SN22, TGase II, PAI-1 (senescence associated gene products) / 9 day - Nuclei slightly large and unequal in size / 3 day; increase micronuclei/6 day; no DNA fragmentation or nuclei condensation (characteristic for apoptosis) -abnormal mitosis=>spindles (tri/quadripolar or asymmetric microtubules) / 3 day -↓ mitosis associated protein Cdc2, CENP-A, Mad2, Bub R1 -Cytochrome *c* release -Cleavage of caspase (3, 9) / 12 days	Low dose killed cells via mitotic catastrophe	(Eom *et al.,* 2005)
18.4 µM (high dose) /0-10 days		*High Dose:* -Apoptotic morphology=> reduced cell volume, blebbing, ↑ nucleus:cytoplasma ratio -↑ in sub-G1 populations -Cytochrome *c* release (in both low and high doses) -↑ phosphorylation p38 and JNK / 1 h-24 h -↑ MEKK3/6 and SEK1 (upstream kinase of p38 and JNK) -Cleavage of caspase (3, 8, 9, 6, 7) -Cleave PARP, FAK, (caspase-3 subst) BID (caspase-8 subst), Lamin B (caspase-6 subst)	High dose killed cells via apoptosis	

Table 1.2 (cont'd)

Concentration/ Incubation time	Cell line/Experimental model	Mechanical Pathway	Type of death	References
2.5-30 µM/24 h CT26 25 µM PROb 30 µM, B16F10 2.5 µM	*In vitro:* CT26 (colon cancer) PROb B16F10 *In vivo:* Mice model with injected cell lines	*In vitro:* -↑ caspase-3 -↑ chromatin condensation *In vivo and ex vivo:* -induced immunogenic cell death mediated by Dendritic cells	Apoptosis	(Casares *et al.,* 2005)
0.11 µM /6, 12, 24 h	Primary ALL cells JURKAT cells U266	-↑ caspase-2 activity -↑ Bak activity -cleave PKCδ (stress activated kinase- respond to DNA damage and apoptosis)- caspase-2 primary cleave, caspase-3 secondary cleave -requires JNK (not upstream to caspase-2 and, PKCδ) Caspase-2, PKCδ , JNK => activated upstream of the mitochondria, leading to ↑ BAK = apoptosis -Cytochrome *c* release	Apoptosis	(Panaretakis *et al.,* 2005)
1 µM / 0, 24, 48,72 h	HeLa U87-MG and LN18 (glioma) HepG2 (hepatoma) PaTu02 (pancreatic adenocarcinoma) Caco2 (colon carcinoma)	-↑ cathepsin B (time- dose- dependent) HeLa cells: -cathepsin B => ↑ caspase-3 activity (time-dependent) - PARP cleavage -loss of mitochondrial membrane potential -Cytochrome *c* and AIF release - ↓ Bcl-2 protein and ↑ Bid - ↓ XIAP (X-chromosome linked inhibitor of apoptosis)- protein mediating degradation of caspase-3 - ↑ phosphorylation of cdk1 (inactivation) - ↑ p21 (cyclin-dependent kinase inhibitor) - ↑ G2/M phase		(Bien *et al.,* 2010)
12.5 µM/ 24 h	Breast cancer cell lines (33 cells) Non-small cell lung cancer (74 cells)	-↓ MCL-1 gene - clinically relevant concentrations act as transcriptional repressors	Apoptosis	(Wei *et al.,* 2012)

Table 1.2 (cont'd)

Concentration/ Incubation time	Cell line/Experimental model	Mechanical Pathway	Type of death	References
0-0.2 µM/ 24, 36, 48, 72 h	AGC (stomach carcinoma)	- changes in mRNA levels of (Dox 0.025 µM): - ↑ Bax (peak at 24 h followed by time-dependent drop) - ↓ Bcl-2 (36-48 h) - ↓ Bcl-2L12 (48-72 h)	Apoptosis	(Florou *et al.,* 2013)
1, 2, 4, 8 µM in 0.05% DMSO/ 24 and 48 h	Breast cancer cell lines: MCF-10F MCF-7 MDA-MB-231	- ↓ Bcl-2 protein - ↓ Bcl-XL gene - ↓ NF-κB gene - ↑ hydrogen peroxide (oxidative stress) - ↑ Bax protein and gene - ↑ Caspase -8 and -3	Apoptosis	(Pilco-Ferreto and Calaf, 2016)
2 µM/ 3, 6, 12 h	NCI-H196 (Small cell lung cancer)	- ↓ in anti-apoptotic mRNA levels of: - ↓ Bcl-2 - ↓ Bcl-XL - ↓ MCL-1	Apoptosis	(Inoue-Yamauchi *et al.,* 2017)

1.4.2 Betulinic acid

Betulinic acid (BetA) ($C_{30}H_{48}O_3$; 3β, hydroxyl-lup-20(29)-en-28-oic acid) is a triterpenoid abundant in the plant kingdom, in particular the outer bark of trees such as the white birch (*Betula pubescent*) (Fulda, 2008; Cichewicz and Kouzi, 2004). BetA is an active constituent in many traditional medicinal formulations and has been reported to promote some health benefits, including having anticancer properties (Yang *et al.,* 2012a).

BetA has been studied largely in solid cancers/cancer of epithelial origin such as head/neck (Thurnher *et al.,* 2003), prostate (Chintharlapalli *et al.,* 2007), ovarian (Fulda, 2008; Zuco *et al.,* 2002), cervix (Fulda, 2008; Zuco *et al.,* 2002), lung (Fulda, 2008; Kessler *et al.,* 2007; Zuco *et al.,* 2002), skin (Hata *et al.,* 2003), liver (Santos *et al.,* 2009), breast (Kessler *et al.,* 2007), colorectal (Kessler *et al.,* 2007) cancers, and multiple myeloma (Yang *et al.,* 2012b; Rzeski *et al.,* 2006). Limited studies have been reported on leukaemia (Faujan *et al.,* 2010; Gopal *et al.,* 2005; Ehrhardt *et al.,* 2004; Hata *et al.,* 2003). Reports on BetA cytotoxic effect *in vitro* was summarised in Table 1.3 Although, studies have shown the broad effectiveness of the compound in cancer cell lines, there are some inconsistencies regarding the mechanism of action of BetA-induced cancer cell death.

Cell death induction by BetA is only partially understood (Vadivelu *et al.,* 2012). Most studies have focused on BetA cell death associated with apoptosis by activating caspases. In addition, mitochondria deregulation has been reported to be the main contributor in the mechanism of action of BetA (Liu and Luo, 2012; Fulda and Kroemer, 2009; Fulda, 2008; Gopal *et al.,* 2005; Ehrhardt *et al.,* 2004; Liu *et al.,* 2004; Tan *et al.,* 2003; Fulda *et al.,* 1997). Therefore, there is a possibility that BetA can modulate the Bcl-2 family of proteins, which interacts with mitochondrial membrane permeability to cause cell death. To date, studies reported on the involvement of the Bcl-2 protein family in BetA cell death execution are inconclusive due to contradictory data (Fig. 1.5) (Shankar *et al.,* 2017; Mullauer, Franziska *et al.,* 2010; Qiuling *et al.,* 2010; Thurnher *et al.,* 2003; Fulda *et al.,* 1997). Thus, BetA may exert varying effects on different cell types, and it is worth investigating Bcl-2 regulation in AML cell lines treated by the compound.

Apoptosis may not be the only pathway triggered by BetA. For instance, in JURKAT (T-cell leukaemia) cell lines, BetA was able to cause cell death despite the blocking of caspase activation by the caspase inhibitor z-VAD.fmk (Kessler *et al.,* 2007). Therefore, BetA may potentially be able to cause cell death in leukaemia by other pathways other than caspase-dependent apoptosis. Some studies have reported the ability of BetA to induce autophagy in cancer cells. However, autophagy by BetA was shown to be protective and inhibitory towards cancer cell death (Potze *et al.,* 2014; Yang *et al.,* 2012b) as well as cause the cells to die

(Martins *et al.,* 2015). Martins *et al*., (2015) attributed death by autophagy induced by BetA to cellular stress induction in the mitochondria. Several modes of cell death feature mitochondrial damage in their pathways (Orrenius *et al.,* 2007), which has a significant role in the mechanism of action of BetA. Thus, BetA may potentially stimulate multiple programmed cell death pathways. A summary of BetA-induced cancer cell death through various mechanisms of action reported by other studies is outlined in Figure 1.5.

To date there are not many reported studies on BetA and alternative cell death (autophagy and necroptosis) on myeloid leukaemia cell lines. It would be interesting to investigate the role of these death pathways in leukaemic cells of relapsed origin, which are known for their refractory nature. Induction of alternative death pathways may be a potential strategy in bypassing chemoresistance in aggressive leukaemic cells.

Additionaly, BetA has been reported to be selectively cytotoxic, showing minimal cytotoxicity in healthy cells when comparing results in cancer cells with normal cells (Aisha *et al.,* 2012; Selzer *et al.,* 2000). BetA has been reported to lack cytotoxic effect on non-leukaemic blood cells, where the compound did not affect the cell growth of normal peripheral blood lymphoblasts (Zuco *et al.,* 2002) and peripheral blood mononuclear cells (Faujan *et al.,* 2010) at relatively high concentrations. Moreover, reports on BetA in combined therapy with other chemotherapy drugs showed enhancement of apoptotic death in cancer cell lines without exerting much negative impact on the normal cells in some of the studies (Gao, Yong *et al.,* 2011; Jung *et al.,* 2007; Fulda and Debatin, 2005; Fulda *et al.,* 2004; Sawada *et al.,* 2004). Since the treatment of leukaemia heavily relies on chemotherapy, novel drug combinations that can molecularly target AML with less systematic side effects are important to study.

Table 1.3 *In vitro* effect of betulinic acid on cancer and normal cells

Histotype	Cancer Cell lines	IC_{50} and ED_{50} of betulinic acid (µM)	Time point/ Assay	References
Melanoma	Me665/2/21	(IC_{50}) 3.3±0.9*	72 h/ MTT	(Zuco *et al.,* 2002)
	Me665/2/60	(IC_{50}) 3.5±0.4*	72 h/ MTT	(Zuco *et al.,* 2002)
	SK-MEL2	(IC_{50}) 5 to 10	6 d/ Coulter Z1 cell counter	(Chintharlapalli *et al.,* 2007)
		(IC_{50}) 14.2	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
	G361	(IC_{50}) 11.4	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
	Mel-1, Mel-2, Mel-3, Mel-4	(ED_{50}) 2.4-10.5	72 h/ Trypan blue exclusion	(Pisha *et al.,* 1995)
Neuroblastoma	SH-EP	(ED_{50}) 4.4-22	72h/ (FACS/PI)	(Fulda *et al.,* 1997)
	GOTO	(IC_{50}) 17.3	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
	NB-1	(IC_{50}) 20.8	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
	SKNAS	(IC_{50}) 3.9	96 h/ MTT	(Rzeski *et al.,* 2006)
Multiple Myeloma	KM3	(IC_{50}) 48.8, 38, 28.6	12 h/ MTT, 38 h/ MTT, 36 h/ MTT	(Yang *et al.,* 2012b)
	RPMI18226	(IC_{50}) 4.3	96 h/ MTT	(Rzeski *et al.,* 2006)
Glioblastoma	SK14, SK17, SK19, SK22, SK37, SK49, SK51, SK55, SK60	(ED_{50}) 15.3-35	72h/ (FACS/PI)	(Fulda *et al.,* 1999)
	U118, U138, U251, U343, U373	(ED_{50}) 10.9-20.8	72h/ (FACS/PI)	(Fulda *et al.,* 1999)
	A172	(ED_{50}) 17.5	72h/ (FACS/PI)	(Fulda *et al.,* 1999)
	Primary cells	(ED_{50}) 4.4-37.2	72h/ (FACS/PI)	(Fulda *et al.,* 1999)
	C6 (Glioma)	(IC_{50}) 7	96 h/ MTT	(Rzeski *et al.,* 2006)
	HPGBM (multiforme)	(IC_{50}) 3.9	96 h/ MTT	(Rzeski *et al.,* 2006)
Head/neck cancer	HNSCC	(ED_{50}) 17.5	72 h/ Casy Cell Counter	(Thurnher *et al.,* 2003)
Ovarian cancer	A2780	(IC_{50}) 3.9±0.4*	72 h/ MTT	(Zuco *et al.,* 2002)
	OVCAR-5	(IC_{50}) 7.2±2.6*	72 h/ MTT	(Zuco *et al.,* 2002)
	IGROV-1	(IC_{50}) 9.8±5.2*	72 h/ MTT	(Zuco *et al.,* 2002)
	HPOC (primary cells)	(IC_{50}) 5.5	96 h/ MTT	(Rzeski *et al.,* 2006)

Table 1.3 (cont'd)

Histotype	Cancer Cell lines	IC_{50} and ED_{50} of betulinic acid (μM)	Time point/ Assay	References
Breast Cancer	MDA-MB-231	(IC_{50}) 127±6.7*	72 h/ MTT	(Yazan *et al.,* 2009)
	MCF-7	(IC_{50}) 44.66±6.4*	72 h/ MTT	(Faujan *et al.,* 2010)
	T47D	(IC_{50}) 2.4	96 h/ MTT	(Rzeski *et al.,* 2006)
Cervical carcinoma	A431	(IC_{50}) 4±0.7*	72 h/ MTT	(Zuco *et al.,* 2002)
	HeLa	(IC_{50}) 36.8±1.3*, 5.5±8.1*	48 h/ MTT, 72 h/ MTT	(Faujan *et al.,* 2010)
		(IC_{50}) 30.4±2.39*, 25.9±2.39*	48 h/ MTT, 72 h/ MTT	(Xu *et al.,* 2014)
		(IC_{50}) 26±2.1*	72 h/ MTT	(Santos *et al.,* 2009)
		(IC_{50}) 0.067±1.73*, 0.04±2.16*, 0.034±2.39*	24 h/ MTT, 48 h/ MTT, 72 h/ MTT	(Xu *et al.,* 2017)
	HPCC (primary cells)	(IC_{50}) 4.5	96 h/ MTT	(Rzeski *et al.,* 2006)
Hepatocellular carcinoma	HepG2	(IC_{50}) 36.4±1.5*	72 h/ MTT	(Santos *et al.,* 2009)
Lung carcinoma	POGB (small cells)	(IC_{50}) 9.2±1.1*	72 h/ MTT	(Zuco *et al.,* 2002)
	POGB/DX^{o}	(IC_{50}) 7±1*	72 h/ MTT	(Zuco *et al.,* 2002)
	H460(Non-small cells)	(IC_{50}) 3.3±0.4*	72 h/ MTT	(Zuco *et al.,* 2002)
	A549	(IC_{50}) 4.3	96 h/ MTT	(Rzeski *et al.,* 2006)
Leukaemia	JURKAT (T-cell)	(IC_{50}) 26.9±2.2*	72 h/ XTT	(Santos *et al.,* 2009)
		(IC_{50}) 6.9	96 h/ MTT	(Rzeski *et al.,* 2006)
	K562 (chronic myeloid)	(IC_{50}) 27.4*	48 h; MTT	(Gopal *et al.,* 2005)
		(IC_{50}) 9.8	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
		(IC_{50}) 46.55±2.78*	24 h; MTT	(Qiuling *et al.,* 2010)
	HL-60 (human myeloid)	(IC_{50}) 45.3±11.8*, 32±5.1*, 5.7±3.3*	24 h/ MTT, 48 h/ MTT, 72 h/ MTT	(Faujan *et al.,* 2010)
		(IC_{50}) 6.6	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
		(IC_{50}) 293.4±10.11*	72 h/ MTT	(Yazan *et al.,* 2009)
	CEM-SS (T4-lymphoblastoid)	(IC_{50}) 11.8±2.32*, 5±2.5* 4.6±1.1*	24 h/ MTT, 48 h/ MTT, 72 h/ MTT	(Faujan *et al.,* 2010)
	Primary acute leukaemia	(ED_{50}) 4.4-32.8	24-48 h/ FACscan	(Ehrhardt *et al.,* 2004)

Table 1.3 (cont'd)

Histotype	Cancer Cell lines	IC_{50} and ED_{50} of betulinic acid (μM)	Time point/ Assay	References
Lymphoma	U937(human lung lymphoblast)	(IC_{50}) 10	72 h/ Trypan blue exclusion	(Hata *et al.,* 2003)
Prostate cancer	LNCaP	(IC_{50}) 1-5	6 d/ Coulter Z1 cell counter	(Chintharlapalli *et al.,* 2007)
		(IC_{50}) 38, 15	24 h/ MTT, 48 h/ MTT	(Shankar *et al.,* 2017)
	DU145	(IC_{50}) 6	48 h/ MTT	(Ganguly *et al.,* 2007)
		(IC_{50}) 38, 15	24 h/ MTT, 48 h/ MTT	(Shankar *et al.,* 2017)
Rhabdomyosarcoma	RMS-13	(IC_{50}) 10.9	72 h/ MTT	(Eichenmüller *et al.,* 2010)
	RH-30	(IC_{50}) 8.5	72 h/ MTT	(Eichenmüller *et al.,* 2010)
	RD	(IC_{50}) 20.8	72 h/ MTT	(Eichenmüller *et al.,* 2010)
	TE671[#]	(IC_{50}) 4.4	96 h/ MTT	(Rzeski *et al.,* 2006)
Thyroid carcinoma	FTC238	(IC_{50}) 5.2	96 h/ MTT	(Rzeski *et al.,* 2006)
Colon cancer	HT-29	(IC_{50}) 2.7	96 h/ MTT	(Rzeski *et al.,* 2006)
Normal cells	Peripheral blood lymphocytes	>110	72 h/ [^{3}H]thymidine incorporation	(Zuco *et al.,* 2002)
	Peripheral blood mononuclear cells	>66	24-72 h/ MTT	(Faujan *et al.,* 2010)

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay; FACS/PI- Flow cytometry using propidium iodide; XTT - 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide colorimetric assay

* Mean ± standard deviation (SD)

º Small cells lung carcinoma cell line resistant to doxorubicin

Rhabdomyosarcoma-medulloblastoma cell line

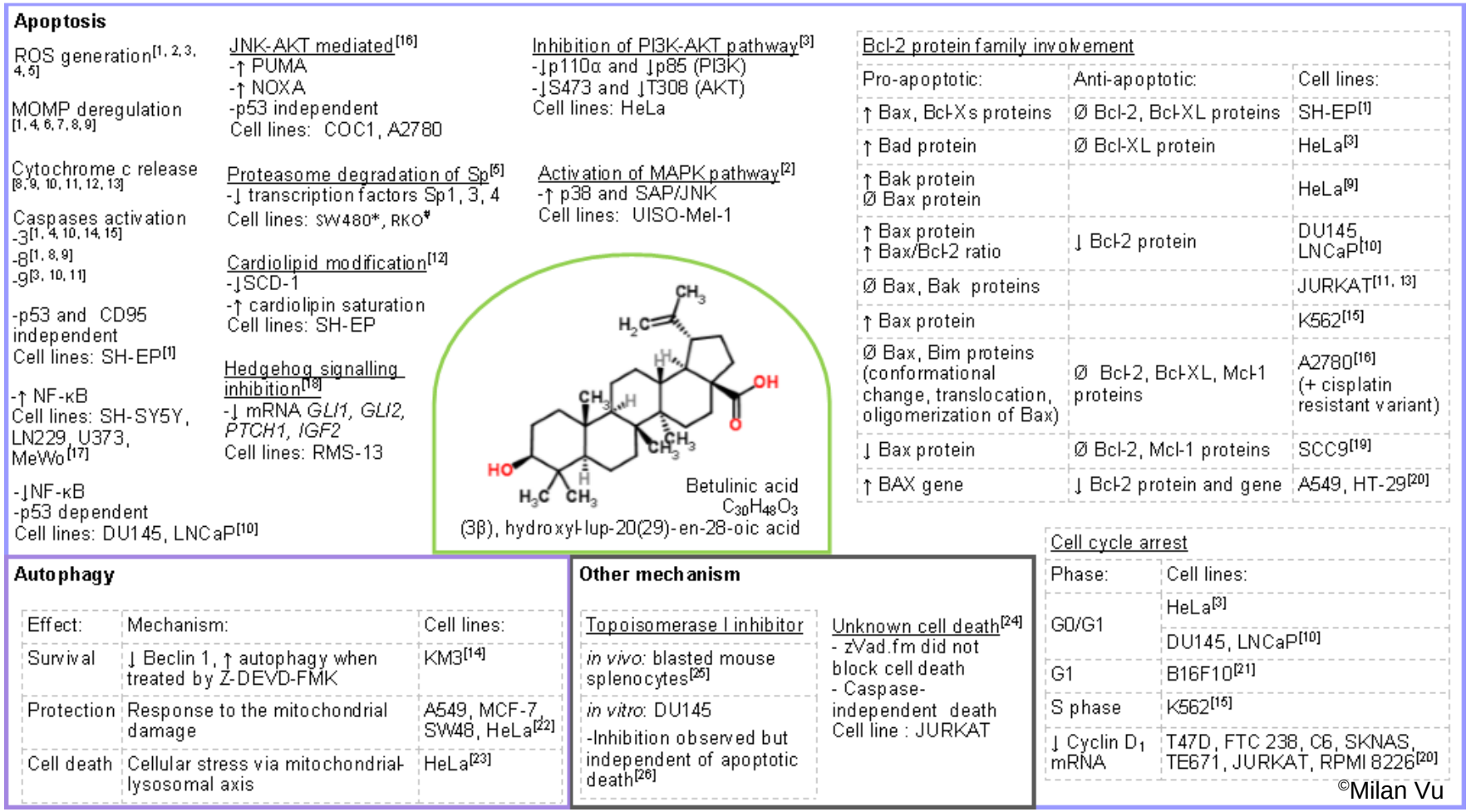


Figure 1.5 Cell death mechanism of action of Betulinic acid reported in cancer cell lines

(Fulda *et al.,* 1997)[1], (Tan *et al.,* 2003)[2], (Xu *et al.,* 2017)[3], (Liu *et al.,* 2004)[4], (Chintharlapalli *et al.,* 2007)[5], (Ehrhardt *et al.,* 2004)[6], (Gopal *et al.,* 2005)[7], (Fulda and Debatin, 2005)[8], (Li *et al.,* 2011)[9], (Shankar *et al.,* 2017)[10], (Liu and Luo, 2012) [11] , (Potze *et al.,* 2016)[12], (Mullauer, Franziska B. *et al.,* 2009)[13], (Yang *et al.,* 2012b)[14], (Qiuling *et al.,* 2010)[15], (Zhao *et al.,* 2012)[16], (Kasperczyk *et al.,* 2005)[17], (Eichenmüller *et al.,* 2010)[18], (Thurnher *et al.,* 2003)[19], (Rzeski *et al.,* 2006)[20], (Sawada *et al.,* 2004)[21], (Potze *et al.,* 2014)[22], (Martins *et al.,* 2015)[23], (Kessler *et al.,* 2007)[24], (Chowdhury *et al.,* 2002)[25], (Ganguly *et al.,* 2007)[26]

Ø—No regulation
ROS—Reactive Oxygen Species
MOMP—Mitochondrial outer membrane permeabilisation
Sp—Specificity protein

Z-DEVD-FMK, caspase-3 inhibitor
*proteasome-dependent (SW480)
#proteasome-independent (RKO)

1.5 Summary of the rationale for the study

Understanding the interplay between cell signalling pathways can potentially help in eliminating refractory leukaemic cells that are commonly developed through cancer relapse. Most anti-cancer drugs target cells by inducing death by apoptosis (Horita *et al.,* 2008). However, only apoptosis sensitive cells, will be killed while cells that are more refractory may acquire additional mutations leading to resistant cells and relapse. Therefore, activation of alternative programmed cell death (non-apoptotic pathways) can be a potential strategy to destroy persistent cancer cells. There is a cross-talk between programmed cell death pathways (Ouyang *et al.,* 2012). For instance, anti-apoptotic Bcl-2 protein of the Bcl-2 protein family can form a complex with Beclin 1, leading to the modulation of autophagy (Li *et al.,* 2016; Tait *et al.,* 2014). The autophagy process exhibits a dual and contradictory role. It can act as a cell survival mechanism and protection of cancer cells from death. However, over-activation of autophagy can lead to cell death (Nikoletopoulou *et al.,* 2013; Ouyang *et al.,* 2012). Consequently, autophagy can be considered as another cell death pathway and its regulation may facilitate alternative cancer cell killing. Another programmed cell death by necrosis, known as necroptosis, can be activated as a back-up mechanism when apoptosis is compromised and fails to induce cell death (Fulda, 2014). These types of programmed cell death pathways may be interconnected and their modulation could lead to enhanced killing of apoptosis-resistant cancer cells or refractory cells that are common for AML relapse.

Utilising a combination of drugs or compounds that can exert synergistic/additive effect might modulate multiple cellular processes simultaneously which may incorporate different modes of programmed cell death pathways such as apoptosis, autophagy, and necroptosis. Therefore, interconnectivity, interaction and modulation of signalling molecules involved in regulating cell death will provide potential strategies in targeting aggressive cancer cells.

1.6 Aims and objectives

The overall aim of this study was to investigate cell death induction in AML cell line from a relapsed MLL-rearranged cell model (MOLM-13), by doxorubicin (Dox) and betulinic acid (BetA), singly and in combination. The objectives were:

- To determine the cytotoxic and anti-proliferative effect of Dox on MOLM-13 cells.
- To evaluate possible mechanisms of action of Dox on leukaemic cell lines by studying its effect on the expression of proteins involved in cell apoptosis, autophagy, as well as the cell survival pathway, PI3K/AKT.
- To investigate and compare the effect of BetA on leukaemic cell lines and its potential to enhance cell death induction by Dox.
- To study the interplay between apoptosis and autophagy through protein and gene expression of apoptotic Bcl-2 family members and autophagic Beclin 1.

The aim of this PhD study was achieved through two related studies described in Chapter 3 and Chapter 4. In Chapter 3, the effect of Dox as a single compound on cell death mechanism and pathways involved in AML model of relapsed cells are discussed and compared to other leukaemic monocytic cell lines. Chapter 4 is a follow up on the findings from Chapter 3 and reports on the potential of BetA to enhance Dox-induced cell death in relapsed AML cells. The modulation of cell death mechanism by the drug combination was studied through the regulation of transcripts and proteins involved in apoptosis and autophagy pathways.

1.2 Outline of the thesis

Background literature detailing the rationale of the study has been provided in this chapter, ending with the aims and objectives. Chapter 2 explains the method development and the experimental procedures used to achieve the objectives of this PhD work. Chapter 3 outlines studies on single Dox and reports it as a selective inducer of apoptosis in MOLM-13 cells associated with inhibition of a novel Bcl-2 isoform, and reduced autophagy inducer protein Beclin 1. Additionally, modulatory effect of Dox on the PI3K-AKT pathway in MOLM-13 cells is reported in Chapter 3. Chapter 4 reports on the effects of combination treatments, assessing the potential of BetA in modulating Dox-induced cell death in MOLM-13 cells. Furthermore, the cell-dependent effects of the drug combinations, compared between AML cells of relapsed origin and leukaemic monocytic cell are reported in Chapter 4. The selective cell death mechanism related to modulation of apoptotic Bcl-2 protein family and autophagy regulation was explored and reported in Chapter 4. Chapter 5 provides the overall summary of the study outcome and further discuss future prospect of this work.

Chapter 2

Methodology, Materials and Experimental Procedures

2 Methodology, Materials and Experimental Procedures

2.1 Rationale for a choice of experimental procedures

2.1.1 In vitro model of study

Studies performed *in vitro* (outside a biological system) and *in vivo* (inside a biological system) are both essential models in cancer research. However, both systems are presented with advantages and limitations in studying the disease (Hartung and Daston, 2009). Human cancer cells can be immortalized into cell lines which are used for investigating molecular pathophysiology and building up the understanding of the disease and its treatment, in fact they serve as an excellent tool to study the behaviour and biological mechanism of cancer (Ferreira *et al.,* 2013; Greshock *et al.,* 2007).

Cell lines can be implanted and maintained in an animal xerograph model, mimicking *in vivo* studies (Greshock *et al.,* 2007). A big advantage of using animal models is the sustenance of the cells within the living tissues where they can be exposed to physiological reactions and interaction, mimicking the cells' microenvironment (Hartung and Daston, 2009). Therefore, this model is useful for testing cells tumorigenecity and metastatic capability (Ferreira *et al.,* 2013). However, there are difficulties with visualisation of the processes and extracting quantitative data of cell mechanism (Katt *et al.,* 2016). Furthermore, *in vivo* studies are usually conducted for more specific pre-clinical evaluations of drugs' toxicity. Initiation of any drug development must go through *in vitro* studies before committing to a large scale and expensive *in vivo* testing or clinical trials (Ferreira *et al.,* 2013).

Cancer cell lines are very similar to the initial tumours and are good representations of a 'pure' population of the cancer cells (Ferreira *et al.,* 2013). Although on their own, the cells in *in vitro* studies lack the microenvironment complexity of *in vivo* studies, thus have reduced physiological relevance, the study model permits control over most experimental variables and quantitative analysis is straightforward (Katt *et al.,* 2016). Cancer cell lines maintained within *in vitro* model systems are easy to grow and thus are appropriate for investigating genetic/epigenetic, cell proliferation deregulation and progression, and for screening and characterisation of cancer therapeutics. Furthermore, the *in vitro* system is crucial for studying molecular mechanisms and cellular pathways, which makes it a robust tool for identifying molecular targets (Katt *et al.,* 2016; Ferreira *et al.,* 2013). This is due to oncogenic pathway activated within cancer cell lines, which still retain the deregulation of transcription of the primary tumour but in simple transcriptome. Their loss of 'unneeded' functions makes them more suitable for anticancer drug testing. In addition, this permits analysis of drug action, drug combination, and screening for resistance or sensitivity (Ferreira *et al.,* 2013).

Therefore, an *in vitro* model was selected for this study because it is suitable for examining cell death pathways and potential markers for therapeutic intervention. It is a valid system as a first approach to the investigation of the mechanism of action of compounds within cancer types, allowing examination of potential drug-induced targets.

2.1.2 Selection of cell lines

Leukaemic cancer cell lines of myeloid origin K562 (CML cells in blast crisis phase) and MOLM-13 (a relapsed AML cancer cell line) were studied to validate the experimental design. For this study, MOLM-13 cell line was selected as the main cell model to investigate the cell death pathways induced by BetA and Dox. MOLM-13 cell line evolved from myelodysplastic syndrome (Matsuo *et al.,* 1997). The cell line would be classified as M5a by an old FAB system of poorly differentiated monoblastic leukaemia of monocytic lineage. The new WHO system, based on recurrent genetic abnormalities, would group MOLM-13 into 'not otherwise specified AML' category which replaced the FAB classification. The MOLM-13 cell line is positive for the FLT3 mutation, presence of FLT3-internal tandem duplications (ITD), which is associated with more aggressive cells (Reiter *et al.,* 2018). In addition, MOLM-13 is derived from the mix lineage leukaemia (MLL)-rearranged cell line. MLL is a genetically distinct form of AML and it is considered to be particularly aggressive, with sufferers having poorer prognosis and early relapse onset (Daigle *et al.,* 2011). Therefore, MOLM-13 serves as an ideal *in vitro* model to study death signalling pathways in a refractory cell line of relapsed origin.

K562 cell lines are CML cells in the fatal phase known as blast crisis, which is associated with an acute transformation (Hoffbrand and Moss, 2011). There are existing studies on the anti-leukaemic effect of BetA on K562 cells (Gopal *et al.,* 2005; Hata *et al.,* 2003). However, there are lack of reported studies conducted on AML cell lines. In the current study, K562 cells were used for preliminary studies alongside MOLM-13 cells to compare and assess the broad toxicity effect of BetA and Dox in myeloid leukaemia cells. Furthermore, studies on cell death induction in AML cells at relapse are required. Therefore, MOLM-13 cell lines were selected for further combination investigations to study if BetA can enhance Dox-induced apoptotic death in AML cells.

Non-cancerous adherent human embryonic kidney cells (HEK293T; cells of epithelial origin) were used as a cell control to examine compounds' selectivity towards cancer cells. Another AML cell line OCI-AML2 was used for protein quantification to assess the constitutive expression of specific apoptosis and autophagy proteins, thus compare the proteomic diversity between leukaemic cell lines.

Non-malignant blood cells (non-leukaemic monocytes cells) were purchased as control cells to test whether the compounds of interest, BetA and Dox, are selective in targeting the cancer cells and non-toxic to non-cancerous blood cells. However, non-leukaemic SC monocytes (supplied from ATCC) used in this study were later confirmed to be co-cultured with leukaemic monocytic cell line U-937 before acquisition from the supplier. Thus, SC/U-937 cells were implemented in this study as a comparative leukaemic monocyte cell line to MOLM-13 cells. Studies on other leukaemic monocytic cell line such as SC/U-937 is of interest with regards to cancer research in providing comparative data between different leukaemic monocytes where one is a result of cancer recurrence (MOLM-13). This would allow examining the distinction in regulating the growth/viability of the cells and determination of genetic alteration/transformation between cancer of relapsed origin and non-relapse cells.

2.2 Materials and Experimental procedures

2.2.1 Assessing normal growth of cells under standard in vitro conditions

Normal cell growth under standard *in vitro* conditions was assessed to determine the doubling time for each cancer cell line. Therefore, the cell density of MOLM-13 and K562 cancerous cell lines were counted over a time period up to 54 h using Trypan blue HyClone® exclusion assay without refreshing the medium. This quick method utilises a dye that penetrates and stains only cells with weak/ruptured cellular membranes (categorised as dead cells) while cells with healthy cell membranes can exclude the dye. The log phase (cells growing exponentially and therefore at maximum activity) of the dividing cells was identified. Drug treatments and other further investigations were carried out on cells growing in the log phase.

2.2.2 Investigating the effect of dimethyl sulfoxide (DMSO) to select vehicle cell control concentration

BetA and Dox both have low solubility in culture medium owing to its high lipophilic characteristic. Organic solutions such as dimethyl sulfoxide (DMSO) are used in order to dissolve lipophilic compounds. Unfortunately, DMSO may become cytotoxic at high concentrations and distort the findings of biological assays. Therefore, studies were conducted to select a non-toxic concentration of DMSO for the dissolution of the test compounds. This DMSO concentration was then standardised in all compound preparations used for cell treatments and was also used as a negative control (vehicle control).

2.2.3 Evaluation of cell viability

Initially, CyQUANT Direct® and alamarBlue™ assays were performed to verify the inhibitory effect of BetA on the growth of the leukaemic cell lines. Both methods showed similar result outcomes (Appendix 1). Cell viability/cytotoxicity assays measures how many cells remain viable after treating them with toxic compounds for a specific period of time. CyQUANT Direct® detection reagent binds to nucleic acids thus measures cell viability based on the DNA/RNA content of life cells. alamarBlue™ dye estimates cell viability by measuring the cell metabolic activity.

The data for cell viability inhibition was used to calculate the IC_{50} of the compounds in the tested cell lines. Only data that were found to be dose- and time- dependent in inhibiting the cell viability were suitable for the estimation of IC_{50} value. The IC_{50} and data from the literature were considered in selecting the concentration of BetA for combination studies (with the established AML chemotherapy drug, Dox).

DNA binding dye (CyQUANT) may be considered a more appropriate approach for detecting cell viability measurement since nucleic acid content in viable cells is highly regulated and directly correlates to the live cells. On the other hand, the metabolic assay (alamarBlue) is an indirect measurement that depends on efficiency of metabolic enzymes of the cells. This introduces numerous variables since metabolic enzymes may vary in different cell life cycles (Quent *et al.,* 2010). In addition, CyQUANT Direct® dye employs reagents that are selectively permeable to live cells. Therefore, the CyQUANT Direct® assay was utilised for further combination studies since cell viability is assessed based on both DNA content and membrane integrity of the cells.

2.2.4 Investigating toxicity of individual treatments of betulinic acid and doxorubicin on leukaemic and non-cancerous cells

BetA cytotoxicity studies were conducted on non-cancerous HEK293T cells to examine if the compound is selective towards the cancer cells. Dox is an established AML chemotherapy drug that is also used in other therapies. However, the drug has been reported to cause toxicity in normal tissues and cancer cells may eventually develop resistance to the drug (Minotti *et al.,* 2004). Dox was also investigated to determine its toxicity on non-cancerous HEK293T cells (used as control cells). Furthermore, the toxicity of the compounds (BetA and Dox) was assessed in range of leukaemic cell lines to evaluate distinct sensitivity of the cytotoxic agents.

2.2.5 Selection of suitable concentrations for combination studies

The plasma concentration for BetA has not yet been determined. However, BetA at 10 µg/ml (22 µM), a non-toxic dose to human cells, *in vitro* (Viji *et al.,* 2010; Ehrhardt *et al.,* 2004) is equivalent to the administration of 100 mg/kg (body weight). This concentration has been found to be non-toxic to experimental animals (Pisha *et al.,* 1995). In this study, based on preliminary cytotoxicity data, BetA 20 µM was an agreed effective concentration (from an average) between MOLM-13 (acute) and K562 (chronic) cell lines (myeloid leukaemia cells) based on the IC_{50} values at 48 h. Therefore, 20 µM BetA was selected as suitable concentration for combination studies with an established AML chemotherapy drug Dox for the currently reported study.

The clinical dose of Dox used in combination with other chemotherapy agents is 40-60 mg/m^2 IV every 21 to 28 days and the maximum recommended cummulative dose is 450 mg/m^2 (Cheesman and Shields, 2014). Dox plasma concentration can be as high as 10 µM during bolus administration with half-time exponentially declining within 24-30 h. The expected concentration of Dox in the patient's plasma within 2 h is 0.1-1 µM which equates to relevant clinical concentration used in *in vitro* studies (McHowat *et al.,* 2001). Thus, in this study the higher end (1 µM) and its half (0.5 µM) was used for the combination studies.

2.3 Method

2.3.1 Cell culture

MOLM-13 is an immortalised AML cell line derived from MLL-rearranged cells from a relapsed patient (with FLIT3 mutation), which has evolved from myelodysplastic syndrome (Matsuo *et al.,* 1997). This cell line allowed for the study of relapsed AML. OCI-AML2 is a cell line established from a patient acute myelomonocytic leukaemia (sub-group of AML) which carries DNMT3A R635W mutation. K562 was established from a patient in the blast crisis stage of chronic myelogenous leukaemia (CML) and positive for the Philadelphia chromosome. Epithelial kidney cell line HEK293T was used as cell control of non-malignant origin to assess compounds cell toxicity and selectivity.

SC (ATCC CRL-9855) cells were purchased from American Type Culture Collection and maintained in Iscove's Modified Dulbecco's Medium (Sigma, UK). SC cells were originally monocyte cells from human peripheral blood but have since been cross contaminated by U-937 (ATCC CRL-1593.2). U-937 cells are derived from histiocytic lymphoma; leukaemia of mononuclear phagocyte system consisting of monocyte/macrophage cells which are myeloid in nature (Usmani *et al.,* 2019). Therefore, the cell line is referred to as SC/U-937 cells in this study. SC/U-937 cells were used as additional leukaemic monocytic cell control, which allowed comparison between leukaemic cells (MOLM-13) representing relapsed AML cell model and monocytes of non-relapsed origin.

Authenticated leukaemic MOLM-13, OCI-AML2 (AML) and K562 (CML) cell lines (purchased from the European Collection of Cell Cultures, Public Health England) were maintained in RPMI 1640 (developed at Roswell Park Memorial Institute; Sigma, UK) medium. Human embryonic kidney cell lines (HEK293T; non-cancer epithelial control cells) purchased from American Type Culture Collection were maintained in Dulbecco's Modified Eagle Medium. Each complete culture medium was supplemented with 1% L-glutamine, 1% penicillin-streptomycin antibiotic and 10% fetal bovine serum (FBS). All cell lines were tested regularly for Mycoplasma using polymerase chain reaction (PCR) and were Mycoplasma-free throughout the experiments. The cells were cultured and grown in a humidified incubator at 5% CO_2 environment and temperature of 37°C. The media for cancerous blood cells was changed every 48-72 h through cell pelleting by centrifugation that was followed by removing the old medium and replenishing with a fresh complete medium. Adherent cells, HEK293T, were subcultured when 80% confluent with Trypsin-EDTA (Gibco, Thermo Fisher Scientifi) for up to 10 minutes. Only cells in logarithmic growth were used in experiments. Trypan blue HyClone® dye assay was also used to determine cell viability and cell density prior to proceeding with other assays. The cells were stained at a 1:1 ratio with the dye and manually

counted using a haemocytometer under light microscopy. Cell viability of at least 97% was accepted as appropriate for experiments.

2.3.2 Determination of cell viability and cytotoxicity

CyQUANT Direct® assay

The CyQUANT Direct® (Invitrogen, Thermo Fisher Scientific) assay was used to determine the viability of the cells after the treatment period. CyQUANT® detection dye was prepared by mixing nucleic acid stain (0.4%), background suppressor I (2%), and culture media (97.6%). CyQUANT dye stains nucleic acids (RNA and DNA) in dividing cells and is used as an indicator of cell number since DNA/RNA content is highly and tightly regulated in live cells (Invitrogen, 2009; Jones *et al.,* 2001).

Stock solutions (20x the final conc.) of BetA (Sigma Aldrich, UK) and doxorubicin (Dox; Sigma Aldrich, UK) were prepared in 1% DMSO (in medium or PBS) and the cell treatments were conducted in 0.05% DMSO. The effect of the drugs were examined using non-cancerous HEK293T cells and leukaemic (CML) K563, (AML) MOLM-13 and SC/U-937 cells.

Cells were seeded at 95 µL ($5x10^5$ cells/mL) in 96-well plates to which 5 µL BetA (final conc. of 10-40 µM) or Dox (final conc. of 0.5-10 µM) was added and incubated for 24 h, 48 h and 72 h. For drug combination analysis, the cells were treated with BetA (final conc. of 20 µM), Dox (final conc. of 0.5 µM or 1 µM) or a combination of both of BetA (final conc. of 20 µM) and Dox (final conc. of 0.5 µM or 1 µM) for 24 h. Cells treated with the DMSO vehicle (final conc. 0.05%) were used as negative control.

After the treatment period, CyQUANT Direct® (Invitrogen, Thermo Fisher Scientific) dye was overlaid in each well and incubated for a further 45 min (in a humidified incubator at 37ºC) to determine the viability of the cells. The fluorescence of the samples was measured using a microplate reader FLUOstar Omega (BMG Labtech) at 1000 gain at 485 nm excitation and 520 nm emission wavelength.

alamarBlue™ assay

Cell treatments were seeded at 90 µL/well in 96-well plate; cell suspension (85.5 µL at $5x10^5$ cells/mL) were treated with individual test compounds (4.5 µL; 20x the final conc.) and then incubated at 37ºC and 5% CO2. The vehicle (DMSO 0.05%) was used as negative control and culture medium with no cells was used as blank samples.

After the incubation period, a ready-to-use alamarBlue™ solution (Thermo Fisher Scientific) at 1:10 dilution (10 µL) was directly added onto treated microplates with a further 6 h incubation in humidified incubator at 37ºC. alamarBlue™ detection dye quantitatively measures cell viability/cytotoxicity by determining the metabolic activity of cells. Viable cells with active

metabolism reduces alamarBlue™ (oxidised blue resazurin product that is non-fluorescent) into resorufin product (reduced red/pinkand fluorescent) (Riss *et al.,* 2013; O'Brien *et al.,* 2000). The fluorescence of the samples was detected using microplate reader FLUOstar Omega (BMG Labtech) at 600 gain at 544 nm excitation and 590 nm emission wavelengths.

2.3.3 Determination of cell proliferation using 5-(and -6) carboxyfluorescein diacetate succinimidyl ester (CFSE)

Labelling cells with CFSE fluorescent dye

MOLM-13 cells were labelled with a cell membrane permeable fluorescent dye, 5-(and -6) carboxyfluorescein diacetate succinimidyl ester (CFSE) prior to cell treatment. Fluorescent intensity of labelled cells was distributed and split with each cell division. The cells were harvested, washed 3 times and re-suspended in phosphate buffer saline (PBS). A stock solution of CFSE (2 µM in DMSO) was freshly diluted 100-fold in PBS before labelling the cell. The cells were incubated in the dark with CFSE at room temperature for 20 min with shaking at 5 min intervals. The reaction was stopped by adding complete RPMI medium followed by pelleting of the cells. The cells were then re-suspended in fresh complete RPMI medium and incubated in the dark at 37°C for 10 min before being harvested and re-suspended at $1x10^6$ cells/mL with fresh complete RPMI medium. Cells not labelled with CFSE and not stained by propidium iodide (PI) were used as a blank control.

Cell treatments and propidium iodide staining

CFSE labelled MOLM-13 cells (at $1x10^6$ cells/mL; 950 µL) were treated with BetA (final conc. 20 µM, 50 µL), Dox (final conc. 0.5 µM or 1 µM, 50 µL) and BetA (final conc. 20 µM, 25 µL) combined with Dox (final conc. 0.5 µM or 1 µM, 25 µL) in a 24-well plate. The plate was incubated in 5% CO_2 at 37°C. CFSE-labelled cells were treated for 24, 48 and 78 h. Unlabelled cells were harvested, washed in PBS and fixed in 0.4% paraformaldehyde (0.5 mL) for 10 min, washed and then re-suspended in PBS at a density of $2x10^6$ cells/mL. These fixed cells (50 µL; equivalent to $0.1x10^6$ cells/mL) were added into each cell treatment (data obtained from the fixed cells were used for normalisation). Cell treatments were then harvested, washed in flow buffer (PBS, 2% FBS, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide), pelleted and stained with 0.5 µg/mL propidium iodide (PI; 100 µL) for 10 min in the dark at room temperature. Finally, flow buffer (400 mL) was added to the samples and the samples were analysed using FACS Calibur flow cytometry (BD CellQuest™ Pro software, Oxford). Fluorescent detection was made at an emission wavelength of 480 nm (FL1 for CFSE) and 630 nm (FL3 for PI) using 488 nm excitation wavelength. Fixed cells stained only with PI (showing dead cells) were used as a control. The data was further analysed by Flowing Software (version 2.5.1).

2.3.4 Cell death population assays using 488 Annexin V and PI: Flow cytometry

MOLM-13 and SC/U-937 cells ($1x10^6$ cells/mL) were treated with BetA (final conc. 20 µM), Dox (final conc. 0.5 µM, 1 µM, 5 µM) and BetA (final conc. 20 µM) combined with chemotherapy drug Dox (final conc. 0.5 µM, 1 µM) for 24 and 48 h in 5% CO_2 and at 37°C. Cells treated with 0.05% DMSO vehicle were used as negative control. After the incubation period, cells were harvested, washed in cold PBS and then stained with Alexa Fluor® 488 Annexin V (5 µL) (Thermo Fisher Scientific) for 10 min followed by PI (100 µg/mL; 1 µL) for a further 10 min. The cells were analysed by FACS Calibur flow cytometry (BD CellQuest™ Pro software, Oxford) at a fluorescent emission at 525 nm (FL1; Alexa Fluor®) and 575 nm (FL3; PI) using 488 nm excitation wavelength. AlexaFluor® uses Annexin V that binds to phosphatidylserine present in apoptotic cell membrane and PI which binds to the DNA of dead cells with damaged cell membrane to differentially stain and estimate the cell population of viable and dead (necrotic and apoptotic) cells.

2.3.5 Reactive Oxygen Species (ROS) formation

DCFDA cellular ROS detection assay (Abcam, UK) was used to measure ROS activity in cells using the fluorogenic dye 2',7'–dichlorofluorescin diacetate (DCFDA). The oxidation product, 2',7'–dichlorofluorescein is highly fluorescent and was measured to estimate ROS levels in the cells. Harvested MOLM-13 cell lines were first washed in cold PBS followed by 1X Buffer wash. The cells were then stained by suspending them in DCFDA solution (20 µM) at a concentration of $1x10^6$ cells/mL and incubated at 37°C for 30 min in the dark. After incubation, the cells were wash in 1X buffer and re-suspended at $1x10^6$ cells/mL in 1X supplement buffer (1X buffer containing 10% FBS) and seeded at 95 µL/well in a 96-well microplate. Cells were then treated by BetA (final conc. 20 µM), Dox (final conc. 0.5 µM, 1 µM, 5 µM; positive control), and BetA (final conc. 20 µM) combined with Dox (final conc. 0.5 µM or 1 µM) and incubated at 37°C in 5% CO_2. Tert-butyl hydrogen peroxide (TBHP) (final conc. 50 µM; positive control) and vehicle (DMSO final conc. 0.05%; negative control) treatments were used as controls. The fluorescence of 2',7'–dichlorofluorescein was measured at 30 min intervals up to 3.5 h using a FLUOstar Omega microplate reader (BMG Labtech) at 1000 gain, 485 nm excitation and 520 nm emission wavelengths.

2.3.6 Investigation of proteins involved in cell death: Western blot analysis

Cell treatment and lysis

The cells were treated in 1:20 dilution with BetA (final con. 20 µM), Dox (final conc. 0.2, 0.5, 0.75 and 1 µM), DMSO (final conc. 0.05%; vehicle control) and two combinations of BetA (final

conc. 20 µM) with Dox (final conc. 0.5 or 1 µM). The treatments were incubated for 48 h at 37°C in humidified atmosphere of 5% CO_2.

After treatment, the cells were harvested, washed twice in ice-cold PBS and pelleted. The cell lysate buffer was prepared by diluting Halt Protease Inhibitor Cocktail 100-fold in RIPA buffer. The lysate buffer at 50 µL/1x10^6 cells (150 µL/3x10^6 cells) was homogenised with the cell pellets and incubated for 15 min with shaking at 5 min intervals. To further extract the proteins from the cells, the samples were sonicated at 50% amplitude thrice for 2 sec with 1 min rest in between on ice and then left on ice for an additional 15 min. The samples were then centrifuged and the supernatant of the lysates were collected.

Bradford assay for the determination of protein concentration

A stock solution of bovine serum albumin (BSA; 2 mg/mL) in PBS was used to prepare BSA standards working solutions (1.5, 1.4, 1.2, 1.0, 0.8, 0.4, 0.2, 0.1, and 0.05 mg/mL). Lysate samples were diluted in PBS (1:10; total 100 µL). The diluted samples, BSA standards, and blank samples (PBS only) were plated in a 96-well plate (20 µL/well) in triplicates. Bradford Coomassie reagent (300 µL) was overlaid onto each well and incubated in the dark for 5 min followed by absorbance measurement using a microplate reader FLOstar Omega (BGM Labtech) at 595 nm wavelength. A regression equation was generated to calculate protein concentration and loading protein volume.

SDS-PAGE electrophoresis and membrane transfer

Loading buffer was prepared using 2x Laemmli (BIO-RAD) with a reducing agent β-mercaptoethanol (5%, 1:20 dilution). Protein lysates (30 µg of whole cell lysate) were loaded into the well by mixing samples 1:1 ratio with the loading buffer. Samples were then boiled by heating block at 95^{O}C for 5 min, and then kept on ice. Lysate samples were separated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using Mini-PROTEAN® TGX™ (4-15%) Precast gels (BIO-RAD) for 30-40 min at constant 100 volts. The lysates were then transferred onto a nitrocellulose membrane by Trans-Blot® Turbo™ Transfer System Transfer Pack (BIO-RAD).

Immunoblotting and visualisation

The transferred membranes were blocked with 5% BSA or 5% skimmed milk in 0.01% Tween20 in PBS (PBS-T) with rocking for 1 h at room temperature and then washed 5 times in PBS-T. The membrane blots were incubated overnight at 4°C with a primary antibody (anti-Bcl-2 (1:1000 in 5% milk; Abcam cat. no. ab32124), anti-Bax (1:1000 in 5% milk; Abcam cat. no. ab32503), anti-Beclin 1 (1:2000 in 5% BSA; Abcam cat. no. ab207612), anti-Cytochrome *c* (1:5000 in 5% milk; Abcam cat. no. ab133504), anti-caspase-8 (1:2000 in 5% milk; Abcam cat. no. ab32397), anti-caspase-9 (1:2000 in 5% milk; Abcam cat. no. ab202068), anti-PI3K

p85α (1:1000 in 5% milk; Abcam cat. no. ab191606), anti-pan-AKT (phospho T308) (1:1000 in 5% BSA; Abcam cat. no. ab38449), and anti-PTEN (1:2000 in 5% milk; Abcam cat. no. ab170941). The blots were then washed 5 times in PBS-T and then probed with a secondary antibody (Goat Anti-Rabbit IgG (H+L) horseradish peroxidase (1:3000 in 5% BSA or 5% milk; BIO-RAD cat. no. 170-6515)) for 1 h at room temperature and then washed 5 times in PBS-T. Primary antibody for β-actin (1:5000 in 5% BSA; Abcam cat. no. ab8226) was used as a housekeeping protein control, which was probed by another secondary antibody, Goat Anti-Mouse IgG (H+L) horseradish peroxidase-labelled secondary antibody (1:3000 in 5% BSA or 5% milk; BIO-RAD cat. no. 170-6516). The membranes were then incubated with ECL using ODYSSEY® FC imager (Li-Cor, Bioscience). A semi-quantitative comparison of protein levels was determined by densitometry using Image Studio™ Lite software.

2.3.7 Investigating gene regulation via RT-PCR

This work was done in collaboration with dissertation students under the supervision of Dr Nick Kassouf.

Cell treatment and RNA isolation

MOLM-13 and SC/U-937 cells at $1x10^6$ cells/ml density (950 µl) were treated with BetA (final con. 20 µM, 50 µl), Dox (final con. 1 µM, 50 µl), or combination treatment of BetA (final con. 20 µM, 25 µl) with Dox (final con. 1 µM, 25 µl) for 48 h. Cells treated with DMSO (final con. 0.05%) were used as a vehicle control. Prior to the RNA isolation, the cells were pelleted via centrifugation, snap frozen in liquid nitrogen and stored at -80°C. The RNA samples were obtained by carrying out a silica membrane binding method utilised within the Isolate II RNA mini assay (Bioline, UK). The RNA concentration and purity were checked using a Nanodrop (NanoDrop 2000/2000c, Thermo Scientific)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A standard amount of RNA at 200 ng for all samples was converted to cDNA by reverse transcription and amplified by PCR utilising a one-step RT-PCR method (Table 2.1); the MyTaq™ One-Step RT-PCR Kit (Bioline, UK) in a thermal cycler (Techne® Prime, Bibby Scientific). The RT-PCR conditions on the thermocycler were as follows: reverse-transcription (1 cycle, 45°C, 20 min), polymerase activation (1 cycle, 95°C, 1 min), denaturation (30 cycles, 95°C, 10s), annealing (30 cycles, 60°C, 10s), and extension (30 cycles, 72°C, 30s). Specific primers for pro-apoptotic BAK and BAX, anti-apoptotic BCL-2 and BCL-XL, autophagy marker ATG5 and BECLIN 1 (Table 2.2; all from Invitrogen, Thermo Fisher Scientific) and for the housekeeping gene GAPDH (Invitrogen, Thermo Fisher Scientific) (Fwd: ACCACAGTCCATGCCATCAC; Rev: TCCACCACCCTGTTGCTGTA) were used for gene targeting. An intercalating DNA binding stain, SafeView (5 µl/100 ml agarose), was added to the 1.5% agarose gel. PCR products were loaded into the gel and the electrophoresis was

carried out at a constant 90 V for 30 minutes. The gel image was visualized under UV illumination using an UVP GelDoc-It® imaging system 2UV transilluminator. Densitometry was carried out using ImageJ software.

Table 2.1 Template of the components used in one-step RT-PCR Table

Reagents	Samples		
	Vehicle ctrl	Treatment	NTC
2x MyTaq One-Step Mix	12.5 µL		
Forward primer*	1 µL		
Reverse primer*	1 µL		
Reverse transcriptase	0.25 µL		
RiboSafe RNase Inhibitor	0.5 µL		
DEPC-H_2O#	Volume to make up final 25 µL		9.75 µL
Template (200 ng)#	X (µL)	X (µL)	Ø
Final volume	25 µL		

DEPC-Diethyl Pyrocarbonate, NTC – non-template control, (Ø) lack of nucleic acid, (*) Different set of primers were used for each investigation of transcript regulation, (#) – specific per sample, (X) – specific volume to standardize 200 ng of RNA, which depended on the RNA concentration of the sample.

Table 2.2 Primer design for apoptotic Bcl-2 family and autophagy genes

Gene	Primer sequence (5'-3')	Product size (bp)
Pro-apoptotic Bcl-2 family		
BAK	Fwd: CTGTTTTTACCGCCATCAGCAGG Rev: CTCTCAAACAGGCTGGTGGCAATC	249
BAX	Fwd: CCGTTCATCTCAGTCCCCTG Rev: GAAGTGTGTCCCGAAGGAGG	396
Anti-apoptotic Bcl-2 family		
BCL-2	Fwd: GACTTCTTCCGCCGCTACCG Rev: GACAGCCAGGAGAAATGAAAC	341
BCL-XL	Fwd: CCCAGAAAGGATACAGCTGG Rev: GCGATCCGACTCACCAATAC	488
Autophagy marker		
ATG5	Fwd: TCTAAGGATGCAATTGAAGCTCA Rev: GGCCCAAAACTGGTCAAATCT	153
BECLIN 1	Fwd: GCTGGAAGACGTGGAAAAGA Rev: TCCAGCTGCTGTCGTTTAAATT	135

Fwd – Forward primer, Rev – Reverse primer

2.3.8 Data and statistical analysis

The data were expressed as a mean percentage ± SE relative to untreated cells or the vehicle control (DMSO 0.05%) from a sample size of at least three replicates. The IC_{50} of the single compounds were calculated by using GraphPad Prism 6 software, based on the mean ± SE cell viability detected by CyQUANT Direct assay (Section 4.2.1) and Annexin V/PI method (Section 3.2.2).

Statistical analyses were performed by using Minitab 18® statistical software. The data were analysed using (independent) two Sample *t*-test (comparison of two means) or one-way ANOVA (comparison of multiple means) after performing Anderson-Darling normality test and equal variances test, all with 95% coefficient interval. Tukey (Honest significant difference) post-hoc tests were then applied. Statistical significance was accepted with p-value$\leq$0.05.

Combination index (CI; value which determines multiple drug interaction) was calculated by the Chou-Thalaya method using CompuSyn software (ComboSyn, Inc.). Data for cell viability and cell inhibition measured by CyQUANT Direct was used to generate a dose-effect curve and a median-effect plot. Data were obtained for combination of non-constant ratio design. CI was calculated by the following formula (CI for mutually exclusive drugs):

Equation 1

$$\mathrm{CI} = \frac{(\mathrm{D})1}{(\mathrm{Dx})1} + \frac{(\mathrm{D})2}{(\mathrm{Dx})2}$$

Where D is the concentration of a single drugs and Dx a dose of D alone which gives x inhibition. Drug synergism was accepted with CI < 1, additive effect with CI = 1, and antagonism with CI > 1.

Data acquired by Flow cytometry (cell death Annexin V/PI and proliferation CFSE) were further analysed by Flowing Software program (version 2.5.1). Cells in apoptosis (FL1-H, x-axis) were detected by Annexin V and necrotic/dead (FL3-H; y-axis) cells by PI staining. Cell population event was distinctively gated and back-gated to compensate for Dox colour and auto-fluorescence. Distribution (cell population, y-axis) of mean fluorescent intensity (MFI) shift within CFSE labelled cells (FL1-H, x-axis) was visualised using histograms. Cell doubling time was calculated using Log MFI and time curve with the following formula:

Equation 2

$$\mathrm{MFI(t)} = \mathrm{MFI(0)}\ \exp(\alpha \mathrm{t})$$

Where MFI(t) represents the measure of MFI at time t, MFI(0) is the fluorescence at the time of labelling (t=0) and α is the growth rate when the cell population grows exponentially.

Chapter 3

Doxorubicin selectively induced apoptosis through an association with a novel isoform of Bcl-2 in acute myeloid leukaemia MOLM-13 cells with reduced Beclin 1

3 Doxorubicin selectively induced apoptosis through an association with a novel isoform of Bcl-2 in acute myeloid leukaemia MOLM-13 cells with reduced Beclin 1

3.1 Introduction

Acute myeloid leukaemia (AML) has a much higher mortality rate compared to other major leukaemia types (Chapter 2; Table 2.1), despite advancement in treating cancerous diseases. For instance, AML chronic counterpart, CML, has generally more favourable prolonged survival rate since the employment of tyrosine kinase inhibitors in the standard treatment (Iqbal and Iqbal, 2014). Unfortunately, AML has not seen this improvement, due in part to the disease heterogeneity and generic standard treatment (Hassan *et al.,* 2017). The current standard chemotherapy treatment regime for AML includes seven days cytarabine infusion combined with anthracycline drugs such as doxorubicin (Dox) or daunorubicin for the first three days. Another poor prognostic factor of AML is the high relapse rate of the disease, which leads to development of more aggressive cells that may acquire chemoresistance to the standard treatment (Ding *et al.,* 2012). In addition, most drug therapies have their limits and can induce cytotoxic effects on non-leukaemic cells. For instance, Dox showed toxicity towards cardiac cells (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004). Nevertheless, Dox is an FDA approved drug for various cancer types (National Cancer Institute, 2019) for its ability to trigger apoptotic cell death (Inoue-Yamauchi *et al.,* 2017; Pilco-Ferreto and Calaf, 2016; Florou *et al.,* 2013; Casares *et al.,* 2005; Wang *et al.,* 2004; Brantley-Finley *et al.,* 2003).

Dox is a topoisomerase II inhibitor, which targets the enzyme that affects DNA structure and the overall cell ability to enter cell cycle thus preventing cell division (Gewirtz, 1999; Fornari *et al.,* 1994). Although this mechanism can subsequently lead to cell death by apoptosis, interference with DNA can also increase risk of developing therapy-related secondary cancer (Rheingold *et al.,* 2003). Dox-induced apoptotic death is often the desirable outcome to achieve when treating the cancer cells. However, a more aggressive genetically distinct form of AML, mixed lineage leukaemia (MLL), has the potential to develop a countering mechanism (Lun *et al.,* 2017; Daigle *et al.,* 2011). Therefore, there is a need for a better understanding of Dox-induced signalling mechanism and its effectiveness on these cells.

It has been proposed that cancer-resistance to apoptotic death could be through modulation of autophagy (Radogna *et al.,* 2015). Autophagy is usually an adaptive response to cellular stress to enhance cell survival but its overproduction can also lead to a distinct programmed cell death from apoptosis (Nikoletopoulou *et al.,* 2013; Ouyang *et al.,* 2012). There is a link between apoptotic cell death and autophagy, where autophagy showed to obstruct apoptosis (Liang *et al.,* 2007), facilitate apoptotic death (Ding *et al.,* 2007), or take over as an alternative

cell death pathway when apoptosis is blocked (Nikoletopoulou *et al.,* 2013). An interplay between anti-apoptotic B-cell lymphoma 2 (Bcl-2) and autophagy initiator Beclin 1 has been elucidated to regulate cells into undergoing apoptosis and/or autophagy (Decuypere *et al.,* 2012; Marquez and Xu, 2012; Lian *et al.,* 2010). When the complex of Bcl-2:Beclin 1 is blocked through Bcl-2 downregulation and subsequent Beclin 1 release, autophagy can be activated. Thus, the anti-apoptotic regulatory protein Bcl-2 is involved in both apoptotic and autophagic mechanism (Lian *et al.,* 2010). Since Bcl-2 is reported to be upregulated in many cases of relapsed AML cell (Su, Y. *et al.,* 2018; Moon *et al.,* 2009; Zinkel *et al.,* 2006; Bensi *et al.,* 1995), targeting the Bcl-2 protein could be a valuable mechanism in fighting refractory cancer cells through modulating other alternative cell death pathways such as autophagy.

Dox capability to inhibit DNA replication makes it an effective anti-proliferative agent, which is another useful anti-cancer mechanism (Tharkar-Promod *et al.,* 2018). Class I phosphoinositide-3 kinases (PI3K), group of intracellular signalling transduction enzymes, promote cell survival, growth and proliferation through activating downstream Protein Kinase B (AKT) pathway (Yuan *et al.,* 2017; Park *et al.,* 2010). Therefore, the rapid progression of refractory AML cells could be further inhibited by targeting the PI3K-AKT pathway and its regulators.

3.1.1 Aims and objectives

The main aim of this study was to determine aspects of selectively induced cell death in AML from a relapsed MLL-rearranged cell model (MOLM-13) by Dox through modulation of apoptosis and autophagy. Furthermore, the cytotoxic effect of Dox on pro-survival PI3K/AKT pathway was examined in this AML cell model.

Objectives

- To compare Dox-induced cell death in AML, MOLM-13 cell lines and SC/U-937 cells, and to determine if death is associated with apoptosis or necrosis
- To evaluate the anti-proliferative effect of Dox on MOLM-13 cells
- To determine constitutive expression level of proteins involved in apoptosis (Bcl-2 protein family, Cytochrome *c*, caspases) and the autophagy protein Beclin 1 in the relapsed leukaemic cell line MOLM-13 and other leukaemic cells (OCI-AML2, K562 and SC/U-937)
- To study the interplay between Bcl-2 and Beclin 1 proteins induced by Dox treatment, comparing MOLM-13 and SC/U-937 cells
- To examine the mechanism of action of Dox in MOLM-13 cells through studying the expressions of proteins involved in apoptotic cell death, autophagy and pro-survival pathway PI3K/AKT

3.2 Results

3.2.1 Method development

Normal cell growth of myeloid leukaemia cell lines under standard conditions

Trypan blue was used to determine the normal pattern of cell growth and the viability of the untreated cells over 54 h in an AML (MOLM-13) and CML (K562) cell lines (Fig. 3.1; Table 3.1). The doubling time for both cell lines was 24 h. The cell density of MOLM-13 increased from 0.10 $x10^6$ cells/mL to 0.2 $x10^6$ cells/mL and 0.39 cells/mL, respectively after 24 h and 48 h incubation periods. K562 increased from 0.15 $x10^6$ cells/mL to 0.32 $x10^6$ cells/mL (24 h incubation) and 0.53 $x10^6$ cells/mL (48 h incubation).

Both cell lines showed a lag (stationary) phase in cell growth from the starting point up to 8 h, where the cell density fluctuated minimally due to the initial adaptation of the cells to the environment. After the 8-hour incubation, there was steep cell growth for both myeloid cell lines, indicating the log phase when the cells utilise the available nutrients and grow exponentially. The cell viability of K562 and MOLM-13 was within the 88-95% and 83-100% range, respectively during the log phase of cell growth. Experiments were designed to be conducted within the log phase of cell growth.

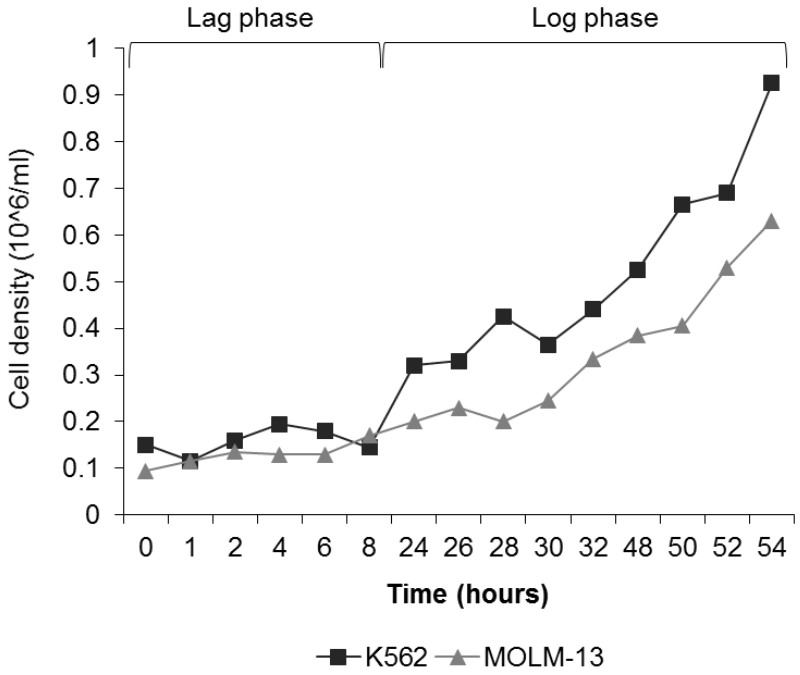


Figure 3.1 The cell growth of K562 and MOLM-13 myeloid leukaemia cell lines within 54 hour

Table 3.1 Cell density and cell viability of AML (MOLM-13) and CML (K562) cell lines during 54-h incubation period

Cell growth period	Incubation period (h)	K562		MOLM-13	
		Cell density ($x10^6$ cells/mL)	Cell viability (%)	Cell density ($x10^6$ cells/mL)	Cell viability (%)
Lag (stationary) period	0	0.15	88	0.10	68
	1	0.12	72	0.12	92
	2	0.16	89	0.14	93
	4	0.20	87	0.13	93
	6	0.18	88	0.13	90
	8	0.15	85	0.17	87
Log (growth) period	24	0.32	93	0.2	85
	26	0.33	88	0.23	100
	28	0.43	89	0.20	83
	30	0.37	94	0.25	91
	32	0.44	90	0.34	93
	48	0.53	88	0.39	87
	50	0.67	88	0.41	91
	52	0.69	94	0.53	99
	54	0.93	95	0.63	93

Cell suspension was stained by trypan blue at 1:1 ratios and counted by using haemocytometer, under the light microscopy. The doubling time for K562 and MOLM-13 cell lines was within 24 h, K562 of 0.15 $x10^6$ cells/mL and MOLM-13 of 0.10 x106 cells/mL increased to 0.32 x106 cells/mL and 0.2 $x10^6$ cells/mL, respectively after 24 h incubation period. (representative data; The experiment was repeated periodically)

The effect of dimethyl sulfoxide (DMSO) on myeloid leukaemia cell lines

To determine the non-cytotoxic concentration range of DMSO suitable for solubilising the test drugs, K562 and MOLM-13 cells were treated with different concentrations of DMSO (final concentrations of 0.05, 0.5, 0.7, 0.8, and 1% prepared in phenol-red free RPMI medium) for 72 hours (Fig. 3.2). Doxorubicin (Dox; final concentrations of 5 µM; prepared in 0.05 % DMSO) the positive control drug, inhibited the growth of K562 and MOLM-13 by 33% ($p < 0.001$) and 76% ($p < 0.001$), respectively compared to the negative control (cells with equivalent volume RPMI). DMSO, between 0.05% and 0.8% did not have an apparent impact on the growth K562 and MOLM-13 cell lines. At the highest concentrations of DMSO (0.8% and 1%) some inhibitory effect of cell growth was observed with high statistical significance ($p < 0.01$ for MOLM-13 and $p < 0.01$ for K562) for the 1% DMSO treatment. For further experiments, the lowest DMSO content (0.05%) was chosen as the final concentration for the dissolution of test compounds, maintaining the same level of DMSO concentration in all the treatments, including those for the negative and positive controls.

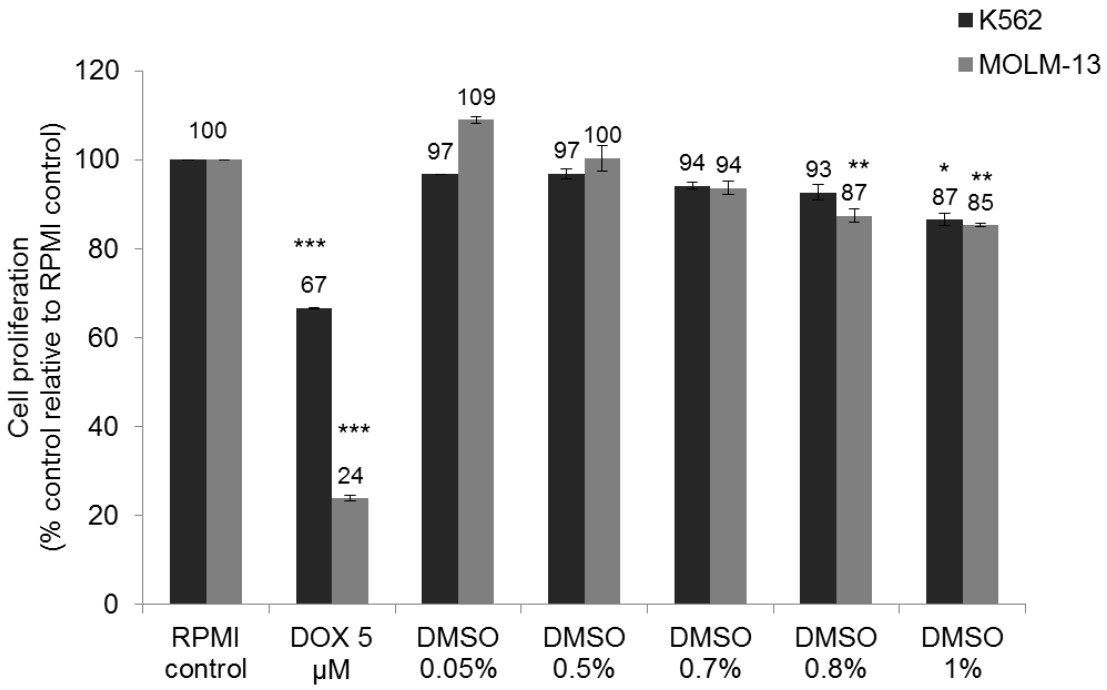


Figure 3.2 Effect of different dimethyl sulfoxide (DMSO) contents on acute myeloid leukaemia (MOLM-13) and chronic myeloid leukaemia (K562) cell lines in 72 hour
Cell proliferation was determined by fluorescence CyQuant assay. The data were expressed as mean ± SE of percentage control; values obtain for cells with RPMI vehicle. Doxorubicin 5 µM was used as positive control. The statistical difference between RPMI and DMSO group was analysed by Minitab 17 software. Statistical difference was accepted as following: $p > 0.05$ no significance, $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant.

3.2.2 Doxorubicin shows a level of selectivity in the induction of cell death of leukaemic cells

Dox-induced death in leukaemic monocytic cells were studied using flow cytometry. Apoptotic and necrotic cells were differentiated by double-staining MOLM-13 and SC/U-937 cells with Annexin V and PI in after 48 h co-incubation of the cells with and without Dox (Fig. 3.3). The effect of therapeutically relevant concentrations of Dox (0.5 and 1 µM) were tested against vehicle control (DMSO 0.05%). A supraclinical dose of 5 µM of Dox was used as a positive control.

After 48 h co-incubation of cells with treatments, the vehicle control treated cells showed comparable cell death, with 25% and 21% dead cells in MOLM-13 and SC/U-937 cells, respectively (with no statistical difference). In MOLM-13 treated cells, including the vehicle control, the cell death populations resided more in early apoptosis ($p = 0.03$ for Dox 0.5 µM and $p < 0.001$ for all other treatments compared to SC/U-937 monocytes) (Fig. 3.3 A). In contrast, compared to MOLM-13, dead cells were predominantly in late apoptosis in the SC/U-937 cells ($p < 0.01$) except for Dox 1 µM which showed a non-significant effect compared to MOLM-13 but was shown to be highly significant ($p < 0.01$) compared to vehicle control (Fig. 3.3 B).

Cell-death by necrosis was less than 2% in all of the MOLM-13 treated cells whereas it ranged from 7-9% (in Dox 1 µM and vehicle control-treated cells) to 37% (in Dox 5 µM-treated cells) in SC/U-937 cells, all with $p < 0.05$ compared to MOLM-13. Thus, Dox induced MOLM-13 cell death in a dose-dependent manner through mainly early apoptotic death with minimal necrosis. However, it is noteworthy that the different concentrations of Dox induced death in the SC/U-937 cells by apparently different mechanisms. Dox 5 µM induced death mainly by necrosis. A lower concentration of Dox, 0.5 µM, caused greater late apoptotic cell death and was more potent than that of 1 µM; the reason for this is currently uncertain. At therapeutically relevant concentrations, Dox 0.5 and 1 µM-treated cells showed 53% ($p < 0.05$) and 89% ($p < 0.001$) dead MOLM-13 cells, respectively, compared to vehicle control (Fig. 3.3). Comparatively, the SC/U-937 monocytic cells exhibited fewer dead cells with a 1.3-fold decrease in 1 µM-treated cells ($p < 0.001$ compared to MOLM-13 cells). The IC_{50} of Dox after 48 h co-incubation with MOLM-13 cells (based on the percentage of viable cell population) was lower compared to that for the SC/U-937 monocytic cells (0.397 µM vs 0.658 µM respectively) (Fig. 3.3).

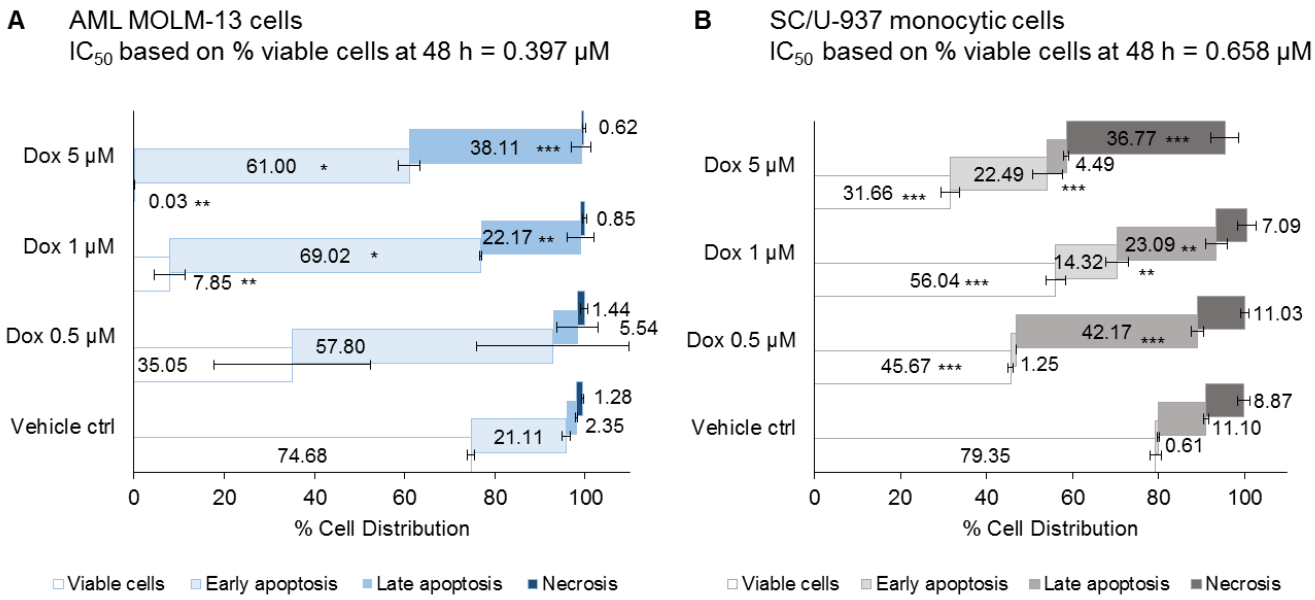


Figure 3.3 Cell death population of MOLM-13 and SC/U-937 monocytic cells treated by Dox for 48 h

Samples were double stained by Annexin V (apoptotic cell dye) and Propidium Iodide (PI, dead cell dye). **(A)** MOLM-13 cell line and **(B)** SC/U-937 monocytic cells were treated with Dox (0.5, 1 and 5 µM) for 48 h. Dox 5 µM was used as the positive control drug and DMSO 0.05% was used as negative (vehicle) cell control. One-Way ANOVA; Tukey post-hoc test was used to compare live cells (-ve Annexin V and –ve PI) of the control and the treatments: $p > 0.05$ no significant difference (NSD), $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant. Dox demonstrated a level of selectivity in its cytotoxicity against MOLM-13 compared to SC/U-937 monocytes ($p < 0.05$). Result were expressed as mean ± SE, n=3. Flowing software (version 2.5.1) was used to construct dot plots and gate cell populations as follows: Live cells (–ve Annexin V and –ve PI), Early apoptosis (+ve Annexin V and –ve PI), Late apoptosis (+ve Annexin V and +ve PI), Necrotic cells (–ve Annexin V and +ve PI)

3.2.3 Doxorubicin decreased the proliferative rate of MOLM-13 cell lines

To further investigate the molecular mechanism of Dox-induced cell death we used a single-cell assay based on CFSE tracker-labelled MOLM-13 cells. CSFE binds covalently and irreversibly to free amino groups on proteins within cells and the fluorescence intensity of the dye halves with each cell division. Combined with live-dead staining this allows not only to determine whether the drug affects the rate of cell division but also how the cells are dying. The number of MOLM-13 cells in the untreated cell samples doubled every 18 h (Fig. 3.4 C). After 24 h incubation period, the control (untreated cells) and the Dox-treated samples all showed similar mean fluorescence intensity (MFI) values. The changes in the MFI at 48 h and 72 h demonstrated that the rate of MOLM-13 cell proliferation was affected by Dox in a time- and concentration-dependent manner (Fig. 3.4 B II & III). In the untreated cells, there was a shift to lower MFI values between 24 h and 72 h incubation time due to halving of the amount of the CFSE stain during each cell division cycle.

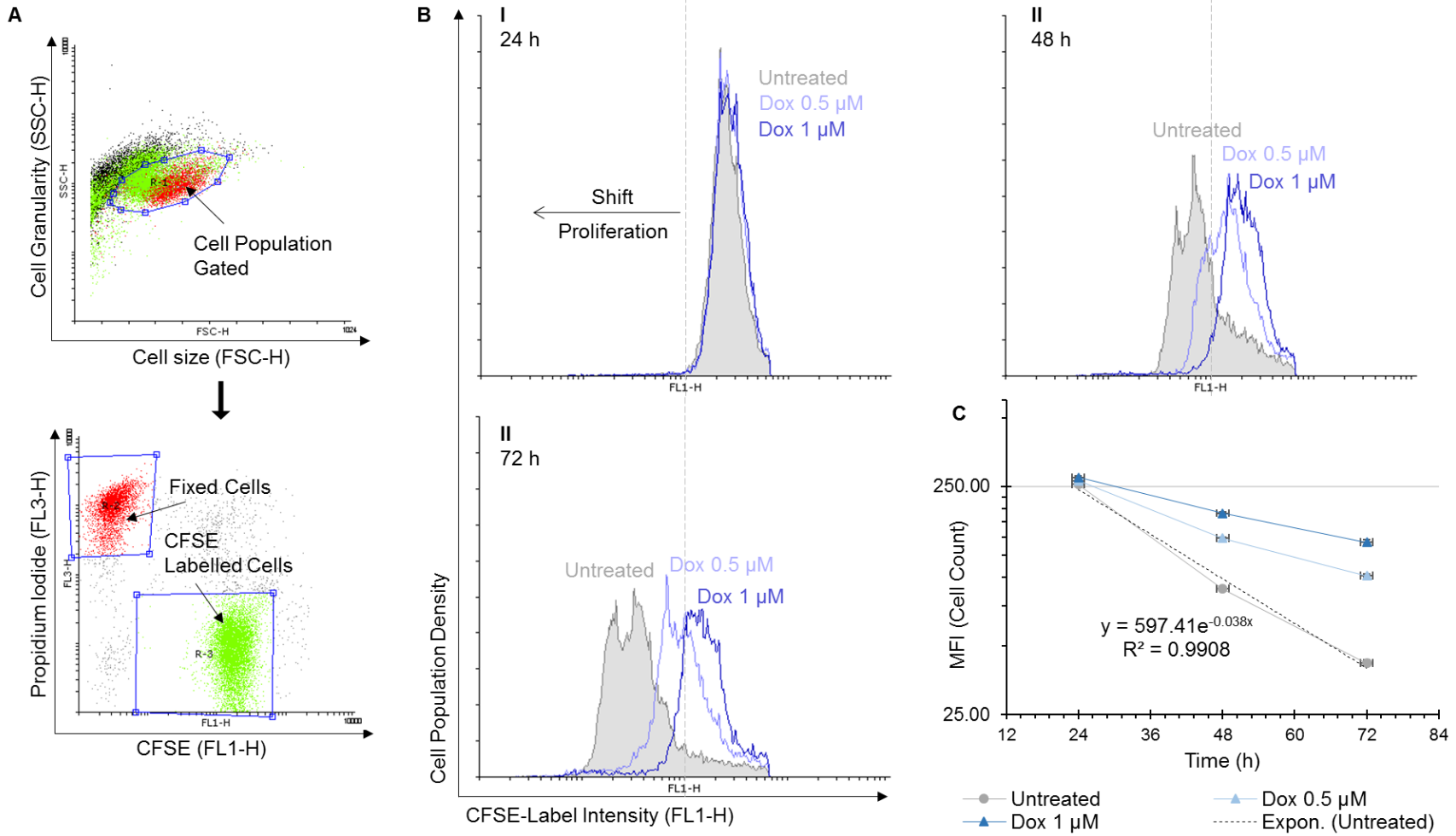


Figure 3.4 The anti-proliferative effect of doxorubicin on MOLM-13

Cell proliferation was measured by CFSE assay. Representative **(A)** flow cytometry dot plots showing gating of CFSE labelled cell population. Flowing software (version 2.5.1) was used to construct **(B)** histogram overlay of treatments at **(I)** 24 h, **(II)** 48 h, and **(II)** 72 h. The MFI values of MOLM-13 after the treatments was plotted to generate **(C)** the equation for doubling time. n=2.

3.2.4 Doxorubicin selectively inhibited a novel Bcl-2 isoform exclusively expressed in AML MOLM-13 cell line

To study the molecular mechanisms of Dox-induced cytotoxicity in MOLM-13 cells, Bcl-2 and Beclin 1 protein levels were quantified using Western blot analysis. Initial studies on the expression of Bcl-2 on MOLM-13 revealed two isoforms of the protein, estimated from the molecular weight of the proteins from the ladder control, to be 26 kDa (p26-Bcl-2-α) and 15-20 kDa (p15-20-Bcl-2). As p26-Bcl-2-α is the functional Bcl-2 protein known to have anti-apoptotic effect and p15-20-Bcl-2 appear to be a novel isoform in MOLM-13, further experiments were conducted to determine if they were both present in other leukaemic cell lines containing different mutations. Unstressed AML cell lines (MOLM-13 and OCI-AML2), as well as CML K562, and SC/U-937 monocytic cells all showed basal protein expressions of the usually reported isoform of Bcl-2, p26-Bcl-2-α (Fig. 3.5). However, the expression of the protein was very low in CML K562 cells. OCI-AML2 cells showed the highest expression ($p < 0.001$) of p26-Bcl-2-α when compared to the other cells. Of the cells tested, only MOLM-13 cells contained the p15-20-Bcl-2 protein but at lower levels compared to the p26-Bcl-2-α protein ($p < 0.01$).

Bcl-2 is an anti-apoptotic protein involved in cell death and interacts with an autophagy pathway through regulating Beclin 1 (Marquez and Xu, 2012). The differences in the basal expressions of Beclin 1 protein were not statistically significant ($p > 0.05$) between MOLM-13, OCI-AML2, CML K562 and SC/U-937 cells.

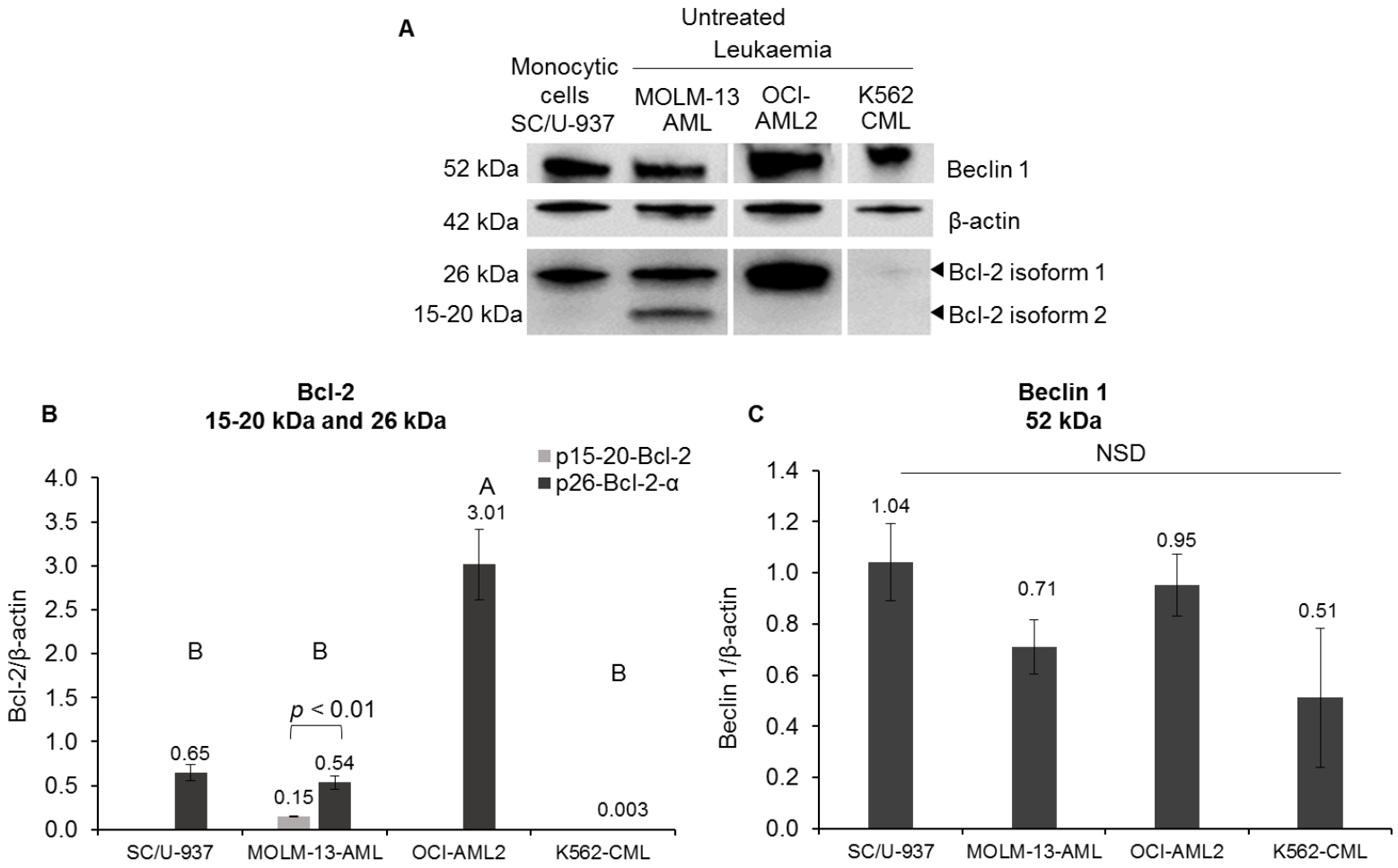


Figure 3.5 Bcl-2 and Beclin 1 protein expressions in untreated cells

Western blot detection of proteins from whole cell lysates using 30 µg protein loading in SC/U-937, AML MOLM-13 and OCI-AML2 cells and CML K562. **(A)** Representative data image of a Western blot. Experimental samples were run on the same gel and shown protein expressions are on the same blot for each protein antibody. β-actin was used as a housekeeping protein. Basal protein expression of **(B)** anti-apoptotic Bcl-2 and **(C)** autophagy Beclin 1. Data expressed as mean ± SE; n=4. One-Way ANOVA; Tukey post-hoc test was used for grouping and pairwise comparison. 2-sample t test was used to compare the Bcl-2 isoforms in MOLM-13 cell line.

Further experiments were conducted to determine if the p15-20-Bcl-2 isoform could be selectively targeted by drug therapy to induce cell death by comparing the effects of Dox on MOLM-13 and the SC/U-937 cells. Vehicle control treatment did not significantly affect ($p > 0.05$) the expression of Bcl-2 and Beclin 1 protein expression in both MOLM-13 cells and SC/U-937 monocytes, when compared to the expression of untreated cells (Fig. 3.6 A I & II).

The expression of this novel p15-20-Bcl-2 protein was selectively inhibited in Dox-treated MOLM-13 cells without any appreciable drug-specific effect on the expression of the p26-Bcl-2-α isoform in either MOLM-13 or SC/U-937 monocytes (Fig. 3.6 A II & B II). p15-20-Bcl-2 protein levels in MOLM-13 cells were significantly reduced by Dox ($p < 0.05$) compared to the untreated control cells.

Conversely, Dox inhibited the autophagic Beclin 1 protein levels by almost half at the higher concentrations tested with statistical significance shown for the effect of the 1 µM concentration ($p < 0.05$) (Fig. 3.6 A III). However, a non-significant ($p > 0.05$) dose-dependent reduction trend of Beclin 1 was observed in treated SC/U-937 cells (Fig. 3.6 B II). The effect of Dox in MOLM-13 cells exhibit decreasing dose-dependent trend of the autophagy initiator marker Beclin 1 protein expression as well as reduction of the anti-apoptotic Bcl-2 protein but only at the novel isoform p15-20-Bcl-2 (Fig. 3.6 A IV).

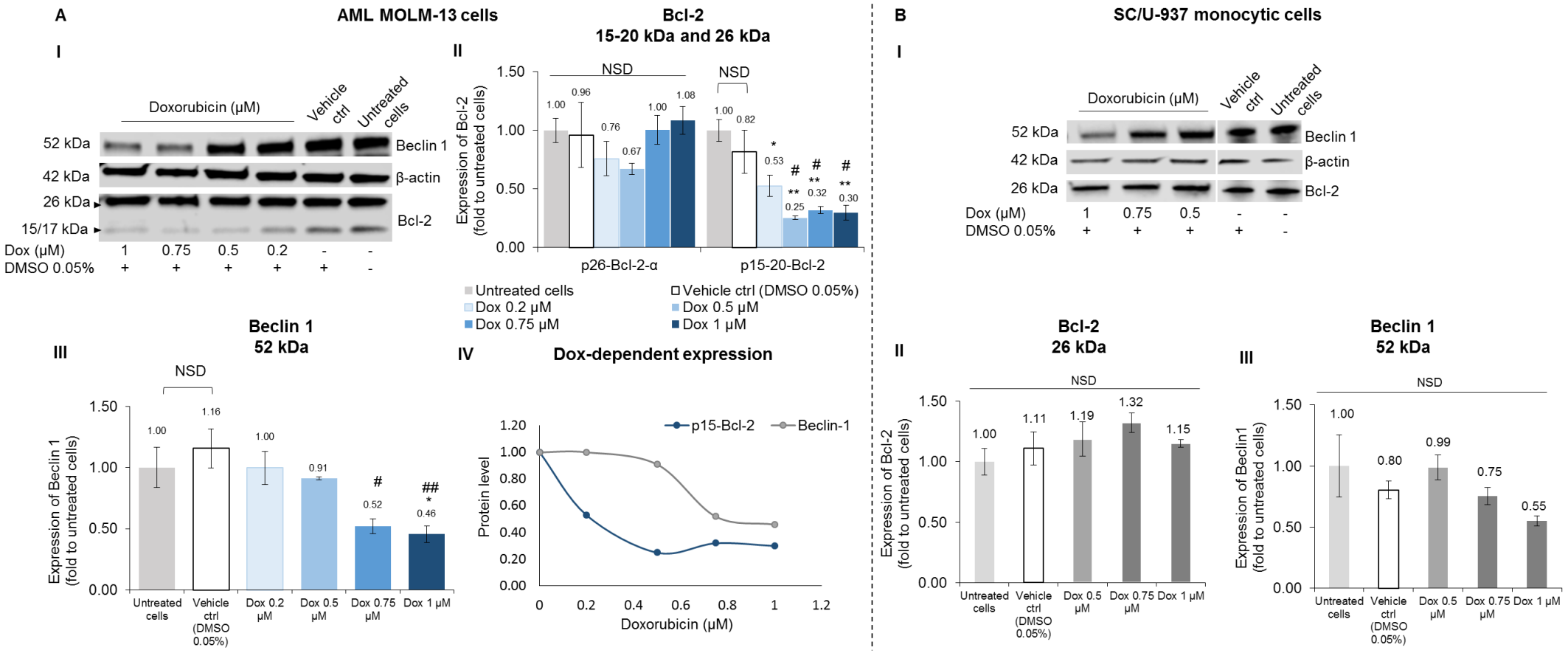


Figure 3.6 Bcl-2 and Beclin 1 protein expressions in doxorubicin-treated SC/U-937 and MOLM-13 cells

(A) MOLM-13 and **(B)** SC/U-937 monocyte cells were treated with Dox for 48 h. **(I)** Representative Western blot of 30 µg protein loading (from whole cell lysates), using β-actin as a housekeeping protein. Experimental samples were run on the same gel and shown protein expressions are on the same blot for each protein antibody. Quantitative analysis of proteins relative to β-actin normalised to untreated control: **(II)** anti-apoptotic Bcl-2 **(III)** pro-autophagy marker Beclin 1, **(IV)** Dox-dependent expression of p15-20-Bcl-2 and Beclin 1 protein levels. Data of proteins were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: p >0.05 no significant difference (NSD), */#$p \leq 0.05$ significant, **/##$p \leq 0.01$ highly significant, ***/###$p \leq 0.001$ very highly significant. (*) Comparison relative to untreated cell and (#) vehicle (DMSO 0.05%) cell controls.

3.2.5 Doxorubicin modulates the regulation of downstream signalling proteins to Bcl-2 family members (Cytochrome c and caspases) in AML MOLM-13 cell lines

When investigating baseline expression of regulatory proteins downstream to Bcl-2 family (Fig 4.7), CML K562 showed significantly higher expression of pro-apoptotic Cytochrome *c* ($p < 0.001$) and pro-caspase-8 ($p < 0.001$) protein levels compared to myeloid cell lines, MOLM-13 and OCI-AML2, and SC/U-937 monocytes in which Cytochrome *c* (Fig 3.7 B) and pro-caspase-8 (Fig 3.7 C) proteins were uniformly expressed. CML K562 also expressed higher levels ($p < 0.001$) of pro-caspase-9 (full length; 46 kDa) when compared to the other cells. Interestingly, MOLM-13 presented minimal amount of pro-caspase-9 ($p < 0.05$) than any other tested cells. On the other hand, cleaved intermediate p35 and fully cleaved p17 kDa of caspase-9 was constitutively expressed in the unstressed MOLM-13 cell with caspase-9 p17 kDa significantly ($p < 0.01$) more abundant. The intermediate p35 of caspase-9 was also present in SC/U-937 monocytic cells

The binding of Bcl-2 to pro-apoptotic Bax prevents mitochondrial membrane pore formation, and hence the release of Cytochrome *c*, hindering the activation of caspase to induce apoptosis. Therefore, change in the regulation of Bax and Cytochrome *c* protein expression was investigated. MOLM-13-treated cells by vehicle control did not statistically change the expression of pro-apoptotic proteins Bax and Cytochrome *c* when compared to the expression of the untreated cells (Fig 3.8). Although, the expressions of vehicle control treatments were relatively lowered ($p > 0.05$) on average. Dox did not significantly alter the level of pro-apoptotic Bax protein (Fig. 3.8 B). However, the expression of Bax in Dox-treated MOLM-13 cells appeared to be greater ($p > 0.05$) next to vehicle control but levelled to untreated cells. Pro-apoptotic Cytochrome *c* protein expression (Fig. 3.8 C), downstream to Bax, was reduced dose-dependently by Dox treatments. The reduction in the expression was significant ($p < 0.05$) at Dox 0.75 µM and 1 µM when compared to the untreated cell control.

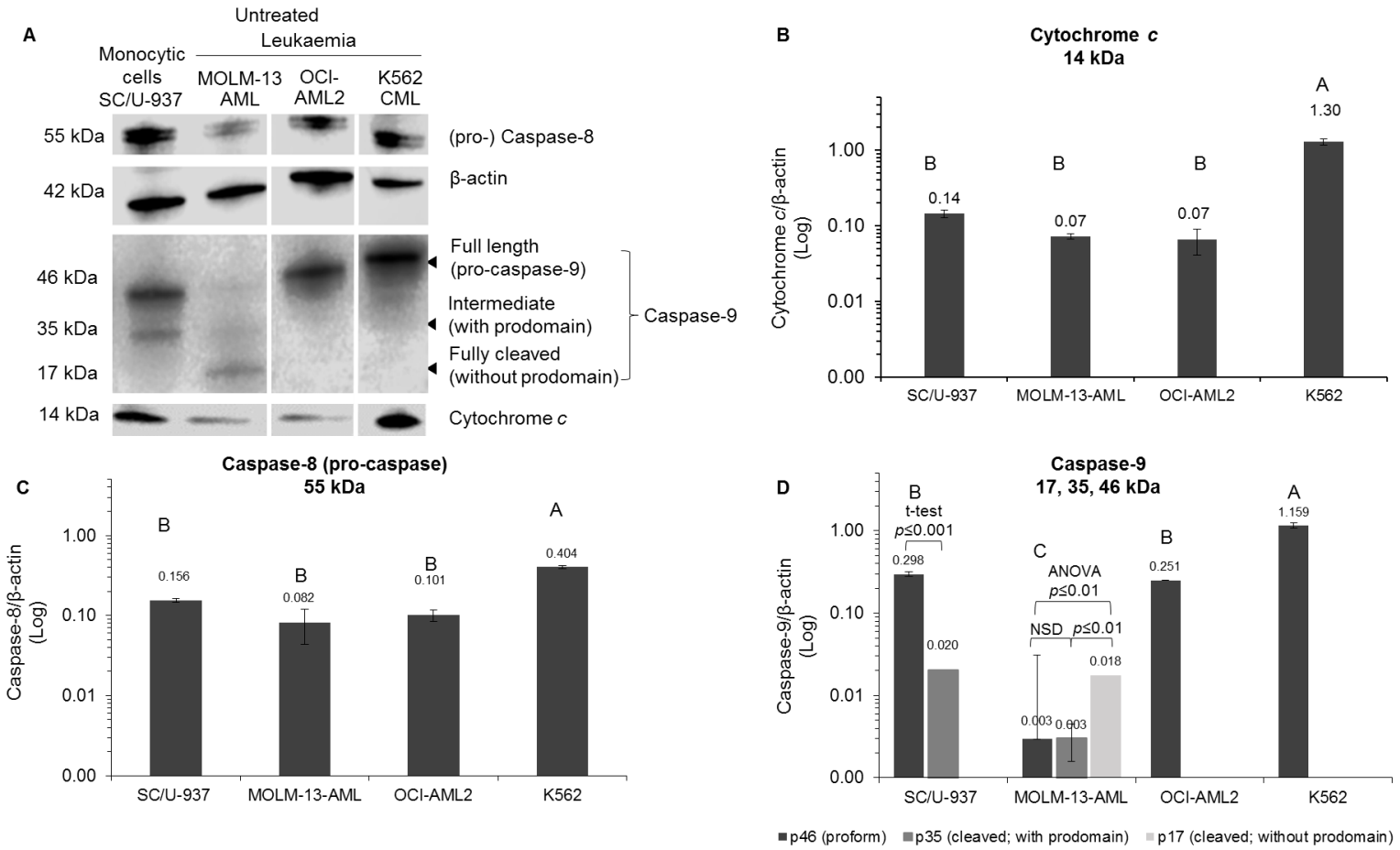


Figure 3.7 Pro-apoptotic protein caspase-8, -9 and Cytochrome c expressions in leukaemic cells

Western blot detection of proteins from whole cell lysates using 30 µg protein loading in SC/U-937, AML MOLM-13, OCI-AML2 cells and CML K562. **(A)** Representative data image of a Western blot. Experimental samples were run on the same gel and shown protein expressions are on the same blot for each protein antibody. β-actin was used as a housekeeping protein. Basal protein expression of **(B)** pro-apoptotic Cytochrome *c,* **(C)** (pro-) caspase-8 and caspase-9. Data expressed as mean ± SE; n=4. One-Way ANOVA; Tukey post-hoc test was used for grouping and pairwise comparison. 2-sample t test was used to compare caspase-9 forms present in SC/U-937 cells.

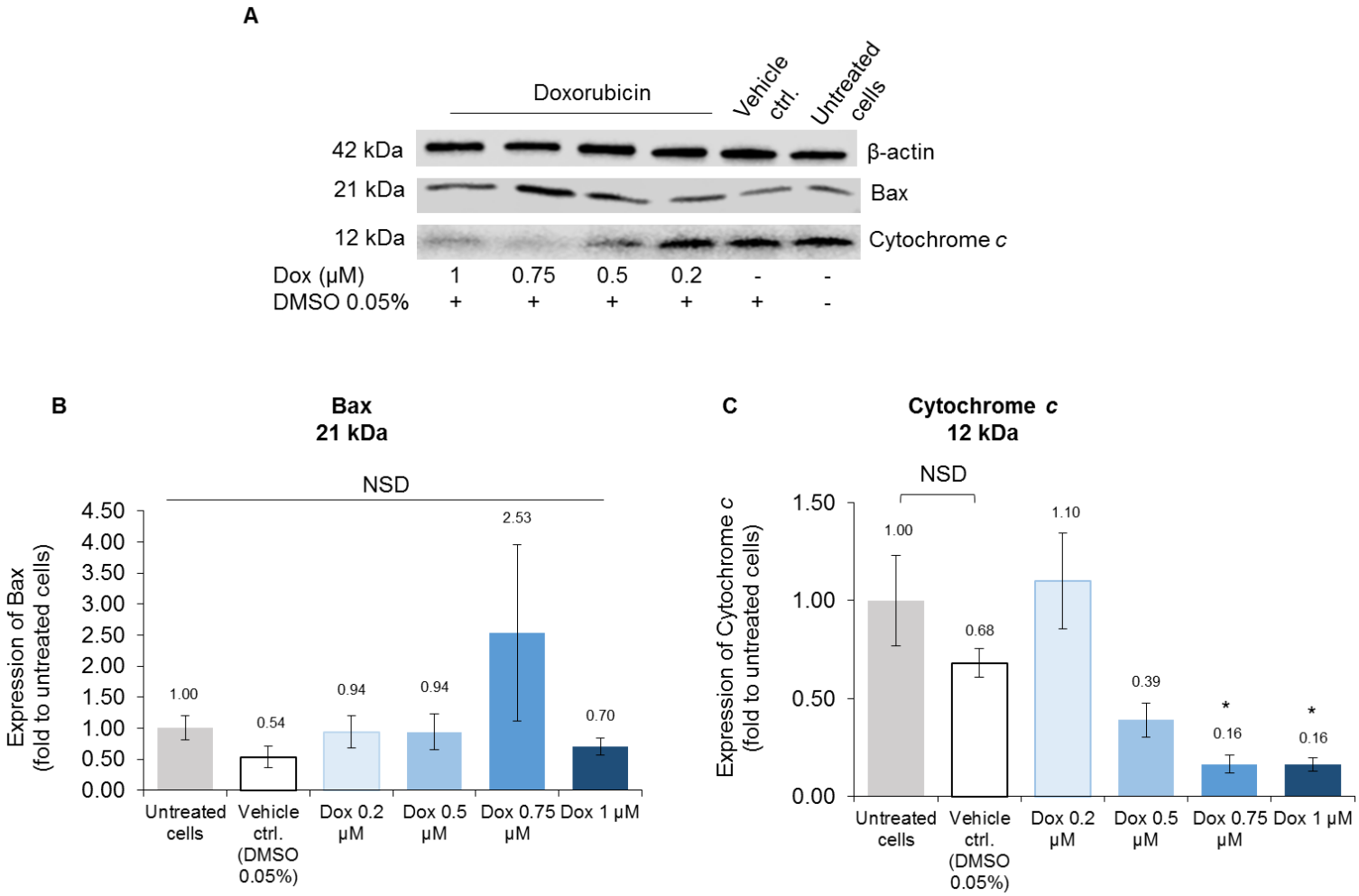


Figure 3.8 Pro-apoptotic Bax and Cytochrome *c* proteins expression in MOLM-13 cells treated by doxorubicin at 48 h

Western blot detection of proteins from whole cell lysates using 30 µg protein loading in AML cells MOLM-13 treated by Dox for 48 h. **(A)** Representative data image of a Western blot. β-actin was used as a housekeeping protein. Quantitative analysis of proteins relative to β-actin normalised to untreated cell control: **(B)** pro-apoptotic Bax and **(C)** Cytochrome *c*. Data of proteins were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), */#$p \leq 0.05$ significant, **/##$p \leq 0.01$ highly significant, ***/###$p \leq 0.001$ very highly significant. (*) Comparison relative to untreated cell and (#) vehicle (DMSO 0.05%) cell controls.

The difference in caspase-8 and -9 protein expression between MOLM-13 untreated cells and vehicle treated cells was not significant (Fig. 3.9 & 4.10), except in cleave intermediate (p35) caspase-9 where vehicle control significantly ($p < 0.001$ compared to untreated cells) elevated the expression (Fig. 3.10 B).

Pro-caspase-8 and its cleaved forms showed a trend in reduced expression by Dox but this was not significant ($p > 0.05$) when compared to the untreated cell control. Except for the shortest cleaved caspase-8 (p18), where Dox 0.75 µM and 1 µM significantly downregulated ($p < 0.05$) the protein levels (compared to untreated cells) (Fig. 3.9 B).

When investigating Dox effect on the ratio of cleaved forms/pro-form of caspase-8, a non-significant dose-dependent increase in the ratio was shown with p41 and p18 forms. In contrast, p30 ratio was significantly reduced ($p < 0.001$) by Dox (0.5-1 µM) when compared to the untreated cell control. Additionally, the cleaved p30/pro-form (p55) ratio was significantly different ($p < 0.001$) between untreated cells and vehicle control (Fig. 3.9 C).

Pro-form of caspase-9 was not detected in MOLM-13 cells after 48 h incubation, while the cleaved forms were prevalently expressed (Fig. 3.10 A). Cleaved p35 caspase-9 protein levels were significantly reduced ($p > 0.05$) by Dox 0.75 and 1 µM compare to untreated cell control. No significant change in protein expression was observed in p25 cleaved form between Dox treatments and the control. Fully cleaved form of caspase-9 (p17) was significantly upregulated by Dox 0.2 µM ($p < 0.001$, compared to untreated cells and vehicle control). However, higher Dox concentrations did not significantly change p17 expression although an inverse proportion trend could be observed (Fig. 3.10 B).

In addition, Dox treatments dose-dependently elevated the p25/p35 ratio of caspase-9 but only Dox 1 µM significantly ($p < 0.01$ compare to untreated cells) increased the ratio by 2.35-fold. Although Dox showed marginal increase (non-dose-dependent) in caspase-9 p17/p35 ratio, there was no significant difference ($p > 0.05$) between the treatments and the untreated cell control (Fig. 3.10 C).

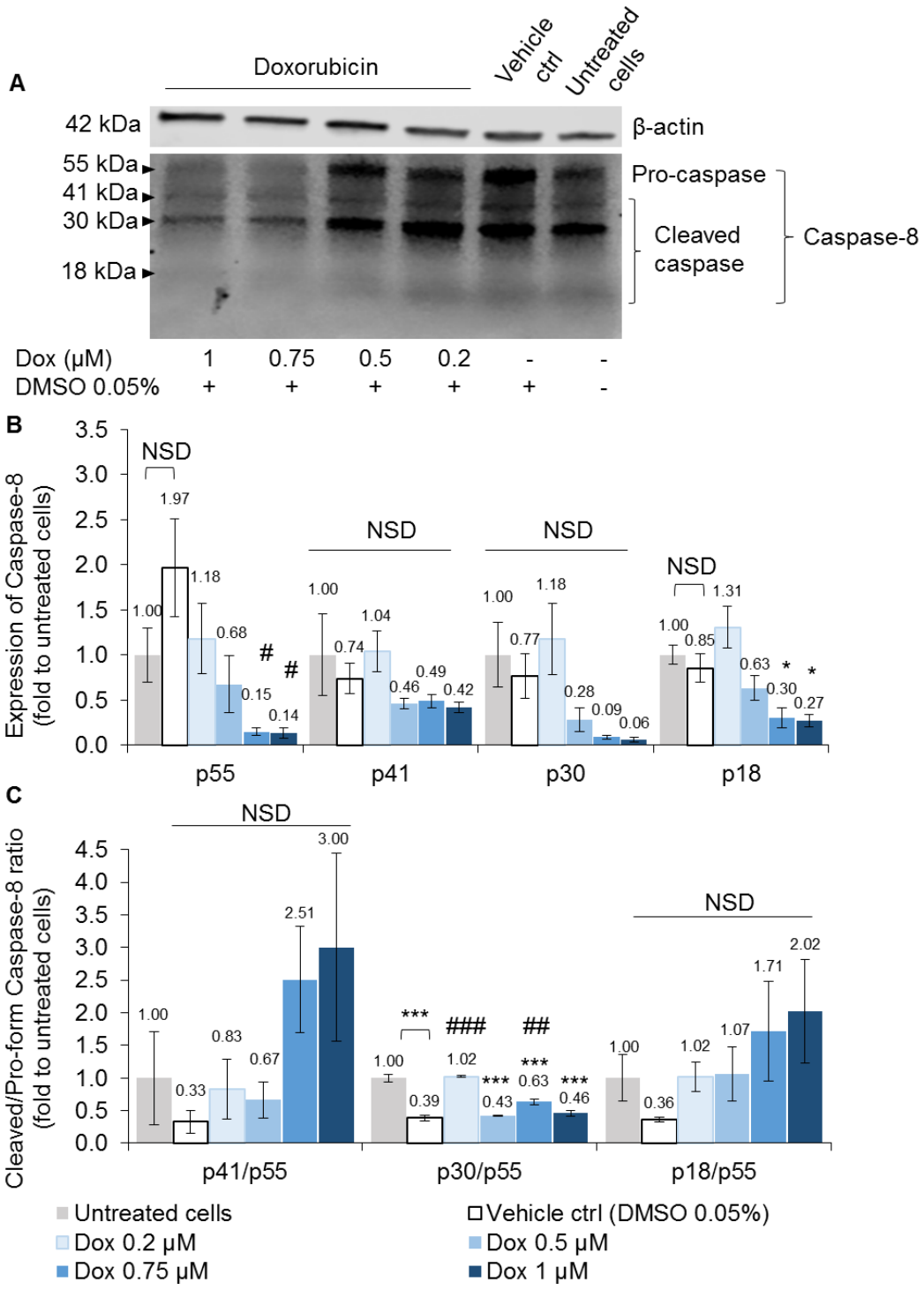


Figure 3.9 Protein expression of pro-form and cleaved-form of Caspase-8 in MOLM-13 cells treated by doxorubicin at 48 h

Western blot detection of proteins from whole cell lysates using 30 μg protein loading in AML cells MOLM-13 treated by Dox for 48 h. **(A)** Representative data image of a Western blot. β-actin was used as a housekeeping protein. Quantitative analysis **(B)** of the pro-form and active-form of Caspase-8 proteins relative to β-actin normalised to untreated cell control. **(C)** Ratio of smaller cleaved form to the pro-caspase-8. Data of proteins were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: p >0.05 no significant difference (NSD), */#$p \leq 0.05$ significant, **/##$p \leq 0.01$ highly significant, ***/###$p \leq 0.001$ very highly significant. (*) Comparison relative to untreated cell and (#) vehicle (DMSO 0.05%) cell controls.

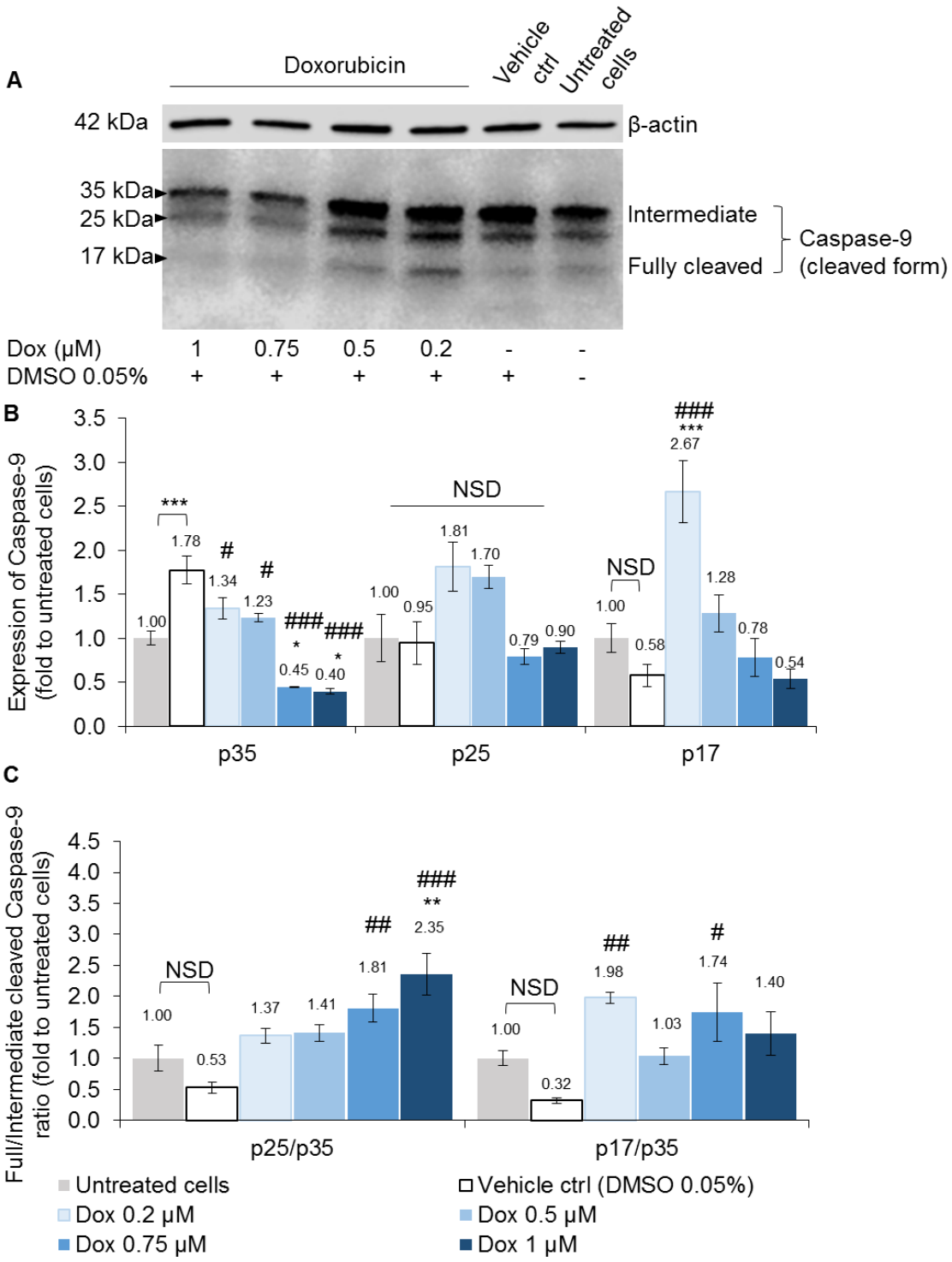


Figure 3.10 Protein expression of Caspase-9 active forms in MOLM-13 cells treated by doxorubicin at 48 h

Western blot detection of proteins from whole cell lysates using 30 µg protein loading in AML cells MOLM-13 treated by Dox for 48 h. **(A)** Representative data image of a Western blot. β-actin was used as a housekeeping protein. Quantitative analysis **(B)** of cleaved-forms of Caspase-9 proteins relative to β-actin normalised to untreated cell control. **(C)** Ratio of smaller cleaved form to the intermediate form of Caspase-9. Data of proteins were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: p >0.05 no significant difference (NSD), */#$p \leq 0.05$ significant, **/##$p \leq 0.01$ highly significant, ***/###$p \leq 0.001$ very highly significant. (*) Comparison relative to untreated cell and (#) vehicle (DMSO 0.05%) cell controls.

3.2.6 Doxorubicin downregulate key pro-survival proteins of PI3K-AKT pathway in MOLM-13 cells, but also reduced its negative regulator PTEN

To further investigate the mechanism of Dox-induced anti-proliferative effect, the key protein modulators of PI3K signalling pathway were studied. There was no significant difference in the expression of key regulating PI3K signalling proteins (PI3K p85, pan-AKT, PTEN) between untreated cells and cells treated by vehicle control (Fig 3.11). Although, marginal increase ($p > 0.05$) by vehicle control was observed in PI3K (p85α) and phosphorylated pan-AKT.

No significant difference in the expression of PI3Kα regulatory subunit p85α (p85-PI3K-α) was shown when MOLM-13 cells were treated by Dox, compared to untreated cells. However, there was significant decline ($p < 0.05$) by Dox 0.2 and 0.75 µM when compared to vehicle control (Fig. 3.11 B). In addition, the antibodies for this specific subunit also detected bands at approximately 55-60 kDa (p55-60-PI3K-α), which were significantly ($p < 0.05$, compared to untreated cells) reduced by Dox (0.75 and 1 µM) treatments. The effect was even more significant when compared to vehicle control.

The activated downstream effector protein of PI3K, pan-AKT phosphorylated at Threonine 308 site (p(T308)) showed no significant change upon Dox treatments when compared to untreated MOLM-13 cells, despite Dox 0.75 and 1 µM showing on average great reduction ($p > 0.05$ compare to untreated cells) (Fig. 3.11 C). However when compared to vehicle control, Dox-induced reductions of pan-AKT p(T308) were significant ($p < 0.01$) from 0.75 µM concentration.

Interestingly, a tumour suppressor of PI3K/AKT pathway, PTEN protein, was significantly downregulated by Dox treatments in a dose-dependent manner. In addition, two bands were detected at approximately 47 kDa and 54 kDa. Nevertheless, a Dox dose-dependent reduction of PTEN was reflected in both forms (Fig 3.11 D).

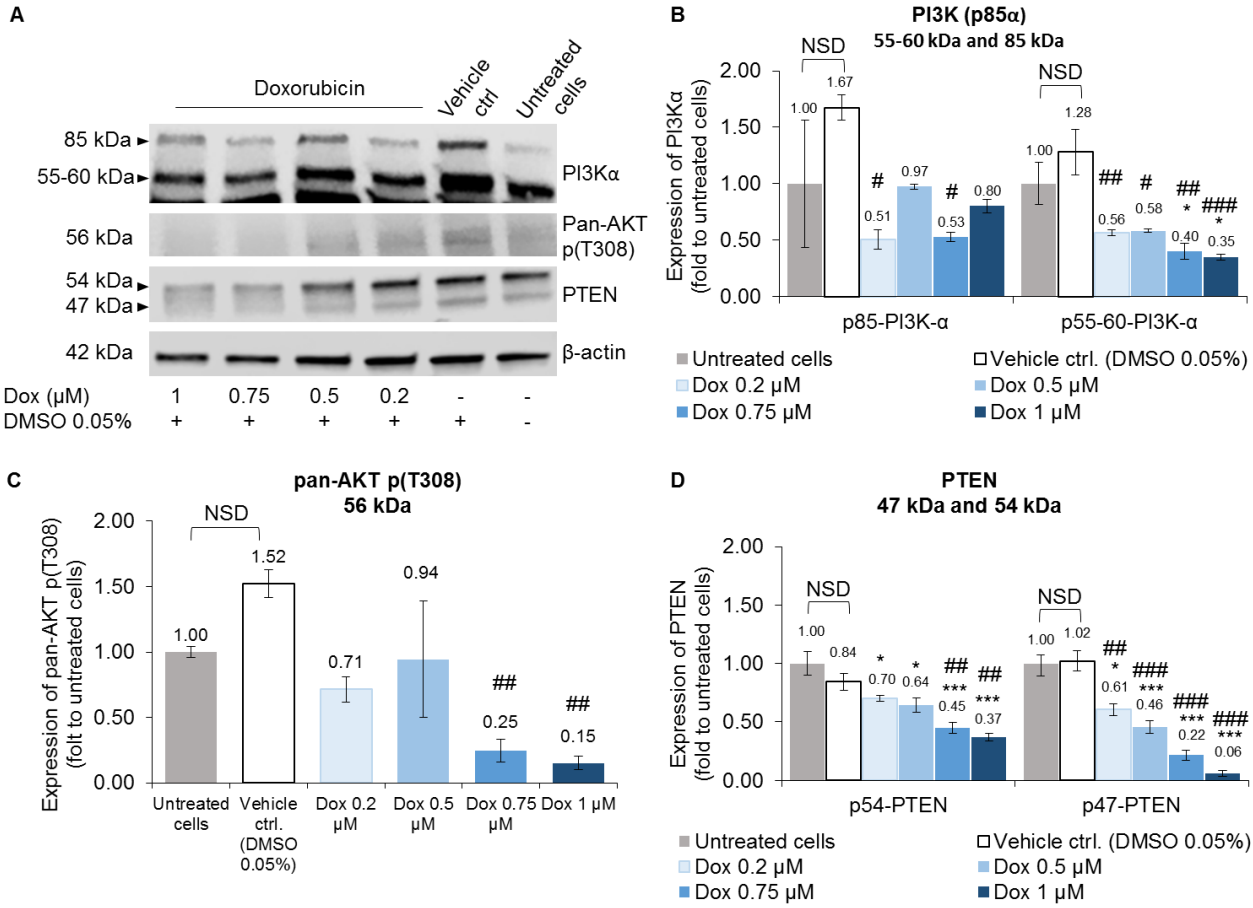


Figure 3.11 Protein expression of PI3K/AKT signalling proteins in MOLM-13 cells treated by doxorubicin at 48 h

Western blot detection of proteins from whole cell lysates using 30 μg protein loading in AML cells MOLM-13 treated by Dox for 48 h. **(A)** Representative data image of a Western blot. β-actin was used as a housekeeping protein. Quantitative analysis of proteins relative to β-actin normalised to untreated cell control: **(B)** regulatory subunit of PI3K p85α, **(C)** active form of AKT, phosphorylated at Threonine 308 and **(D)** PTEN, inhibitor of PI3K/AKT pathway. Data of proteins were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), */#$p \leq 0.05$ significant, **/##$p \leq 0.01$ highly significant, ***/###$p \leq 0.001$ very highly significant. (*) Comparison relative to untreated cell and (#) vehicle (DMSO 0.05%) cell controls.

3.3 Discussion

The deregulation of the Bcl-2 proteins in leukaemia may be one of the contributing factors of drug resistance against apoptotic cell death. Treatment of haematological malignancies have been shown to benefit from BH3-mimetics (Henz *et al.,* 2019), which are compounds that promote the release of pro-apoptotic proteins from anti-apoptotic Bcl-2 effects (Gomez-Bougie *et al.,* 2018). This leads to induction of cell apoptosis (Vogler *et al.,* 2013). For instance, BH3-mimetic drugs such as venetoclax (ABT-199) enhance the effectiveness of available therapies for AML by inhibiting the overexpression of Bcl-2 seen in refractory leukaemia (Campos and Pinto, 2019). Beclin 1 (an autophagy protein), is also a BH3-containing protein and, similar to some pro-apoptotic Bcl-2 members, it binds to the hydrophobic groove of Bcl-2, making this interaction one of the regulatory mechanisms of autophagy (Decuypere *et al.,* 2012). In this study, doxorubicin (Dox) was evaluated on its potential to induce selective apoptotic cell death in AML MOLM-13 and to modulate autophagy by regulating the expressions of Bcl-2 and Beclin 1 protein. In addition, the mechanism of action of Dox was studied in the leukaemic cell lines through investigating key apoptotic proteins and regulatory proteins involved in PI3K-AKT pathway.

3.3.1 Doxorubicin inhibited cell proliferation and showed a level of selective cell-death induction in MOLM-13 cells

The concentration of Dox in a patient's plasma within about 2 h of intravenous administration of the drug is 0.1-1 µM (McHowat *et al.,* 2001). The documented effect of Dox as an anti-proliferative agent (Rudolfová *et al.,* 2014) has been confirmed in this study at 0.5 and 1 µM. However, Dox may not have a major effect on MOLM-13 during the first 24 h. Dox-treated MOLM-13 population decreased (relative to drug-free cell population) through a drug-induced reduction in the rate of proliferation in a time- and concentration-dependent manner (Fig. 3.4). This study reports that Dox induced more cell death in MOLM-13 than in SC/U-937 monocytes ($p < 0.05$; Fig. 3.3) indicating a certain level of selectivity of Dox toxicity on MOLM-13 cells, and this may be due to cell-dependent action of Dox.

In the literature, Dox has been reported to demonstrate a pleiotropic effect on cells ranging from affecting cell proliferation by binding to topoisomerase II (Fornari *et al.,* 1994) to inducing apoptosis by activating caspases (Inoue-Yamauchi *et al.,* 2017; Florou *et al.,* 2013; Panaretakis *et al.,* 2002) and disrupting mitochondrial membrane potential (Gamen *et al.,* 2000). In the search for the molecular mechanisms of Dox, further studies were conducted to investigate the effect of the drug on members of the Bcl-2 family, the anti-apoptotic Bcl-2 and Beclin 1, an initiator of autophagy.

3.3.2 Doxorubicin inhibited the expression of a novel Bcl-2 protein variant in MOLM-13 cells

The BCL-2 gene has been reported to be upregulated in 84% of AML patients at diagnosis and the cases increase at relapse to 95% (Moon *et al.,* 2009). MOLM-13, the cell line investigated in this study, is derived from cells of a relapsed AML patient (Matsuo *et al.,* 1997). A novel Bcl-2 isoform variant of 15-20 kDa (p15-20-Bcl-2), found in this study, was expressed in unstimulated leukaemic MOLM-13 cells (in addition to the usual 26 kDa Bcl-2 protein) but not in other AML and CML cells tested in this study (which only expressed p26-Bcl-2-α) (Fig. 3.5). Anti-apoptotic Bcl-2 protein reduction by Dox has also been reported in HeLa cells (Bien *et al.,* 2010) and breast cancer cells (Pilco-Ferreto and Calaf, 2016). In previous studies, the apoptosis inhibitory properties of Bcl-2 have been credited to the main p26-Bcl-2-α isoform, consisting of a hydrophobic transmembrane region that can regulate mitochondrial permeability (Guillem *et al.,* 2015; Akgul *et al.,* 2004).

This study reports a Bcl-2 isoform similar in size as that reported by Messingerova *et al*., (2015) and show that the isoform is a functional protein, which is selectively sensitive to Dox treatment in MOLM-13 cells. There is a possibility that the proteomic diversity of anti-apoptotic Bcl-2 in MOLM-13 cell lines may contribute to the oncogenic behaviour of the cancer. Understanding the different isoforms of Bcl-2, especially those that are preferentially expressed in cancer cells, may be useful for developing specific drugs to target cells to induce cancer cell death.

3.3.3 Doxorubicin modulated Beclin 1 leading to cell death of MOLM-13 cells

The protein expression of Beclin 1 was reduced by Dox, but only at concentrations above 0.5 µM, with statistical significance for 1 µM ($p < 0.001$) (Fig. 3.6 A III). It has been reported that depleting Beclin 1 by gene silencing augmented mitochondrial permeabilisation and enhanced Dox-induced apoptosis (Daniel *et al.,* 2006) and treatment with pharmacological and genetic inhibitors of autophagy augmented Dox-induced apoptotic cell death (Pan *et al.,* 2011). In the currently reported study Dox induced a greater number of cells in apoptosis in MOLM-13 compared to SC/U-937 cells, with an inhibition of p15-20-Bcl-2 and a concurrent inhibition of Beclin 1.

The interplay between Beclin 1 and Bcl-2 to regulate apoptosis and autophagy has also been documented by other researchers. Dissociation of this complex allows the activation of Beclin 1 to bind and form a complex with various proteins in initiating pre-autophagosomal structure (Lian *et al.,* 2010). Due to the ability of Bcl-2 protein to form a complex with Beclin 1, it also performs a role in regulating autophagy (Marquez and Xu, 2012). Beclin 1 plays a crucial role

in autophagy by forming vesicle nucleation and promoting the isolation membrane development through a complex of phosphatidyl inositol-3 kinase (PI3K) class III. Bcl-2 blocks autophagy by preventing Beclin 1 from facilitating the formation of the pro-autophagosome promoting complex. This leads to a decreased PI3K activity of Beclin 1-associated binding partner (Pattingre *et al.,* 2005). Beclin 1 is also regulated by other autophagy proteins such as Rubicon and Ambra 1 (Nikoletopoulou *et al.,* 2013; Marquez and Xu, 2012). Similar to the findings in this study, some researchers have reported that reduction in autophagic proteins causes an increase in cell apoptosis (Boya *et al.,* 2005). In contrast, Smuder *et al*., (2011) have reported that Dox treatments increased markers of autophagy, including Beclin 1 mRNA and protein levels in muscle tissues, which may have contributed to Dox-induced muscle toxicity. In addition, Beclin 1 levels increased time-dependently in multiple myeloma cell lines when treated by Dox (Pan *et al.,* 2011). Therefore, increases in autophagy proteins in some cells could be an adaptive response to drug-induced stress for survival initiated by dying cells and inhibition of these proteins result in death (Pan *et al.,* 2011). Although the role of autophagy in cancer is yet to be confirmed, there is possibility of its modulation and usefulness in cancer therapy.

3.3.4 Doxorubicin apoptotic mechanism of action in MOLM-13 cells

The mode of action of Dox is mostly reported through induction of apoptosis by Cytochrome *c* release from mitochondria (Eom *et al.,* 2005; Panaretakis *et al.,* 2005; Panaretakis *et al.,* 2002) and cleavage/activation of caspases (Bien *et al.,* 2010; Panaretakis *et al.,* 2005; Gamen *et al.,* 2000). Although Dox reduced protein expression of the anti-apoptotic Bcl-2 in MOLM-13 (Fig. 3.6 A II), the pro-apoptotic Bax expression was not significantly change by the drug (Fig. 3.8 B). The Bcl-2 family of proteins regulates the release of Cytochrome *c* from mitochondrial membrane. Cytochrome *c* activates caspase-9 once in cytosol, leading to cell death by mitochondrial (intrinsic) apoptotic pathway (Ouyang *et al.,* 2012). However, the expression of Cytochrome *c* significantly declined when MOLM-13 cells were treated by Dox 0.75 µM and 1 µM (Fig. 3.8 C) despite Dox inducing apoptotic death observed by live-dead staining (Fig. 3.3). The protein profile between different cancer types and variants can have variable constitutive protein expression (Irish *et al.,* 2004). Therefore, the diversity in the protein pattern of cancer may potentially affect mechanism of action of drugs in cellular context-dependent manner.

Unstressed MOLM-13 cells expressed similar constitutive level of Cytochrome *c* and caspase-8 when compared between AML cell lines, OCI-AML2 and SC/U-937 cells (Fig. 3.7 B & C). However, caspase-9 expression in MOLM-13 cells showed a distinct protein profile compared to the other unstimulated cells, where the pro-form (p46) of caspase-9 was minimally detected in MOLM-13 but the cleaved forms (p35 and p17) were highly expressed (Fig 3.7 A). There

are two conventional cascades of pro-caspase activation by proteolytic processing of either extrinsic (caspase-8) or intrinsic (caspase-9) apoptotic pathway. Both ways would eventually lead to activation of executioner caspase-3 or -7 through internal cleavage of the caspases, which results in chromatin condensation and DNA fragmentation (Su, Z. *et al.,* 2015). The distinctive expression of cleaved caspase-9 forms in unstimulated MOLM-13 cells could potentially affect the caspase cascade activation of apoptotic death in a different manner.

A trend in pro- and cleaved caspase-8 protein reduction was observed in Dox-treated MOLM-13 cells (Fig. 3.9 B), although cleaved/pro-caspase-8 ratio (p41/p55 and p18/p55) appeared to rise dose-dependently (Fig. 3.9 C), suggesting caspase-8 activation that is involved in cell death is associated with apoptosis (Kallenberger *et al.,* 2014). However, it is not certain if the effect truly contributed to Dox-induced MOLM-13 death or is by chance due to its non-statistical significance.

Overexpression of caspase-9 has been reported to indicate activation of the caspase (Druskovic *et al.,* 2006). However, in this study caspase-9 cleaved protein form p35 was reduced by Dox in MOLM-13 cells while the smaller form p17 was significantly induced at Dox 0.2 µM but then dose-dependently declined (Fig. 3.10 B). Studies often determine caspase-9 activity by proteolytic cleavage but unlike other caspases, proteolysis of caspase-9 is not essential for its activation (Renatus *et al.,* 2001; Zhivotovsky *et al.,* 1999). Although, the autocatalytic cleavage of caspase-9 may still accompany the apoptotic process (Twiddy and Cain, 2007). The full scope of caspase-9 activation is not fully understood (Li *et al.,* 2017a; Li *et al.,* 2017b). However, two main activation pathways have been described. First, activation through apoptosome complex consisting of Apaf-1, Cytochrome *c* and dATP leading to conformational changes (Li *et al.,* 2017b; Zhivotovsky *et al.,* 1999). Second, activation through dimerisation of inactive zymogens/monomers (Li *et al.,* 2017a; Renatus *et al.,* 2001). Cytochrome *c* is not always required for caspase-9 activation, as reported by Morishima *et al*., (2002) where activation of caspase-9 was independent of Cytochrome *c* through direct proteolytic processing by another upstream signalling enzyme caspase-12. Reduction of Cytochrome *c* expression by Dox (Fig. 3.8 C) in this study would conflict with caspase-9 activation associated with apoptosome formation pathway. However, this should be taken with caution since this was a protein expression from a whole cell lysate. The localisation of Cytochrome *c* protein is more indicative since caspase-9 co-localise with Cytochrome *c* in the cytosol (Li *et al.,* 2016; Ouyang *et al.,* 2012). Therefore, the difference in mitochondrial and cytosolic Cytochrome *c* expression should be considered. The proposed role of cleaved-caspase-9 is to initiate and tightly regulate the caspase cascade (Twiddy and Cain, 2007).

Interestingly, this study showed dose-dependent increase in the small/large (p25/p35) cleaved-caspase-9 ratio by Dox treatments in MOLM-13 cells (Fig. 3.10 C).

Further investigations could evaluate if the effect is linked to the distinct pattern expression of caspase-9 protein in MOLM-13 cells. In addition, protein quantification and expression at specific site of the cell using additional investigations such as immunofluorescence staining or specific lysate extraction could provide better insight on its regulation. Dox possibly regulated the caspase cascade in the AML MOLM-13 cell lines through shifting the cleaved-form ratio of caspase-9 to induce cell death. However, further studies are warranted to determine the mechanism of proteolytic cascade regulation and activation contributing to apoptotic death.

3.3.5 Dox inhibits pro-survival pathway PI3K-AKT pathway in MOLM-13 cells but with a downregulation of the tumour suppressor PTEN

Intracellular signalling transduction pathway, PI3K-AKT, activates translational machinery of cell growth. The signalling network of this pathway is commonly hyperactive in cancer, allowing the cells to over-proliferate (Lynch *et al.,* 2016). The pathway contributes to AML pathogenesis and is frequently active in the immature leukaemia population, with activation in 50%-80% of AML cases (Park *et al.,* 2010). Dox is a topoisomerase II inhibitor and thus have anti-proliferative properties (Rudolfová *et al.,* 2014; Bidwell and Raucher, 2006). This effect was confirmed through tracking the cell division rate of MOLM-13 treated by Dox (Fig. 3.4). The PI3K-AKT pathway regulation in MOLM-13 cells was studied to determine its involvement in the mechanism of Dox proliferation inhibition.

The pro-survival PI3K-AKT pathway in MOLM-13 cells was inhibited by Dox treatment (Fig. 3.11). Tyrosine kinase receptors upon ligand binding will go through dimerisation and auto-phosphorylation to activate phosphoinositide 3-kinase (PI3Ks) enzymes (Chen *et al.,* 2016; Park *et al.,* 2010). Class IA PI3Ks, heterodimers made up of one regulatory subunit (p50, p55, p85) and catalytic subunit (p110α, p110β, p110δ), are family of lipid kinases that catalyse phosphoinositides at 30-hydroxyl group (Yuan *et al.,* 2017; Wang *et al.,* 2016; Park *et al.,* 2010). Their activation phosphorylates the lipid (phosphoinositide) which leads to conversion of phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3), re-localising the enzyme product to the plasma membrane. PIP3 is a vital second messenger that recruits downstream signalling proteins such as phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (AKT) (Wang *et al.,* 2016; Park *et al.,* 2010). The PI3K p85α, regulatory subunit of PI3K class IA, protein was decreased by Dox treatments in MOLM-13 cells at p85 ($p > 0.05$) and with statistical significance ($p < 0.05$) of the p55-60 protein (Fig. 3.11 B). This regulatory subunit is one of the variants belonging to p85 type (p50α, p55α, p55γ, p85α, p85β) which is recruited through activated tyrosine kinase receptor

(through SH2 domain binding) and it is essential for activating the catalytic PI3K subunit p110 (α/β/δ) which allows PIP2 to PIP3 conversion (Park *et al.,* 2010). Thus, loss of the PI3K regulatory subunit would disrupt the effectiveness of the catalytic subunit and affect the enzymatic activity.

Recruitment of AKT (a serine/threonine kinase) to PI3K through pleckstrin homology domain leads to activation of AKT through phosphorylation by PDK-1. Phosphorylated AKT (at threonine 308 and serine 473) can then translocate to the cytoplasm and activate downstream pathways of cell growth, survival, proliferation, migration, and cell cycle progression (Chen *et al.,* 2016). Dox treatments in MOLM-13 cells reduced on average the protein level of phosphorylated AKT at threonine 308 (significant compared to vehicle control but not to untreated cells) (Fig. 3.11 B), suggesting a reduction in cell survival mechanism induced by the drug. Inhibition of activated/phosphorylated AKT enhances cell death by apoptosis. Normally, active AKT can target the pro-apoptotic BAD protein by serine 136 phosphorylation, which halts the binding of Bad to anti-apoptotic Bcl-2 family members. Furthermore, activation of AKT promotes MDM2, which is a negative regulator of tumour suppressor p53 (Song *et al.,* 2005). Therefore, in this study, Dox negatively affected the key enzymes that are crucial in PI3K-AKT pathway, inhibiting a range of cell survival mechanisms to promote apoptotic death of MOLM-13 cells.

Phosphatase and tensin (PTEN) homolog deleted on chromosome ten is a tumour suppressor and plays an important role in regulating the PI3K pathway (Mashayekhi *et al.,* 2019; Boosani *et al.,* 2019). PTEN, a lipid 3-phosphatase, acts as a negative regulator to PI3K-AKT pathway by removing the phosphate (D3) on inositol ring of PIP3, converting the phospholipid back to inactive PIP2, thus preventing AKT recruitment and activation (Wang *et al.,* 2016). Interestingly, PTEN function to dephosphorylate PIP3 is compromised when the protein itself is phosphorylated; making non-phosphorylated PTEN the more active form (Boosani *et al.,* 2019). This study reports that PTEN in non-phosphorylated form was dose-dependently reduced by Dox treatment in MOLM-13 cells (Fig. 3.11 C). Loss of PTEN function would lead to continuous AKT activity. However, AKT phosphorylation (p308) was reduced by Dox treatment in this study (Fig. 3.11 B). Schöndorf *et al*., (2001) tested three different anti-cancer drugs (cisplatin, paclitaxel and andromycin) *in vitro* and reported that at low drug concentrations, PTEN activity was initially increased but decreased activity was observed at higher drug concentrations. A study by Lin *et al*., (2014) reported Dox (1 μM)-induced decrease of PTEN level in the breast (MDA-MB157) and colon (HCT116) cancer cell lines. In addition, the same authors reported increased levels of phosphorylated AKT upon Dox treatment that would suggest cell pro-survival, which was in contrast to this study on AML

MOLM-13 cells. In agreement with Lin *et al*., (2014) group, another study on oestrogen receptor positive breast cancer cell lines (MCF7 and T47D) showed increase of phosphorylated AKT upon Dox treatment, but the same study reported no effect on oestrogen receptor negative breast cancer cell lines (MDA-MB231) (Yndestad *et al.,* 2017). Thus, the cellular response to drug may be context dependent. Downregulation of PTEN is associated with unfavourable tumour prognosis and development of multi-drug resistance in cancer (Mashayekhi *et al.,* 2019). Current reports suggest that this effect may be a tumour cell response to protect against cytotoxic drugs and PTEN inhibition could be a mechanism for developing more refractory cells (Lin *et al.,* 2014; Schöndorf *et al.,* 2001). In this study, PTEN protein reduction may be a defence feedback of MOLM-13 cells to Dox exposure, where the PI3K-AKT pathway is downregulated and cells die by apoptosis. This could be an early mechanical development of drug resistance to Dox chemotherapy in MOLM-13 cells. The scope of this cancer counteraction is yet to be determined.

Chapter 4

Betulinic acid selectively enhanced doxorubicin-induced apoptotic death in MOLM-13 cells, but rescued SC/U-937 cells

4 Betulinic acid selectively enhanced doxorubicin-induced apoptotic death in MOLM-13 cells, but rescued SC/U-937 cells

4.1 Introduction

The standard treatment for acute myeloid leukaemia (AML) utilises potent chemotherapy drugs such as doxorubicin (Dox) (National Cancer Institute, 2019). However, the disease remains to maintain high mortality rate among other main leukaemia types (Cancer Research, 2019). This could be due to its rapid progression and high-relapse onset, which is associated with poorer prognoses (Ding *et al.,* 2012), an example of a genetically distinct form of AML cells, mixed lineage leukaemia (MLL), is particularly aggressive (Daigle *et al.,* 2011). Despite Dox being a strong cytotoxic cancer drug, it is more challenging to treat these cells as some may develop or acquire resistance against the chemotherapy (Ding *et al.,* 2012). In fact, some potential signs of drug resistance was observed in AML cells treated by Dox (Chapter 3; Section 3.3.5). Simply increasing the chemotherapy dose is not optional since Dox has been reported to affect to some extent non-cancerous cells (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004; Zuco *et al.,* 2002). It is desirable that alternative treatment should be obtained which can kill these cells if standard drugs are no longer effective or further facilitate the potency of the current drug without inducing advese effects.

Ehrhardt *et al*. (2004) tested the effects of ten drugs on acute primary leukaemia cells. They reported that the most effective drug against relapsed cells was betulinic acid (3β,hydroxyl-lup-20(29)-en-28-oic acid, BetA). This effect was more potent than those exerted by standard AML chemotherapeutics such as doxorubicin and cytarabine (tested at clinically relevant concentrations). Other than this report, there is a paucity of literature on the effect of BetA on AML. There are however, some published data on the *in vitro* effect of BetA on myeloid leukaemic K562 cells (CML in blast crisis) (Qiuling *et al.,* 2010; Gopal *et al.,* 2005; Hata *et al.,* 2003) and promyelocytic leukaemia HL-60 cells (Faujan *et al.,* 2010; Hata *et al.,* 2003). Most other studies regarding BetA describe its effect against cancers of epithelial origin. Studies in the 1990s showed the potential of BetA as an anticancer agent against neuroectodermal tumours (Fulda *et al.,* 1997), melanoma and brain tumour (Fulda *et al.,* 1999) cells, *in vitro*. Later studies demonstrated anti-cancer activities of BetA in head/neck (Thurnher *et al.,* 2003), prostate (Chintharlapalli *et al.,* 2007), ovarian (Fulda, 2008; Zuco *et al.,* 2002), cervix (Fulda, 2008; Zuco *et al.,* 2002), lung (Fulda, 2008; Kessler *et al.,* 2007), breast (Kessler *et al.,* 2007) and colorectal cancer (Kessler *et al.,* 2007). Furthermore, it has been reported that BetA exerts some selectivity towards cancer cells while being less cytotoxic towards non-cancerous cells. This effect was described in both epithelial and blood cells such as human primary melanocytic cells (Selzer *et al.,* 2000), normal colon cells (Aisha *et al.,* 2012), peripheral blood

mononuclear cells (Faujan *et al.,* 2010), and normal peripheral blood lymphoblast (Zuco *et al.,* 2002).

Combining novel compounds with effective chemotherapy may enhance drug-induced cell death mechanism or sensitize the cancer cells to the drug (Fulda and Debatin, 2005). Therefore, this could be a potential strategy in eliminating aggressive AML variants without the need to increase or prolong drug dosage. Most studies have reported that BetA-induced cell death in cancer cells is through apoptotic death, specifically by affecting the intrinsic (mitochondrial) pathway (Gopal *et al.,* 2005; Liu *et al.,* 2004; Ehrhardt *et al.,* 2004; Fulda *et al.,* 1997). Therefore, the compound may modulate the Bcl-2 family and possibly enhance targeting of the novel Bcl-2 variant p15-20-Bcl-2 in MOLM-13 cells, which was described in Chapter 3 (Section 3.3.4). However, due to inconsistent reports between different cell types on the role of Bcl-2 family regulation by BetA (Shankar *et al.,* 2017; Liu and Luo, 2012; Mullauer *et al.,* 2009; Thurnher *et al.,* 2003), studies are warranted to examine BetA mechanism in sensitising relapsed monocytic AML cells to chemotherapy drugs. Although drug combination is a promising approach for improving cancer treatment, possible drug interference in the mechanism of action may impede its benefit (Jaeger *et al.,* 2017). Therefore, the synergism of multiple drugs and their mechanism of drug interaction should be established first. Combination of BetA with standard chemotherapy such as Dox may potentially endorse the apoptotic-autophagic interplay and provide valuable mechanistic information on cross-talk of cell death signalling in leukaemic cells.

4.1.1 Aims and objectives

This study aimed to determine if combining betulinic acid (BetA) to the established AML drug, doxorubicin (Dox), could enhance the cytotoxicity of doxorubicin to MOLM-13 cells, and compare the drug selectivity between AML monocytic cells. Studies were conducted to determine combination effect of the drugs on inhibition of cell division and/or induction of mitochondrial-mediated cell apoptosis is associated with the stimulation of reactive oxygen species. The mechanism of BetA and Dox combination were further investigated and compared to the single treatment through studying the regulatory genes and proteins involved in modulating programmed cell death such as apoptosis and autophagy.

Objectives

- To determine if BetA exerts selective cytotoxicity against myeloid leukaemia cell lines (compared to other leukaemic, monocytic and non-cancerous cells). Additionally, to determine a suitable concentration of BetA for combinational studies with the established chemotherapy drug Dox

- To evaluate cell death population (apoptotic and necrotic) induced between single and combination treatment. In addition, to investigate the cell death population difference between AML MOLM-13 cells of relapsed origin and SC/U-937 monocytic cells

- To investigate if reactive oxygen species stimulation contributed to drug-induced cell death in MOLM-13 cells

- To study the mechanism of action induced by the drugs, singly and combined, by examining protein and gene expressions involved in apoptotic death and autophagy modulation. Furthermore, to compare this effect between relapsed AML cell model MOLM-13 and SC/U-937 monocytic cells.

4.2 Results

4.2.1 Betulinic acid, but not doxorubicin, showed selective cytotoxic effect on AML MOLM-13 leukaemic cell lines

In order to evaluate if Dox and BetA affect the viability of leukaemic and non-cancerous cells, the drugs were tested on a cell line of non-cancerous epithelial origin (human embryonic kidney cell line, HEK293T) alongside leukaemic blood cells (leukaemic cell line - AML, MOLM-13; CML, K562 and SC/U-937 cells). CyQUANT® assay was used which utilises a detection dye that can penetrate viable cells and estimate cell number by binding to nucleic acid of live cells. Metabolic based viability assay, alamarBlue™, was performed as part of preliminary experiments to compare and select appropriate method for cell viability measurements (Table 4.1).

Dox (0.1 - 5 µM) showed potent inhibition of cell viability in a dose- and time-dependent manner on all tested cell lines (Fig. 4.1), including non-cancerous HEK293T cells. The viability of leukaemic blood cells was significantly inhibited at 24 h by Dox treatments in MOLM-13 and K562 cells, except for SC/U-937 cells showing significant cells suppression only at 48 h. At 48 h, HEK293T cells already showed sensitivity to Dox at 0.5 µM with a significant ($p < 0.01$) decrease of 49% in cell number.

BetA treatment did not show any statistically different decrease in non-cancerous HEK293T cell growth at any of the concentrations tested, compared to the vehicle control (DMSO 0.05%) (Fig 4.1 A). Instead, BetA 1.5 µM and 20 µM significantly stimulated HEK293T cell viability by 21% ($p < 0.01$) at 72 h and 28% ($p < 0.05$) at 48 h, respectively. Conversely, BetA at 10 µM and above showed significant induced cell viability inhibition when co-treated with leukaemic cells K562 and MOLM-13 (Fig. 5.1 C & D), revealing some cell selectivity of BetA. However, no significant difference was shown between treated SC/U-937 monocytic cells with vehicle control and BetA treatments (Fig. 4.1 B). Moreover, BetA 20 µM and 40 µM slightly increased cell viability of the monocytic SC/U-937 cells by 11% and 17%, respectively ($p > 0.05$)

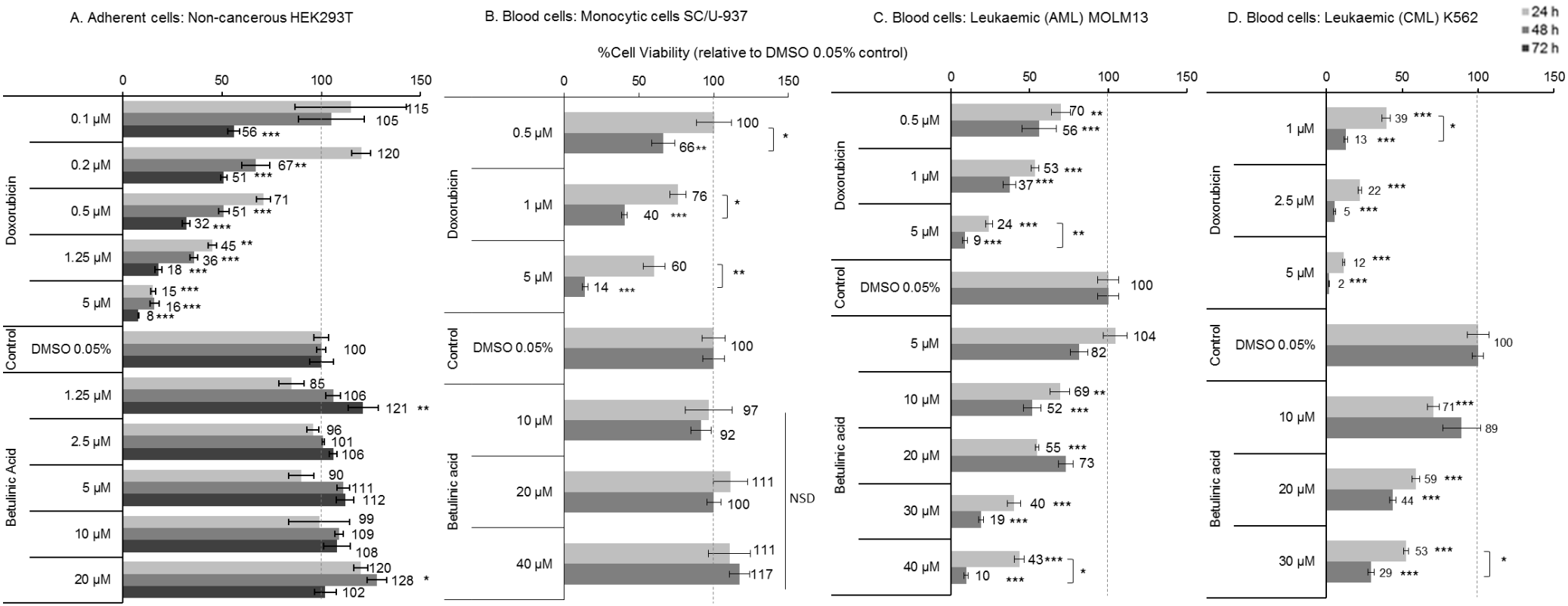


Figure 4.1 The effect of betulinic acid and doxorubicin on non-cancerous cells and leukaemic cells

Non-cancerous human embryonic kidney cells **(A)** HEK293T and leukaemic **(B)** SC/U-937 monocytes, **(C)** AML MOLM-13 and **(D)** CML K562 were treated with different concentrations of BetA (1.25-40 µM) and an established cancer drug, Dox (0.1-5 µM). n=3-4. Cell viability was determined by fluorescence using CyQUANT® Direct assay. The data was analysed using One-Way ANOVA with Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant.

When comparing CyQUANT Direct® and alamarBlue™ cell viability measurements, both methods yield similar results as indicated by IC_{50} of BetA in K562 cells (Table 5.1). However, CyQUANT Direct® was selected for further experiments over alamarBlue™ due to its robustness as described in Chapter 2 (Section 2.2.4).

In contrast to the effect observed for BetA, Dox did not exhibit selective inhibitory effect on the viability of leukaemic cells but showed similar inhibitory effects on both the leukaemic cells (K562, MOLM-13, SC/U-937) and the non-cancerous (HEK293K) cells. The IC_{50} of Dox after 48 h co-treatment with K562, MOLM-13, SC/U-937 and HEK293K was 0.18 µM, 0.62 µM, 0.81 µM and 0.64 µM, respectively (Table 4.1). Interestingly, similar to the IC_{50} values assessed by Annexin V/PI (Chapter 3; Fig. 3.3), MOLM-13 cells were more sensitive to Dox compared to monocytic SC/U-937 cells when IC_{50} was evaluated through measurement of nucleic acid content in viable cells.

Furthermore, tested BetA concentrations also showed selectivity towards different monocytic cells. No significant inhibitory effect was observed in BetA-treated SC/U-937 cells but the compound significantly inhibited cell viability of MOLM-13 cell line with IC_{50} at 24.3±0.4 µM.

Table 4.1 The IC_{50} value of single BetA and Dox in leukaemic cell lines and non-cancerous cells

	IC_{50} (µM)			
	Blood cells			Adherent Cells
	K562 (CML)		MOLM-13 (AML)	SC/U-937 Monocytic cells	HEK293T Non-cancerous
BetA (48 h)	17.65* 19.24* (Average = 18.45±0.80)	19.63# 18.33# (Average = 18.98±0.65)	23.97* 23.72* 25.13* (Average = 24.27±0.43)*	NSD*	NSD*
Dox	0.65 (24h)* 0.18 (48 h)*		1.25 (24 h)* 0.62 (48 h)* 0.40 (48 h)+	0.81 (48 h)* 0.66 (48 h)+	1.14 (24 h)* 0.64 (48 h)* 0.17 (72 h)*

Cell viability was measured in fluorescence by (*) CyQUANT Direct® (at 1000 gain), (#) alamarBlue™ (at 600 gain) assay, (+) Annexin V/PI double-staining (Live cells population; –ve Annexin V and –ve PI). (n=3-6). The IC_{50} values were calculated using GraphPad Prism 8 software.

4.2.2 Combination of betulinic acid and doxorubicin synergistically reduced cell viability in MOLM-13 AML cell line, but did not negatively affect cell viability of monocytic SC/U-937 cells

Experiments were conducted to determine if BetA could enhance the cytotoxic effect of Dox on leukaemic cells of relapsed origin (MOLM-13) and compare this effect to other monocytic cells (SC/U-937).

Sole treatments of Dox 0.5 µM and 1 µM significantly reduced the cell viability of MOLM-13 ($p < 0.01$ and $p < 0.001$, respectively (Fig. 4.2 A)), compared to vehicle control. No statistically significant effect was shown in SC/U-937 monocytes treated by Dox (0.5 µM and 1 µM) at 24 h, despite the suppression of SC/U-937 cell growth by 24% when co-treated with Dox (1 µM). BetA (20 µM) showed selective cytotoxicity by inhibiting MOLM-13 cell growth at 24 h by 26% ($p < 0.05$) without growth inhibitory effect on SC/U-937 (Fig. 4.2 A & B).

Co-treatment of BetA and Dox at 24 h showed selective cytotoxicity towards the MOLM-13 cell line, the relapsed AML cell model (Fig. 4.2 A). BetA 20 µM combined with Dox 0.5 µM or Dox 1 µM showed highly significant cell growth inhibition in MOLM-13 by 30% ($p < 0.01$) and 60% ($p < 0.001$), respectively at 24 h. No statistical effect on cell growth was observed on SC/U-937 monocytes by the same combination treatments, despite an increase in cell numbers by 20% at BetA 20 µM and Dox 0.5 µM combination (Fig. 4.2 B). Therefore, combination treatments were cytotoxic to leukaemic relapsed cell model but did not affect the cell viability of monocytic SC/U-937.

Of the two combination treatments studied, only BetA 20 µM with Dox 1 µM showed greater cell growth suppression (60%) on MOLM-13 at 24 h compared to the single compound treatments (reduction of 26% and 47% by BetA 20 µM and Dox 1 µM alone, respectively). However, the effect of the combined drug was not statistically significant when compared to the sole treatment with Dox 1 µM based on ANOVA ($p > 0.05$), while significant ($p < 0.05$) with 2 sample T-test analysis (Fig. 4.2 A II).

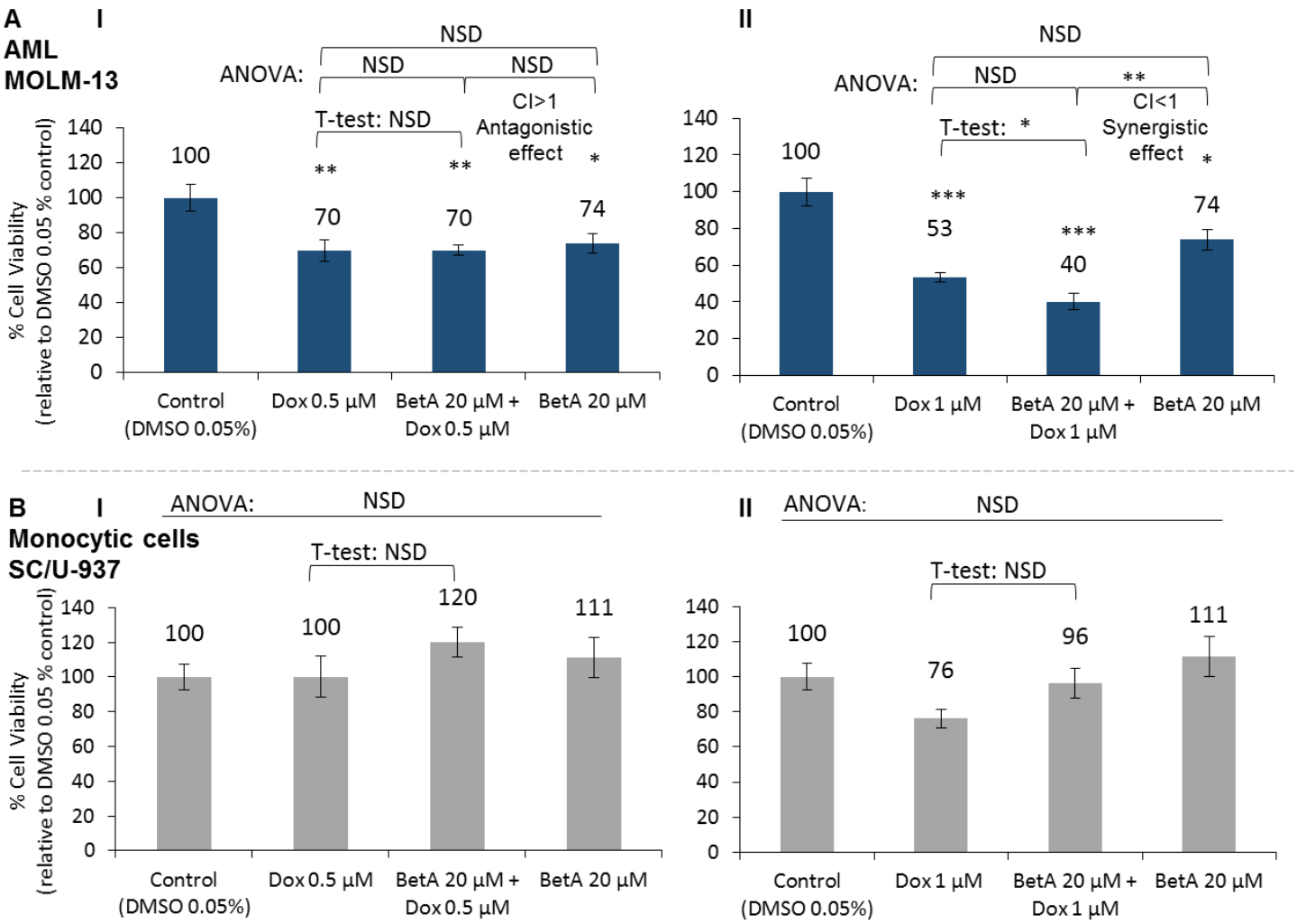


Figure 4.2 Effect on cell viability of betulinic acid and doxorubicin combination treatments on leukaemic MOLM-13 and SC/U-937 cells

MOLM-13 **(A)** and SC/U-937 **(B)** cells were treated for 24 h by Dox 0.5 µM with BetA 20 µM **(I)** and Dox 1 µM with BetA 20 µM **(II)**, and the respective individual drugs. The data were expressed as mean ± SE of percentage relative to control, values obtained for control cells (cells with DMOS 0.05% vehicle). Cell viability was determined by fluorescence using CyQuant Direct assay. The data set was analysed by One-Way ANOVA with Tukey post-hoc test. Single Dox and combination drugs were compared by 2 samples T-test. n=4. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant.

Comparison of statistical mean difference between single drugs and combination treatments gave contradicting outcomes when using parametric statistical tests comparing variances (ANOVA; post-hoc Tukey and 2 sample T-test) (Fig. 4.2 A). However, Chou-Talaya method using combination index (CI) has been reported to be a more reliable way to interpret results for combinational studies over analysis of variances (Chou, 2011; Chou, 2010). Based on the CI value, the inhibition effect of BetA combined with Dox 1 µM was synergistic (CI < 1) in suppressing the viability of MOLM-13 at 24 h incubation. However, BetA combined with 0.5 µM Dox was antagonistic (CI > 1) in their inhibitory effect (Table 4.2).

Table 4.2 Synergistic effect of betulinic acid and doxorubicin on cell viability of MOLM-13 cell lines

Single Drugs				Drug Combination			
Betulinic acid		Doxorubicin		% inhibition	*p*-value Dox vs Comb	CI	Effect
µM	% inhibition	µM	% inhibition				
20	26 ± 5.45	0.5	30 ± 6.04	30 ± 3.16	>0.05	1.94	Antagonistic
		1	47 ± 2.39	60 ± 4.47	<0.05	0.87	Slightly synergistic

Cells were treated by BetA, Dox and their combinations for 24 h. Cell viability was measured in fluorescence by CyQuant assay. The data were expressed as cell growth inhibition and presented as mean ± SE (n=4) of percentage control, values obtained for % cells treated with vehicle and subtracted by % viable cells for each treatment. Synergy quantification was based on Chou-Talaya method by Combination Index (CI) using CompuSyn (non-constant ratio design). Additive effect (CI = 1), Synergism (CI < 1), Antagonism (CI > 1)

4.2.3 Betulinic acid did not affect the antiproliferative effect of doxorubicin on MOLM-13 cells

Cell division was measured by CSFE assay to investigate if BetA affects the ability of Dox to inhibit cell proliferation of AML cells. CFSE, a non-toxic fluorescent dye, penetrates cells and binds covalently to free amino acids on proteins in cell cytoplasm. The intensity of the dye halves with each cell division, thus allowing the rate off cell proliferation to be tracked.

In this study, the doubling time of untreated MOLM-13 cells was 18 h. At 24 h, the control (untreated cells) sample and drug treated samples showed similar mean florescence intensity (MFI) values (Fig. 4.4 A). However, by 72 h there was an evident shift, a decrease in the MFI, demonstrating a different rate of proliferation, where Dox treatments and combination treatments inhibited MFI in a concentration-dependent manner (Fig. 4.4 B, C). In contrast, BetA (20 µM) had no marked effect on the rate of cell growth when tested alone nor affect the anti-proliferative effect of Dox in the combined drug treatments.

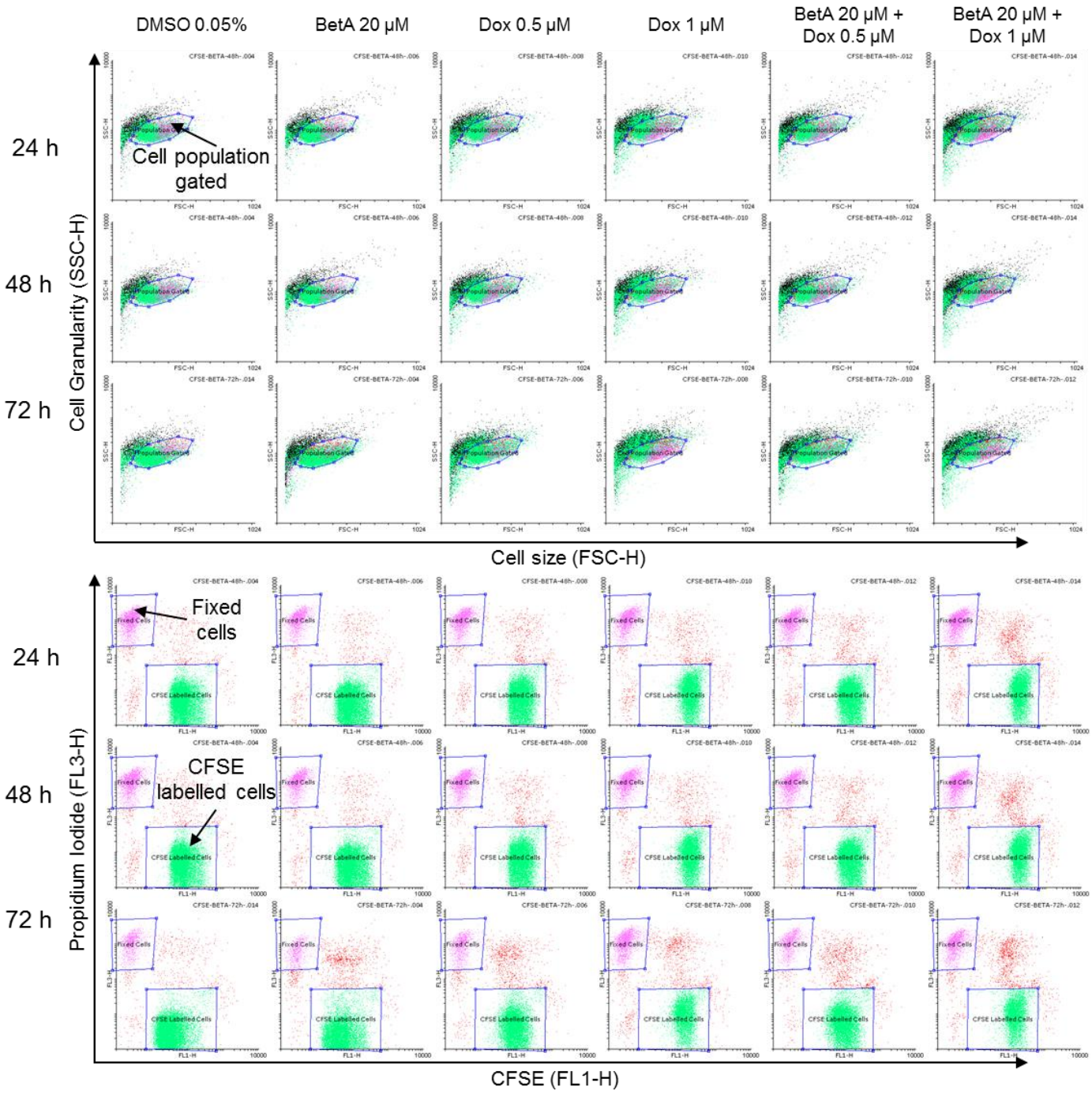


Figure 4.3 Proliferation rate of treated MOLM-13 assessed by 5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) fluorescent dye

Representative (n=2) flow cytometry dot plots of CFSE labelled MOLM-13 cell treated for 24, 48 and 72 h by BetA, Dox and combination of BetA and Dox. Upper panels showing selection of cell population in the granularity vs size (SSC/FSC). Lower panels showing gating of CFSE labelled cells (FL1) and fixed cells (FL3). Fixed cells were used to normalised the data and MFI from CFSE labelled cells were used to track the shift in proliferation.

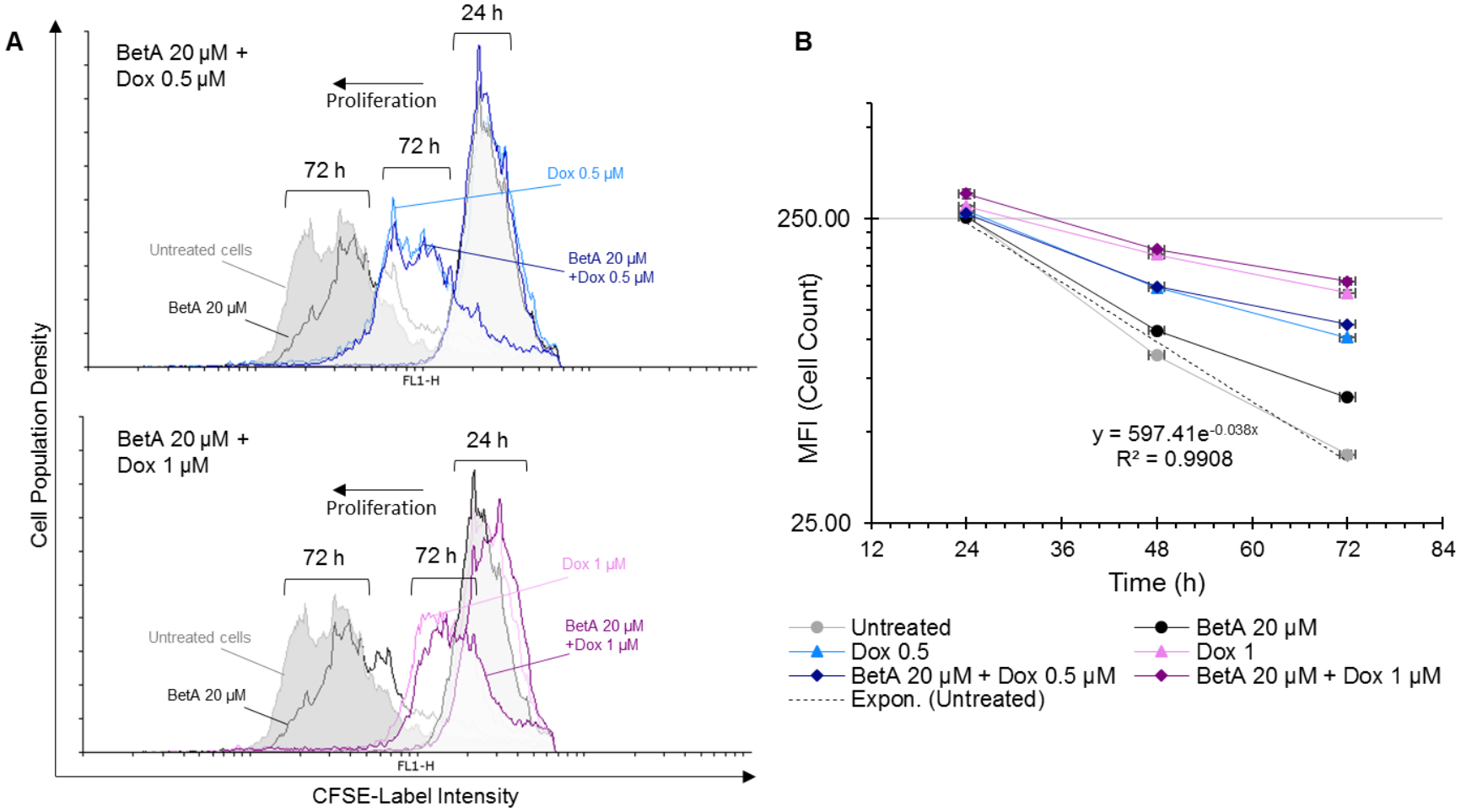


Figure 4.4 The anti-proliferative effect of betulinic acid and doxorubicin alone and in combination on MOLM-13 for 3 days
Cell proliferation was measured by CFSE assay. Flowing software was used for histogram overlay of treatments at 24 h **(A)**, 48 h **(B)**, and 72 h **(C)**. The MFI values of MOLM-13 after the treatments was plotted to generate the equation for doubling time **(D)** n=2.

5.2.4 Combination treatments induced apoptotic death in MOLM-13 AML cell line, but rescued SC/U-937 cells from doxorubicin-induced cell death

Annexin V and Propidium Iodide (PI) double staining was used to examine the effect of drug-induced cell death and to determine the proportion of cells in different apoptotic stages. Annexin V binds to phosphatidylserine, an apoptotic marker, on the membrane of the cells that are undergoing apoptosis, while PI intercalates with DNA of dead cells with compromised membrane integrity.

Cell death was observed in single Dox treatments as well as Dox and BetA co-treatments in MOLM-13 cell line at 24 h and 48 h incubation with very highly statistical differences ($p < 0.001$) compared to vehicle control (DMSO 0.05%). However, BetA (20 µM) alone induced similar apoptotic death profile when compared to DMSO control with $p > 0.05$ at both time points (Fig. 4.5 A).

Co-treatments of the combined drugs with MOLM-13 showed a slight shift in cell death population when compared to single Dox. At 24 h, about 10% ($p > 0.05$) more cells resided in reversible early apoptotic stage (+ve Annexin V, -ve PI) and 8% ($p > 0.01$) less cells in irreversible late apoptosis (+ve Annexin V, +ve PI) with Dox 0.5 µM when compared to the equivalent Dox co-treatment with BetA. In addition, combination treatment of Dox 1 µM and BetA had significantly more (11%, $p < 0.05$) cells in late apoptosis compare to Dox 1 µM alone at 24 h. On the contrary, the same combination did not show any statistical difference in cell death population when compared to the relevant Dox concentration at 48 h. However, the lower combination (Dox 0.5 µM and BetA 20 µM) at 48 h had significantly more (17%, $p < 0.05$) cells present in late apoptosis and less (23%; $p > 0.05$) viable cells compared to 0.5 µM Dox (Fig. 4.6 A).

In SC/U-937 cells at 24 h incubation, Dox treatments showed no statistical significance in cell population shift compared to DMSO 0.05% control cells. Although Dox 0.5 µM appeared to have more cells in necrosis (12%; $p > 0.05$) and Dox 1 µM more cells in early apoptosis (24%; $p > 0.05$). BetA (20 µM) alone showed no significant change in cell death induction or cell viability suppression when compared to the control, nor enhanced the cell killing ability of Dox treatments (Fig. 4.6 B). Combination treatments on average have more viable cells present compared to their single Dox counterparts. However, the effect was not significantly different.

At 48 h incubation, cell death induced in SC/U-937 cells by both concentrations of Dox (0.5 and 1 µM) was more pronounced compared to the control treatment (viable cell reduction; $p < 0.001$), with the majority of cells residing in late apoptosis ($p < 0.001$; 42% in Dox 0.5 µM

and 23% in Dox 1 µM). Although, sole BetA 20 µM in SC/U-937 cells at 48 h showed statistically less viable cells (9%; $p < 0.05$) than the control, none of the cell death populations were significantly different. Moreover, BetA with Dox co-treatment attenuated Dox-induced cell death in SC/U-937 cells by roughly 25% and 13% compared to equivalent single Dox 0.5 µM ($p < 0.001$) and 1 µM ($p < 0.01$), respectively (Fig. 4.6 B).

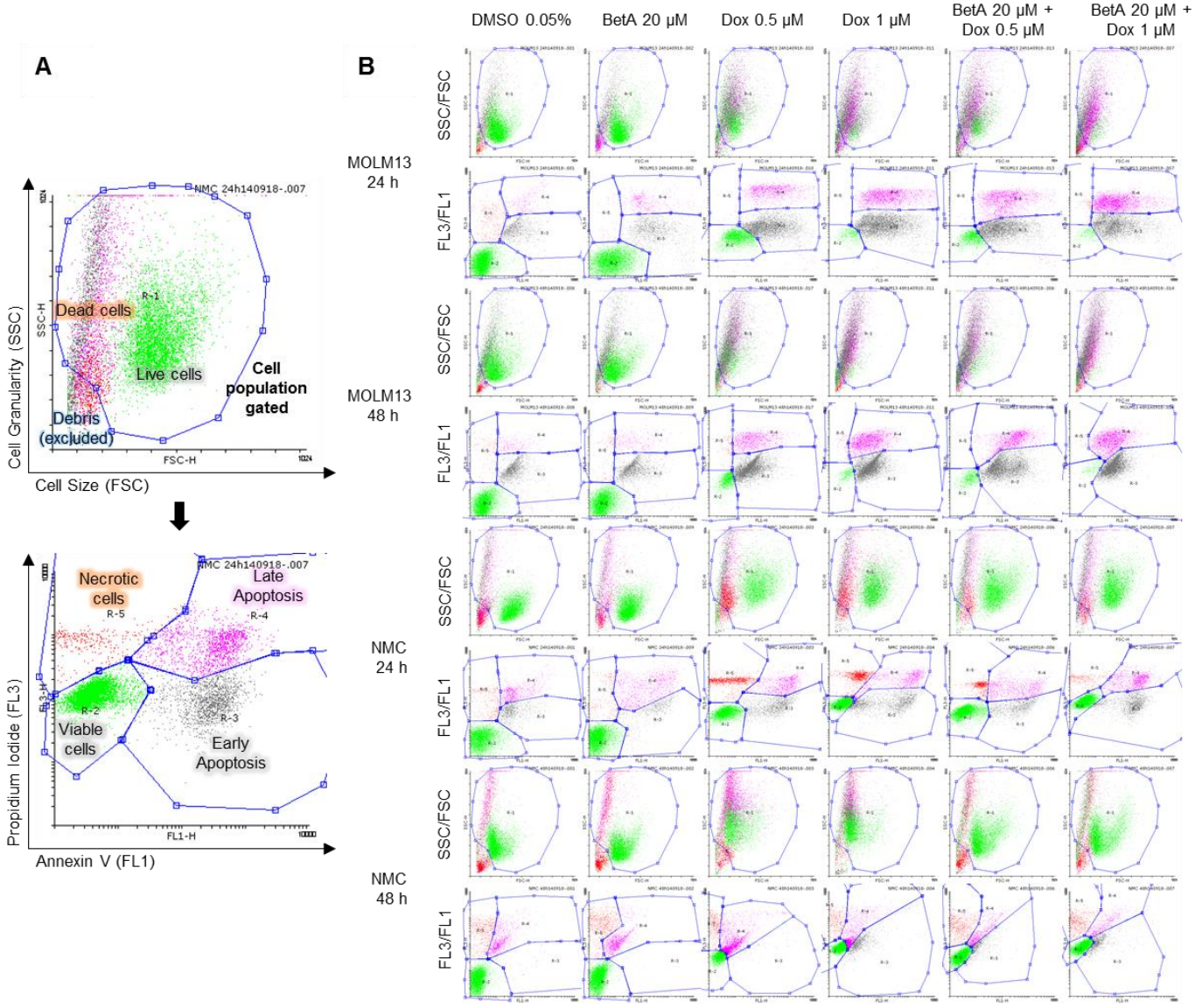


Figure 4.5 Gating of treated MOLM-13 and SC/U-937 cells to determine cell death population

(A) Example of gating process. **(B)** Representative (n=3) flow cytometry dot plots of cells treated for 24 and 48 h by single BetA, Dox, and combination of BetA and Dox double stained by Annexin V (FL1) and PI (FL3). Population of interest was selected by cell granularity and size (SSC/FSC). Backgating (FL1/FL3) was applied to determine viable (-ve Annexin V and -ve PI), early apoptotic (+ve Annexin V and -ve PI), late apoptotic (+ve Annexin V and +ve PI) and necrotic (-ve Annexin V and +ve PI) cell population.

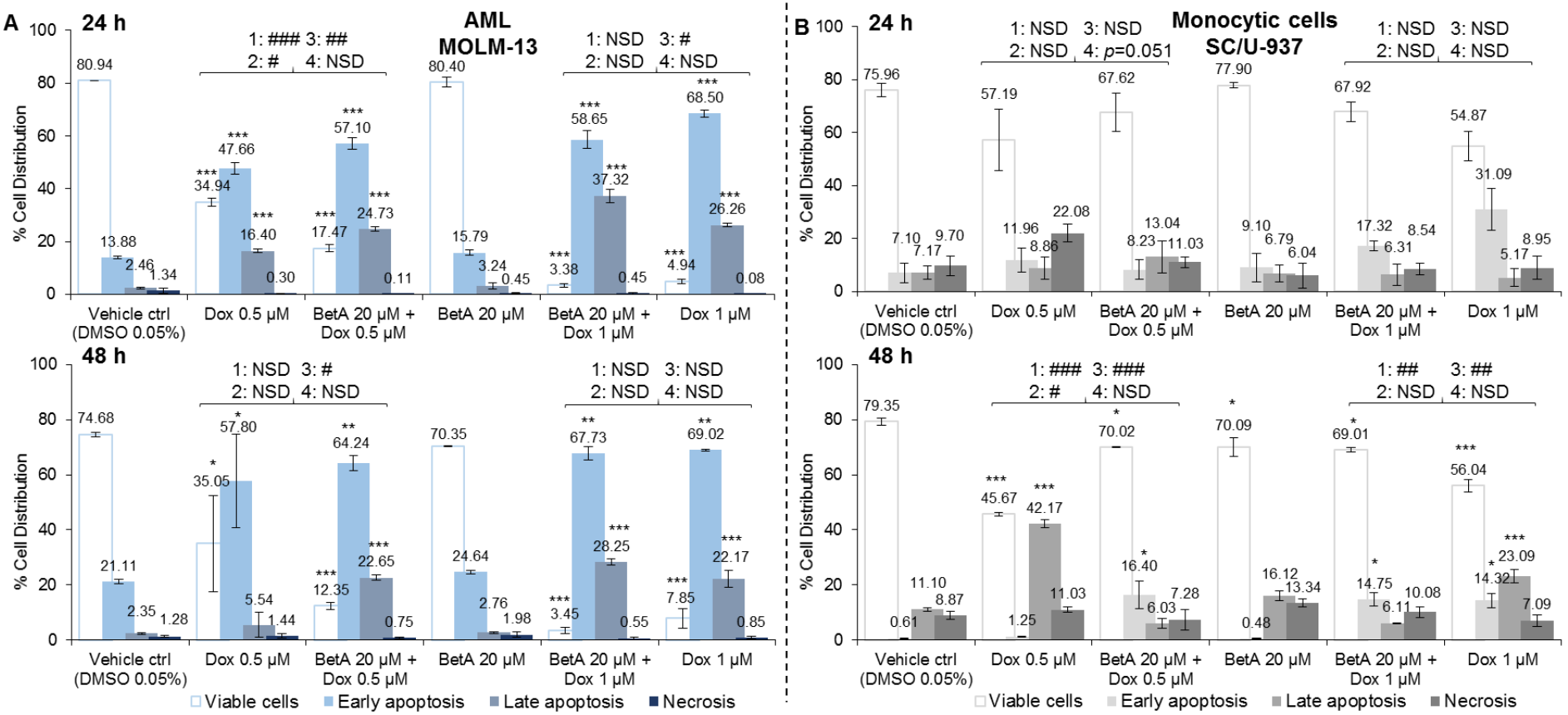


Figure 4.6 Cell death population of MOLM-13 and SC/U-937 treated by individual drugs and combined therapy for 24 and 48 h

Samples were double stained by Annexin V (apoptotic cell dye) and Propidium Iodide (PI, dead cell dye). **(A)** MOLM-13 and **(B)** SC/U-937 cells were treated with BetA (20 µM), Dox (0.5 and 1 µM), and BetA (20 µM) combination with Dox (0.5/1 µM) for 24 h and 48 h incubation. DMSO 0.05% was used as vehicle cell control. One-Way ANOVA; Tukey post-hoc test was used to compare the cell population between the vehicle (DMSO 0.05%) control and the treatments. Shift in different cell death population between single Dox and combination was compared using 2 samples T-test analysis. The data were expressed as a mean ± SE; n=3. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant.

Combination and single Dox cell population compared by 2 sample T-test: $p > 0.05$ (NSD), $\#p \leq 0.05$, $\#\#p \leq 0.01$, $\#\#\#p \leq 0.001$.

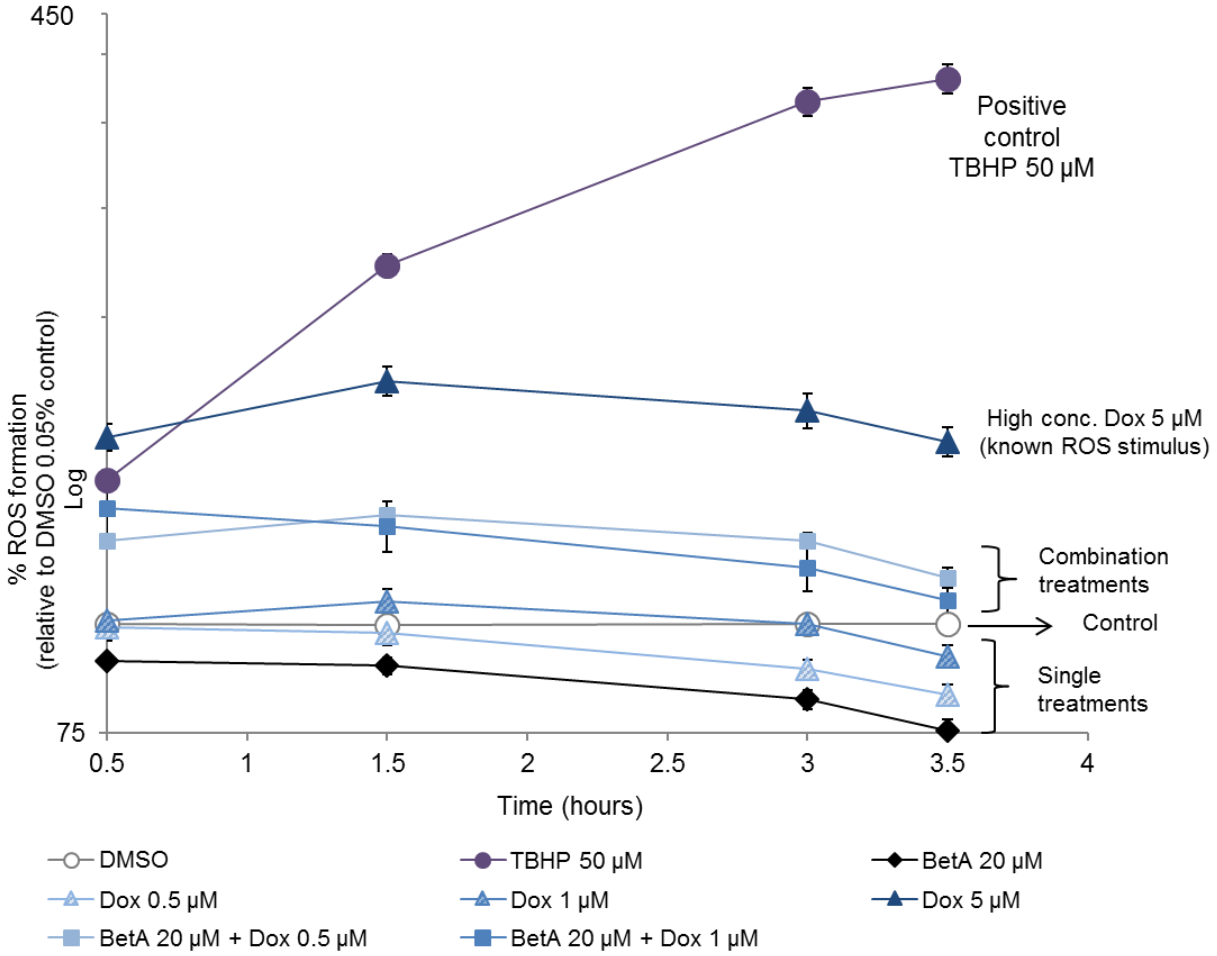
1: Live cells (–ve Annexin V and –ve PI) 2: Early apoptosis (+ve Annexin V and –ve PI)

3: Late apoptosis (+ve Annexin V and +ve PI) 4: Necrotic cells (–ve Annexin V and +ve PI)

4.2.5 Betulinic acid and doxorubicin combination enhanced the formation of reactive oxygen species in MOLM-13 cell lines.

To determine which type of apoptotic pathway (extrinsic or intrinsic) is utilised by the drug treatments, reactive oxygen species (ROS) stimulation was measured. ROS is associated with intrinsic (mitochondrial) apoptotic cell death pathway.

In this study, MOLM-13 cells individually treated with Dox (5 µM) or TBHP (50 µM) significantly ($p < 0.001$) stimulated ROS generation. Although single treatments of low dose Dox (0.5 or 1 µM) induced cell apoptosis in MOLM-13, they failed to exhibit an increase in ROS levels after treatment of MOLM-13 cells with the drugs for up to 3.5 h (Fig. 4.7). Single BetA (20 µM) treatment did not significantly alter the levels of ROS when compared to the control. However, the combined treatments stimulated ROS production within 1.5 h after cell treatment ($p < 0.001$ when compared to vehicle (DMSO 0.05%) control). In addition, the combination treatments significantly enhanced ROS generation when compared to single Dox treatments. BetA augmented Dox 0.5 µM and 1 µM ROS levels to 1.26-1.40 fold within 0.5-3 h ($p < 0.05$) and 1.35 fold at 0.5 h ($p < 0.001$), respectively.



Control (DMSO 0.05%)	
TBHP 50 µM	$p < 0.001^{\#}$
BetA 20 µM	NSD#
Dox 0.5 µM	NSD#
Dox 1 µM	NSD#
Dox 5 µM	$p < 0.001^{\#}$
BetA 20 µM + Dox 0.5 µM	$p < 0.01$ (0.5-1.5 h)
BetA 20 µM + Dox 1 µM	$p < 0.01$ (0.5-1.5 h)

Single Dox	
	Dox 0.5 µM
BetA 20 µM + Dox 0.5 µM	$p < 0.05$ (0.5-3 h)
	Dox 1 µM
BetA 20 µM + Dox 1 µM	$p < 0.001$ (0.5 h)

# = statistically significant at all-time points
NSD = no statistical difference

Figure 4.7 Reactive oxygen species stimulation in MOLM-13 after treatment with doxorubicin, betulinic acid and their combination
The cells were stained with 20 µM DCFDA following 0.5, 1.5, 3 or 3.5 h co-treatment with BetA (20 µM), Dox (0.5 or 1 µM) or a combination of the drugs. TBHP (50 µM) was used as a positive control. ROS stimulation was measured as the fluorescence of the treatments using FLUOstar Omega (BMG Labtech) at 485/520 nm, excitation and emission, respectively.
Data are expressed as the mean ± SE of four replicate (n=4) measurements in one representative experiment. The data were analysed by One-Way ANOVA using Tukey post-hoc analysis. Dox and the combined treatments were compared by Two Sample *t*-Test.

4.2.6 Doxorubicin, alone and in combination with betulinic acid, inhibits a novel isoform of Bcl-2 in AML MOLM-13 cells without a potent effect on the main Bcl-2 isoform

Dox and combination treatments reduced the level of Bcl-2 anti-apoptotic protein in MOLM-13. However, the decrease was statistically significant only in the smaller (and novel) Bcl-2 of 15-20 kDa (p15-20-Bcl-2) and not in the major 26 kDa Bcl-2 isoform (p26-Bcl-2-α) when compared to vehicle control (DMSO 0.05%). Therefore, the treatments potently affected the novel truncated p15-20-Bcl-2. When Bcl-2 isoforms were combined, statistical reduction ($p < 0.05$, compared to vehicle control) was shown only with the combination treatment of Dox 1 µM and BetA 20 µM (Fig. 4.8 B). The expression level of Bax pro-apoptotic protein was increased by single Dox and combination treatments, but this protein elevation was not statistically different ($p > 0.05$) between the treatments and vehicle control, except for 1 µM Dox treatment that increased Bax level by 2.08-fold ($p < 0.05$) (Fig. 4.8 C).

Sole BetA 20 µM did not significantly alter the expression of Bcl-2 or Bax protein. Additionally, the effect of Dox on apoptosis-regulating proteins and the isoforms was not enhanced when combined with BetA. Therefore, BetA did not enhance but also did not interfere with the effect of Dox in regulating the pro- and anti-apoptotic proteins Bax and Bcl-2 in MOLM-13 (Fig. 4.8).

To assess the susceptibility of the AML cells undergoing apoptosis, Bax/Bcl-2 ratio was evaluated (Fig. 4.8 C). All treatments, except for sole BetA, increased the ratio in both Bcl-2 isoforms, with the Bax/Bcl-2 ratio of p15-20-Bcl-2 being much greater. Moreover, the ratio difference when comparing single Dox and the combination with BetA was similar regardless of the Bcl-2 isoform. Combination of Dox 0.5 µM and BetA 20 µM slightly enhanced the ratio more than single Dox 0.5 µM treatment: an increase by 1.21-1.25-fold. On the contrary, the ratio increase of Dox 1 µM and BetA 20 µM combination was marginally lower when compared to Dox 1 µM treatment, a reduction by 1.18-1.20-fold.

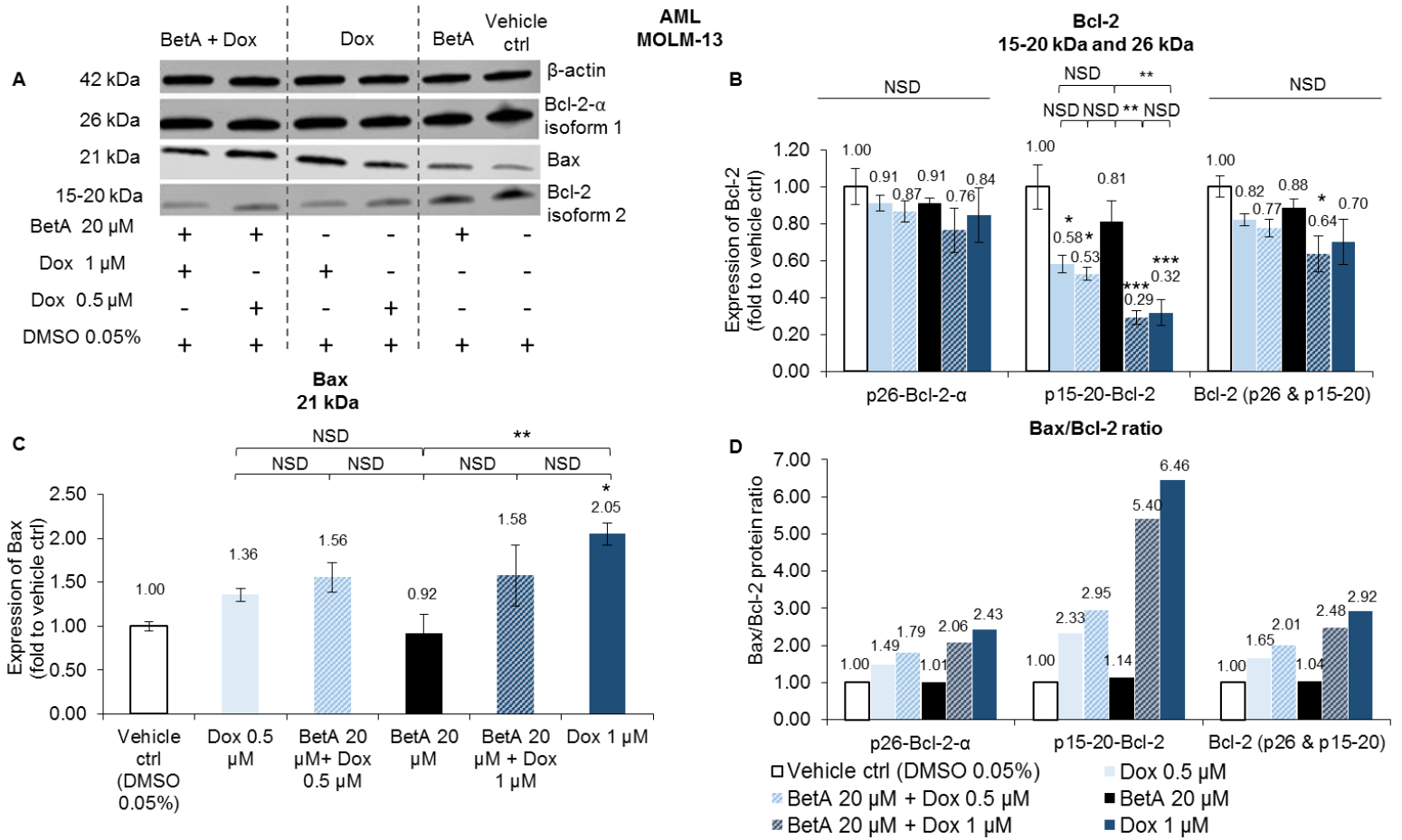


Figure 4.8 The effect of BetA, Dox and combination on pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression on MOLM-13 cell line

The protein expressions of Bcl-2 (26 kDa and 15-20 kDa) and Bax (21 kDa) were estimated by Western blot **(A)**. Bcl-2 **(B)** and Bax **(C)** protein levels were determined by normalising quantified protein intensity relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and β-actin (housekeeping protein). Data were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant. Relative change in the ratio of Bax/Bcl-2 **(D)** were expressed as the fraction of the mean protein levels.

➔ The difference in the graph layout and sample arrangement on the Western blot was to facilitate a clear display of statistical comparison

4.2.7 Autophagy marker Beclin 1 was reduced by doxorubicin and betulinic acid co-treatment in AML cell line

Expression of Beclin 1 protein, an autophagy marker, was significantly decreased in MOLM-13 cells when treated with Dox alone (0.5; $p < 0.001$ and 1 µM; $p < 0.001$) and Dox combined with BetA 20 µM (Dox 0.5; $p < 0.01$ and 1 µM; $p < 0.001$) when compared to the expression of vehicle control (DMSO 0.05%) (Fig. 4.9 B). The protein downregulation of Beclin 1 was in dose-dependent manner. Single BetA 20 µM slightly reduced the protein expression but with no statistical significance when compared to the control. In addition, there was no apparent effect of BetA on Dox activity in regulating the Beclin 1 expression.

To evaluate autophagy-apoptosis regulatory pathway and explore its association with cell death, Beclin 1/Bcl-2 ratio was calculated. The ratio was dose-dependently reduced by Dox and combination treatments with the established p26-Bcl-2-α, but no marked change was shown with BetA treatment. Moreover, the pronounce reduction in Beclin 1/Bcl-2 at 26 kDa Bcl-2 by Dox and combination treatment was in contrast to the novel Bcl-2 at 15-20 kDa isoform where reduction of the ratio by the treatments was hindered (Fig. 4.9 C).

The effect of the drugs (singly and combined) on the Beclin 1/Bcl-2 ratio across p26-Bcl-2-α, p15-20-Bcl-2, and combined isoforms (Bcl-2, p26 + p15-20) was similar. Dox 0.5 µM and BetA 20 µM combination marginally increased Beclin 1/Bcl-2 ratio by 1.48-1.54-fold compared to single Dox 0.5 µM. In contrast, combination of Dox 1 µM and BetA 20 µM slightly reduced the ratio by 1.36-1.42-fold compared to single Dox 1 µM treatment (Fig. 4.9 C).

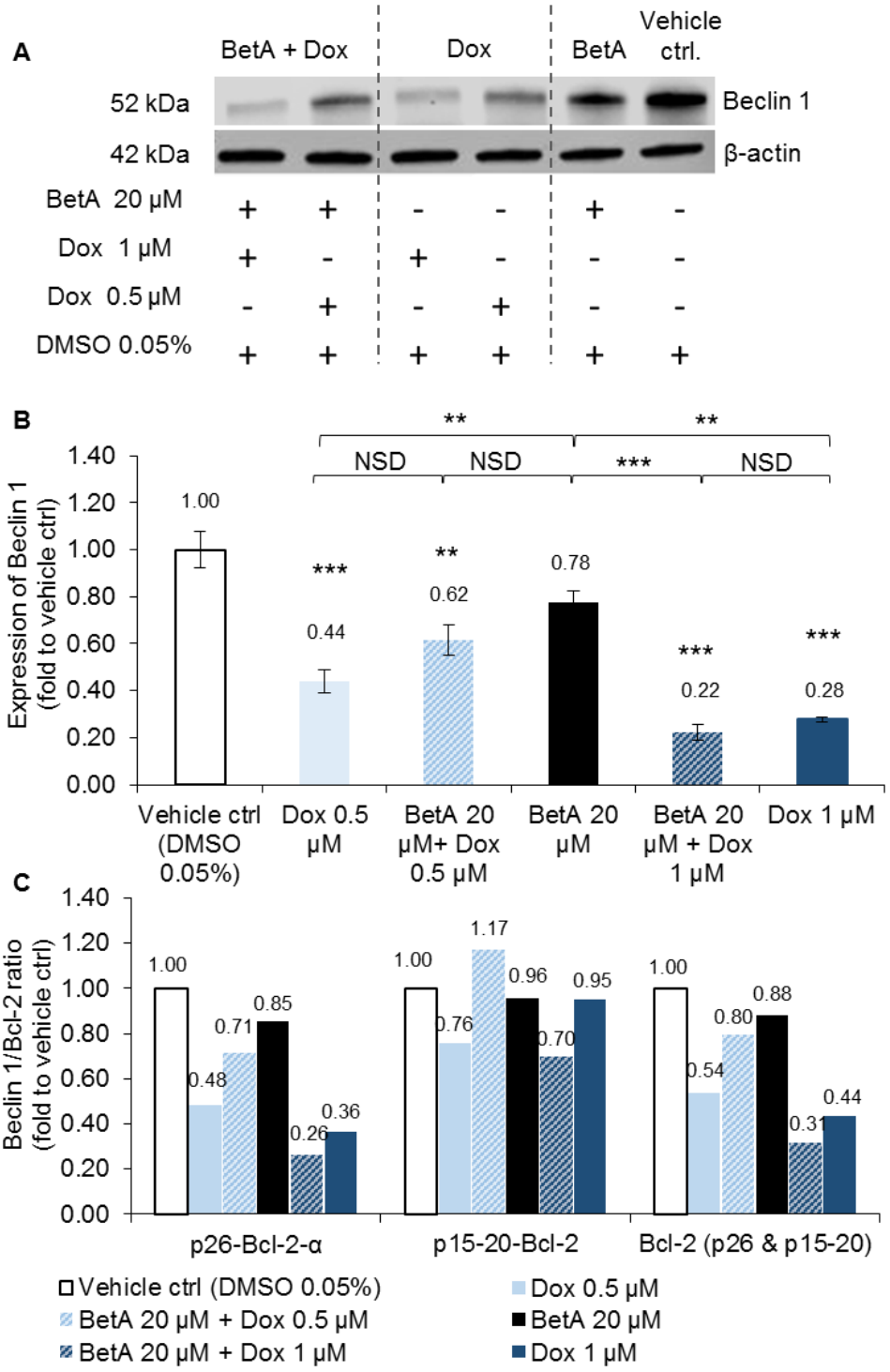


Figure 4.9 The effect of the single drugs and combination treatment on pro-autophagy protein expression on MOLM-13 cell line

The protein expressions of Beclin 1(**A**) (52 kDa) were estimated by Western blot. Beclin 1 protein level (**B**) was determined by normalising quantified protein intensity relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and β-actin (housekeeping protein). Result were expressed as mean ± SE, n=3. The data set was analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant. Relative change in the ratio of Beclin 1/Bcl-2 (**C**) were expressed as the fraction of the mean protein levels.

➔ The difference in the graph layout and sample arrangement on the Western blot was to facilitate a clear display of statistical comparison

4.2.8 Apoptotic and autophagy signalling protein levels were not altered by the treatments in CML cell lines K562 and SC/U-937 cells.

The levels of anti-apoptotic Bcl-2 protein in K562 cell lines were not significantly different when co-treated by Dox, BetA, drug combination or vehicle control (DMSO 0.05%) after 48 h, although Dox 1 µM and BetA 20 µM combination showed on average a non-significant 2.2-fold increase ($p > 0.05$) (Fig. 4.10 A II). In addition, overall expression of the Bcl-2 protein in K562 cells was minimal (Fig. 4.10 A I). On the other hand, Cytochrome *c* was greatly expressed in the CML cell lines. However, Cytochrome *c* protein showed no significant change in the expression between any of the single treatments or combinations and vehicle control, despite non-significant reductions by single Dox 0.5 µM and the combination treatments with BetA 20 µM. Conversely, non-significant ($p > 0.05$) Cytochrome *c* upregulation of 2.2-fold (on average) was observed in K562 treated by Dox 1 µM (Fig. 4.10 A III).

Similarly, in SC/U-937 monocyte cells, none of the treatments altered significantly ($p > 0.05$) the expression levels of apoptotic Bcl-2 and Cytochrome *c* proteins after 48 h treatment when compared to the vehicle control (Fig. 4.10 B). For instance, BetA-treated SC/U-937 cells showed on average 1.96-fold increase in Bcl-2 expression, but without significance ($p > 0.05$) (Fig. 4.10 B II). Single treatments of Dox and BetA did not noticeably change the pro-apoptotic Cytochrome *c* protein level of SC/U-937 cells when compared to vehicle control treatment. Interestingly, Cytochrome *c* level was on average non-significantly (compared to vehicle control) increased by 2.00-fold by Dox 0.5 µM and BetA 20 µM combination (Fig. 4.10 B III). In contrast, combination of Dox 1 µM and BetA 20 µM non-significantly decreased ($p > 0.05$ compared to vehicle control) Cytochrome *c* protein expression to 0.75-fold (on average).

Beclin 1 expression level was not significantly changed ($p > 0.05$) by any tested treatments in K562 or SC/U-937 cells when compared to vehicle-treated cells (Fig. 4.11). However, SC/U-937 cells treated by single Dox 1 µM marginally declined Beclin 1 protein level (0.69-fold; $p > 0.05$ compared to vehicle control), which was significantly ($p < 0.01$) lower compared to BetA 20 µM treatment that slightly increased Beclin 1 expression (1.36-fold; $p > 0.05$ compared to vehicle control) (Fig. 4. 11 B II).

Overall, the co-treatments of CML K562 cell line and monocytic SC/U-937 cells with single Dox and BetA as well as drug combinations did not significantly affect the expressions of apoptotic proteins (Bcl-2 and Cytochrome *c*) and autophagy marker Beclin 1 tested in this study.

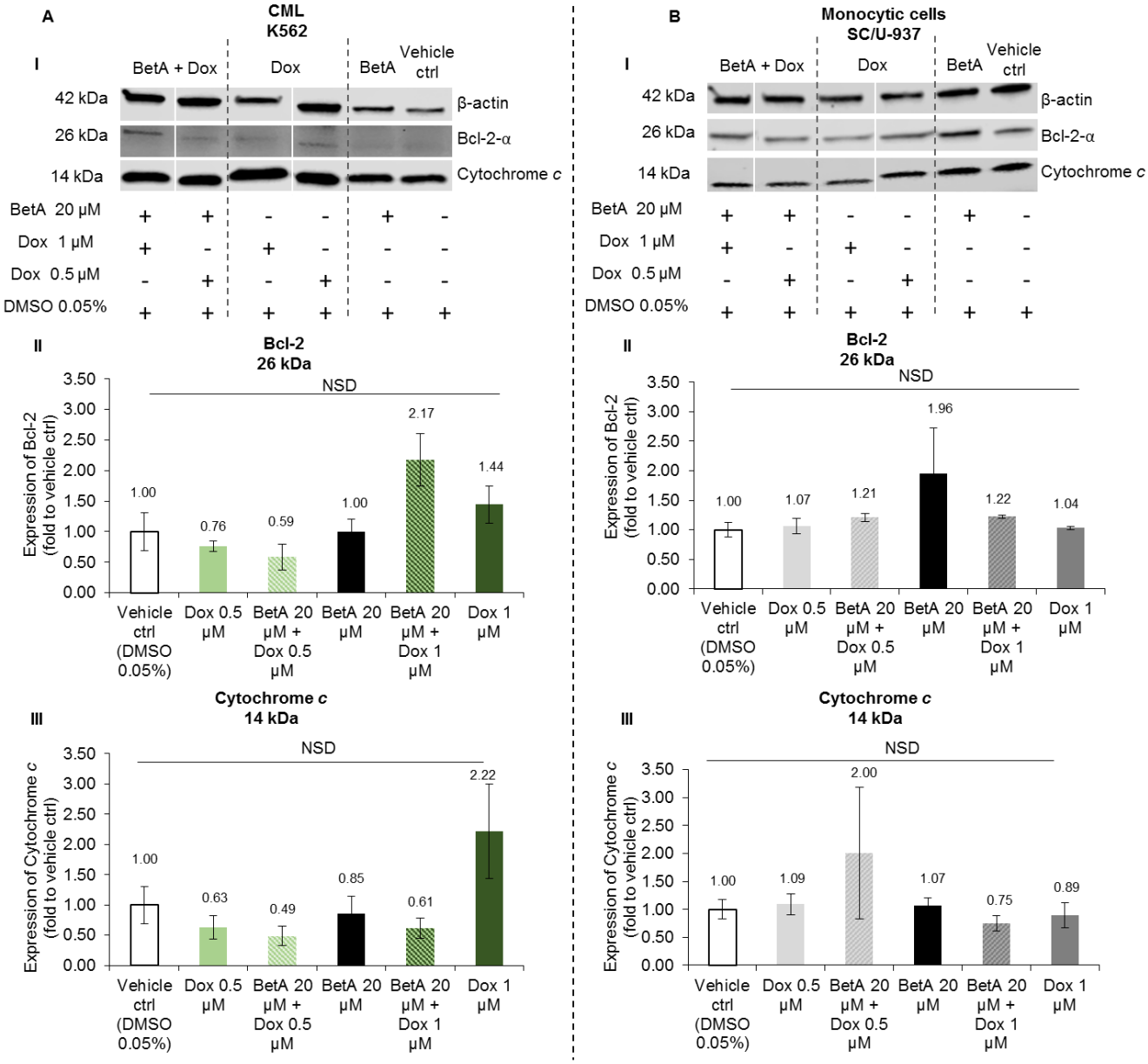


Figure 4.10 Apoptosis regulating proteins Bcl-2 and Cytochrome c expression in CML K562 and SC/U-937 monocytes treated by single and combination drug

Leukaemic cell line **(A)** K562 and **(B)** SC/U-937 cells were treated by single Dox or combination of BetA and Dox for 48 h. **(I)** The protein expressions of anti-apoptotic Bcl-2 (26 kDa) and pro-apoptotic Cytochrome *c* (14 kDa) were estimated by Western blot. Experimental samples were run on the same gel and shown protein expressions are on the same blot for each protein antibody. **(II)** Bcl-2 and **(III)** Cytochrome *c* protein levels were determined by normalising quantified protein intensity relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and β-actin (housekeeping protein). Data were expressed as mean ± SE (n=3) and analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant.

➔ The difference in the graph layout and sample arrangement on the Western blot was to facilitate a clear display of statistical comparison

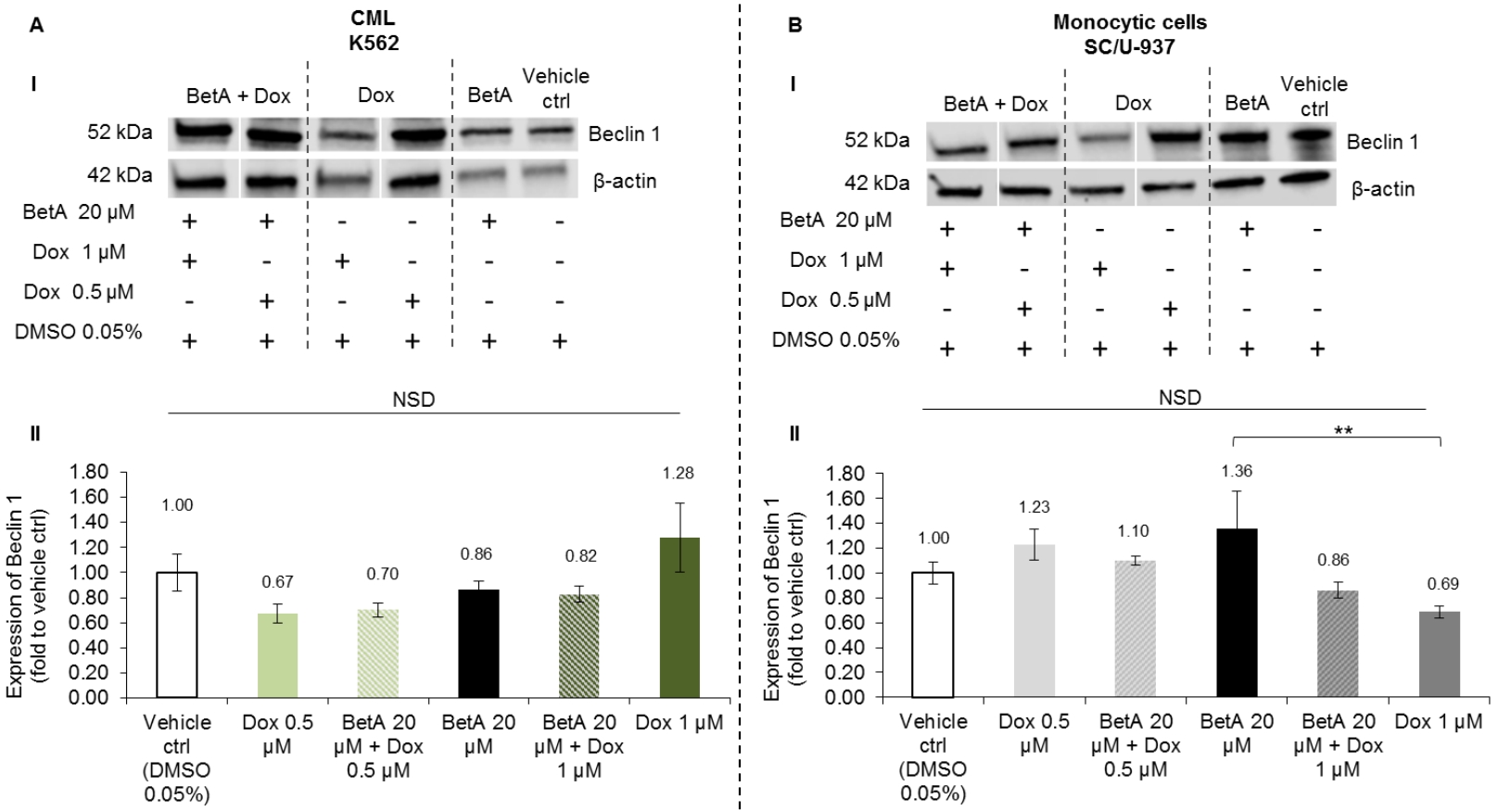


Figure 4.11 Expression of autophagy protein Beclin 1 in CML and SC/U-937 cell lines after single and combination drug treatment
Leukaemic cell line **(A)** K562 and **(B)** SC/U-937 cells were treated by single Dox or combination of BetA and Dox for 48 h. The protein expressions of Beclin 1 (52 kDa) **(I)** were estimated by Western blot. Experimental samples were run on the same gel and shown protein expressions are on the same blot for each protein antibody. Beclin 1 protein level **(II)** was determined by normalising quantified protein intensity relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and β-actin (housekeeping protein). Result were expressed as mean ± SE, n=3. The data set was analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant.

➔ The difference in the graph layout and sample arrangement on the Western blot was to facilitate a clear display of statistical comparison

4.2.9 Combination treatment altered mRNA expression of Bcl-2 family members and autophagy towards cell death in MOLM-13 cells, but survival in SC/U-937 cells

The Central dogma of biology tightly links molecular species of DNA, RNA and protein. Alteration in gene level could potentially be associated with the protein expression change. Therefore, the gene transcript (mRNA) was quantified to further examine the mechanism of action and the pathways involved in cell death induction by the drugs.

Dox and BetA alone marginally but not significantly ($p > 0.05$) increased the mRNA expression of pro-apoptotic BAK and BAX in MOLM-13 cells compared to vehicle control. Combination treatments further increased the mRNA of pro-apoptotic proteins when compared to the control, but only the higher combination (BetA 20 µM + Dox 1 µM) significantly upregulated both Bak (2.7-fold increase, $p < 0.05$) and BAX (6.2-fold increase, $p < 0.01$). Despite combinations showing higher upregulation of BAK and BAX mRNA levels, the effect was not significantly different when compared to the relative single Dox treatment (Fig. 4.12 A)

Anti-apoptotic BCL-XL mRNA expression was not statistically changed by any of the tested treatments in MOLM-13 cells, there was no significant difference ($p > 0.05$) between treatments and the vehicle control. Conversely, the anti-apoptotic mRNA BCL-2 expression was downregulated by the drug treatments. Single Dox treatments dose-dependently reduced ($p < 0.05$) BCL-2 expression by more than half in MOLM-13 cells, while BetA alone also showed on average decline (0.62-fold relative to vehicle control) but with no significance. Although both combination co-treatments with MOLM-13 cells reduced (on average) the BCL-2 level, it was significant ($p < 0.05$) only with BetA 20 µM and Dox 0.5 µM combination. In addition, there was no difference in the mRNA expression between single drugs and combination treatments (Fig. 4.12 B).

The band expression of autophagy genes ATG5 and BECLIN 1 was clearly detectable in MOLM-13 cells treated by the vehicle control and single BetA treatment. BetA 20 µM alone did not change the regulation of ATG5 and BECLIN 1 genes in MOLM-13 cells, from vehicle control expression. Conversely, ATG5 and BECLIN 1 mRNA expression in MOLM-13 cells were markedly downregulated to almost undetectable levels by single Dox treatments and combination treatments compared to vehicle control levels (Fig. 4.12 C).

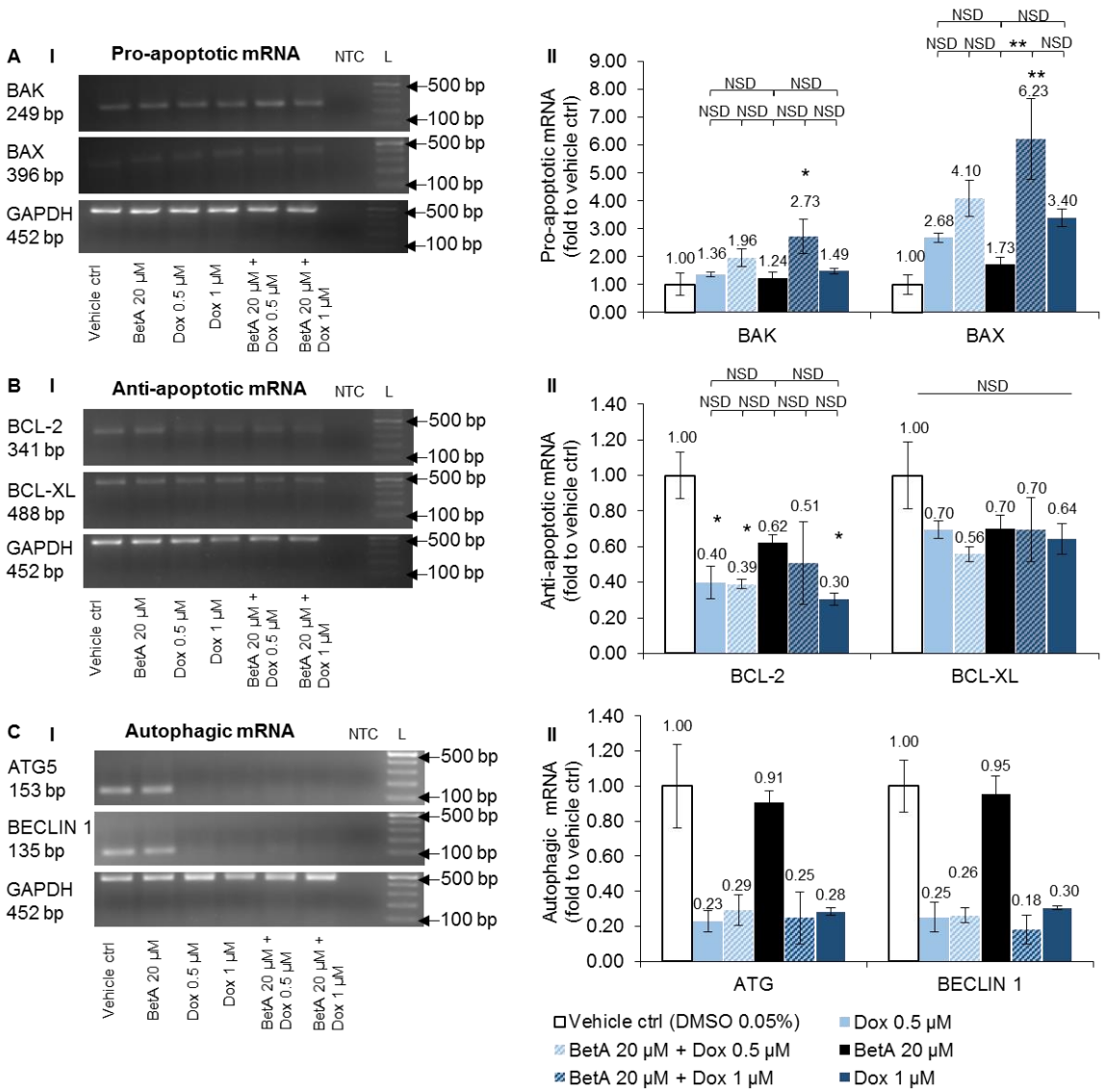


Figure 4.12 Expression of apoptotic Bcl-2 family members and autophagy genes in MOLM-13 cell lines co-treatment with single and combination drugs after 48 h

RT-PCR gene expression detection by gel electrophoresis **(I)** of MOLM-13 treated cells were quantified **(II)** using ImageJ software. The mRNA expression of pro-apoptotic **(A)** BAK and BAX (n=3), anti-apoptotic **(B)** BCL-2 and BCL-XL (n=3), and autophagy **(C)** ATG5 and BECLIN 1 (n=2) is relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and GAPDH (housekeeping gene). NTC-non-template control. L-molecular ladder PCRBIO Ladder IV (100-1500 bp). Result were expressed as mean ± SE. The data set was analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant.

➔ The difference in the graph layout and sample arrangement on the gel was to facilitate a clear display of statistical comparison

In SC/U-937 monocyte cells, no significant ($p > 0.05$) change in the expression of pro-apoptotic BAK mRNA was observed between the treatments and the vehicle control. The pro-apoptotic BAX was significantly ($p < 0.001$) upregulated in SC/U-937 by single Dox treatments, but single BetA 20 µM did not change ($p > 0.05$) the expression when compared to vehicle control (Fig. 4.13 A).

Interestingly, the effect of Dox treatments (0.5 µM and 1 µM) on BAX upregulation was mitigated to the expression level of vehicle control when combined with BetA 20 µM in SC/U-937 cell co-treatments. The difference in BAX mRNA between single Dox and their relevant BetA combination was very highly significant ($p < 0.001$) (Fig. 4.13 A II).

Anti-apoptotic BCL-2 mRNA expression was not detected in SC/U-937-treated cells when the same experimental conditions that were used for other genes and different cells were applied. Anti-apoptotic BCL-XL was dose-dependently downregulated in SC/U-937 monocytic cells by single Dox treatments with statistical significance ($p < 0.01$) at 1 µM. BCL-XL mRNA levels were not changed in SC/U-937 cells treated by BetA 20 µM alone when compared to vehicle-treated cells. However, BetA 20 µM upregulated BCL-XL expression of SC/U-937 cells when co-treated with Dox 1 µM. This combination treatment hindered the significant downregulating effect of Dox 1 µM and significantly increased ($p > 0.001$) regulation of BCL-XL mRNA level Fig. 4.13 B).

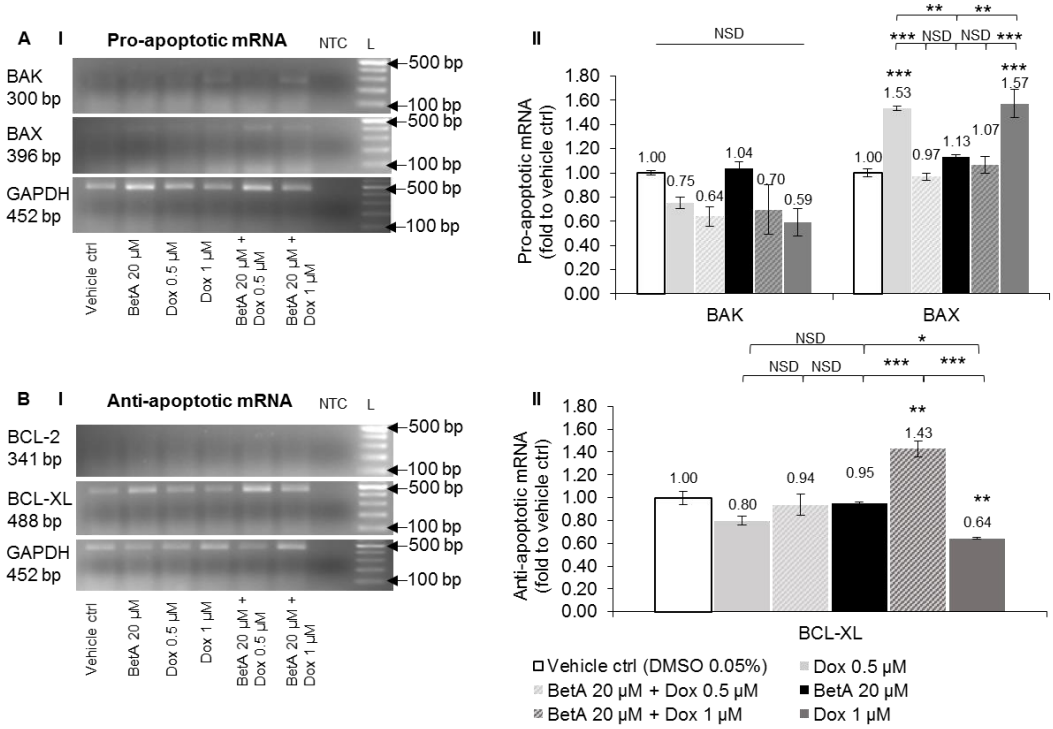


Figure 4.13 Expression of pro- and anti-apoptotic Bcl-2 family member genes in SC/U-937 cells after co-treatment with single and combination drug for 48 h

Gene expression by gel electrophoresis **(I)** of SC/U-937 treated cells quantified **(II)** using ImageJ software. The mRNA expression of pro-apoptotic **(A)** BAK and BAX (n=3) and ant-apoptotic **(B)** BCL-2 and BCL-XL (n=3) is relative to vehicle control (DMSO 0.05%, representing as 1.00 fold) and ratio between normalised data and GAPDH (housekeeping gene). NTC-non-template control. L-molecular ladder PCRBIO Ladder IV (100-1500 bp). Result were expressed as mean ± SE. The data set was analysed by One-Way ANOVA; Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), $*p \leq 0.05$ significant, $**p \leq 0.01$ highly significant, $***p \leq 0.001$ very highly significant.

➔ The difference in the graph layout and sample arrangement on the gel was to facilitate a clear display of statistical comparison

4.3 Discussion

The heterogenous nature of acute myeloid leukaemia (AML) has hindered some aspects of the prognostic improvement of the disease (Erba, 2007). Although standard chemotherapy drugs such as doxorubicin (Dox) can potently eliminate AML cells, Dox exhibited certain selectivity toward leukaemic monocytic cells (Chapter 3; Section 3.2.2). There are still reports of Dox-induced toxicity on non-transformed/non-malignant cell lines (Zuco *et al.,* 2002). In addition, AML is prone to relapse, producing more refractory cells that may develop resistance to chemotherapy and avoid apoptosis (Ding *et al.,* 2012). For instance, a sign of resisting mechanism was observed in AML cell line MOLM-13 when exposed to Dox treatment (Chapter 3; Section 3.3.5).

Effective treatment for AML should be able to target the leukaemic cell by inducing cell death while the toxicity to healthy cells should be minimal. The Bcl-2 family of proteins mainly regulates apoptotic cell death and the anti-apoptotic member Bcl-2 has been reported to be upregulated in AML cells (Zinkel *et al.,* 2006; Bensi *et al.,* 1995; Campos *et al.,* 1993) with the BCL-2 gene being upregulated in 95% of AML cases at relapse (Moon *et al.,* 2009). Bcl-2 protein can affect the autophagy process, which is a potential alternative cell death pathway (Shimizu *et al.,* 2004). Targeting the Bcl-2 in AML cells can possibly enhance chemosensitivity and may be a valuable mechanism in fighting refractory cancer cells.

In this study, the effect of betulinic acid (BetA) was investigated as a single treatment or in combination with established AML drug doxorubicin in inducing apoptotic cell death in acute myeloid leukaemia cell line MOLM-13 (relapsed cells derived from MLL-rearrangement). The cytotoxic effect of BetA and Dox was determined in myeloid leukaemic cell lines (chronic: K562 and acute: MOLM-13 and SC/U-937) and non-cancerous cells (human embryonic kidney: HEK293T). Based on the cytotoxic results and literature review, a suitable concentration of BetA for combinational studies with Dox was established. The anti-proliferative effect of the compounds, singly and in combination, was investigated as well as their potential to raise reactive oxygen species generation (which is linked to causing cell death in cancer) in AML cell lines. Pro- and anti- apoptotic Bcl-2 family and autophagy proteins and genes involvement in apoptotic death and autophagy modulation by BetA, Dox, and their combination was examined.

4.3.1 Cytotoxic effect of betulinic acid on leukaemia cell lines

Antitumor potency of BetA was studied mostly in cancers of epithelial origin (Xu *et al.,* 2014; Fulda, 2008; Chintharlapalli *et al.,* 2007; Kessler *et al.,* 2007; Thurnher *et al.,* 2003; Zuco *et al.,* 2002; Fulda *et al.,* 1999; Fulda *et al.,* 1997) but some studies on blood cancer exist (Faujan

et al., 2010; Ehrhardt *et al.,* 2004; Fernandes *et al.,* 2003). In this study, BetA suppressed cell growth of leukaemia cell lines MOLM-13 and K562 in a dose- and time-dependent manner (Fig. 4.1 C & D), showing cytotoxic effects on both acute and chronic myeloid leukaemia cell types. This is in agreement with studies carried out by Fernandes *et al*., (2003) and Gopal *et al*., (2005) that also experimented on the same K562 cell line and other papers investigating HL-60 (human promyelocytic leukaemia cells), another type of myeloid cell line, (Hata *et al.,* 2003) where BetA similarly demonstrated reduction of cell viability in these cancer cells. Thus, BetA demonstrated the capability to suppress cell viability of several myeloid leukaemic cells. However, this effect was not seen in SC/U-937 cells (Fig. 4.1 B), thus BetA displayed some selectivity between leukaemic monocytic cells as well.

In reported papers, BetA induced IC_{50} at 32 µM and 27.4 µM in leukaemic HL-60 and K562 cell lines at 48 h, respectively (Faujan *et al.,* 2010; Gopal *et al.,* 2005). In parallel to this study, MOLM-13 inhibited cell growth with IC_{50} at 24.3 µM and K562 at 18.5 µM after 48 h treatments based on the cell viability of cytotoxic assay that measured the nucleic acid content of live cells (Table 4.1). These concentrations are relatively higher than a clinical concentration of established AML chemotherapy drugs such as doxorubicin as expected concentration of doxorubicin in the patient's plasma within 2 h is 0.1-1 µM (McHowat *et al.,* 2001). BetA seems to have a lower potency than doxorubicin. However, it is suspected that the two compounds utilise different mechanisms in inducing cell death. This may also determine the selectivity of BetA against cancer cell lines. Due to the scarcity of reports on the effect of the drug on relapsed AML cells, MOLM-13 cells were selected for onward investigations.

4.3.2 Cell inhibition by betulinic acid is selectively cytotoxic to cancer cells, but also showed selectivity between leukaemic monocytic cells

Doxorubicin (Dox), but not BetA significantly suppressed the viability of non-cancerous cells. The potent cytotoxic effect of the drug was documented in normal healthy cells and is linked to cardiotoxicity (Takemura and Fujiwara, 2007; Minotti *et al.,* 2004). This study tested Dox clinical concentrations (0.1-1 µM) as well as a supraclinical concentration (5 µM) which significantly decreased the cell growth of both blood and those of epithelial origin and did not discriminate between leukaemic or non-cancerous cells, thus supporting this phenomenon. On the other hand, BetA-tested concentrations did not suppress cell viability of HEK293T (non-cancerous cells) for up to tested 72 h (Fig. 4.1 A). In line with this study, Zuco *et al*., (2002) demonstrated that Dox induced toxicity on variety of tumour cells (IC_{50} 0.026-0.602 µM) which was similar to non-cancerous peripheral blood lymphoblast cells (PBL; IC_{50} 0.037 µM) and dermal fibroblasts (IC_{50} 0.699 µM), whereas BetA toxic effect in PBL was much less (1000-fold) compared to Dox.

Studies have been done comparing BetA cytotoxic effects between cancer cells and their non-cancer cell counterparts. Experiments comparing human primary melanocytic cells and melanoma showed that normal cells were less susceptible to BetA (Selzer *et al.,* 2000). In another study, BetA demonstrated minimal cytotoxic effect in normal colon cells while potently inhibiting the growth of colorectal carcinoma (HCT 116) cells in a dose-dependent manner (Aisha *et al.,* 2012). Relevant to this study, in normal blood cells BetA tested up to a high dose over 110 µM on PBL (Zuco *et al.,* 2002) and 66 µM on peripheral blood mononuclear cells (Faujan *et al.,* 2010) did not affect or suppress cell growth.

Interestingly, evidence from the literature indicates that BetA seems to be targeting mainly cancer cell lines but the mechanism of this selective toxicity remains to be determined. Furthermore, this study reports BetA selectivity between leukaemic monocytes where cell viability of AML MOLM-13 (relapsed cell model) were significantly inhibited by BetA, but no inhibitory effect was observed in SC/U-937 cells.

4.3.3 The effect of drug combination on cell viability, proliferation and apoptosis induction in AML cell line MOLM-13

AML treatment plan usually involves combination drug therapy and the management of the disease still heavily relies on intensive chemotherapy (Dombret and Gardin, 2016). There is a need for more efficient drug combination with the standard drugs. However, it must be established whether the compounds can work synergisticly and their mechanism of action has to be determined.

There are several reports of BetA combination with other forms of therapy that showed some anti-cancer potential. For instance, BetA had shown to have an additive effect in combination treatment with irradiation therapy in melanoma cells (Selzer *et al.,* 2000). Studies co-treating BetA with other phytochemicals such as α-Mangostin (Aisha *et al.,* 2012) or ginsenoside Rh2 (Li *et al.,* 2011) showed enhanced cytotoxicity and apoptotic death in cancer cell lines of epithelial origin. Another study combined BetA with TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) and demonstrated enhanced apoptotic activity in various cell lines (neuroblastoma, medulloblastoma, glioblastoma and melanoma), but did not affect normal human fibroblasts, showing exclusive effectiveness in tumour cells (Fulda *et al.,* 2004). Consequently, it was feasible to integrate the compound with a chemotherapy agent.

BetA 20 µM was selected as suitable concentration for combination studies since BetA 10 µg/ml (22 µM) was non toxic in human cells *in vitro* studies (Viji *et al.,* 2010; Ehrhardt *et al.,* 2004) and is relative to 100 mg/kg administration which was non-toxic in *in vivo* studies (Pisha *et al.,* 1995). In addition, BetA 20 µM was a compromised IC_{50} value at 48 h between K562

and MOLM-13 cell lines (Table 4.1). Dox 0.5 μM and 1 μM were selected concentrations for combination with BetA 20 μM. The Dox concentrations chosen were within a 0.1-1 μM range which is equivalent to clinical concentrations (McHowat *et al.,* 2001).

Fulda & Debatin, (2005) indicated that BetA enhances the drug treatment activity by sensitising tumour cells. The authors rationalized that distinct compounds in combination therapy amplify weaker death signals in apoptosis, which leads to a potential strategy to enhance induction of cell death and overcome drug-resistance. However, it must be taken into consideration that anthracylines can block transcriptional induction and thus may potentially block the pro-apoptotic Bcl-2 activation stimulated by other therapies. For instance, Dox was shown to be able to rescue cancer cell lines from cell-induced death by bortezomib and vorinostat (Wei *et al.,* 2012). Therefore, it is essential to assess multiple drug effect interaction (by combination index (CI)) of the combination therapy that utilise anthracyline class of drugs to prevent antagonistic action between the compounds. In this study, BetA (20 μM) and Dox (1 μM) combination reduced cell viability of AML (MOLM-13) more than the single Dox treatment (Fig. 4.2 A II), which was statistically different (by the comparison of variances using 2 sample T-test; $p < 0.05$), and the interaction between the compounds was synergistic (CI<1) in suppressing cell viability (Table 4.2).

The cytotoxicity assay revealed that BetA appeared to cooperate well with Dox and therefore may potentially enhance Dox-induced reduction of clonogenic activity in leukaemic MOLM-13 cell lines. Although BetA (20 μM) singly did not show a great anti-proliferative trend, assessed by CFSE staining, the compound in combination with Dox did not negatively affect drug-induced cell growth suppression of MOLM-13 cells (Fig. 4.4). However, based on the live-dead staining method for cell death differentiation using Annexin V/PI, combination treatments significantly shifted the cell death population ratio by inducing more late apoptotic death compare to the single Dox treatment in MOLM-13 cells (Fig 4.6 A). Apoptotic cell marker phosphatidylserine (PS), identified by Annexin V staining, exists in the inner plasma membrane but will be exposed on the outer surface to signal for phagocytosis upon apoptosis. Although cells are being signalled to be cleared and are in process of dying, anastasis (cellular recovery from death) can take place even when PS is activated (Tang and Tang, 2018). Cells in both early and late apoptosis express PS but late apoptotic cells, unlike cells in early apoptosis, have a compromised cell membrane (Poon *et al.,* 2010; Patel *et al.,* 2006). Loss of membrane integrity will expose other intracellular molecules, which can further promote the signalling effect and facilitate cell clearance (Poon *et al.,* 2010). Therefore, cells in late apoptosis are more prone to clearance and anastasis is less likely to occur in these cells.

In this study, death induction by BetA and Dox drug combination showed more cells in late

apoptosis compared to the single drugs, indicating BetA enhancing Dox-induced cell death of MOLM-13 cells. This is in accordance with another study where BetA was combined with Dox as well as other anticancer drugs such as taxol, cysplatin, VP16, actinomycin D; showing increased apoptosis in meduloblastoma, glioblastoma and melanoma tumour cell lines (Fulda and Debatin, 2005). Other combination treatments of BetA and chemotherapy drugs (5-fluorouracil, irinotecan, oxaliplatin) were studied in colon cancer cell lines SNU-C5 and their drug resistant variants. The drug with BetA combination treatments showed cytotoxicity in 5-fluorouracil (BetA + irinotecan or oxaliplatin) and oxaliplatin (BetA + 5-fluorouracil, irinotecan or oxaliplatin) resistant SNU-C5 cells, demonstrating BetA tumour chemosensitising capability in bypassing drug resistance in combination studies (Jung *et al.,* 2007).

4.3.4 Betulinic acid hindered cytotoxic effects of doxorubicin in SC/U-937 monocytic cells

Although drug combinations could be a potential strategy to enhance cell death, this toxic effect should not extend to non-cancerous cells. A report by Selzer *et al*., (2000) suggested that due to BetA cancer cell specificity the supplementation of BetA in combination therapy may be more potent in cancer without enhancing the side-effects in normal cells. In fact, BetA tested from 0.3-22 µM (*in vivo*) showed no genotoxicity and was able to reduce chromosomal damage by mutagenic agent methyl methanesulfonate in fibroblast cells (Acésio *et al.,* 2016). Thus, the compound exhibits some protective mechanisms in certain cells.

This study demonstrated that the combination treatment of BetA (20 µM) with Dox (0.5 or 1 µM), significantly suppressed cell viability of leukaemic MOLM-13 cell lines but not the SC/U-937 cells (Fig. 4.2 B), demonstrating selectivity of BetA towards certain types of leukaemic monocytes/myeloid cells. Since HEK293T cell viability was also not affected by BetA, there is a possibility that the non-cytotoxic selectivity of the combination treatment could extend to non-cancerous cells. However, this warrants further investigations. Nevertheless, there are several studies where combination of BetA with chemotherapeutic drugs appeared to be less toxic towards non-cancerous cells. A study done *in vitro* and *in vivo* by Sawada *et al*., (2004) combined BetA with chemotherapy drug vincristine on a murine melanoma cell line. The study showed induced cell cycle arrest and apoptosis *in vitro* and inhibition of metastasis to lungs *in vivo* by the combination therapy. In addition, the combined BetA and vincristine treatment showed side effects of negligible magnitude *in vivo*, suggesting suitable treatment regime (Sawada *et al.,* 2004). Another *in vivo* study combined non-toxic doses of BetA (10 mg/kg) with mythramycin A (0.05 mg/kg) which suppressed antitumour activity (proliferation, invasion and angiogenesis) of pancreatic cancer cell lines without any systemic side effect (Gao *et al.,* 2011).

It should be noted that single Dox treatments at clinically relevant concentrations (0.5 and 1 μM) did not significantly inhibit cell growth of SC/U-937 cells at 24 h, based on cell viability measurements (Fig. 4.2 B), thus showing some degree of Dox selectivity. However, the viability measurement also showed that combination treatments non-significantly increased (20% on average, $p > 0.05$) the cell viability when compared to the single Dox. This possible trend in viability stimulation by BetA was further confirmed by experiments assessing cell death population (using Annexin V/PI double staining) in co-treated SC/U-937 cells with Dox and BetA, which revealed that Dox-induced apoptotic cell death at 48 h was hindered by BetA combination and increased the population of viable cells (Fig. 4.6 B). Therefore, it was evident that the combination treatment worked effectively in decreasing MOLM-13 cell viability and enhanced apoptotic death while not affecting monocytic SC/U-937 cells and even hampering the effect of Dox-induced apoptosis. The selective effect of the combination treatment between the leukaemic monocytic cells may provide some insight on cell targeting. Thus, studies on cell death mechanism of action should further proceed to determine the difference between the molecular pathway inductions in a wider panel of cell lines.

4.3.5 Betulinic acid enhanced anticancer drug activity of doxorubicin by sensitising the cancer cell lines to apoptosis and ROS formation

A study by Ehrhardt *et al*., (2004) demonstrated that BetA was capable of killing AML cancer cells through apoptosis. When investigating cell death differentiation by Annexin V/PI double staining, BetA at 20 μM concentration as a single treatment did not significantly induce apoptotic cell death in MOLM-13 cells that was different from the control (vehicle control) treatment. However, BetA augmented Dox-induced apoptotic death in the combination treatments through increase of late apoptotic population in MOLM-13 cells (Fig. 4.6 A), which is more irreversible cell death (Poon *et al.,* 2010). A similar study by Fulda & Debatin, (Fulda and Debatin, 2005) experimented on neuroblastoma cells and tested co-treatments of BetA (6.6, 8.8 or 10.9 μM) and Dox (0.05, 0.2 or 0.4 μM) which triggered cell death in a dose- and time-dependent manner. The authors determined the induced-cell death to be apoptotic based on the presence of DNA fragmentation, elevation of Smac (mitochondria-derived caspase activator), Cytochrome *c* release from mitochondria and activation of caspase-8, -3 and poly-ADP-ribose polymerase (PARP) (Fulda and Debatin, 2005).

PARP family of proteins respond to DNA damage and are involved in maintaining genomic stability (Chou *et al.,* 2010). Cellular insults such as an increase in ROS formation have been reported to activate PARP as ROS is known to cause oxidative DNA lesions (Ganguly *et al.,* 2007). ROS are by-products of normal cellular metabolism and in small amounts are necessary for maintaining cellular homeostasis (Reczek and Chandel, 2017). However,

cancer cells are known to have elevated ROS levels, which contribute to its tumorigenesis (Galadari *et al.,* 2017). Therefore, an altered redox environment in cancer cells makes them more sensitive to ROS/redox manipulation (Galadari *et al.,* 2017; Reczek and Chandel, 2017). Thus, to determine if ROS generation contributes to cell death induced in MOLM-13 by the drugs and the combination treatments, DCFDA fluorogenic dye was used to identify ROS product formation in cells after exposure to the drugs.

In this study, single BetA (20 µM) and clinical concentrations of Dox (0.5 and 1 µM), as single drugs, had no significant effect on ROS production in MOLM-13. On the other hand, higher Dox (5 µM) significantly increased ROS (Fig. 4.7). This is in contrast to other reports where BetA was capable of ROS stimulation in other cancer cell lines, which the authors linked to the compounds ability to induce cellular death associated with mitochondrial membrane permabilisation (Xu *et al.,* 2017; Liu and Luo, 2012; Fulda and Kroemer, 2009; Fulda, 2008; Gopal *et al.,* 2005; Ehrhardt *et al.,* 2004; Liu *et al.,* 2004; Tan *et al.,* 2003; Fulda *et al.,* 1997). Disproportional formation of ROS in cancer cells is linked to mitochondrial membrane deregulation; this mitochondrial damage causes several events leading to apoptosis (Ganguly *et al.,* 2007). An increase in ROS is also associated with other modes of cell death pathways (Orrenius *et al.,* 2007).

Dox is a known ROS inducer. However only supraclinical concentrations are capable to show this effect (Gewirtz, 1999; Fornari *et al.,* 1994) which is in line with this study. Interestingly, BetA (20 µM) and low Dox concentrations (0.5 µM and 1 µM), which alone did not alter ROS levels in MOLM-13, together significantly elevated ROS stimulation in MOLM-13 cell lines, showing potential mutual collaboration in the mechanism of action (Fig. 4.7). BetA could have sensitized the cells to enhance Dox-induced cell death by shifting ROS balance in the cells. According to a study by Acésio *et al*., (2016), BetA and Dox co-treatments have shown to potentiate DNA damage. This leads to an increase in programmed cell death activation as shown in this study where BetA and Dox together stimulated ROS formation. This effect possibly led to the rise in apoptotic death of MOLM-13 cells, where combination treatment increased the late apoptotic population (Fig. 4.6 A). Interestingly, single Dox at low concentrations (0.5 µM and 1 µM) independent of ROS elevation (Fig. 4.7) still showed some cell death by apoptosis in MOLM-13 (Fig. 4.6 A). Therefore, a different or multiple mechanism of action in cell death induction may be suspected between the single and the combination treatments. The mechanical pathways affected by Dox treatment in MOLM-13 cells has been elucidated (Chapter 3). Further investigations have been conducted to determine the regulatory mechanism contributing to cell death induced by the combination treatment of BetA and Dox in MOLM-13 cells.

4.3.6 Bcl-2 protein family regulation by the combination treatment in apoptotic cell death

Overall, the anti-apoptotic and pro-apoptotic profile of Bcl-2 family of proteins in the cells are essential in the induction of intrinsic apoptotic pathways by regulating the permeability of the mitochondria membrane. A shift in their balance within the cells can activate downstream caspases, the key effectors of apoptosis (Nikoletopoulou *et al.,* 2013; Adams and Cory, 2007).

The regulation of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins were not affected by the single BetA (20 µM) treatments on MOLM-13 cells (Fig. 4.8 B & C) in this study. On the contrary, a study on chronic (CML) type showed Bax protein up-regulation in K562 by BetA treatment (Qiuling *et al.,* 2010). From the literature, the involvement of Bcl-2 family is not conclusive in BetA induced cell death. A study in the late 90's tested the regulation of some Bcl-2 protein family by BetA in neuroblastoma cells and showed up-regulation of pro-apoptotic members Bax and Bcl-Xs but no alteration in anti-apoptotic Bcl-2 and Bcl-XL (Fulda *et al.,* 1997). Similarly, the Bcl-2 and Mcl-1, another anti-apoptotic protein, levels were unaffected by BetA in tongue squamous carcinoma cell lines (Thurnher *et al.,* 2003). However, a later study on various other cancer cells showed that BetA significantly suppressed the Bcl-2 protein, which was also mirrored by downregulation of BCL-2 and upregulation of BAX gene levels (Rzeski *et al.,* 2006). In this study, the effect of BetA at 20 µM on Bcl-2 protein was constant across other tested cells, K562 and SC/U-937, where the compound did not significantly affect the protein regulation (Fig. 4.10).

Contradicting data showed that in human nasopharyngeal carcinoma cells (Liu and Luo, 2012) and in JURKAT leukaemia cells (Mullauer *et al.,* 2009) the BetA-induced cell death was Bax/Bak independent since BetA was able to kill Bax/Bak deficient cell variants. Furthermore, overexpression of Bcl-2 in JURKAT cells have shown to only delay apoptosis by BetA (Mullauer *et al.,* 2009). Interestingly, BetA-induced death in tongue squamous carcinoma cell lines showed to decrease pro-apoptotic protein Bax (Thurnher *et al.,* 2003). As suggested by the authors, this could be either an adaptive response or Bax was not directly involved in the cell death mechanism. Increase in pro-apoptotic Bax is associated with disruption of mitochondrial membrane and release of Cytochrome *c*, which would activate caspase cascade to induce apoptotic cell death (Su *et al.,* 2015). Cytochrome *c* protein expression was not changed upon BetA treatment in SC/U-937 cells (Fig. 4.10 B III), indicating that BetA does not have detrimental effect on these particular monocytic blood cells. However, BetA treatment did not affect Cytochrome *c* expression of K562 cells (Fig. 4.10 A III), although there is a report on BetA-induced apoptotic cell death in K562 (Qiuling *et al.,* 2010). Therefore, it is evident that BetA regulates and affects the cell death mechanism differently from cell to cell basis.

More studies with variable results on BetA regulated Bcl-2 protein family have been reported in cancers of epithelial origin. A study by Zhao *et al*., (2012) looked at the Bcl-2 family protein profile where the levels of both anti-apoptotic (Mcl-1, Bcl-2, Bcl-XL) and pro-apoptotic (Bax and Bim) proteins exhibited no major alteration by BetA treatment on ovarian cancer cells. However, the authors showed conformational change, translocation, and oligomerization of Bax by BetA treatment in these cells, demonstrating some involvement of the Bcl-2 family in BetA-induced death. A study on HeLa cells by Xu *et al*., (2017), showed that BetA did not change the regulation of anti-apoptotic Bcl-XL protein; however, pro-apoptotic Bad protein was increased. This was linked to AKT (protein involved in cell growth and survival pathway) inactivation and together with enhanced ROS formation up-regulated Bad protein, which caused a decrease in mitochondrial membrane potential, releasing Cytochrome *c* and activating caspase-9 that ultimately lead to apoptotic cell death (Xu *et al.,* 2017). A study carried out on prostate cancer cells showed that BetA induced apoptosis by enhancing Bax/Bcl-2 ratio, which was due to increasing Bax protein and decreasing Bcl-2 protein (Shankar *et al.,* 2017).

From the literature, several members of regulatory Bcl-2 protein family seems to be involved in the mechanism of action of BetA but the effect varies and is cell-dependent. The Bcl-2 regulation of Bcl-2 and Bax proteins was not observed in MOLM-13 cells when treated solely by BetA (20 µM).

On the other hand, the tested Dox concentrations (0.5 and 1 µM) as single treatments elevated the protein expression of Bax (compared to vehicle control) and statistically decreased truncated Bcl-2 isoform (p15-20-Bcl-2) in MOLM-13 cell lines (Fig. 4.8). Studies have shown that pro-apoptotic molecules such as Bax, Bak (Pilco-Ferreto and Calaf, 2016; Panaretakis *et al.,* 2002) and Bid (Bien *et al.,* 2010) proteins are upregulated and activated upon Dox treatments in cancer cell lines. Anti-apoptotic Bcl-2 protein reduction by Dox has been reported in HeLa cells (Bien *et al.,* 2010) and breast cancer cells (Pilco-Ferreto and Calaf, 2016). In relation to gene expression, mRNA levels of anti-apoptotic molecules MCL-1, BCL-2, and BCL-XL have been shown to be repressed by Dox in several cancer cell lines (Inoue-Yamauchi *et al.,* 2017; Wei *et al.,* 2012). However, Dox did not significantly affect the anti-apoptotic Bcl-2 protein expression in leukaemic K562 and SC/U-937 cells (Fig. 4.10), unlike in MOLM-13 cells. Therefore, it is proposed that other Bcl-2 members may be involved or death-induction is through Bcl-2 family-independent mechanism in these cells. This study on MOLM-13 cells and other reported literature on various cancer cells showed that Dox might activate apoptosis by regulating the Bcl-2 family in cancer cells. Nevertheless, drug effects on different Bcl-2 protein family members may not be inclusive to all cells.

In this study, treatments of BetA in combination with Dox marginally ($p > 0.05$) increased pro-apoptotic Bax protein expression in MOLM-13 cells (Fig. 5.8 C). Meanwhile, the combination treatments did not alter the 'usual' anti-apoptotic p26-Bcl-2-α with any significance but significantly suppressed the protein isoform p15-20-Bcl-2 (Fig. 4.8 B). However, the effect on protein expression between the drug combinations was not significantly different compared to equivalent single Dox treatments. Thus, BetA did not enhance the regulation of Bcl-2 apoptotic protein family but also did not negatively affect the Dox-induced effect in MOLM-13 cells. Interestingly, neither combination treatment nor Dox alone modified the expression of Bcl-2 or Cytochrome *c* proteins in SC/U-937 or K562 cells (Fig. 4.10), further indicating some selectivity in mechanism of action by the drugs.

Furthermore, data in this study showed that the Bax/Bcl-2 ratio in MOLM-13 AML cell lines was elevated from the control when exposed to single Dox or combination treatments. The increase in the ratio was much more prevalent in the novel Bcl-2 isoform p15-20-Bcl-2 (Fig. 4.8 D). An increase in Bax/Bcl-2 ratio suggests that cells are more prone to undergo cell death associated with apoptosis (Raisova *et al.,* 2001). The ratio of Bax/Bcl-2 was only weekly different between the single Dox and combination treatments. Therefore, there may be other underlying mechanism, not associated with Bcl-2 family regulation, that contributes to the enhanced cell death induction by the combination treatment. Nevertheless, apoptosis induced by the drugs showed a greater inhibitory effect on the novel p15-20-Bcl-2 protein that was exclusively present in MOLM-13 cell lines. This interesting finding may propose some specificity in targeting the MOLM-13 cancer cells of relapsed origin.

4.3.7 Autophagic Beclin 1 protein regulation upon exposure to betulinic acid and doxorubicin drug combination

The relationship between apoptotic death and autophagy has been elucidated in the past but there are some inconsistencies regarding the role of autophagy. Autophagy has been described to initiate both cell survival/protection and cell death mechanism (Radogna *et al.,* 2015; Ouyang *et al.,* 2012). Nevertheless, it was proposed that modulating this cellular process could be useful in fighting cancerous cells (Nikoletopoulou *et al.,* 2013; Marquez and Xu, 2012). This cellular process is tightly linked to apoptosis through Beclin 1:Bcl-2 complex regulation (Marquez and Xu, 2012). Studies have showed that Beclin 1, a marker and an initiator of autophagy, is being regulated by Dox treatment through upregulation (Smuder *et al.,* 2011) or downregulation (Schott *et al.,* 2018; Pizarro *et al.,* 2016). In MOLM-13 cells, Dox decreased the expression of Beclin 1 protein parallel to cell death induction (Chapter 3; Section 3.3.3). However, no significant change in Beclin 1 expression was observed in K562 and SC/U-937 cells when treated with Dox (Fig. 4.11). Therefore, modulatory effect of Dox on Beclin 1 protein expression appears to be context and cell-dependent.

On the other hand, BetA (20 µM) concentration tested in this study did not show any considerable effect on Beclin 1 regulation in leukaemic MOLM-13, K562 and SC/U-937 cells, which was also reflected in Dox combination treatments (Fig. 4.11). However, a study by Yang *et al*., (2012) demonstrated a decrease of Beclin 1 protein using higher concentrations of BetA (32.8 µM and above), which also inhibited autophagic flux and induced apoptosis in myeloma cell line, KM3. Nevertheless, Beclin 1 downregulation by Dox in MOLM-13 cells was not significantly affected by passive BetA effect in combination treatments (Fig. 4.9 B)

Increases in autophagy markers and subsequent autophagy activation is sometimes expected with chemotherapy. The exact reason is not clearly defined but it could be a cell response to drug-induced stress. Therefore, if autophagy acts as a survival response against chemotherapy drugs, cancer cells can potentially utilise the process for pro-survival and tumorigenic function (Kimmelman, 2011). In this study, treatments (except single BetA) significantly reduced the autophagy marker Beclin 1 protein in AML cell line MOLM-13, which could indicate reduction in autophagy. The exact role of autophagy in these cells is not defined, although decrease of autophagy marker Beclin 1 was observed alongside significant decline of anti-apoptotic Bcl-2 (p15-20-Bcl-2) and a slight increase in pro-apoptotic Bax protein (Fig. 4.8), suggesting cell death associated with mitochondrial apoptotic pathway.

Beclin 1 is negatively regulated by anti-apoptotic Bcl-2 protein and is activated by dissociating from the Beclin 1:Bcl-2 complex (Nikoletopoulou *et al.,* 2013; Marquez and Xu, 2012). The ratio between autophagic Beclin 1 and anti-apoptotic Bcl-2 determines sensitivity to autophagy stimuli (Gao, Yuan *et al.,* 2018; Hseu *et al.,* 2017). Thus, an increase in the ratio denotes autophagy activation (Hseu *et al.,* 2017). In this study, Beclin 1/Bcl-2 ratio was decreased in MOLM-13 cells by the treatments (single Dox and combination drugs) (Fig. 4.9 C), suggesting inhibition of autophagy process but the drop in the ratio was more prominent with the usual p26-Bcl-2-α and only negligibly altered with p15-20-Bcl-2. Although, Bcl-2 protein regulates Beclin 1 activity, a study by Ciechomska *et al*., (2009) demonstrated inability of Beclin 1 to inhibit Bcl-2 anti-apoptotic function. Thus, the binding of Beclin 1 to Bcl-2 would not affect the anti-apoptotic activity of Bcl-2 but Bcl-2 can suppress the autophagic function of Beclin 1. However, Bcl-2 (p15-20-Bcl-2) protein decline was accompanied with decline in Beclin 1 expression in this study. It is yet to be evaluated whether reduction of autophagy initiator supported the induction of apoptotic death in MOLM-13 cell line.

4.3.8 Change in expression of mRNA level complemented the protein level regulation by the treatment

The basic understanding of molecular information transfer from gene to protein would imply that the mRNA level corresponds to the protein amount as mRNA transcript is eventually

translated into amino acids and subsequently forming a protein (Maier *et al.,* 2009). Thus, this study quantified the mRNA expression of apoptotic and autophagy genes to illuminate on the mechanical action of the drugs in regulating cell death.

The Bcl-2 family of proteins have a well-established role in regulating apoptosis and are encoded by various genes from the Bcl-2 gene family (Shamas-Din *et al.,* 2013). Altered expression of genes encoding for various Bcl-2 family proteins have been documented in many human cancers including leukaemia (Yip and Reed, 2008). Thus, transcriptional regulation of the Bcl-2 family gene profile was further investigated. BetA and Dox 1 µM combination treatment significantly upregulated the mRNA expression of pro-apoptotic BAK and BAX in MOLM-13 cells (Fig. 4.12 A). In regards to the anti-apoptotic genes, the treatments did not significantly change BCL-XL mRNA regulation but significantly suppressed BCL-2 mRNA level (Fig. 4.12 B). This drug-induced change in gene regulation closely resembled the protein profile of Bax and Bcl-2 of treated MOLM-13 cells, but the significance in BAX mRNA by combination treatment was not observed at protein level.

BetA selectivity towards cancer cells was reported in this study and by others from the literature (Section 4.3.2). Although BetA singly did not affect Bcl-2 family mRNA expression, combination treatment defended SC/U-937 cells from apoptotic death by inhibiting ($p < 0.001$) Dox-induced pro-apoptotic BAX upregulation (Fig. 4.13 A) and increased (BetA + Dox 1 µM; $p < 0.01$) anti-apoptotic BCL-XL mRNA level which was significantly suppressed ($p < 0.01$) by Dox (1 µM) (Fig. 4.13 B). However, the effect was not evident when assessing SC/U-937 cells' Bcl-2 protein regulation, which was not detectible at mRNA level (Fig. 4.13 B I). Nevertheless, the difference in the regulation between MOLM-13 and SC/U-937 monocytic cells has been shown. Thus, the drug combination presented some targeting potential. Based on the gene regulation studies, the selective effect of BetA was shown to exert protective mechanisms towards SC/U-937 monocytic cells when co-treated with Dox, which was associated with Bcl-2 family gene regulation. This is in line with live-dead staining investigation, where Dox-induced apoptotic death in SC/U-937 cells was alleviated with BetA combinations (Fig. 4.6).

ATG5 and BECN1/ATG6 (BECLIN 1) genes encodes for Atg5 and Beclin 1 proteins, respectively. In this study, expression of ATG5 and BECLIN 1 was only clearly detected in MOLM-13 treated by vehicle control and single BetA (20 µM) (Fig. 4.12 C), thus signifying strong downregulation of autophagy by Dox and combination treatments, which was parallel to the protein investigation. Both ATG5 and BECLIN 1 transcript expressions represents key proteins involved in induction of autophagy. Atg5 protein forms a complex with other Atg proteins (ATG12-ATG5:ATG16) that elongates the autophagosome vesicle and conjugate LC3-I to LC3-II (Pyo *et al.,* 2013). Unlike LC3-I, LC3-II binds to the scaffold of autophagosome,

making it a good protein marker and reliable indicator of autophagy (Tanida *et al.,* 2008). Beclin 1 protein is an autophagy promoter, inactive when in a complex with anti-apoptotic protein Bcl-2 (Pattingre *et al.,* 2005). Thus, mRNA upregulation would imply increase in the protein level and possible activation of the autophagy pathway.

Some similarity between protein and mRNA transcript expressions was observed in this study, which may further reinforce the mechanism of action induced by the treatments where stimulation of apoptosis was conveyed with autophagy reduction in MOLM-13 cells. Interestingly, pro-apoptotic effect of BetA combined with Dox was selective towards MOLM-13 cells but relieved SC/U-937 cells from cytotoxic effect of Dox. Both mRNA and protein measurements are considered fundamental to understand cell behaviour and their changes should be complementary (Greenbaum *et al.,* 2003). However, weak or lack of correlation between mRNA and protein exists (Maier *et al.,* 2009; Greenbaum *et al.,* 2003). Although some studies showed general correlation between the mRNA and protein level, the correlation strength varies among genes (Guo, Yanfang *et al.,* 2008). For instance, study by Gygi *et al*., (1999) reported that the mRNA expression could be accompanied by variation of more than 20-fold difference of protein abundance. Various biological and technical factors will influence the correlation such as post-translational protein modification and imminent noise/errors in quantifying mRNA and proteins that limits accuracy of the measurement (Guo *et al.,* 2015; Maier *et al.,* 2009; Greenbaum *et al.,* 2003). Nevertheless, the proteins are a direct executor of life processes and their interactions are the causative force of action within the cell (Guo *et al.,* 2008; Greenbaum *et al.,* 2003). Thus, the mRNA level is only informative and not predictive of protein abundance (Guo *et al.,* 2008). However, the complementary regulation of mRNA and protein, shown in this study, is still valuable in comprehending the mechanical pathway.

Chapter 5

Overall discussion and future work

5 Overall discussion and future work

5.1 Mechanism of action of doxorubicin in MOLM-13 cell death induction

MOLM-13 cells, an AML model of relapsed MLL-rearranged cells, were sensitive to Doxorubicin (Dox)-induced death and inhibition of cell proliferation. The apoptotic death was associated with selective inhibition of a novel Bcl-2 isoform (p15-20-Bcl-2) that was exclusively present in MOLM-13 cells. Interestingly, the pro-apoptotic Cytochrome *c* protein of MOLM-13 was depleted upon Dox treatment. In addition, Dox reduced expression of some cleaved forms of caspses-8 and -9 in these cells. Nevertheless, there was a significant shift in the ratio of the cleaved caspase-9 forms, which could indicate caspase cascade regulation by Dox. The aberrant mechanism of apoptotic protein regulation warrants for examination. However, the distinct proteomic profile (Bcl-2 and caspase-9) of MOLM-13 from other leukaemic cells could be associated with this outcome. The effect of Dox in reducing Beclin 1 protein expression could indicate a modulation of autophagy in MOLM-13 cells but it is not clear whether this confers a protective or cell-death inducing effect. The pro-survival pathway of MOLM-13 cells was inhibited by Dox which was associated with reduction of the PI3K regulatory subunit p85α (p85/p55-60) and active AKT (phosphorylated T308) proteins, conferring Dox cytotoxicity. However, the negative regulator of this pathway, PTEN, was also reduced in its (non-phosphorylated) active form in MOLM-13 when exposed to Dox treatments. This could be a response of MOLM-13 to the cytotoxic drug and may imply development of resistance mechanism against Dox (Fig. 5.1)

In this study, apoptotic cell death was observed in MOLM-13 cells when co-incubated with Dox, despite dose-dependent reduction in caspases. Functional loss of caspases is one of the possible signs of cancer cell developing chemoresistance (Liu *et al.,* 2009; Horita *et al.,* 2008; Sebens Mueerkoester *et al.,* 2006; Devarajan *et al.,* 2002). However, inhibition of caspase-8 could also indicate stimulation of an alternative (non-apoptotic) cell death mechanism. Necrosis-like programmed cell death, necroptosis, is propagated by caspase-8 inhibition. This halts the active caspase from cleaving Receptor-Interacting serine/threonine-Protein Kinases (RIPK1 and RIPK3) (Tait *et al.,* 2014). RIPK1 and RIPK3 are key modulators of necroptotic cell death and when activated the RIPK protein kinases trans-phosphorylate and recruit other signalling proteins to execute necroptosis (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). A Study by Horita *et al*., (2008) demonstrated a possibility of inducing two types of programmed cell death in AML cell line by certain toxins, regulating both caspase-dependent apoptosis and RIPK-dependent necroptosis. It would be interesting to explore the possibility of an alternative necroptotic pathway involvement in Dox-cell death induction in future studies.

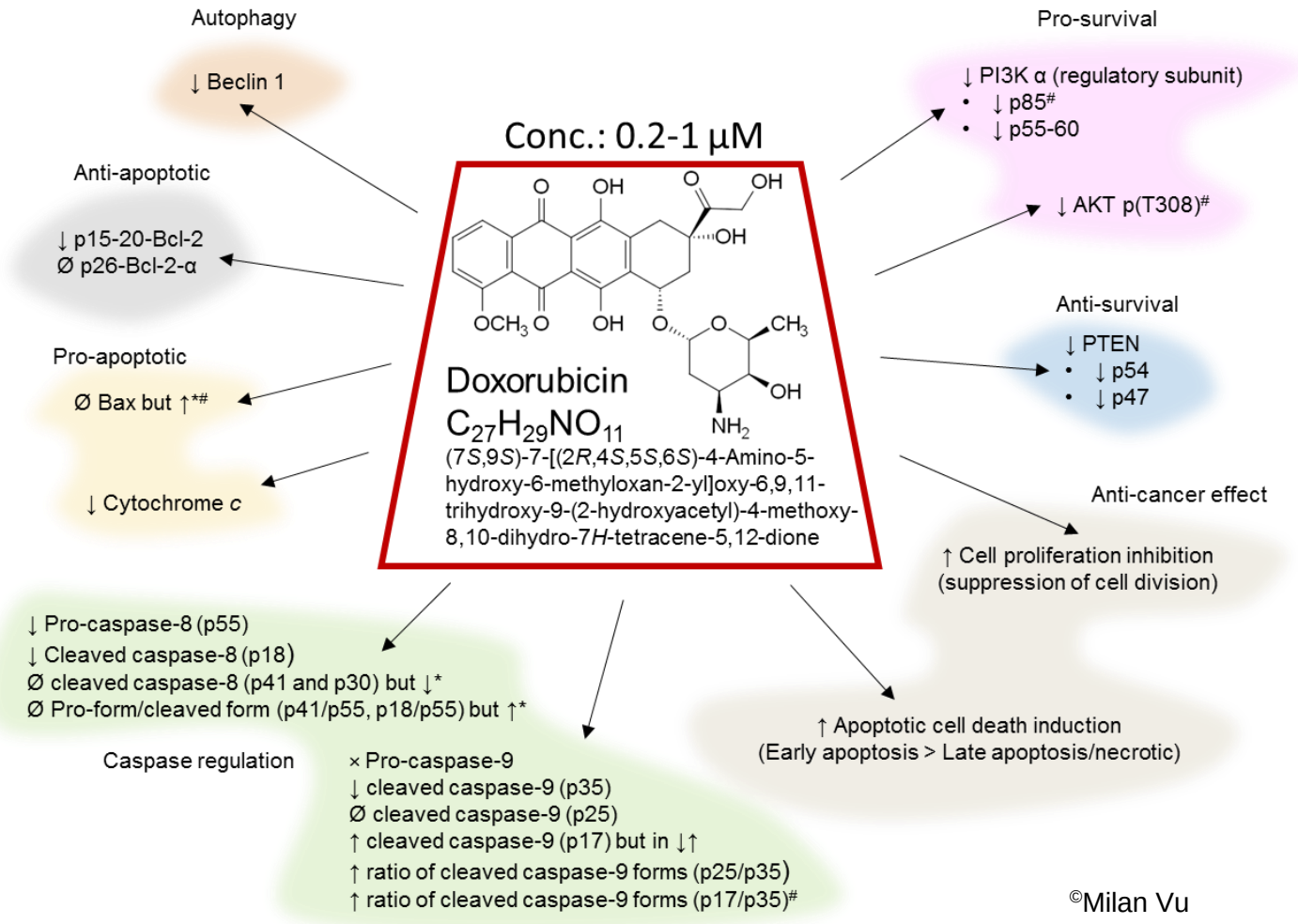


Figure 5.1 Summary of the effect of doxorubicin and its mechanism of action at clinically relevant concentrations in MOLM-13 cell lines

The diagram is adapted from the resultsdescribed in Chapter 4. (↑) increase, (↓) decrease, (Ø) no significant change, (↑↓) inverse proportion, (*) non-statistical effect, (#) compared to vehicle control but not untreated cells, (×) not detectable.

5.2 Betulinic acid in combination with doxorubicin modulated a cell death mechanism that was cell type dependent

Betulinic acid (BetA) treatments showed selective cytotoxic effect towards myeloid leukaemia MOLM-13 and K562 but did not suppress cell viability of non-cancerous HEK293T. Although the compound displayed cytotoxicity in leukaemic MOLM-13, AML monocytes from a relapsed disease, this effect was not seen in other leukaemic/monocytic cell line SC/U-937 cells. Thus, BetA tumour cytotoxicity is extended to some but not all leukaemic cells. Interestingly, when BetA was combined with Dox, a synergistic reduction in cell viability and enhanced cell death associated with an increase in late apoptotic population was observed in MOLM-13 cells. In addition, ROS formation in MOLM-13 was enhanced by the BetA and Dox combination treatments, which is associated with mitochondrial (intrinsic) apoptotic pathway cell death. Although enhanced cytotoxicity in MOLM-13 cells was shown by the combination treatment, Dox anti-proliferative effect and protein regulation of Bcl-2 family and Beclin 1 was not markedly affected by BetA combination. However, increase in ROS generation by the combination treatment could be a mechanical factor that enhanced cell death, resulting in drug synergism in MOLM-13 cells. Therefore, despite no apparent observation in the difference of the Bcl-2 family and Beclin 1 protein regulation between single Dox and its combination with BetA, treatments of Dox and BetA together could have utilised high ROS activity in affecting the mitochondrial integrity, which led to an increase in cell death mechanism by more cells undergoing late apoptosis. The trend in mRNA and protein expressions were similar, although drug combinations on average increased ($p > 0.05$) pro-apoptotic BAK and BAX mRNA levels more compared to single drugs and the combination treatment with higher Dox concentration (BetA 20 μM + Dox 1 μM) statistically enhanced these pro-apoptotic transcripts (Fig. 5.2 A).

On the contrary, BetA in combination treatment hindered Dox effect in leukaemic/monocytic cell line SC/U-937. Thus, the drug combination exhibited selective cell death as well as cell-dependent protective effects. In addition, the treatments did not affect the proteins in K562 or SC/U-937 cells as opposed to MOLM-13-treated cell where the proteins were evidently modulated. Therefore, the mechanism of action of the compounds (single and combined) was cell type dependent. Moreover, mRNA level of the Bcl-2 family showed pro-survival regulation of SC/U-937 cells when treated by the drug combination. BetA co-treatment with Dox upregulated anti-apoptotic BCL-XL and downregulated pro-apoptotic BAX, combating the effect of Dox and further demonstrated salvage of monocytic SC/U-937 cells by BetA from cell death by Dox (Fig. 5.2 B)

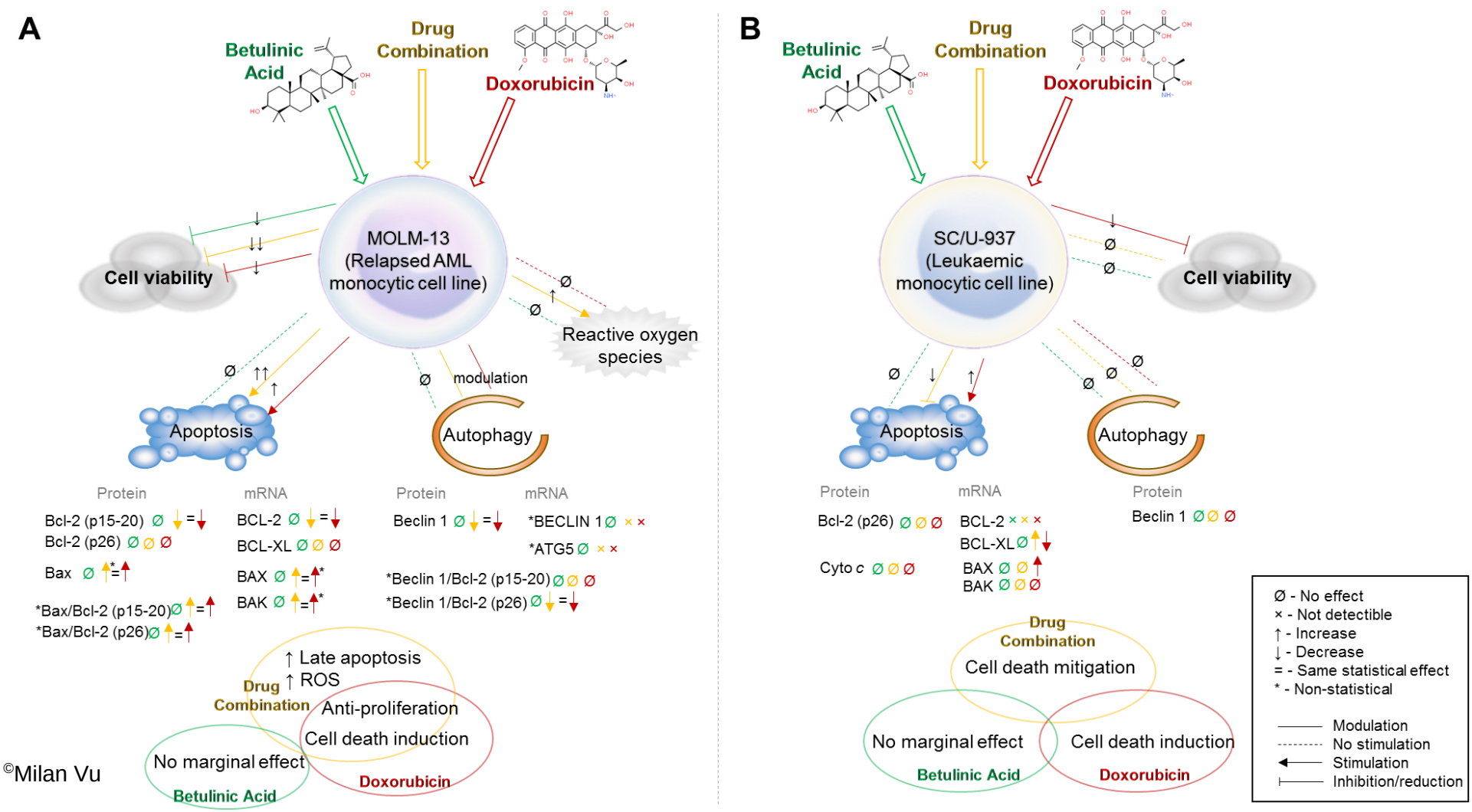


Figure 5.2 Summary of the mechanism of action of single and combination drugs in AML monocytic cell lines
The effect of the single and combination treatments on MOLM-13 cell lines (**A**) and SC/U-937 (**B**). Diagram adapted from the result outcome described in Chapter 4. Effect of sole BetA (Green), effect of sole Dox (Red), effect of BetA and Dox combination treatment (Yellow).

It is apparent that BetA, in combination with Dox selectively reinforced cell death in MOLM-13 but this does not seem to involve enhancement of Dox regulation of Bcl-2 family. Therefore, further investigations are required to identify the exact mechanism that sensitize the cells and facilitates the cell death in MOLM-13. Moreover, there appears to be a paradoxical effect where SC/U-937 cells were being protected by the combination treatment from Dox induced death. SC cells were non-leukaemic blood monocytes, which are suspected to be contaminated with leukaemic U-937 cells. This suspicion has not been formally verified through testing.

Some other compounds such as arsenic are employed in targeting tumours by inducing apoptosis as chemotherapy but exposure to this compound is also associated with development of cancer (Bode and Dong, 2002). In fact, cell death pathways are not linearly defined and the programmed cell death mechanism is extremely dependent on the cell type and the stimuli (Soengas and Lowe, 2003). The context within the cell may play a role in the outcome of drug targeting. For instance, it has been documented that some antioxidants can impede or promote tumorigenesis (Mendelsohn and Larrick, 2014). Although enhancement of ROS formation can induce cell death, ROS can also cause DNA damage, which may contribute to cancer initiation or progression (Galadari *et al.,* 2017). Therefore, depending on the context, protective effect of antioxidants may hinder ROS stimulated cell death or reduce ROS mediated cell transformation (Mendelsohn and Larrick, 2014).

Antioxidant ability to scavenge ROS has been reported for BetA, which inhibited apoptosis in some non-cancerous cells (Yi *et al.,* 2016; Yi *et al.,* 2014; Zheng *et al.,* 2011). However, it has been documented from several studies that BetA utilise ROS formation in cell death mechanism against various cancer cells (Xu *et al.,* 2017; Chintharlapalli *et al.,* 2007; Liu *et al.,* 2004; Tan *et al.,* 2003; Fulda *et al.,* 1997). The switch in the mechanism between different cell types is not fully understood but the cellular environment could be involved. ROS levels are usually upregulated in cancer cells with an increased abundance of antioxidant enzymes when compared to non-cancerous cells (Galadari *et al.,* 2017). Therefore, manipulation of the redox system in the cancer could differently affect the cells based on the ROS context. Tested BetA concentration alone did not increase the ROS level in MOLM-13 cells but when combined with Dox at non-stimulatory ROS concentrations, ROS levels were significantly stimulated at an early stage of treatment incubation. There is evidently a selective mechanism that targets MOLM-13 cells. However, BetA capability to enhance MOLM-13 death by Dox as well as mitigate Dox-induced death in SC/U-937 cells remains to be evaluated.

5.3 Targeting novel anti-apoptotic Bcl-2 protein isoform in MOLM-13 cell lines

Apoptosis regulation by Bcl-2 family of proteins is often deregulated in cancer cells where pro-apoptotic (Bax, Bak, Bad, BH3 and t-Bid) molecules are downregulated and anti-apoptotic (Bcl-2, Bcl-X_L, and Mcl-1) molecules are upregulated, allowing cancer cells to evade apoptosis (Hu & Xuan 2007). It was reported that up to 80% of cellular Bak is oligomerised and bound to anti-apoptotic Bcl-2 in unstimulated AML cells (Dai *et al.,* 2015). In fact, AML cells have been documented to have an upregulated level of anti-apoptotic Bcl-2, which contributes to their oncogenesis (Zinkel *et al.,* 2006; Bensi *et al.,* 1995). To confirm this phenomenon, this study compared the constitutive Bcl-2 protein levels by Western blot analysis between unstimulated cells (Chapter 3, Section 3.2.4). In agreement to the literature, Bcl-2 protein levels were significantly more upregulated in AML cell line OCI-AML2 over the CML cell line K562. The AML cell lines of interest MOLM-13 expressed similar Bcl-2 level to SC/U-937 cells and the protein bands were clearly detectable as opposed to K562 cells. Interestingly, MOLM-13 cells in this study expressed two Bcl-2 protein isoforms, an expected p26-Bcl-2-α and a novel p15-20-Bcl-2 that was not present in other cells including SC/U-937 cells.

Both cell survival and death regulation by the apoptotic Bcl-2 family of proteins is activated through phosphorylation. This depends on the type of protein member, phosphorylated location and other post-translational changes, which can be enhanced or inhibited by various stimuli. However, post-transcriptional changes such as alternative splicing can result in several versions of the same protein, thus creating distinct isoforms, which may also affect the characteristic and function of the molecule (Akgul *et al.,* 2004). Several alternative protein variants or isoforms of the Bcl-2 family members had been identified. Two main Bcl-2 proteins encoded by the BCL-2 gene are documented, p26-Bcl-2-α and p22-Bcl-2-β (Fig 5.3), which are mainly distinguished by their carboxyl tails difference (Guillem *et al.,* 2015; Tanaka *et al.,* 1993).

Bcl-2 α, the 26 kDa protein size form, is the standard protein variant and is quantitatively more abundant in cells. The protein has 239 amino acid residues, its subcellular localisation is in mitochondria, and can target mitochondrial membrane by its hydrophobic transmembrane domain (stretch of 17 hydrophobic amnion acids). On the other hand, Bcl-2 β, a truncated version of 22 kDa size, is rarely reported in cells. This shortened protein has 205 amino acid residues, resides mainly in the cytoplasm, and lacks a hydrophobic transmembrane domain, which is essential for membrane stimulation (Guillem *et al.,* 2015; Tanaka *et al.,* 1993). In terms of mRNA levels, three transcripts for BCL-2 exist; BCL-2 α1 and α2 which encodes for the same protein (Bcl-2 α) and BCL-2 β encoding for truncated protein (Bcl-2 β) without the transmembrane region due to retaining some introns instead of exons (Ghassemifar *et al.,*

2012). Therefore, the anti-apoptotic activity is always attributed to the Bcl-2 α form (Guillem *et al.,* 2015; Akgul *et al.,* 2004).

This study showed that MOLM-13 cell lines express two distinct Bcl-2 protein variants of different sizes (p26-Bcl-2-α and p15-20-Bcl-2). A study carried out on AML cross-resistance using the same MOLM-13 cell line showed that the Bcl-2 protein shifted and migrated to approximately 19 kDa in azacytidine (cancerostatic agent) resistant MOLM-13 cells, indicating possibility of a structural difference of the Bcl-2 protein in the cells (Messingerova *et al.,* 2015). The authors did not report further on the protein alteration. In addition, there was no presence of the standard p26-Bcl-2-α (Messingerova *et al.,* 2015). However, proteomic diversity of the anti-apoptotic Bcl-2 in MOLM-13 cells seen in this study may contribute to the tumorigenicity of the cells, which could be used as a target for drugs.

Elevated or overexpressed levels of Bcl-2 have been found to protect cancer cells from apoptosis (Hu and Xuan, 2007). In general, the apoptosis inhibitory properties were credited to the main isoform, an 'α' form of 26 kDa size (p26-Bcl-2-α), which can regulate mitochondrial permeability due to its hydrophobic transmembrane region (Guillem *et al.,* 2015; Akgul *et al.,* 2004). The other established Bcl-2 form, p22-Bcl-2-β, was described as functionless and lacking any capability to prolong survival or apoptosis in the cells (Akgul *et al.,* 2004; Tanaka *et al.,* 1993). However, another study showed that the Bcl-2 β form was capable of enhancing the tumourigenicity in fibroblast NIH-3T3 cells (Reed *et al.,* 1988). A later study by Schinkothe *et al*., (2006) demonstrated that the p22-Bcl-2-β has a linkage function between intrinsic and extrinsic apoptotic pathways (by binding to cytoplasmic Bid) and may play a role in hematopoietic malignancies. Furthermore, the authors observed that overexpression of p22-Bcl-2-β in the cells displayed chemo-resistance (Schinkothe *et al.,* 2006). The gene expression of BCL-2 β isoform levels was found to be higher in chronic lymphocytic leukaemia patients compared to healthy patients, although gene expression of the α form remained dominant (Ghassemifar *et al.,* 2012). Few other studies have documented different Bcl-2 isoforms that were present in cancer cells. For instance, the Bcl-2 isoform at 21 kDa was expressed alongside the p26-Bcl-2-α in thyroid tumours (Manetto *et al.,* 1997). Huang *et al*., (2003) found in invasive prostate cancer another truncated isoform of Bcl-2, which was named Bcl-2 Ψ (psi). Bcl-2 Ψ isoform prevented pro-apoptotic Bax and Bid molecules from dimerisation since the protein lacked BH-3 domain, which protected the cancerous cells from apoptotic death (Huang *et al.,* 2003). Therefore, other Bcl-2 isoforms beside the usual p26-Bcl-2-α could carry some cell survival and tumorigenic functions.

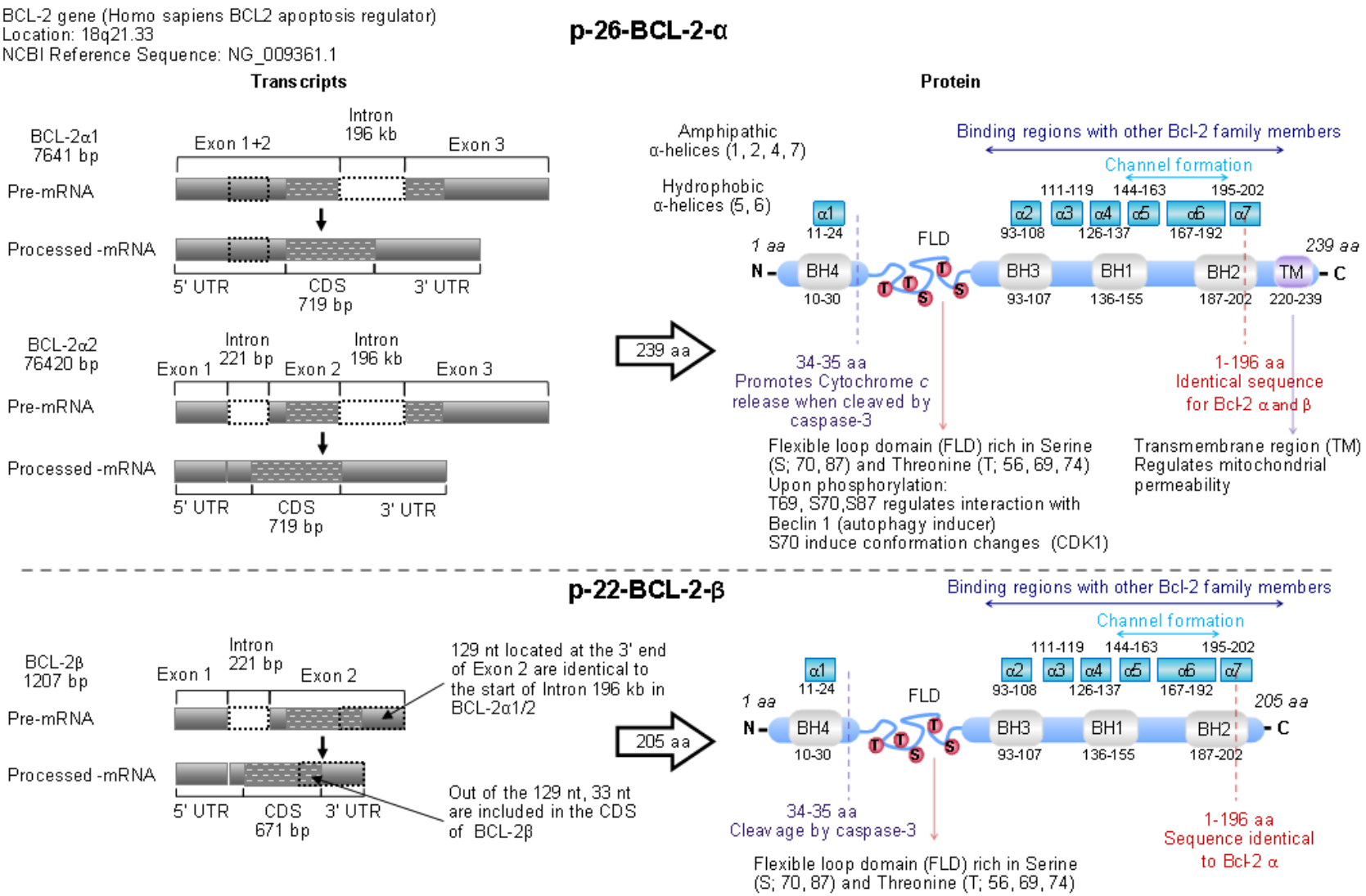


Figure 5.3 Schematic representation of p26-Bcl-2-α and p22-Bcl-2-β protein and their mRNA transcripts
Diagram adapted from Warren *et al*., (2019); Mohamed *et al*., (2018); Correia *et al*., (2015); Ghassemifar *et al*., (2012)
UTR – Untranslated region, CDS – Coding sequence, bp (base pari), kb (kilobases; 1000 bp), aa (amino acids), nt (nucleotides)

Understanding these alternative Bcl-2 isoforms specifically present in cancer cells may be useful for selective cell targeting leading to cell eradication as well as modulation of several cell pathways. The protein ratio between anti-apoptotic Bcl-2 and pro-apoptotic Bax shifted when MOLM-13 cells were exposed to Dox treatment (Chapters 3 & 4) or drug combination (Chapter 4). Changes in this ratio is indicative of whether cells are more likely to undergo apoptosis or resist death induction since Bcl-2 can prevent Bax from downstream initiation of apoptosis (Raisova *et al.,* 2001). While Bcl-2 protein was downregulated at p15-20-Bcl-2 isoform, the regulation of Bax was only marginally upregulated by the treatments. Interestingly, the subtle increase in pro-apoptotic Bax protein was only seen when compared against the vehicle control, which showed statistical ($p < 0.05$) increase at Dox 1 μM (Fig. 4.8). This still resulted in overall enhancement of Bax/Bcl-2 ratio (at both Bcl-2 isoforms), which is associated with induction of apoptosis. Since activated Bax migrates from the cytosolic space to mitochondrial membrane (Shamas-Din *et al.,* 2013), other investigative work on Bax localisation could provide a better understanding of its activation and involvement in cell death induced by the compounds.

Since Bcl-2 can regulate autophagy through inhibition of Beclin 1 activity by a complex formation (Marquez and Xu, 2012), further studies on the role of distinct Bcl-2 isoform variants could provide some additional insight on apoptosis-autophagy interaction. The novel Bcl-2 isoform p15-20-Bcl-2 exclusive to MOLM-13 in this study was sensitive to treatments while p26-Bcl-2-α, that was present in all cell lines, remained relatively intact by the drugs. Reduction in anti-apoptotic p15-20-Bcl-2 was corresponding to apoptotic death induced by Dox and combination treatment in MOLM-13, which was also parallel to decline of Beclin 1 autophagy protein marker. Decrease in Beclin 1 resulted in Beclin 1/Bcl-2 ratio being markedly reduced at p26-Bcl-2-α, which could indicate reduction in autophagy (Gao *et al.,* 2018; Hseu *et al.,* 2017). However, the autophagic decline in the ratio was not seen with p15-20-Bcl-2. Thus, depending on the Bcl-2 isoform, apoptosis induction and autophagy modulation mechanism was depicted in MOLM-13 cell lines. Better clarity on the modulation of cell death mechanism in the cross talk between apoptosis and autophagy is required. Nevertheless, the novel Bcl-2 isoform p15-20-Bcl-2 was inhibited by the drugs and although it is not conclusive whether this led to the cell death, SC/U-937 cells that lack this isoform were less sensitive to the drugs. Thus, it is worth further investigation to define its role as a potential selective target for cell death induction.

5.4 Potential of betulinic acid to selectively sensitise relapsed/refractory cancer cells to chemotherapy treatment

Although selected BetA concentration for combination studies did not significantly enhance suppression of the p15-20-Bcl-2 or Beclin 1 inhibition by Dox, the combination treatment in

general exhibited greater cell death in MOLM-13 cells. Thus, BetA showed potential in sensitising MOLM-13 cells to Dox chemotherapy drug. It is important to note that relapsed cells (such as represented by MOLM-13) are usually considered to be more aggressive with additional mutations that makes them refractory (Ding *et al.,* 2012). In this study, BetA as a single treatment showed cytotoxicity towards MOLM-13 and inhibited viability of the cells, thus demonstrating efficacy in combating cells of relapsed origin.

BetA effectiveness against drug resistance cells was reported in the literature. A study by Ehrhardt *et al*., (2004) investigated cross-resistance between BetA and cytotoxic drugs, which was established on drug-resistant T-cell leukaemia (JURKAT) cell line. No cross-resistance was observed since BetA-induced apoptosis did not differ in drug-resistant and non-resistant cells. An early study by Fulda *et al*., (1997) showed that BetA was more responsive in apoptosis induction of Dox and anti-CD95 resistant neuroblastoma cells compared to Dox, demonstrating the potential to bypass the cancer mechanism of Dox resistance in cells. In fact, BetA has been reported to be effective against several cancer cell lines resistant to conventional antitumor drugs (Fernandes *et al.,* 2003; Zuco *et al.,* 2002). Furthermore, BetA was effective against cancer cell lines with induced chemoresistance such as in cisplatin-resistant ovarian cancer cells (Zhao *et al.,* 2012) and 5-fluorouracil and oxaliplatin resistant colon cancer cells (Jung *et al.,* 2007). Ehrhardt *et al*., (2004) compared BetA to ten cytotoxic agents used in current treatment protocol such as Dox, dexamethasone, cytarabine and asparaginase on primary leukaemia cells, and 9/10 tested drugs were less potent in apoptosis induction (in median) then BetA. Moreover, the study showed that BetA was the most effective in cases of relapse when compared with those cytotoxic drugs (Ehrhardt *et al.,* 2004). However, it should be noted that the authors used plasma concentrations of the drugs, which were comparatively lower than the tested BetA concentration, which does not have formally established plasma concentration.

BetA seems to possess the ability to bypass cancer drug resistance as well as affect relapsed cells which are considered to be more refractory, based on other reports. However, BetA cytotoxicity in this study was also highly selective as lack of cell viability reduction was observed in non-cancerous HEK293T and monocytic SC/U-937 cells. No selectivity towards non-malignant cells is a desirable effect for cancer treatment, yet BetA was non-selective towards leukaemic/monocytic SC/U-937 cells. Therefore, the compound performed much better against monocytic AML cell line of relapsed origin. Although, BetA selective enhancement of cell death is appreciated in Dox-treated MOLM-13 cells, its potential to protect other leukaemic monocytes raises some concerns. The dual effect of BetA could be further exploited for cell death modulation but the underlying mechanism of BetA to switch roles between different cell types needs to be addressed.

5.5 Further Studies

5.5.1 Investigating necroptotic cell death induction in MOLM-13

Certain stimuli can induce multiple programmed cell death, although one mechanism usually dominates the others (Su *et al.,* 2015). Necroptosis may be a possible option for future studies since the mechanism is usually triggered as a back-up to ensure cell death (Fulda, 2014; Ouyang *et al.,* 2012). Since caspases, in particular caspase-8, were depleted upon Dox exposure, necroptotic cell death could have taken place or supported apoptotic death. This would not be a first time when apoptosis and necroptosis were simultaneously stimulated in AML cell lines (Horita *et al.,* 2008). Thus, further studies could investigate possible involvement of other non-apoptotic programmed cell death pathways.

There is no reliable FACS staining method or morphological characteristic that would distinguish necrosis from necroptotic cells. However, necroptosis is a highly regulated event (Vanden Berghe *et al.,* 2010). The presence of this alternative programmed cell death pathway can be studied through expression of key markers of necroptosis molecules, investigating the mRNA transcript regulation and detection of proteins using antibodies for immunoblotting or immunostaining. Receptor-Interacting serine/threonine-Protein Kinases (RIPK), RIPK1 and RIPK3 are key regulators of necroptosis, making them excellent markers of the pathway (Wang *et al.,* 2017). These protein kinases are typically cleaved by activated caspase-8 but when caspase-8 is inactivated, RIPK1 and RIPK3 can trans-phosphorylate and recruit pro-necrosome protein (Li *et al.,* 2016; Nikoletopoulou *et al.,* 2013). Mixed lineage kinase domain-like protein (MLKL) is the 'executioner' protein of necroptosis due to its ability to translocate to cell membrane and disrupt its integrity upon activation (by phosphorylation to form oligomers) (Wang *et al.,* 2017). Thus, phosphorylated MLKL would be another useful marker for necroptosis detection.

5.5.2 Verifying cell pathway modulation using specific inhibitors of programmed cell death

In order to confirm that a programmed cell death is taking place, the said pathway should be blocked if a key regulator of the signalling mechanism is suppressed. This is to ensure that the mechanism of action initiated by the stimuli is dependent on that specific interaction in cell signalling transduction. Therefore, a method to validate intracellular changes in response to stimuli can be evaluated by an addition of a specific inhibitor. Inhibitors that can block imperative molecules in a specific signalling transduction pathway provides information on cell response as well as determining the sequence of the events or possible interference of another mechanism (Hartford Svoboda and Reenstra, 2002).

In this study, MOLM-13 cells died upon Dox exposure regardless of caspase upregulation. Caspase dependency is not essential for cell death induction, although activation of caspases may be considered as a major predictor defying apoptotic death (Kroemer *et al.,* 2005). Regardless, the network cascade activation involves many types of caspases and not all have been explored in this study. In addition, some regulatory effect was shown since the ratio of cleaved caspase-9 markedly shifted under Dox stimulation. Therefore, broad-spectrum (pan) caspase inhibitors could serve as a tool to identify caspase-dependent vs caspase-independent death (Chauvier *et al.,* 2007). There are many examples of inhibitors capable of caspase suppression, ranging from viral, cellular to chemical agents. Synthetic caspase inhibitors, which have been widely used in cell death studies, are designed to react with the catalytic cysteine of caspases (cysteine proteases), and thus can be very specific to a caspase or target a wider range of caspases. An example of the latter (synthetic pan-caspase inhibitor) is N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), which is a tri-peptide that suppresses all the major caspases (Callus and Vaux, 2007). Inclusion of inhibitor such as z-VAD-fmk in treatments for future studies could underline cell death induction by apoptotic mechanism and its caspase dependency in MOLM-13 cells.

Autophagy modulation associated with decline in Beclin 1 was observed by Dox in MOLM-13 cells but this does not conclusively confirm autophagic role in cell protection or cell-death mechanism. Autophagy is not easily measured or quantified since it is a dynamic process and the presence of the autophagosomes does not correlate or define autophagic activity (Pasquier, 2016). Autophagic flux, the degradative rate of autophagy, must be defined to properly assessed presence of the autophagy process (Yang *et al.,* 2013). Therefore, establishing the outcome of the mechanism upon inhibition of the autophagic flux can confer the role of autophagy in cell survival or cell death. The autophagic flux can be suppressed at any stage and most inhibitors target the initial stage of autophagy machinery or affect the late stage by targeting the lysosomal function (Pasquier, 2016). Already approved anti-rheumatoid and anti-malarial drugs, chloroquine and hydroxychloroquine, are clinically relevant inhibitors that are suitable for late stage inhibition of the autophagy flux by mediated lysosomal acidification that hinders activity of degradative enzymes (Pasquier, 2016; Yang *et al.,* 2013).

To establish whether necroptosis contributes to cell death, inhibition of this signalling pathway should be also evaluated. 5-(1H-indol-3-ylmethyl)-2-thiohydantoin, necrostatin-1 was proposed as an appropriate inhibitor of necroptotic signalling due to its specificity which does not affect other cellular pathways such as apoptosis or autophagy. Necrostatin-1 inhibits necroptotic cell death by interacting with the T-loop of RIPK1 and allosterically inhibits the kinase activity (Han *et al.,* 2009; Korkina *et al.,* 2008). The outcome of the inhibitor work would

further elucidate the necroptotic role in the signalling mechanism and possible interplay between other cell pathways.

Experiments with inhibitors can provide a better understanding of programmed cell death and signalling pathway interaction but there are several foreseen obstacles and limitations. For instance, pan-caspases do not inhibit systematically all caspases; they do not share the same efficiency or specificity toward each caspase; and they may inhibit other cysteine proteases apart from caspases (Chauvier *et al.,* 2007). Inhibitors may not exclusively suppress molecules that belong to a particular pathway and may affect other cellular processes (Pasquier, 2016). In fact, inhibitors at certain concentration can cause nonspecific toxic effect (Callus and Vaux, 2007). Therefore, the addition of an inhibitor dose that can repress a pathway but should not induce cytotoxicity needs thorough optimisation according to the experimental conditions and cell type to minimise confounding variables.

5.5.3 Further examination of the novel Bcl-2 variant p15-20-Bcl-2

In this study, the AML cell line, MOLM-13, expressed a Dox-regulated p15-20-Bcl-2 isoform and also the usual p26-Bcl-2-α isoform of which expression levels are unaffected. This novel p15-20-Bcl-2 isoform that was present in MOLM-13 cells but not in other tested cells has not been documented in the past and thus more accurate determination of this isoform is required. Proteomic and genomic studies may further validate this Bcl-2 variant.

A method to determine a protein could be done by a conventional two-dimensional electrophoresis using isoelectric focusing to separate the proteins on a sodium dodecyl sulfate- polyacrylamide gel followed by Coomassie staining, excision of the protein and enzymatic digestion, the peptides can be identified using time-of-flight mass spectrometry (Maier *et al.,* 2009; Greenbaum *et al.,* 2003). This method approach can detect only proteins that are expressed at relatively high levels with a long half-life. The detection is also limited to proteins that are neither too acidic nor too basic or hydrophobic. In addition, the method requires high level of expertise to obtain reproducibility and the protein size should be between 10-200 kDA for reliable separation on gel (Greenbaum *et al.,* 2003). Nevertheless, the low cost of this method approach and the potential to separate several samples on the same gel would provide direct comparison between the two Bcl-2 isoforms, which were clearly different in size.

Knockout, knockdown or silencing the BCl-2 gene that encodes for p15-20-Bcl-2 isoform could provide some insight on the role of the protein. Reduction of the gene expression and function could be achieved through BCL-2 targeted siRNA transfection (Inao *et al.,* 2018) and complete gene deletion using genetic manipulation such as CRISPR plasmids (O'Neill *et al.,* 2016).

However, it must be established whether p15-20-Bcl-2 is a result of post-transcription by alternative slicing or other possible processing such as post-translational modification. Thus, an approach to find the potential gene transcript may require to be mapped out through the protein product.

A wider panel of cell lines as well as primary cells should be investigated for Bcl-2 isoforms. To further evaluate the novel p15-20-Bcl-2 isoform, qPCR analysis with a different set of primers may be used to study the relative gene expression levels of BCL-2 in MOLM-13 and other cell lines. In addition, cloning and transfecting the p15-20-Bcl-2 into other suitable cell lines may be used to assess its sensitivity towards apoptosis. Protein purification could further help with characterisation of the structure, function and association with other proteins.

5.5.4 Defining the mechanism of betulinic acid in sensitising cells to chemotherapy

From the literature, BetA is being praised for its many potential pharmacological prospects (Ríos and Máñez, 2018). True to this claim, this study showed that BetA did not inhibit cell viability in non-cancerous cells (HEK293T) and induced cytotoxicity in some leukaemic cells, including cells of relapsed AML model MOLM-13, which were also more sensitive to Dox by BetA combination. Future studies could investigate whether this effect of drug sensitisation could reverse the signs of resistance development seen in MOLM-13 when treated by single Dox.

Although BetA and Dox combination selectively eradicate MOLM-13 cells, the only identified mechanism, which possibly enhanced the apoptotic effect of the drug, in this study was demonstrated by an increased in ROS generation at early incubation. Excessive production of ROS is linked to mitochondrial dysfunction, which is associated with the intrinsic apoptotic pathway (Ouyang *et al.,* 2012; Orrenius *et al.,* 2007) as well as necroptotic death as a form of bioenergetics crisis (Galluzzi and Kroemer, 2008) or in autophagy as a response to cellular stress (Martins *et al.,* 2015). Thus, future investigations could put more emphasis on mitochondrial permeability and dysfunction induced by the drug combination.

Several methods could assess mitochondrial function under stress and therefore compare effect of the drugs, singly and in combination. Measurement of mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial mass can be obtained through fluorescent probes using flow cytometry (Zorova *et al.,* 2018; Doherty and Perl, 2017) and mitochondrial morphology and fragmentation could be observed under confocal microscopy (Redmann *et al.,* 2018). These approaches could validate whether the enhancement of cell death was through increase of mitochondrial dysfunction in the cells. Furthermore, employment of specific

inhibitors of programmed cell death in the combination study could reveal any possible enhancement, interference or interaction between different cell pathways.

Although ROS was being enhanced by combination treatments, no statistical difference was shown to indicate that there was a modulation of apoptotic Bcl-2 family (Bax and Bcl-2) and autophagic Beclin 1 (BH3-containing) protein expressions between single Dox and combination treatment. Nevertheless, Dox was not negatively affected by BetA in the drug combination, although synergism and enhanced cell death was reported based on cell viability and cell death population, respectively. Bcl-2 protein family regulates cell death through manipulating the outer mitochondrial membrane integrity, while ROS activity can affect the fragile redox environment in cancer cells and thus can distress mitochondria directly (Galadari *et al.,* 2017). Therefore, ROS could be a mechanism that was independent from Bcl-2 family of protein regulation in affecting the mitochondria. Although BetA alone did not directly affect ROS regulation, the compound could have primed the cells to make them susceptible to ROS, thus sensitising cells to Dox activity.

It should be noted that detection of the protein expressions was done from a whole cell lysate extract. In order to evaluate relevant interaction of Bcl-2 and Beclin 1, organelle specific protein detection should be compared as well. Localisation of anti-apoptotic Bcl-2 in endoplasmic reticulum is associated with the repression of autophagy by Beclin 1 binding as opposed to mitochondrial Bcl-2 (Decuypere *et al.,* 2012). Therefore, the difference in protein expression between endoplasmic reticulum and mitochondria may provide better clarity on Bcl-2 and Beclin 1 interaction as well as their possible enhancement by drug combination. Further work using antigen-antibody techniques such as immuno-precipitation can provide insight on protein binding and Beclin 1:Bcl-2 complex.

The contrasting effect where BetA combination is rescuing Dox-treated SC/U-937 cells is not fully understood. However, differential mechanisms based on cell context appears to be involved. Selective and cell-dependent mechanism of BetA was documented in another combination study testing cell lines of breast cancer, where BetA combination with Andrographolide showed inhibition of cell cycling genes in one cancerous cell line but not in the other (Weber *et al.,* 2017). Prospective studies should test other cell lines of cancerous origin, non-malignant cells as well as primary cells to identify factors contributing to this mechanical switch.

Dox cytotoxicity may contribute to this phenomenon since Dox apoptotic cell death was reported to utilise a distinct mechanism between cells to which BetA could respond differently. Study by Wang *et al*., (2004) demonstrated Dox-induced apoptosis to be H_2O_2-dependent in

cardiomyocytes and aortic endothelial cells while p53-dependent in ovarian and breast cancer cell lines. In this study, Dox showed some selectivity between eradication of MOLM-13 and SC/U-937 cells as well as a different outcome in cell death populations. Since Dox reduced expression of anti-apoptotic p15-20-Bcl-2 (exclusive to MOLM-13) isoform but not the p26-Bcl-2-α present in both cells, future studies could investigate whether transfection of p15-20-Bcl-2 would change the mechanism of action of Dox in SC/U-937 cells, which could be potentially further sensitised by BetA.

5.6 Concluding comments

Conclusively, AML MOLM-13 cell death induction by Dox was associated with suppression of pro-survival pathway PI3K-AKT and a novel pro-apoptotic Bcl-2 isoform p15-20-Bcl-2 as well as autophagic modulation by reduction of Beclin 1. It is not clear whether the modulation of autophagy facilitated protective or death-inducing effects. However, some possible sign of mechanical drug resistance development such as downregulation of PTEN and apoptotic Cytochrome *c* and caspases was observed in MOLM-13 as a response to Dox exposure. Regardless, Dox-induced cell death in MOLM-13 cell lines was enhanced by BetA combination at least in part due to increase in cellular ROS formation. However, this effect was not seen in SC/U-937 cells and instead, BetA diminished Dox-induced death.

This study is first to report a novel p15-20-Bcl-2 variant of anti-apoptotic Bcl-2, which was expressed in MOLM-13 cells alongside p26-Bcl-2-α that is present in all cells. It is proposed that p15-20-Bcl-2 could be a potential target for cell death induction due to its sensitivity to Dox as opposed to p26-Bcl-2-α, which was demonstrated in this study. However, this hypothesis requires to be further tested.

Future studies should provide more accurate determination of the novel Bcl-2 variant by employing arrays of proteomic and genomic investigations with could lead to its potential cloning and transfection into other cells. The work should expand to more cells, including a range of immortalised cell lines as well as primary cells. The interaction between Bcl-2 and Beclin 1 in apoptosis-autophagy interplay could be further assessed by detection of organelle specific protein as well as immuno-precipitation work. Additionally, a wider panel of other signalling proteins, including markers of necroptosis are to be evaluated to determine possible modes of programmed cell death involvement. For better understanding of multiple programmed cell death interaction and the possible mechanism of drug combination, recommended future work should include pathway specific inhibitors.

References

Acésio, N., Oliveira, P., Mastrocola, D., Lima, I., Munari, C., Sato, V., Souza, A., Faluzino, L., Cunha, W. and Tavares, D. (2016) 'Modulatory Effect of Betulinic Acid on the Genotoxicity Induced by Different Mutagens in V79 Cells', *Evidence-based complementary and alternative medicine : eCAM,* 2016, pp. 8942730-6. doi: 10.1155/2016/8942730.

Adams, J.M. and Cory, S. (2007) 'The Bcl-2 apoptotic switch in cancer development and therapy', *Oncogene,* 26(9), pp. 1324-1337. doi: 10.1038/sj.onc.1210220.

Aisha, A.F.A., Abu-Salah, K.M., Ismail, Z. and Majid, Amin Malik Shah Abdul (2012) 'α-Mangostin Enhances Betulinic Acid Cytotoxicity and Inhibits Cisplatin Cytotoxicity on HCT 116 Colorectal Carcinoma Cells', *Molecules (Basel, Switzerland),* 17(3), pp. 2939-2954. doi: 10.3390/molecules17032939.

Akgul, C., Moulding, D.A. and Edwards, S.W. (2004) 'Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications', *Cellular and Molecular Life Sciences,* 61(17), pp. 2189-2199. doi: 10.1007/s00018-004-4001-7.

American Cancer Society (2015) *Treating chronic myeloid leukemia by phase.* Available at: http://www.cancer.org/cancer/leukemia-chronicmyeloidcml/detailedguide/leukemia-chronic-myeloid-myelogenous-treating-treating-by-phase (Accessed: October 20).

Arber, D.A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M.J., Le Beau, M.M., Bloomfield, C.D., Cazzola, M. and Vardiman, J.W. (2016) 'The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia', *Blood,* 127(20), pp. 2391-2405. doi: 10.1182/blood-2016-03-643544.

Ashkenazi, A. (2008) 'Targeting the extrinsic apoptosis pathway in cancer', *Cytokine and Growth Factor Reviews,* 19(3), pp. 325-331. doi: 10.1016/j.cytogfr.2008.04.001.

Banerjee Mustafi, S., Chakraborty, P.K. and Raha, S. (2010) 'Modulation of Akt and ERK1/2 Pathways by Resveratrol in Chronic Myelogenous Leukemia (CML) Cells Results in the Downregulation of Hsp70', *PloS one,* 5(1), pp. e8719. doi: 10.1371/journal.pone.0008719.

Bensi, L., Longo, R., Vecchi, A., Messora, C., Garagnani, L., Bernardi, S., Tamassia, M.G. and Sacchi, S. (1995) 'Bcl-2 oncoprotein expression in acute myeloid leukemia', *Haematologica,* 80(2), pp. 98-102.

Bidwell, G.L. and Raucher, D. (2006) 'Enhancing the antiproliferative effect of topoisomerase II inhibitors using a polypeptide inhibitor of c-Myc', *Biochemical Pharmacology,* 71(3), pp. 248-256. doi: 10.1016/j.bcp.2005.10.041.

Bien, S., Rimmbach, C., Neumann, H., Niessen, J., Reimer, E., Ritter, C.A., Rosskopf, D., Cinatl, J., Michaelis, M., Schroeder, H.W.S. and Kroemer, H.K. (2010) 'Doxorubicin-induced cell death requires cathepsin B in HeLa cells', *Biochemical Pharmacology,* 80(10), pp. 1466-1477. doi: 10.1016/j.bcp.2010.07.036.

Bode, A.M. and Dong, Z. (2002) 'The paradox of arsenic: molecular mechanisms of cell transformation and chemotherapeutic effects', *Critical Reviews in Oncology and Hematology,* 42(1), pp. 5-24. doi: 10.1016/S1040-8428(01)00215-3.

Boosani, C.S., Gunasekar, P. and Agrawal, D.K. (2019) 'An update on PTEN modulators - a patent review', *Expert Opinion on Therapeutic Patents,* 29(11), pp. 881-889. doi: 10.1080/13543776.2019.1669562.

Boya, P., Gonzalez-Polo, R., Casares, N., Perfettini, J., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P. and Kroemer, G. (2005) 'Inhibition of Macroautophagy Triggers Apoptosis', *Molecular and Cellular Biology,* 25(3), pp. 1025-1040.

Brantley-Finley, C., Lyle, C.S., Du, L., Goodwin, M.E., Hall, T., Szwedo, D., Kaushal, G.P. and Chambers, T.C. (2003) 'The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide', *Biochemical Pharmacology,* 66(3), pp. 459-469. doi: 10.1016/S0006-2952(03)00255-7.

Callus, B.A. and Vaux, D.L. (2007) 'Caspase inhibitors: viral, cellular and chemical', *Cell Death and Differentiation,* 14(1), pp. 73-78. doi: 10.1038/sj.cdd.4402034.

Campo, E., Swerdlow, S.H., Harris, N.L., Pileri, S., Stein, H. and Jaffe, E.S. (2011) 'The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications', *Blood,* 117(19), pp. 5019-5032. doi: 10.1182/blood-2011-01-293050.

Campos, E.d.V. and Pinto, R. (2019) 'Targeted therapy with a selective BCL-2 inhibitor in older patients with acute myeloid leukemia', *Hematology, Transfusion and Cell Therapy,* 41(2), pp. 169-177. doi: 10.1016/j.htct.2018.09.001.

Campos, L., Rouault, J.P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.P. and Guyotat, D. (1993) 'High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy', *Blood,* 81(11), pp. 3091-3096. doi: 10.1182/blood.V81.11.3091.3091.

Cancer Research, U.K. (2019) *Leukaemia (all subtypes combined) statistics.* Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia#dgg72fy48VWYawLQ.99 (Accessed: June 24).

Casares, N., Pequignot, M.O., Tesniere, A., Ghiringhelli, F., Roux, S., Chaput, N., Schmitt, E., Hamai, A., Hervas-Stubbs, S., Obeid, M., Coutant, F., Métivier, D., Pichard, E., Aucouturier, P., Pierron, G., Garrido, C., Zitvogel, L. and Kroemer, G. (2005) 'Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death', *The Journal of experimental medicine,* 202(12), pp. 1691-1701. doi: 10.1084/jem.20050915.

Chauvier, D., Ankri, S., Charriaut-Marlangue, C., Casimir, R. and Jacotot, E. (2007) 'Broad-spectrum caspase inhibitors: from myth to reality?', *Cell Death and Differentiation,* 14(2), pp. 387-391. doi: 10.1038/sj.cdd.4402044.

Cheesman, S. and Shields, A. (2014) *Maximum Anthracycline Doses Guidance.* Available at: http://www.londoncancer.org/media/75901/140214-Maximum-Anthracycline-doses-Guideline-v1.pdf (Accessed: April 2).

Chen, H., Zhou, L., Wu, X., Li, R., Wen, J., Sha, J. and Wen, X. (2016) 'The PI3K AKT pathway in the pathogenesis of prostate cancer', *Frontiers in bioscience (Landmark edition),* 21(5), pp. 1084-1091. doi: 10.2741/4443.

Chintharlapalli, S., Papineni, S., Ramaiah, S.K. and Safe, S. (2007) 'Betulinic Acid Inhibits Prostate Cancer Growth through Inhibition of Specificity Protein Transcription Factors', *Cancer Research,* 67(6), pp. 2816-2823. doi: 10.1158/0008-5472.CAN-06-3735.

Chou, D., Adamson, B., Dephoure, N., Tan, X., Nottke, A., Hurov, K., Gygi, S., Colaiácov, M. and Elledge, S. (2010b) 'A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage', *Proceedings of the National Academy of Sciences of the United States of America,* 107(43), pp. 18475-18480. doi: 10.1073/pnas.1012946107.

Chou, T. (2011) 'The mass-action law based algorithms for quantitative econo-green bio-research', *Integrative biology : quantitative biosciences from nano to macro,* 3(5), pp. 548-559. doi: 10.1039/c0ib00130a.

Chou, T. (2010) 'Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method', *Cancer Research,* 70(2), pp. 440-446. doi: 10.1158/0008-5472.CAN-09-1947.

Chowdhury, A.R., Mandal, S., Mittra, B., Sharma, S., Mukhopadhyay, S. and Majumder, H.K. (2002) 'Betulinic acid, a potent inhibitor of eukaryotic topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives', *Medical science monitor : international medical journal of experimental and clinical research,* 8(7), pp. BR254.

Cichewicz, R. and Kouzi, S. (2004) 'Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection', *Medicinal Research Reviews,* 24(1), pp. 90-114. doi: 10.1002/med.10053.

Ciechomska, I.A., Goemans, G.C., Skepper, J.N. and Tolkovsky, A.M. (2009) 'Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function', *Oncogene,* 28(21), pp. 2128-2141. doi: 10.1038/onc.2009.60.

Cools, J. (2012) 'Primetime for chemotherapy in acute myeloid leukemia', *Haematologica,* 97(12), pp. 1775. doi: 10.3324/haematol.2012.081414.

Correia, C., Lee, S., Meng, X.W., Vincelette, N.D., Knorr, K.L.B., Ding, H., Nowakowski, G.S., Dai, H. and Kaufmann, S.H. (2015) 'Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment', *BBA - Molecular Cell Research,* 1853(7), pp. 1658-1671. doi: 10.1016/j.bbamcr.2015.03.012.

Cragg, Gordon M.;kingston, David G. I.;newman and David, J. (2005) *Anticancer Agents from Natural Products.* 1st edn. Baton Rouge: CRC Press Inc - M.U.A.

Dai, H., Ding, H., Meng, X.W., Peterson, K.L., Schneider, P.A., Karp, J.E. and Kaufmann, S.H. (2015) 'Constitutive BAK activation as a determinant of drug sensitivity in malignant lymphohematopoietic cells', *Genes & development,* 29(20), pp. 2140-2152. doi: 10.1101/gad.267997.115.

Daigle, S., Olhava, E., Therkelsen, C., Majer, C., Sneeringer, C., Song, J., Johnston, L. ., Scott, M., Smith, J., Xiao, Y., Jin, L., Kuntz, K., Chesworth, R., Moyer, M., Bernt, K., Tseng, J., Kung, A., Armstrong, S., Copeland, R., Richon, V. and Pollock, R. (2011) 'Selective Killing of Mixed Lineage Leukemia Cells by a Potent Small-Molecule DOT1L Inhibitor', *Cancer Cell,* 20(1), pp. 53-65. doi: 10.1016/j.ccr.2011.06.009.

Daniel, F., Legrand, A., Pessayre, D., Vadrot, N., Descatoire, V. and Bernuau, D. (2006) 'Partial Beclin 1 silencing aggravates doxorubicin- and Fas-induced apoptosis in HepG2 cells', *World journal of gastroenterology,* 12(18), pp. 2895-2900. doi: 10.3748/wjg.v12.i18.2895.

Decuypere, J., Parys, J.B. and Bultynck, G. (2012) 'Regulation of the Autophagic Bcl-2/Beclin 1 Interaction', *Cells,* 1(3), pp. 284-312. doi: 10.3390/cells1030284.

Deschler, B. and Lübbert, M. (2006) 'Acute myeloid leukemia: Epidemiology and etiology', *Cancer,* 107(9), pp. 2099-2107. doi: 10.1002/cncr.22233.

Devarajan, E., Sahin, A.A., Chen, J.S., Krishnamurthy, R.R., Aggarwal, N., Brun, A., Sapino, A., Zhang, F., Sharma, D., Yang, X., Tora, A.D. and Mehta, K. (2002) 'Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance', *Oncogene,* 21(57), pp. 8843-8851. doi: 10.1038/sj.onc.1206044.

Ding, L., Ley, T.J., Larson, D.E., Miller, C.A., Koboldt, D.C., Welch, J.S., Ritchey, J.K., Young, M.A., Lamprecht, T., McLellan, M.D., McMichael, J.F., Wallis, J.W., Lu, C., Shen, D., Harris, C.C., Dooling, D.J., Fulton, R.S., Fulton, L.L., Chen, K., Schmidt, H., Kalicki-Veizer, J., Magrini, V.J., Cook, L., McGrath, S.D., Vickery, T.L., Wendl, M.C., Heath, S., Watson, M.A., Link, D.C., Tomasson, M.H., Shannon, W.D., Payton, J.E., Kulkarni, S., Westervelt, P., Walter, M.J., Graubert, T.A., Mardis, E.R., Wilson, R.K. and DiPersio, J.F. (2012) 'Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing', *Nature,* 481(7382), pp. 506-510. doi: 10.1038/nature10738.

Ding, W., Ni, H., Gao, W., Hou, Y., Melan, M., Chen, X., Stolz, D., Shao, Z. and Yin, X. (2007) 'Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival', *The Journal of biological chemistry,* 282(7), pp. 4702-4710.

Doherty, E. and Perl, A. (2017) 'Measurement of Mitochondrial Mass by Flow Cytometry during Oxidative Stress', *Reactive oxygen species (Apex, N.C.),* 4(10), pp. 275-283. doi: 10.20455/ros.2017.839.

Dombret, H. and Gardin, C. (2016) 'An update of current treatments for adult acute myeloid leukemia', *Blood,* 127(1), pp. 53-61. doi: 10.1182/blood-2015-08-604520.

Druker, B.J. (2008) 'Translation of the Philadelphia chromosome into therapy for CML', *Blood,* 112(13), pp. 4808-4817. doi: 10.1182/blood-2008-07-077958.

Druskovic, M., Suput, D. and Milisav, I. (2006) 'Overexpression of caspase-9 triggers its activation and apoptosis in vitro', *Croatian medical journal,* 47(6), pp. 832-840.

Ehrhardt, H., Fulda, S., Führer, M., Debatin, K.M. and Jeremias, I. (2004) 'Betulinic acid-induced apoptosis in leukemia cells', *Leukemia,* 18(8), pp. 1406-1412. doi: 10.1038/sj.leu.2403406.

Eichenmüller, M., Hemmerlein, B., von Schweinitz, D. and Kappler, R. (2010) 'Betulinic acid induces apoptosis and inhibits hedgehog signalling in rhabdomyosarcoma', *British Journal of Cancer,* 103(1), pp. 43-51. doi: 10.1038/sj.bjc.6605715.

Eom, Y., Kim, M.A., Park, S.S., Goo, M.J., Kwon, H.J., Sohn, S., Kim, W., Yoon, G. and Choi, K.S. (2005) 'Two distinct modes of cell death induced by doxorubicin: apoptosis and

cell death through mitotic catastrophe accompanied by senescence-like phenotype', *Oncogene,* 24(30), pp. 4765-4777. doi: 10.1038/sj.onc.1208627.

Erba, H.P. (2007) 'Prognostic Factors in Elderly Patients with AML and the Implications for Treatment', *Hematology. American Society of Hematology. Education Program,* 2007(1), pp. 420-428. doi: 10.1182/asheducation-2007.1.420.

Estey, E. and Döhner, H. (2006) 'Acute myeloid leukaemia', *The Lancet,* 368(9550), pp. 1894-1907. doi: 10.1016/S0140-6736(06)69780-8.

Faujan, N., H, Alitheen, N., B, Yeap, S., K, Ali, A., Muhajir, A., H and Ahmad F, B., H (2010) 'Cytotoxic effect of betulinic acid and betulinic acid acetate isolated from Melaleuca cajuput on human myeloid leukemia (HL-60) cell line', *African Journal of Biotechnology,* 9(38), pp. 6387-6396.

Feng, Y., Yao, Z. and Klionsky, D.J. (2015) 'How to control self-digestion: transcriptional, post-transcriptional, and post-translational regulation of autophagy', *Trends in Cell Biology,* 25(6), pp. 354-363. doi: 10.1016/j.tcb.2015.02.002.

Fernandes, J., Castilho, R.O., da Costa, M.R., Wagner-Souza, K., Coelho Kaplan, M.A. and Gattass, C.R. (2003) 'Pentacyclic triterpenes from Chrysobalanaceae species: cytotoxicity on multidrug resistant and sensitive leukemia cell lines', *Cancer Letters,* 190(2), pp. 165-169. doi: 10.1016/S0304-3835(02)00593-1.

Ferreira, D., Adega, F. and Chaves, R. (2013) *The Importance of Cancer Cell Lines as in vitro Models in Cancer Methylome Analysis and Anticancer Drugs Testing* InTech.

Fleming, D.R. (2012) 'Leukemia: understanding its types and treatments: this review describes the four major groups of leukemia, plus diagnostic tests and current and emerging treatment regimens for each group', *Oncology Nurse Advisor,* , pp. 16.

Florou, D., Patsis, C., Ardavanis, A. and Scorilas, A. (2013) 'Effect of doxorubicin, oxaliplatin, and methotrexate administration on the transcriptional activity of BCL-2 family gene members in stomach cancer cells', *Cancer Biology & Therapy,* 14(7), pp. 587-596. doi: 10.4161/cbt.24591.

Fornari, F., Randolph, J., Yalowich, J., Ritke, M. and Gewirtz, D. (1994) 'Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells', *Molecular Pharmacology,* 45(4), pp. 649-656.

Fulda, S. (2014) 'Therapeutic exploitation of necroptosis for cancer therapy', *Seminars in Cell and Developmental Biology,* 35, pp. 51-56. doi: 10.1016/j.semcdb.2014.07.002.

Fulda, S. (2008) 'Betulinic Acid for Cancer Treatment and Prevention', *International journal of molecular sciences,* 9(6), pp. 1096-1107. doi: 10.3390/ijms9061096.

Fulda, S. and Debatin, K. (2005) 'Sensitization for Anticancer Drug-Induced Apoptosis by Betulinic Acid', *Neoplasia,* 7(2), pp. 162-170. doi: 10.1593/neo.04442.

Fulda, S., Friesen, C., Los, M., Scaffidi, C., Mier, W., Benedict, M., Nunez, G., Krammer, P.H., Peter, M.E. and Debatin, K. (1997) 'Betulinic Acid Triggers CD95 (APO-1/Fas)- and p53-independent Apoptosis via Activation of Caspases in Neuroectodermal Tumors', *Cancer Research,* 57(21), pp. 4956.

Fulda, S., Jeremias, I. and Debatin, K. (2004) 'Cooperation of betulinic acid and TRAIL to induce apoptosis in tumor cells', *Oncogene,* 23(46), pp. 7611-7620. doi: 10.1038/sj.onc.1207970.

Fulda, S., Jeremias, I., Steiner, H.H., Pietsch, T. and Debatin, K. (1999) 'Betulinic acid: A new cytotoxic agent against malignant brain-tumor cells', *International Journal of Cancer,* 82(3), pp. 435-441. doi: 10.1002/(SICI)1097-0215(19990730)82:33.0.CO;2-1.

Fulda, S. and Kroemer, G. (2009) 'Targeting mitochondrial apoptosis by betulinic acid in human cancers', *Drug Discovery Today,* 14(17), pp. 885-890. doi: 10.1016/j.drudis.2009.05.015.

Galadari, S., Rahman, A., Pallichankandy, S. and Thayyullathil, F. (2017) 'Reactive oxygen species and cancer paradox: To promote or to suppress?', *Free Radical Biology and Medicine,* 104, pp. 144-164. doi: 10.1016/j.freeradbiomed.2017.01.004.

Galluzzi, L. and Kroemer, G. (2008) 'Necroptosis: A Specialized Pathway of Programmed Necrosis', *Cell,* 135(7), pp. 1161-1163. doi: 10.1016/j.cell.2008.12.004.

Gamen, S., Anel, A., Pérez-Galán, P., Lasierra, P., Johnson, D., Piñeiro, A. and Naval, J. (2000) 'Doxorubicin Treatment Activates a Z-VAD-Sensitive Caspase, Which Causes ΔΨm Loss, Caspase-9 Activity, and Apoptosis in Jurkat Cells', *Experimental Cell Research,* 258(1), pp. 223-235. doi: 10.1006/excr.2000.4924.

Ganguly, A., Das, B., Roy, A., Sen, N., Dasgupta, S.B., Mukhopadhayay, S. and Majumder, H.K. (2007) 'Betulinic Acid, a Catalytic Inhibitor of Topoisomerase I, Inhibits Reactive Oxygen Species Mediated Apoptotic Topoisomerase I DNA Cleavable Complex Formation in Prostate Cancer Cells but Does Not Affect the Process of Cell Death', *Cancer Research,* 67(24), pp. 11848-11858. doi: 10.1158/0008-5472.CAN-07-1615.

Gao, Y., Jia, Z., Kong, X., Li, Q., Chang, D.Z., Wei, D., Le, X., Suyun, H., Huang, S., Wang, L. and Xie, K. (2011) 'Combining Betulinic Acid and Mithramycin A Effectively Suppresses Pancreatic Cancer by Inhibiting Proliferation, Invasion, and Angiogenesis', *Cancer research,* 71(15), pp. 5182-5193. doi: 10.1158/0008-5472.CAN-10-2016.

Gao, Y., Zhang, M., Wang, T., Fan, Y., Yu, L., Ye, G., Wang, Z., Gao, C., Wang, H., Luo, C. and Tao, L. (2018) 'IL-33/ST2L Signaling Provides Neuroprotection Through Inhibiting Autophagy, Endoplasmic Reticulum Stress, and Apoptosis in a Mouse Model of Traumatic Brain Injury', *Frontiers in cellular neuroscience,* 12, pp. 95. doi: 10.3389/fncel.2018.00095.

Gewirtz, D. (1999) 'A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin', *Biochemical Pharmacology,* 57(7), pp. 727-741. doi: 10.1016/S0006-2952(98)00307-4.

Ghassemifar, R., Forster, L., Finlayson, J., Calogero, T., Augustson, B., Joske, D. and Cull, G. (2012) 'Differential expression of the Bcl-2 and Bax isoforms in CD19 positive B-lymphocytes isolated from patients diagnosed with chronic lymphocytic leukaemia', *Pathology,* 44(7), pp. 632-637. doi: 10.1097/PAT.0b013e32835a0142.

Gomez-Bougie, P., Maïga, S., Tessoulin, B., Bourcier, J., Bonnet, A., Rodriguez, M., Le Gouill, S., Touzeau, C., Moreau, P., Pellat-Deceunynck, C. and Amiot, M. (2018) 'BH3-mimetic toolkit guides the respective use of BCL2 and MCL1 BH3-mimetics in myeloma treatment', *Blood,* 132(25). doi: 10.1182/blood-2018-03-836718.

Goodall, M., Fitzwalter, B., Zahedi, S., Wu, M., Rodriguez, D., Mulcahy-Levy, J., Green, D., Morgan, M., Cramer, S. and Thorburn, A. (2016) 'The Autophagy Machinery Controls Cell Death Switching between Apoptosis and Necroptosis', *Developmental Cell,* 37(4), pp. 337-349. doi: 10.1016/j.devcel.2016.04.018.

Gopal, R., D.V, Archana, N., A, Badrinath, Y., Mishra, K.P. and Joshi, D.S. (2005) 'Betulinic acid induces apoptosis in human chronic myelogenous leukemia (CML) cell line K-562 without altering the levels of Bcr-Abl', *Toxicology Letters,* 155(3), pp. 343-351. doi: 10.1016/j.toxlet.2004.06.015.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003) 'Comparing protein abundance and mRNA expression levels on a genomic scale', *Genome biology,* 4(9), pp. 117.

Greshock, J., Nathanson, K., Martin, A., Zhang, L., Coukos, G., Weber, B.L. and Zaks, T.Z. (2007) 'Cancer Cell Lines as Genetic Models of Their Parent Histology: Analyses Based on Array Comparative Genomic Hybridization', *Cancer Research,* 67(8), pp. 3594-3600. doi: 10.1158/0008-5472.CAN-06-3674.

Gross, A. and Katz, S.G. (2017) 'Non-apoptotic functions of BCL-2 family proteins', *Cell death and differentiation,* 24(8), pp. 1348-1358. doi: 10.1038/cdd.2017.22.

Guillem, V., Amat, P., Collado, M., Cervantes, F., Alvarez-Larran, A., Martinez, J., Tormo, E., Eroles, P., Solano, C. and Hernandez-Boluda, J.C. (2015) 'BCL2 gene polymorphisms and splicing variants in chronic myeloid leukemia', *Leukemia Research,* 39(11), pp. 1278-1284. doi: 10.1016/j.leukres.2015.08.014.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G.G., Liu, Y., Chen, X., Li, L., Wu, S., Chen, Y., Jiang, H., Tan, L., Xie, J., Zhu, X., Liang, S. and Deng, H. (2008) 'How is mRNA expression predictive for protein expression A correlation study on human circulating monocytes', *Acta Biochimica et Biophysica Sinica,* 40(5), pp. 426-436. doi: 10.1111/j.1745-7270.2008.00418.x.

Guo, Y., Li, Y., Shan, Q., He, G., Lin, J. and Gong, Y. (2015) 'Curcumin potentiates the anti-leukemia effects of imatinib by downregulation of the AKT/mTOR pathway and BCR/ABL gene expression in Ph+ acute lymphoblastic leukemia', *International Journal of Biochemistry and Cell Biology,* 65, pp. 1-11. doi: 10.1016/j.biocel.2015.05.003.

Gygi, S., Rochon, Y., Franza, R. and Aebersold, R. (1999) 'Correlation between Protein and mRNA Abundance in Yeast', *Molecular and Cellular Biology,* 19(3), pp. 1720-1730. doi: 10.1128/MCB.19.3.1720.

Han, W., Li, L., Qiu, S., Lu, Q., Pan, Q., Gu, Y., Luo, J. and Hu, X. (2007) 'Shikonin circumvents cancer drug resistance by induction of a necroptotic death', *Molecular Cancer Therapeutics,* 6(5), pp. 1641-1649. doi: 10.1158/1535-7163.MCT-06-0511.

Han, W., Xie, J., Li, L., Liu, Z. and Hu, X. (2009) 'Necrostatin-1 reverts shikonin-induced necroptosis to apoptosis', *Apoptosis,* 14(5), pp. 674-686. doi: 10.1007/s10495-009-0334-x.

Hanahan, D. and Weinberg, R. (2011) 'Hallmarks of Cancer: The Next Generation', *Cell,* 144(5), pp. 646-674. doi: 10.1016/j.cell.2011.02.013.

Hartford Svoboda, K.K. and Reenstra, W.R. (2002) 'Approaches to studying cellular signaling: A primer for morphologists', *The Anatomical Record,* 269(2), pp. 123-139. doi: 10.1002/ar.10074.

Hartung, T. and Daston, G. (2009) 'Are In Vitro Tests Suitable for Regulatory Use?', *Toxicological Sciences,* 111(2), pp. 233-237. doi: 10.1093/toxsci/kfp149.

Hassan, C., Afshinnekoo, E., Li, S., Wu, S. and Mason, C.E. (2017) 'Genetic and epigenetic heterogeneity and the impact on cancer relapse', *Experimental Hematology,* 54, pp. 26-30. doi: 10.1016/j.exphem.2017.07.002.

Hata, K., Hori, K., Ogasawara, H. and Takahashi, S. (2003) 'Anti-leukemia activities of Lup-28-al-20(29)-en-3-one, a lupane triterpene', *Toxicology letters,* 143(1), pp. 1-7. doi: 10.1016/S0378-4274(03)00092.

Hehlmann, R. (2012) 'How I treat CML blast crisis', *Blood,* 120(4), pp. 737-747. doi: 10.1182/blood-2012-03-380147.

Henz, K., Al-Zebeeby, A., Basoglu, M., Fulda, S., Cohen, G.M., Varadarajan, S. and Vogler, M. (2019) 'Selective BH3-mimetics targeting BCL-2, BCL-XL or MCL-1 induce severe mitochondrial perturbations', *Biological Chemistry,* 400(2), pp. 181-185. doi: 10.1515/hsz-2018-0233.

Hoffbrand, V. and Moss, P. (2011) *Essential Haematology.* 6. Aufl. edn. Hoboken: Wiley-Blackwell.

Horita, H., Frankel, A.E. and Thorburn, A. (2008) 'Acute Myeloid Leukemia-Targeted Toxin Activates Both Apoptotic and Necroptotic Death Mechanisms', *PloS one,* 3(12), pp. e3909. doi: 10.1371/journal.pone.0003909.

Hseu, Y., Tsai, T., Korivi, M., Liu, J., Chen, H., Lin, C., Shen, Y. and Yang, H. (2017) 'Antitumor properties of Coenzyme Q0 against human ovarian carcinoma cells via induction of ROS-mediated apoptosis and cytoprotective autophagy', *Scientific Reports,* 7(1), pp. 1-21. doi: 10.1038/s41598-017-08659-7.

Hu, X. and Xuan, Y. (2007) 'Bypassing cancer drug resistance by activating multiple death pathways – A proposal from the study of circumventing cancer drug resistance by induction of necroptosis', *Cancer Letters,* 259(2), pp. 127-137. doi: 10.1016/j.canlet.2007.11.007.

Huang, J., Lin, S., Lin, T., Chang, D. and Ying, S. (2003) 'Truncated Bcl-2, a potential pre-metastatic marker in prostate cancer', *Biochemical and Biophysical Research Communications,* 306(4), pp. 912-917. doi: 10.1016/S0006-291X(03)01072-6.

Inao, T., Iida, Y., Moritani, T., Okimoto, T., Tanino, R., Kotani, H. and Harada, M. (2018) 'Bcl-2 inhibition sensitizes triple-negative human breast cancer cells to doxorubicin', *Oncotarget,* 9(39), pp. 25545-25556. doi: 10.18632/oncotarget.25370.

Inoue-Yamauchi, A., Jeng, P.S., Kim, K., Chen, H., Han, S., Ganesan, Y.T., Ishizawa, K., Jebiwott, S., Dong, Y., Pietanza, M.C., Hellmann, M.D., Kris, M.G., Hsieh, J.J. and Cheng, E.H. (2017) 'Targeting the differential addiction to anti-apoptotic BCL-2 family for cancer therapy', *Nature communications,* 8(1), pp. 16078. doi: 10.1038/ncomms16078.

Invitrogen (2009) *CyQUANT® Direct Cell Proliferation Assay Kit.* Available at: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp35011.pdf (Accessed: August 12).

Iqbal, N. and Iqbal, N. (2014) 'Imatinib: A Breakthrough of Targeted Therapy in Cancer', *Chemotherapy research and practice,* 2014, pp. 357027-9. doi: 10.1155/2014/357027.

Irish, J.M., Hovland, R., Krutzik, P.O., Perez, O.D., Bruserud, Ø, Gjertsen, B.T. and Nolan, G.P. (2004) 'Single Cell Profiling of Potentiated Phospho-Protein Networks in Cancer Cells', *Cell,* 118(2), pp. 217-228. doi: 10.1016/j.cell.2004.06.028.

Jabbour, E., Kantarjian, H. and Cortes, J. (2015) 'Use of Second- and Third-Generation Tyrosine Kinase Inhibitors in the Treatment of Chronic Myeloid Leukemia: An Evolving Treatment Paradigm', *Clinical Lymphoma, Myeloma & Leukemia,* 15(6), pp. 323-334. doi: 10.1016/j.clml.2015.03.006.

Jaeger, S., Igea, A., Arroyo, R., Alcalde, V., Canovas, B., Orozco, M., Nebreda, A.R. and Aloy, P. (2017) 'Quantification of Pathway Cross-talk Reveals Novel Synergistic Drug Combinations for Breast Cancer', *Cancer research,* 77(2), pp. 459-469. doi: 10.1158/0008-5472.CAN-16-0097.

Jia, Y., Li, J., Qin, Z. and Liang, Z. (2009) 'Autophagic and apoptotic mechanisms of curcumin-induced death in K562 cells', *Journal of Asian Natural Products Research,* 11(11), pp. 918-928. doi: 10.1080/10286020903264077.

Jiao, G., Yan, L., Qiang, L., Xia, G., Ling, G., Zhi Gui, M. and Yi Ping, Z. (2013) 'Resveratrol Induces Apoptosis and Autophagy in T-cell Acute Lymphoblastic Leukemia Cells by Inhibiting Akt/mTOR and Activating p38-MAPK', *Biomed Environ Sci,* 26(11), pp. 902-911.

Jones, L.J., Gray, M., Yue, S.T., Haugland, R.P. and Singer, V.L. (2001) 'Sensitive determination of cell number using the CyQUANT® cell proliferation assay', *Journal of Immunological Methods,* 254(1), pp. 85-98. doi: 10.1016/S0022-1759(01)00404-5.

Jung, G., Kim, K., Choi, C., Lee, T., Han, S.I., Han, H. and Lim, S. (2007) 'Effect of Betulinic Acid on Anticancer Drug-Resistant Colon Cancer Cells', *Basic & Clinical Pharmacology & Toxicology,* 101(4), pp. 277-285. doi: 10.1111/j.1742-7843.2007.00115.x.

Kale, J., Osterlund, E.J. and Andrews, D.W. (2018) 'BCL-2 family proteins: changing partners in the dance towards death', *Cell death and differentiation,* 25(1), pp. 65-80. doi: 10.1038/cdd.2017.186.

Kallenberger, S.M., Beaudouin, J., Claus, J., Fischer, C., Sorger, P.K., Legewie, S. and Eils, R. (2014) 'Intra- and Interdimeric Caspase-8 Self-Cleavage Controls Strength and Timing of CD95-Induced Apoptosis', *Science signaling,* 7(316), pp. ra23. doi: 10.1126/scisignal.2004738.

Kasperczyk, H., La Ferla-Brühl, K., Westhoff, M.A., Behrend, L., Zwacka, R.M., Debatin, K. and Fulda, S. (2005) 'Betulinic acid as new activator of NF-κB: molecular mechanisms and implications for cancer therapy', *Oncogene,* 24(46), pp. 6945-6956. doi: 10.1038/sj.onc.1208842.

Katt, M.E., Placone, A.L., Wong, A.D., Xu, Z.S. and Searson, P.C. (2016) 'In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform', *Frontiers in Bioengineering and Biotechnology,* 4. doi: 10.3389/fbioe.2016.00012/full.

Kawthalkar, S., M (2013) *Essentials of Haematology.* 2nd edn.Jaypee Brothers Medical.

Kessler, J.H., Mullauer, F.B., de Roo, G.M. and Medema, J.P. (2007) 'Broad in vitro efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types', *Cancer Letters,* 251(1), pp. 132-145. doi: 10.1016/j.canlet.2006.11.003.

Kimmelman, A.C. (2011) 'The dynamic nature of autophagy in cancer', *Genes & development,* 25(19), pp. 1999-2010. doi: 10.1101/gad.17558811.

Korkina, O., Germscheid, M., Wagner, G., Ch'en, I.L., Teng, X., Hedrick, S.M., Gerber, S.A., Lugovskoy, A., Hitomi, J., Cuny, G.D., Yuan, J., Abbott, D., Yuan, C. and Degterev, A. (2008) 'Identification of RIP1 kinase as a specific cellular target of necrostatins', *Nature Chemical Biology,* 4(5), pp. 313-321. doi: 10.1038/nchembio.83.

Kroemer, G., El-Deiry, W.S., Golstein, P., Peter, M.E., Vaux, D., Vandenabeele, P., Zhivotovsky, B., Blagosklonny, M.V., Malorni, W., Knight, R.A., Piacentini, M., Nagata, S. and Melino, G. (2005) 'Classification of cell death: recommendations of the Nomenclature Committee on Cell Death', *Cell Death and Differentiation,* 12(S2), pp. 1463-1467. doi: 10.1038/sj.cdd.4401724.

Ku, B., Liang, C.Y., Jung, J. and Oh, B. (2011) 'Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX', *Cell Res,* 21(4), pp. 627-641.

Li, J., Yang, Z., Li, Y., Xia, J., Li, D., Li, H., Ren, M., Liao, Y., Yu, S., Chen, Y., Yang, Y. and Zhang, Y. (2016) 'Cell apoptosis, autophagy and necroptosis in osteosarcoma treatment', *Oncotarget,* 7(28), pp. 44763-44778. doi: 10.18632/oncotarget.8206.

Li, P., Zhou, L., Zhao, T., Liu, X., Zhang, P., Liu, Y., Zheng, X. and Li, Q. (2017a) 'Caspase-9: structure, mechanisms and clinical application', *Oncotarget,* 8(14), pp. 23996-24008. doi: 10.18632/oncotarget.15098.

Li, Q., Li, Y., Wang, X., Fang, X., He, K., Guo, X., Zhan, Z., Sun, C. and Jin, Y. (2011) 'Co-treatment with ginsenoside Rh2 and betulinic acid synergistically induces apoptosis in human cancer cells in association with enhanced capsase-8 activation, bax translocation, and cytochrome c release', *Molecular Carcinogenesis,* 50(10), pp. 760-769. doi: 10.1002/mc.20673.

Li, T., Wang, W., Chen, H., Li, T. and Ye, L. (2010) 'Evaluation of anti-leukemia effect of resveratrol by modulating SATA3 signaling', *International Immunopharmacology,* 10(1), pp. 18-25. doi: 10.1016/j.intimp.2009.09.009.

Li, Y., Zhou, M., Hu, Q., Bai, X., Huang, W., Scheres, S. and Shi, Y. (2017b) 'Mechanistic insights into caspase-9 activation by the structure of the apoptosome holoenzyme', *Proceedings of the National Academy of Sciences of the United States of America,* 114(7), pp. 1542.

Lian, J., Karnak, D. and Xu, L. (2010) 'The Bcl-2-Beclin 1 interaction in (-)-gossypol-induced autophagy versus apoptosis in prostate cancer cells', *Autophagy,* 6(8), pp. 1201-1203. doi: 10.4161/auto.6.8.13549.

Liang, J., Shao, S.H., Xu, Z., Hennessy, B., Ding, Z., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L., Slingerland, J.M. and Mills, G.B. (2007) 'The energy sensing LKB1–AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis', *Nature Cell Biology,* 9(2), pp. 218-224. doi: 10.1038/ncb1537.

Lin, Y., Kang, T. and Zhou, B.P. (2014) 'Doxorubicin enhances Snail/LSD1-mediated PTEN suppression in a PARP1-dependent manner', *Cell Cycle,* 13(11), pp. 1708-1716. doi: 10.4161/cc.28619.

Liu, J., Uematsu, H., Tsuchida, N. and Ikeda, M. (2009) 'Association of caspase-8 mutation with chemoresistance to cisplatin in HOC313 head and neck squamous cell carcinoma cells', *Biochemical and Biophysical Research Communications,* 390(3), pp. 989-994. doi: 10.1016/j.bbrc.2009.10.090.

Liu, W., Ho, J.C.K., Cheung, F.W.K., Liu, B.P.L., Ye, W. and Che, C. (2004) 'Apoptotic activity of betulinic acid derivatives on murine melanoma B16 cell line', *European Journal of Pharmacology,* 498(1), pp. 71-78. doi: 10.1016/j.ejphar.2004.07.103.

Liu, Y. and Luo, W. (2012) 'Betulinic acid induces Bax/Bak-independent cytochrome c release in human nasopharyngeal carcinoma cells', *Molecules and Cells,* 33(5), pp. 517-524. doi: 10.1007/s10059-012-0022-5.

Lun, Y., Yang, J.J. and Wu, Y. (2017) 'Complete molecular remission in relapsed and refractory acute myeloid leukaemia with MLL-AF9 treated with chidamide-based chemotherapy', *Journal of Clinical Pharmacy and Therapeutics,* 42(6), pp. 786-789. doi: 10.1111/jcpt.12577.

Lüpertz, R., Wätjen, W., Kahl, R. and Chovolou, Y. (2010) 'Dose- and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells', *Toxicology,* 271(3), pp. 115-121. doi: 10.1016/j.tox.2010.03.012.

Lynch, J.T., McEwen, R., Crafter, C., McDermott, U., Garnett, M.J., Barry, S.T. and Davies, B.R. (2016) 'Identification of differential PI3K pathway target dependencies in T-cell acute lymphoblastic leukemia through a large cancer cell panel screen', *Oncotarget,* 7(16), pp. 22128-22139. doi: 10.18632/oncotarget.8031.

Macdonald, F., Ford, C. and Casson, A., G (2004) *Molecular Biology of Cancer.* 2nd edn. London: BIOS Scientific, Taylor & Francis group.

Madanat, Y.F., Kalaycio, M.E. and Nazha, A. (2019) 'Advances in Acute Myeloid Leukemia Genomics, Where Do We Stand in 2018?', *Acta medica academica,* 48(1), pp. 35-44. doi: 10.5644/ama2006-124.240.

Maier, T., Güell, M. and Serrano, L. (2009) 'Correlation of mRNA and protein in complex biological samples', *FEBS Letters,* 583(24), pp. 3966-3973. doi: 10.1016/j.febslet.2009.10.036.

Manetto, V., Lorenzini, R., Cordon-Cardo, C., Krajewski, S., Rosai, J., Reed, J. and Eusebi, V. (1997) 'Bcl-2 and Bax expression in thyroid tumours An immunohistochemical and Western blot analysis', *Virchows Archiv,* 430(2), pp. 125-130. doi: 10.1007/BF01008033.

Mao, X., Yu, C.R., Li, W.H. and Li, W.X. (2008) 'Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells', *Cell Research,* 18(8), pp. 879-888. doi: 10.1038/cr.2008.86.

Marcucci, G., Perrotti, D. and Caligiuri, M.A. (2003) 'Understanding the molecular basis of imatinib mesylate therapy in chronic myelogenous leukemia and the related mechanisms of resistance', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 9(4), pp. 1248.

Marquez, R.T. and Xu, L. (2012) 'Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch', *American journal of cancer research,* 2(2), pp. 214-221.

Martins, W.K., Costa, ÉT., Cruz, M.C., Stolf, B.S., Miotto, R., Cordeiro, R.M. and Baptista, M.S. (2015) 'Parallel damage in mitochondrial and lysosomal compartments promotes efficient cell death with autophagy: The case of the pentacyclic triterpenoids', *Scientific reports,* 5(1), pp. 12425. doi: 10.1038/srep12425.

Mashayekhi, S., Yousefi, B., Tohidi, E., Darband, S.G., Mirza-Aghazadeh-Attari, M., Sadighparvar, S., Kaviani, M., Shafiei-Irannejad, V., Kafil, H.S., Karimian, A., Jadidi-Niaragh, F. and Majidinia, M. (2019) 'Overexpression of tensin homolog deleted on chromosome ten (PTEN) by ciglitazone sensitizes doxorubicin-resistance leukemia cancer cells to treatment', *Journal of Cellular Biochemistry,* 120(9), pp. 15719-15729. doi: 10.1002/jcb.28841.

Matsuo, Y., MacLeod, R.A., Uphoff, C.C., Drexler, H.G., Nishizaki, C., Katayama, Y., Kimura, G., Fujii, N., Omoto, E., Harada, M. and Orita, K. (1997) 'Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23)', *Leukemia,* 11(9), pp. 1469-1477. doi: 10.1038/sj.leu.2400768.

McHowat, J., Swift, L.M., Arutunyan, A. and Sarvazyan, N. (2001) 'Clinical concentrations of doxorubicin inhibit activity of myocardial membrane-associated, calcium-independent phospholipase A(2)', *Cancer research,* 61(10), pp. 4024-4029.

Medline Plus (2012) *Doxorubicin.* Available at: https://medlineplus.gov/druginfo/meds/a682221.html#skip (Accessed: January 9).

Mendelsohn, A.R. and Larrick, J.W. (2014) 'Paradoxical Effects of Antioxidants on Cancer', *Rejuvenation Research,* 17(3), pp. 36-311. doi: 10.1089/rej.2014.1577.

Messingerova, L., Imrichova, D., Kavcova, H., Turakova, K., Breier, A. and Sulova, Z. (2015) 'Acute myeloid leukemia cells MOLM-13 and SKM-1 established for resistance by azacytidine are crossresistant to P-glycoprotein substrates', *Toxicology in Vitro,* 29(7), pp. 1405-1415. doi: 10.1016/j.tiv.2015.05.011.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004) 'Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity', *Pharmacological Reviews,* 56(2), pp. 185-229. doi: 10.1124/pr.56.2.6.

Mohamed, A.N. (2018) 'BCL2 (B-Cell Leukemia/Lymphoma 2)', *Atlas of Genetics and Cytogenetics in Oncology and Haematology,* (9). doi: 10.4267/2042/68938.

Moon, J.H., Sohn, S.K., Lee, M., Jang, J.H., Kim, K., Jung, C.W. and Kim, D.H. (2009) 'BCL2 gene polymorphism could predict the treatment outcomes in acute myeloid leukemia patients', *Leukemia Research,* 34(2), pp. 166-172. doi: 10.1016/j.leukres.2009.05.009.

Morishima, N., Nakanishi, K., Takenouchi, H., Shibata, T. and Yasuhiko, Y. (2002) 'An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12', *The Journal of biological chemistry,* 277(37), pp. 34287.

Mullauer, F.B., Kessler, J.H. and Medema, J.P. (2009) 'Betulinic acid induces cytochrome c release and apoptosis in a Bax/Bak-independent, permeability transition pore dependent fashion', *Apoptosis,* 14(2), pp. 191-202. doi: 10.1007/s10495-008-0290-x.

Mullauer, F., Kessler, J. and Medema, J. (2010) 'Betulinic acid, a natural compound with potent anticancer effects', *Anti-Cancer Drugs,* 21(3), pp. 215-227. doi: 10.1097/CAD.0b013e3283357c62.

National Cancer Institute (2019) *Drugs Approved for Leukemia.* Available at: https://www.cancer.gov/about-cancer/treatment/drugs/leukemia (Accessed: November 25).

Nikoletopoulou, V., Markaki, M., Palikaras, K. and Tavernarakis, N. (2013) 'Crosstalk between apoptosis, necrosis and autophagy', *BBA - Molecular Cell Research,* 1833(12), pp. 3448-3459. doi: 10.1016/j.bbamcr.2013.06.001.

O'Brien, J., Wilson, I., Orton, T. and Pognan, F. (2000) 'Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity', *European Journal of Biochemistry,* 267(17), pp. 5421-5426. doi: 10.1046/j.1432-1327.2000.01606.x.

O'Neill, K.L., Huang, K., Zhang, J., Chen, Y. and Luo, X. (2016) 'Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane', *Genes & development,* 30(8), pp. 973-988. doi: 10.1101/gad.276725.115.

Orrenius, S., Gogvadze, V. and Zhivotovsky, B. (2007) 'Mitochondrial Oxidative Stress: Implications for Cell Death', *Annual review of pharmacology and toxicology,* 47(1), pp. 143-183. doi: 10.1146/annurev.pharmtox.47.120505.105122.

Ouyang, L., Shi, Z., Zhao, S., Wang, F.-, Zhou, T.-, Liu, B. and Bao, J.- (2012) 'Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis', *Cell Proliferation,* 45(6), pp. 487-498. doi: 10.1111/j.1365-2184.2012.00845.x.

Pan, Y., Gao, Y., Chen, L., Gao, G., Dong, H., Yang, Y., Dong, B. and Chen, X. (2011) 'Targeting Autophagy Augments In Vitro and In Vivo Antimyeloma Activity of DNA-Damaging Chemotherapy', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 17(10), pp. 3248-3258. doi: 10.1158/1078-0432.CCR-10-0890.

Panaretakis, T., Laane, E., Pokrovskaja, K., Björklund, A., Moustakas, A., Zhivotovsky, B., Heyman, M., Shoshan, M.C. and Grandér, D. (2005) 'Doxorubicin Requires the Sequential Activation of Caspase-2, Protein Kinase Cδ, and c-Jun NH2-terminal Kinase to Induce Apoptosis', *Molecular Biology of the Cell,* 16(8), pp. 3821-3831. doi: 10.1091/mbc.e04-10-0862.

Panaretakis, T., Pokrovskaja, K., Shoshan, M., C. and Grander, D. (2002) 'Activation of Bak, Bax, and BH3-only Proteins in the Apoptotic Response to Doxorubicin', *Journal of Biological Chemistry,* 277(46), pp. 44317-44326. doi: 10.1074/jbc.M205273200.

Panno, J. (2004) *Cancer.* New York: Infobase Learning.

Park, S., Chapuis, N., Tamburini, J., Bardet, V., Cornillet-Lefebvre, P., Willems, L., Green, A., Mayeux, P., Lacombe, C. and Bouscary, D. (2010) 'Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia', *Haematologica,* 95(5), pp. 819-828. doi: 10.3324/haematol.2009.013797.

Pasquier, B. (2016) 'Autophagy inhibitors', *Cellular and Molecular Life Sciences,* 73(5), pp. 985-1001. doi: 10.1007/s00018-015-2104-y.

Patel, V.A., Longacre, A., Hsiao, K., Fan, H., Meng, F., Mitchell, J.E., Rauch, J., Ucker, D.S. and Levine, J.S. (2006) 'Apoptotic cells, at all stages of the death process, trigger characteristic signaling events that are divergent from and dominant over those triggered by necrotic cells: Implications for the delayed clearance model of autoimmunity', *The Journal of biological chemistry,* 281(8), pp. 4663-4670. doi: 10.1074/jbc.M508342200.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D. and Levine, B. (2005) 'Bcl-2 Antiapoptotic Proteins Inhibit Beclin 1-Dependent Autophagy', *Cell,* 122(6), pp. 927-939. doi: 10.1016/j.cell.2005.07.002.

Paul, M.K. and Mukhopadhyay, A.K. (2004) 'Tyrosine kinase – Role and significance in Cancer', *International journal of medical sciences,* 1(2), pp. 101-115. doi: 10.7150/ijms.1.101.

Pilco-Ferreto, N. and Calaf, G.M. (2016) 'Influence of doxorubicin on apoptosis and oxidative stress in breast cancer cell lines', *International Journal of Oncology,* 49(2), pp. 753-762. doi: 10.3892/ijo.2016.3558.

Pisha, E., Kinghorn, A.D., Lee, I., Pezzuto, J.M., Hieken, T.J., Brown, D.M., Wall, M.E., Das Gupta, T.K., Chagwedera, T.E., Wani, M.C., Cordell, G.A., Beecher, C.W.W., Farnsworth, N.R., Chai, H. and Fong, H.H.S. (1995) 'Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis', *Nature Medicine,* 1(10), pp. 1046-1051. doi: 10.1038/nm1095-1046.

Pizarro, M., Troncoso, R., Martínez, G.J., Chiong, M., Castro, P.F. and Lavandero, S. (2016) 'Basal autophagy protects cardiomyocytes from doxorubicin-induced toxicity', *Toxicology,* 370, pp. 41-48. doi: 10.1016/j.tox.2016.09.011.

Poon, I.K.H., Hulett, M.D. and Parish, C.R. (2010) 'Molecular mechanisms of late apoptotic necrotic cell clearance', *Cell Death and Differentiation,* 17(3), pp. 381-397. doi: 10.1038/cdd.2009.195.

Potze, L., Di Franco, S., Grandela, C., Pras-Raves, M.L., Picavet, D.I., van Veen, H.A., van Lenthe, H., Mullauer, F.B., van der Wel, N. N, Luyf, A., van Kampen, A. H. C, Kemp, S., Everts, V., Kessler, J.H., Vaz, F.M. and Medema, J.P. (2016) 'Betulinic acid induces a novel cell death pathway that depends on cardiolipin modification', *Oncogene,* 35(4), pp. 427-437. doi: 10.1038/onc.2015.102.

Potze, L., Mullauer, F.B., Colak, S., Kessler, J.H. and Medema, J.P. (2014) 'Betulinic acid-induced mitochondria-dependent cell death is counterbalanced by an autophagic salvage response', *Cell death & disease,* 5(4), pp. e1169. doi: 10.1038/cddis.2014.139.

Puissant, A., Robert, G., Fenouille, N., Luciano, F., Cassuto, J., Raynaud, S. and Auberger, P. (2010) 'Resveratrol Promotes Autophagic Cell Death in Chronic Myelogenous Leukemia Cells via JNK-Mediated p62/SQSTM1 Expression and AMPK Activation', *Cancer Research,* 70(3), pp. 1042-1052. doi: 10.1158/0008-5472.CAN-09-3537.

Pyo, J., Yoo, S., Ahn, H., Nah, J., Hong, S., Kam, T., Jung, S. and Jung, Y. (2013) 'Overexpression of Atg5 in mice activates autophagy and extends lifespan', *Nature communications,* 4(1), pp. 2300. doi: 10.1038/ncomms3300.

Qiu, Y., Li, P. and Ji, C. (2015) 'Cell Death Conversion under Hypoxic Condition in Tumor Development and Therapy', *International journal of molecular sciences,* 16(10), pp. 25536-25551. doi: 10.3390/ijms161025536.

Qiuling, W., Jing, H., Jun, F. and Mei, H. (2010) 'Antitumor effect of betulinic acid on human acute leukemia K562 cells in vitro', *Journal of Huazhong University of Science and Technology [Medical Sciences],* 30(4), pp. 453-457. doi: 10.1007/s11596-010-0448-y.

Quent, V.M.C., Loessner, D., Friis, T., Reichert, J.C. and Hutmacher, D.W. (2010) 'Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research', *Journal of Cellular and Molecular Medicine,* 14(4), pp. 1003-1013. doi: 10.1111/j.1582-4934.2010.01013.x.

Radogna, F., Dicato, M. and Diederich, M. (2015) 'Cancer-type-specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target', *Biochemical Pharmacology,* 94(1), pp. 1-11. doi: 10.1016/j.bcp.2014.12.018.

Raisova, M., Hossini, A.M., Eberle, J., Riebeling, C., Orfanos, C.E., Geilen, C.C., Wieder, T., Sturm, I. and Daniel, P.T. (2001) 'The Bax/Bcl-2 Ratio Determines the Susceptibility of Human Melanoma Cells to CD95/Fas-Mediated Apoptosis', *Journal of Investigative Dermatology,* 117(2), pp. 333-340. doi: 10.1046/j.0022-202x.2001.01409.x.

Rao, J., Xu, D., Zheng, F., Long, Z., Huang, S., Wu, X., Zhou, W., Huang, R. and Liu, Q. (2011) 'Curcumin reduces expression of Bcl-2, leading to apoptosis in daunorubicin-insensitive CD34+ acute myeloid leukemia cell lines and primary sorted CD34+ acute myeloid leukemia cells', *Journal of translational medicine,* 9(1), pp. 71. doi: 10.1186/1479-5876-9-71.

Reczek, C.R. and Chandel, N.S. (2017) 'The Two Faces of Reactive Oxygen Species in Cancer', *Annual Review of Cancer Biology,* 1(1), pp. 79-98. doi: 10.1146/annurev-cancerbio-041916-065808.

Redmann, M., Benavides, G.A., Wani, W.Y., Berryhill, T.F., Ouyang, X., Johnson, M.S., Ravi, S., Mitra, K., Barnes, S., Darley-Usmar, V.M. and Zhang, J. (2018) 'Methods for assessing mitochondrial quality control mechanisms and cellular consequences in cell culture', *Redox Biology,* 17, pp. 59-69. doi: 10.1016/j.redox.2018.04.005.

Reed, J.C. (2008) 'Bcl-2-family proteins and hematologic malignancies: history and future prospects', *Blood,* 111(7), pp. 3322-3330. doi: 10.1182/blood-2007-09-078162.

Reed, J.C., Cuddy, M., Slabiak, T., Croce, C.M. and Nowell, P.C. (1988) 'Oncogenic potential of bcl -2 demonstrated by gene transfer', *Nature,* 336(6196), pp. 259-261. doi: 10.1038/336259a0.

Reiter, K., Polzer, H., Krupka, C., Maiser, A., Vick, B., Rothenberg-Thurley, M., Metzeler, K.H., Dörfel, D., Salih, H.R., Jung, G., Nößner, E., Jeremias, I., Hiddemann, W., Leonhardt, H., Spiekermann, K., Subklewe, M. and Greif, P.A. (2018) 'Tyrosine kinase inhibition increases the cell surface localization of FLT3-ITD and enhances FLT3-directed immunotherapy of acute myeloid leukemia', *Leukemia,* 32(2), pp. 313-322. doi: 10.1038/leu.2017.257.

Renatus, M., Stennicke, H., Scott, F., Liddington, R. and Salvesen, G. (2001) 'Dimer Formation Drives the Activation of the Cell Death Protease Caspase 9', *Proceedings of the National Academy of Sciences of the United States of America,* 98(25), pp. 14250-14255. doi: 10.1073/pnas.231465798.

Rheingold, S., Neugut, A. and Meadows, A. (2003) 'Therapy-Related Secondary Cancer', in Kufe, D., Pollock, R., Weichselbaum, R., Bast, R., Gansler, T., Holland, J. and Frei, E. (eds.) *Holland-Frei Cancer Medicine*. 6th edn. Ontario, Hamilton: BC Decker.

Ríos, J.L. and Máñez, S. (2018) 'New Pharmacological Opportunities for Betulinic Acid', *Planta Medica,* 84(1), pp. 8-19. doi: 10.1055/s-0043-123472.

Riss, T., Moravec, R., Niels, A., Benink, H., Worzella, T. and Minor, L. (2013) *Cell Viability Assays.* Available at: https://www.ncbi.nlm.nih.gov/books/NBK144065/ (Accessed: July 1).

Rudolfová, P., Hanušová, V., Skálová, L., Bártíková, H., Matoušková, P. and Boušová, I. (2014) 'Effect of selected catechins on doxorubicin antiproliferative efficacy and hepatotoxicity in vitro', *Acta Pharmaceutica,* 64(2), pp. 199-209. doi: 10.2478/acph-2014-0018.

Rzeski, W., Stepulak, A., Szymański, M., Sifringer, M., Kaczor, J., Wejksza, K., Zdzisińska, B. and Kandefer-Szerszeń, M. (2006) 'Betulinic acid decreases expression of bcl-2 and cyclin D1, inhibits proliferation, migration and induces apoptosis in cancer cells', *Naunyn-Schmiedeberg's Archives of Pharmacology,* 374(1), pp. 11-20. doi: 10.1007/s00210-006-0090-1.

Safarzadeh, E., Sandoghchian Shotorbani, S. and Baradaran, B. (2014) 'Herbal Medicine as Inducers of Apoptosis in Cancer Treatment', *Advanced pharmaceutical bulletin,* 4(Suppl 1), pp. 421-427. doi: 10.5681/apb.2014.062.

Santos, R.C., Salvador, J.A.R., Marín, S. and Cascante, M. (2009) 'Novel semisynthetic derivatives of betulin and betulinic acid with cytotoxic activity', *Bioorganic & Medicinal Chemistry,* 17(17), pp. 6241-6250. doi: 10.1016/j.bmc.2009.07.050.

Sawada, N., Kataoka, K., Kondo, K., Arimochi, H., Fujino, H., Takahashi, Y., Miyoshi, T., Kuwahara, T., Monden, Y. and Ohnishi, Y. (2004) 'Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice', *British Journal of Cancer,* 90(8), pp. 1672-1678. doi: 10.1038/sj.bjc.6601746.

Scheinberg, D., A and Jurcic, J., C (2004) *Treatment of Leukemia and Lymphoma.* California: Elsevier Academic Press.

Schinkothe, T., Leistert, C. and Staib, P. (2006) 'Functional Characterization of the Bcl-2 Splice Variant Beta', *Blood,* 108(11), pp. 1451-1451.

Schöndorf, T., Becker, M., Göhring, U., Wappenschmidt, B., Kolhagen, H. and Kurbacher, C. (2001) 'Interaction of cisplatin, paclitaxel and adriamycin with the tumor suppressor PTEN', *Anti-Cancer Drugs,* 12(10), pp. 797-800. doi: 10.1097/00001813-200111000-00002.

Schott, C.R., Ludwig, L., Mutsaers, A.J., Foster, R.A. and Wood, G.A. (2018) 'The autophagy inhibitor spautin-1, either alone or combined with doxorubicin, decreases cell survival and colony formation in canine appendicular osteosarcoma cells', *PLoS ONE,* 13(10), pp. e0206427. doi: 10.1371/journal.pone.0206427.

Sebens Mueerkoester, S., Lust, J., Arlt, A., Haesler, R., Witt, M., Sebens, T., Schreiber, S., Foelsch, U.R. and Schaefer, H. (2006) 'Acquired chemoresistance in pancreatic carcinoma cells: induced secretion of IL-1b and NO lead to inactivation of caspases', *Oncogene,* 25(33), pp. 4628. doi: 10.1038/sj.onc.1209806.

Sellers, W., R and Fisher, D., E (1999) 'Apoptosis and cancer drug targeting', *The Journal of clinical investigation,* 104(12), pp. 1655-1661. doi: 10.1172/JCI9053.

Selzer, E., Pimentel, E., Wacheck, V., Schlegel, W., Pehamberger, H., Jansen, B. and Kodym, R. (2000) 'Effects of Betulinic Acid Alone and in Combination with Irradiation in Human Melanoma Cells', *Journal of Investigative Dermatology,* 114(5), pp. 935-940. doi: 10.1046/j.1523-1747.2000.00972.x.

Shah, U., Shah, R., Acharya, S. and Acharya, N. (2013) 'Novel anticancer agents from plant sources', *Chinese Journal of Natural Medicines,* 11(1), pp. 16-23. doi: 10.1016/S1875-5364(13)60002-3.

Shamas-Din, A., Kale, J., Leber, B. and Andrews, D.W. (2013) 'Mechanisms of Action of Bcl-2 Family Proteins', *Cold Spring Harbor perspectives in biology,* 5(4), pp. a008714. doi: 10.1101/cshperspect.a008714.

Shankar, E., Zhang, A., Franco, D. and Gupta, S. (2017) 'Betulinic Acid-Mediated Apoptosis in Human Prostate Cancer Cells Involves p53 and Nuclear Factor-Kappa B (NF-κB) Pathways', *Molecules (Basel, Switzerland),* 22(2), pp. 264. doi: 10.3390/molecules22020264.

Shi, S. and Cao, H. (2014) 'Shikonin promotes autophagy in BXPC-3 human pancreatic cancer cells through the PI3K/Akt signaling pathway', *Oncology letters,* 8(3), pp. 1087-1089.

Shimizu, S., Kanaseki, T., Mizuta, T., Thompson, C.B., Tsujimoto, Y., Mizushima, N. and Arakawa-Kobayashi, S. (2004) 'Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes', *Nature Cell Biology,* 6(12), pp. 1221-1228. doi: 10.1038/ncb1192.

Smuder, A.J., Kavazis, A.N., Min, K. and Powers, S.K. (2011) 'Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle', *Journal of Applied Physiology,* 111(4), pp. 1190-1198. doi: 10.1152/japplphysiol.00429.2011.

Soengas, M.S. and Lowe, S.W. (2003) 'Apoptosis and melanoma chemoresistance', *Oncogene,* 22(20), pp. 3138-3151. doi: 10.1038/sj.onc.1206454.

Song, G., Ouyang, G. and Bao, S. (2005) 'The activation of Akt/PKB signaling pathway and cell survival', *Journal of Cellular and Molecular Medicine,* 9(1), pp. 59-71. doi: 10.1111/j.1582-4934.2005.tb00337.x.

Su, Y., Li, X., Ma, J., Zhao, J., Liu, S., Wang, G., Edwards, H., Taub, J.W., Lin, H. and Ge, Y. (2018) 'Targeting PI3K, mTOR, ERK, and Bcl-2 signaling network shows superior antileukemic activity against AML ex vivo', *Biochemical Pharmacology,* 148, pp. 13-26. doi: 10.1016/j.bcp.2017.11.022.

Su, Z., Yang, Z., Xu, Y., Chen, Y. and Yu, Q. (2015) 'Apoptosis, autophagy, necroptosis, and cancer metastasis', *Molecular Cancer,* 14(1), pp. 48. doi: 10.1186/s12943-015-0321-5.

Szwed, M. and Jozwiak, Z. (2014) 'Genotoxic effect of doxorubicin–transferrin conjugate on human leukemia cells', *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 771, pp. 53-63. doi: 10.1016/j.mrgentox.2014.06.007.

Tait, S.W.G., Ichim, G. and Green, D.R. (2014) 'Die another way – non-apoptotic mechanisms of cell death', *Journal of cell science,* 127(Pt 10), pp. 2135-2144. doi: 10.1242/jcs.093575.

Takemura, G. and Fujiwara, H. (2007) 'Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management', *Progress in cardiovascular diseases,* 49(5), pp. 330-352.

Tallman, M.S., Gilliland, D.G. and Rowe, J.M. (2005) 'Drug therapy for acute myeloid leukemia', *Blood,* 106(4), pp. 1154-1163. doi: 10.1182/blood-2005-01-0178.

Tan, Y., Yu, R. and Pezzuto, J. (2003) 'Betulinic Acid-induced Programmed Cell Death in Human Melanoma Cells Involves Mitogen-activated Protein Kinase Activation', *Clinical Cancer Research,* 9(7), pp. 2866.

Tanaka, S., Saito, K. and Reed, J.C. (1993) 'Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival', *Journal of Biological Chemistry,* 268(15), pp. 10920-10926.

Tang, H.M. and Tang, H.L. (2018) 'Correction to: 'Anastasis: recovery from the brink of cell death'', *Royal Society open science,* 5(10), pp. 181629. doi: 10.1098/rsos.181629.

Tanida, I., Ueno, T. and Kominami, E. (2008) 'LC3 and Autophagy', *Methods in molecular biology (Clifton, N.J.),* 445, pp. 77-88. doi: 10.1007/978-1-59745-157-4_4.

Tharkar-Promod, S., Johnson, D.P., Bennett, S.E., Dennis, E.M., Banowsky, B.G., Jones, S.S., Shearstone, J.R., Quayle, S.N., Min, C., Jarpe, M., Mosbruger, T., Pomicter, A.D., Miles, R.R., Chen, W.Y., Bhalla, K.N., Zweidler-McKay, P.A., Shrieve, D.C., Deininger, M.W., Chandrasekharan, M.B. and Bhaskara, S. (2018) 'HDAC1,2 inhibition and doxorubicin impair Mre11-dependent DNA repair and DISC to override BCR-ABL1-driven DSB repair in Philadelphia chromosome-positive B-cell precursor acute lymphoblastic leukemia', *Leukemia,* 32(1), pp. 49-60. doi: 10.1038/leu.2017.174.

Theml, H. (2004) *Color atlas of hematology : practical microscopic and clinical diagnosis.* 2nd edn. Germany: Thieme Flexibook.

Thurnher, D., Turhani, D., Pelzmann, M., Wannemacher, B., Knerer, B., Formanek, M., Wacheck, V. and Selzer, E. (2003) 'Betulinic acid: A new cytotoxic compound against malignant head and neck cancer cells', *Head & Neck,* 25(9), pp. 732-740. doi: 10.1002/hed.10231.

Twiddy, D. and Cain, K. (2007) 'Caspase-9 cleavage, do you need it?', *The Biochemical journal,* 405(1), pp. e1. doi: 10.1042/BJ20070617.

Usmani, S., Sivagnanalingam, U., Tkachenko, O., Nunez, L., Shand, J.C. and Mullen, C.A. (2019) 'Support of acute lymphoblastic leukemia cells by nonmalignant bone marrow stromal cells', *Oncology letters,* 17(6), pp. 5039-5049. doi: 10.3892/ol.2019.10188.

Vadivelu, R., Yeap, S., Ali, A., Hamid, M. and Alitheen, N. (2012) 'Betulinic Acid Inhibits Growth of Cultured Vascular Smooth Muscle Cells In Vitro by Inducing G1 Arrest and Apoptosis', *Evidence - Based Complementary and Alternative Medicine,* 2012. doi: 10.1155/2012/251362.

Vanden Berghe, T., Vanlangenakker, N., Parthoens, E., Deckers, W., Devos, M., Festjens, N., Guerin, C.J., Brunk, U.T., Declercq, W. and Vandenabeele, P. (2010) 'Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features', *Cell Death and Differentiation,* 17(6), pp. 922-930. doi: 10.1038/cdd.2009.184.

Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellström-Lindberg, E., Tefferi, A. and Bloomfield, C.D. (2009) 'The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes', *Blood,* 114(5), pp. 937-951. doi: 10.1182/blood-2009-03-209262.

Viji, V., Shobha, B., Kavitha, S.K., Ratheesh, M., Kripa, K. and Helen, A. (2010) 'Betulinic acid isolated from Bacopa monniera (L.) Wettst suppresses lipopolysaccharide stimulated interleukin-6 production through modulation of nuclear factor-κB in peripheral blood mononuclear cells', *International Immunopharmacology,* 10(8), pp. 843-849. doi: 10.1016/j.intimp.2010.04.013.

Vogler, M., Dinsdale, D., Dyer, M.J.S. and Cohen, G.M. (2013) 'ABT-199 selectively inhibits BCL2 but not BCL2L1 and efficiently induces apoptosis of chronic lymphocytic leukaemic cells but not platelets', *British Journal of Haematology,* 163(1), pp. 139-142. doi: 10.1111/bjh.12457.

Vogler, M., Walter, H.S. and Dyer, M.J.S. (2017) 'Targeting anti-apoptotic BCL2 family proteins in haematological malignancies – from pathogenesis to treatment', *British Journal of Haematology,* 178(3), pp. 364-379. doi: 10.1111/bjh.14684.

Wang, S., Konorev, E.A., Kotamraju, S., Joseph, J., Kalivendi, S. and Kalyanaraman, B. (2004) 'Doxorubicin Induces Apoptosis in Normal and Tumor Cells via Distinctly Different Mechanisms', *Journal of Biological Chemistry,* 279(24), pp. 25535-25543. doi: 10.1074/jbc.M400944200.

Wang, T., Jin, Y., Yang, W., Zhang, L., Jin, X., Liu, X., He, Y. and Li, X. (2017) 'Necroptosis in cancer: An angel or a demon?', *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine,* 39(6), pp. 1010428317711539.

Wang, Y., Chen, B., Wang, Z., Zhang, W., Hao, K., Chen, Y., Li, K., Wang, T., Xie, Y., Huang, Z. and Tong, X. (2016) 'Marsdenia tenacissimae extraction (MTE) inhibits the proliferation and induces the apoptosis of human acute T cell leukemia cells through inactivating PI3K/AKT/mTOR signaling pathway via PTEN enhancement', *Oncotarget,* 7(50), pp. 82851-82863. doi: 10.18632/oncotarget.12654.

Warren, C.F.A., Wong-Brown, M.W. and Bowden, N.A. (2019) 'BCL-2 family isoforms in apoptosis and cancer', *Cell death & disease,* 10(3), pp. 177. doi: 10.1038/s41419-019-1407-6.

Weber, D., Zhang, M., Zhuang, P., Zhang, Y., Wheat, J. and Currie, G. (2017) 'The Efficacy of Andrographolide and its Combination with Betulinic Acid in the Treatment of Triple Negative Breast Cancer', *Cancer therapy & Oncology International Journal,* 4(1). doi: 10.19080/CTOIJ.2017.04.555628.

Wei, G., Margolin, A., Haery, L., Brown, E., Cucolo, L., Julian, B., Shehata, S., Kung, A., Beroukhim, R. and Golub, T. (2012) 'Chemical Genomics Identifies Small-Molecule MCL1 Repressors and BCL-xL as a Predictor of MCL1 Dependency', *Cancer Cell,* 21(4), pp. 547-562. doi: 10.1016/j.ccr.2012.02.028.

Whitaker, D. and Green, D. (2014) 'What are the long-term effects of treatment on survivors of childhood leukaemia? A review of the literature', *Journal of Radiotherapy in Practice,* 13(3), pp. 365-370. doi: 10.1017/S146039691300040X.

WHO (2014) *Chronic Myelogenous Leukemia.* Available at: https://www.who.int/selection_medicines/committees/expert/20/applications/cancer/en/ (Accessed: December 10).

Wu, J., Lai, C., Badmaev, V., Nagabhushanam, K., Ho, C. and Pan, M. (2011) 'Tetrahydrocurcumin, a major metabolite of curcumin, induced autophagic cell death through coordinative modulation of PI3K/Akt-mTOR and MAPK signaling pathways in human leukemia HL-60 cells', *Molecular Nutrition & Food Research,* 55(11), pp. 1646-1654. doi: 10.1002/mnfr.201100454.

Xu, T., Pang, Q., Wang, Y. and Yan, X. (2017) 'Betulinic acid induces apoptosis by regulating PI3K/Akt signaling and mitochondrial pathways in human cervical cancer cells', *International Journal of Molecular Medicine,* 40(6), pp. 1669-1678. doi: 10.3892/ijmm.2017.3163.

Xu, T., Pang, Q., Zhou, D., Zhang, A., Luo, S., Wang, Y. and Yan, X. (2014) 'Proteomic Investigation into Betulinic Acid-Induced Apoptosis of Human Cervical Cancer HeLa Cells', *PloS one,* 9(8), pp. e105768. doi: 10.1371/journal.pone.0105768.

Yang, F., Teves, S.S., Kemp, C.J. and Henikoff, S. (2014) 'Doxorubicin, DNA torsion, and chromatin dynamics', *BBA - Reviews on Cancer,* 1845(1), pp. 84-89. doi: 10.1016/j.bbcan.2013.12.002.

Yang, L., Chen, Y., He, J., Yi, S., Wen, L., Zhao, J., Zhang, B., P and Cui, G., H (2012b) 'Betulinic acid inhibits autophagic flux and induces apoptosis in human multiple myeloma cells in vitro', *Acta Pharmacologica Sinica,* 33(12), pp. 1542-1548. doi: 10.1038/aps.2012.102.

Yang, S., Liang, N., Li, H., Xue, W., Hu, D., Jin, L., Zhao, Q. and Yang, S. (2012a) 'Design, synthesis and biological evaluation of novel betulinic acid derivatives', *Chemistry Central Journal,* 6(1), pp. 1-9. doi: 10.1186/1752-153X-6-141.

Yang, Y., Hu, L., Zheng, H., Mao, C., Hu, W., Xiong, K., Wang, F. and Liu, C. (2013) 'Application and interpretation of current autophagy inhibitors and activators', *Acta Pharmacol Sin,* 34(5), pp. 625-635. doi: 10.1038/aps.2013.5.

Yazan, L., Ahmad, F., Li, O., Rahim, R., Hamid, H. and Sze, L. (2009) 'Betulinic acid was more cytotoxic towards the human breast cancer cell line MDA-MB-231 than the human promyelocytic leukaemia cell line HL-60', *Malaysian Journal of Pharmaceutical Sciences,* 7(1), pp. 23-37.

Yi, J., Xia, W., Wu, J., Yuan, L., Wu, J., Tu, D., Fang, J. and Tan, Z. (2014) 'Betulinic acid prevents alcohol-induced liver damage by improving the antioxidant system in mice', *Journal of Veterinary Science,* 15(1), pp. 141-148. doi: 10.4142/jvs.2014.15.1.141.

Yi, J., Zhu, R., Wu, J., Wu, J., Xia, W., Zhu, L., Jiang, W., Xiang, S. and Tan, Z. (2016) 'In vivo protective effect of betulinic acid on dexamethasone induced thymocyte apoptosis by reducing oxidative stress', *Pharmacological Reports,* 68(1), pp. 95-100. doi: 10.1016/j.pharep.2015.07.003.

Yip, K.W. and Reed, J.C. (2008) 'Bcl-2 family proteins and cancer', *Oncogene,* 27(50), pp. 6398-6406. doi: 10.1038/onc.2008.307.

Yndestad, S., Austreid, E., Svanberg, I.R., Knappskog, S., Lønning, P.E. and Eikesdal, H.P. (2017) 'Activation of Akt characterizes estrogen receptor positive human breast cancers which respond to anthracyclines', *Oncotarget,* 8(25), pp. 41227-41241. doi: 10.18632/oncotarget.17167.

Yoon, Y., Kim, Y.O., Lim, N.Y., Jeon, W.K. and Sung, H.J. (1999) 'Shikonin, an Ingredient of Lithospermum erythrorhizon Induced Apoptosis in HL60 Human Premyelocytic Leukemia Cell Line', *Planta medica,* 65(6), pp. 532-535. doi: 10.1055/s-1999-14010.

Yuan, T., Yang, Y., Chen, J., Li, W., Li, W., Zhang, Q., Mi, Y., Goswami, R.S., You, J.Q., Lin, D., Qian, M.D., Calin, S., Liang, Y., Miranda, R.N., Calin, G.A., Zhou, X., Ma, L., Zweidler-McKay, P.A., Liu, B., Weng, A.P., Medeiros, L.J., Zhang, Y. and You, M.J. (2017) 'Regulation of PI3K signaling in T-cell acute lymphoblastic leukemia: a novel PTEN/Ikaros/miR-26b mechanism reveals a critical targetable role for PIK3CD', *Leukemia,* 31(11), pp. 2355-2364. doi: 10.1038/leu.2017.80.

Zahreddine, H. and Borden, K.L.B. (2013) 'Mechanisms and insights into drug resistance in cancer', *Frontiers in pharmacology,* 4, pp. 28. doi: 10.3389/fphar.2013.00028.

Zhao, Z., Wang, J., Tang, J., Liu, X., Zhong, Q., Wang, F., Hu, W., Yuan, Z., Nie, C. and Wei, Y. (2012) 'JNK- and Akt-mediated Puma expression in the apoptosis of cisplatin-resistant ovarian cancer cells', *The Biochemical journal,* 444(2), pp. 291-301. doi: 10.1042/BJ20111855.

Zheng, Z., Song, S., Wu, Y., Lian, L., Wan, Y. and Nan, J. (2011) 'Betulinic acid prevention of d-galactosamine/lipopolysaccharide liver toxicity is triggered by activation of Bcl-2 and antioxidant mechanisms', *Journal of Pharmacy and Pharmacology,* 63(4), pp. 572-578. doi: 10.1111/j.2042-7158.2010.01239.x.

Zhivotovsky, B., Samali, A., Gahm, A. and Orrenius, S. (1999) 'Caspases: their intracellular localization and translocation during apoptosis', *Cell death and differentiation,* 6(7), pp. 644-651. doi: 10.1038/sj.cdd.4400536.

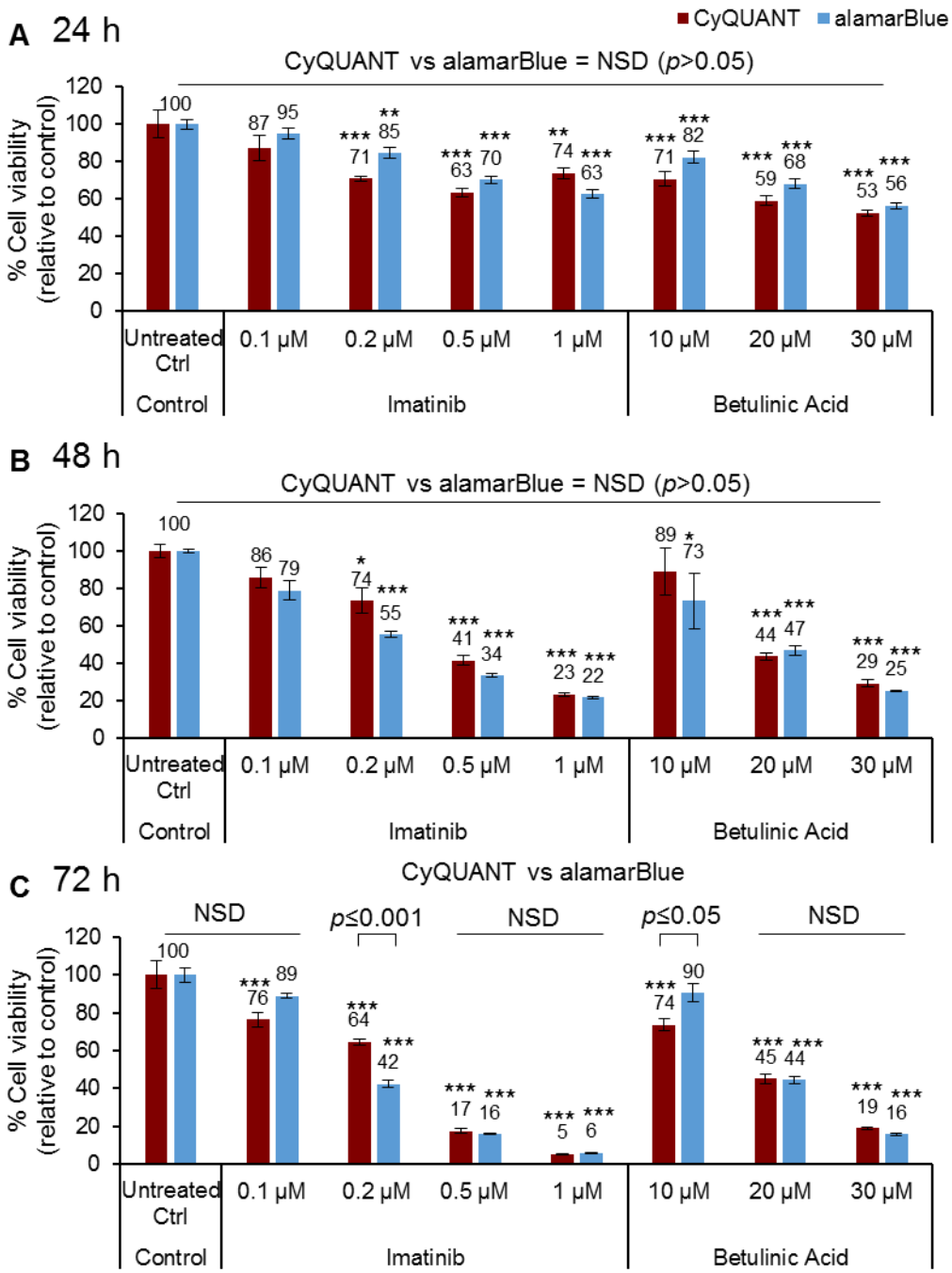
Zinkel, S., Gross, A. and Yang, E. (2006) 'BCL2 family in DNA damage and cell cycle control', *Cell Death and Differentiation,* 13(8), pp. 1351-1359. doi: 10.1038/sj.cdd.4401987.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., Sollott, S.J. and Zorov, D.B. (2018) 'Mitochondrial membrane potential', *Analytical biochemistry,* 552, pp. 50-59. doi: 10.1016/j.ab.2017.07.009.

Zuco, V., Supino, R., Righetti, S.C., Cleris, L., Marchesi, E., Gambacorti-Passerini, C. and Formelli, F. (2002) 'Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells', *Cancer Letters,* 175(1), pp. 17-25. doi: 10.1016/S0304-3835(01)00718-2.

Appendix

Appendix 1 Comparison between CyQUANT Direct® and alamarBlue™ cell viability measurements



Supplementary Figure 1 K562 cell viability after imatinib and betulinic acid treatments
Representative preliminary data of CML cell line K562 treated with TKI drug imatinib (0.1-1 µM) and BetA (10-30 µM) for 24 h **(A)**, 48 h **(B)**, and 72 h **(C)**. n=5. Cell viability was determined by fluorescence using CyQUANT® Direct assay and alamarBlue™ assay. The data was analysed using One-Way ANOVA with Tukey post-hoc test. Statistical difference was accepted as following: $p > 0.05$ no significant difference (NSD), *$p \leq 0.05$ significant, **$p \leq 0.01$ highly significant, ***$p \leq 0.001$ very highly significant.