



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

**The apoptotic effect of copper-imidazo[1,2-*a*]pyridine
complexes on leukaemic cells**

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A dissertation submitted to the Faculty Health Sciences, University of the Witwatersrand,
Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy
(Pharmacology)

Johannesburg, 2020

Declaration

I, Zeenat Ismail, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

Zeenat Ismail

On this.....day of.....2020

Dedication

I proudly dedicate this dissertation to my loving parents and siblings

For their endless love and faith in me

This is only by the Grace of my Lord

“Seek knowledge from the Cradle to the Grave.”

-Prophet Muhammad (PBUH)

Abstract

From the total numbers of leukaemia cases globally, 78% occur in developing countries. In South Africa, haematological malignancies account for 6% of new cancer cases and 8% of cancer deaths yearly. Despite the availability of targeted therapy, current treatments for acute promyelocytic leukaemia and chronic myelocytic leukaemia are still met with challenges. Therapeutic failure due to primary and acquired drug resistance as well as severe adverse effects warrants the search for new anti-leukaemic agents. Small molecules like imidazo[1,2-*a*]pyridines are considered to be biologically active scaffolds. These molecules complexed to metals such as copper and platinum are being actively explored for their anti-cancer potential.

A series of imidazo[1,2-*a*]pyridine derivatives were synthesized and complexed to either copper or zinc to form metal complexes. These imidazo[1,2-*a*]pyridine derivatives were evaluated for their ability to inhibit leukaemic cell growth and induce apoptosis using two leukaemic cell lines; HL-60 and K562. This part of the study identified imidazo[1,2-*a*]pyridines complexed with copper as having superior anti-cancer activity. Three copper-imidazo[1,2-*a*]pyridines; JD88, JD47R and JD49R were most active against HL-60 and K562 cells at low micromolar concentrations ranging between 1.0 and 6.0 μ M.

Morphological evaluation indicated that cell death was caused by apoptosis. The ability of the active compounds to cause apoptosis was confirmed by the Annexin-V binding assay and the fluorimetric caspase-3/7 assay. Following this, the ability of the compounds to activate the intrinsic apoptotic pathway was investigated and confirmed with the following tests: mitochondrial membrane depolarisation, caspase-8 activity and caspase-9 activity assays. Changes to the mitochondrial membrane potential were determined with the JC-1 cationic dye. An increase in Annexin-V binding, caspase-3/7, caspase-9 activity and a loss of mitochondrial membrane potential by the copper complexes indicated activation of the intrinsic apoptotic pathway.

In order to better understand the cell death pathways by which these compounds cause cell death, apoptotic regulatory proteins and the apoptotic transcriptome were investigated in HL-60 and K562 leukaemic cell lines. A Proteome Profiler™ Human Apoptosis Array measured the protein expression levels of 35 apoptotic-regulatory proteins. Proteomic analysis showed an increase in cleaved caspase-3 protein, confirming apoptosis. In both cell lines, the expression of pro-apoptotic proteins Bax and Smac/DIABLO were increased. The expression

of inhibitor of apoptosis proteins, XIAP, cIAP-1 and survivin, were decreased, thereby reducing the apoptotic threshold.

A real-time quantitative polymerase chain reaction (RT-qPCR) array was used to quantify the mRNA levels of apoptotic regulators in the presence of the copper complexes. A decrease in Akt gene expression and nuclear factor-kappa B (NF- κ B) suggested the inhibition of NF- κ B activity. The mRNA transcript levels of non-canonical NF- κ B regulated target genes were decreased, suggesting an inhibitory effect on NF- κ B inducing kinase (NIK), preventing non-canonical NF- κ B activation. Furthermore, expression of various key components of tumour necrosis factor signalling like TNF- α , TRADD, TNF-R1 TNF-R2, TRAF2, TRAF3 and RIPK was suppressed.

The copper complexes caused intrinsic apoptosis in both leukaemic cell lines and decreased the expression levels of key cell pro-survival proteins while increasing pro-apoptotic protein expression. This appears to be mediated by inhibiting the Akt kinase activity and the transcriptional activity of the NF- κ B pathway, which may be central to the apoptotic effect of the copper complexes. These copper complexes serve as valuable tools to better understand the intricacy of the death signalling pathways in leukaemic cells. The data presented here supports the further pre-clinical evaluation of the copper-imidazo[1,2-*a*]pyridines. This should include evaluation of efficacy in animal models of leukaemia, acute and chronic toxicity studies and the determination of pharmacokinetic properties.

Acknowledgments

In the name of Allah, The Most Gracious, The Most Merciful,

I would like to acknowledge the following people who have been fundamental in my journey towards completing this dissertation:

- To my supervisor, Dr Leonie Harmse, thank you for your continuous support and unwavering guidance. Your sage advice, constructive criticism and encouragement aided the completion of this thesis in numerous ways. Thank you for your patience, understanding and commitment. There are not enough words to express my gratitude towards you.
- To my co-supervisor, Dr Clement Penny, thank you for your insight and knowledge into the field of molecular biology. I am grateful for your efforts.
- Professor Charles de Koning, from the Wits School of Chemistry, thank you for your continuous financial support throughout this research project.
- To my fellow colleagues over the years, I am extremely grateful to all of you for our constructive discussions and for keeping me sane. We shared this experience together. Each and every one of you has become a lifelong friend.
- To all my friends who have become family; Bhavani, Razeena, Raeesa, Saajida and Nadia thank you for being my favourite source of comfort, focus and entertainment throughout this journey.
- To my brothers, Kabeer and Taahir, thank you for being there throughout this experience, for always protecting me and for your unconditional love. I love and cherish you guys!
- To my “ride-or-die’s”, my travel buddies, my parents, without whom none of my success would be possible.
 - Thank you Dad, for the many sacrifices you have made in order to give me the best education. Your unconditional love, support and faith in me has driven me to set high standards and, given me the confidence to achieve them.
 - Thank you Mom, for teaching me that even the largest task can be accomplished as long as I have God by my side. Your strength and resilience inspires me every day. Thank you for showering my life with undying love, support and encouragement.
- Above all, to Almighty Allah, the all-encompassing, author of knowledge and wisdom, for without Him none of this would have been possible. All my success is due to His will and I am very grateful for all that He has blessed me with.

Funding

A huge thank you to all the funding bodies that contributed to this research project:

- The University of the Witwatersrand for the Postgraduate Merit Award Scholarship
- The National Research Foundation, Blue Skies Grant
- The McGill Bequest to Wits Pharmacology department
- The University of the Witwatersrand Faculty Research Committee

Research output

Conferences contributions

Ismail Z, de Koning CB, Dam J, Penny C and Harmse L. Poster presentation titled: Copper-imidazo[1,2-*a*]pyridines decrease the transcription of NF- κ B target genes in leukaemic cells. Oral presentation titled: Copper-imidazo[1,2-*a*]pyridines stimulate the intrinsic apoptotic pathway and inhibit the protein expression of negative apoptotic regulators in leukaemic cells. Presented at the South African Society of Basic and Clinical Pharmacology (SASBCP) 2019 in Kievits Kroon, Kameeldrift, Pretoria, South Africa, *5-7 October 2019*

Ismail Z, de Koning CB, Dam J, Penny C and Harmse L. Poster presentation titled: Copper-imidazo[1,2-*a*]pyridines decrease the transcription of NF- κ B target genes in leukaemic cells. Oral presentation titled: Copper-imidazo[1,2-*a*]pyridines stimulate the intrinsic apoptotic pathway and inhibit the protein expression of negative apoptotic regulators in leukaemic cells. Presented at the School of Therapeutic Sciences Biennial Research day at the Public health building, Wits Education Campus, Johannesburg, South Africa, *10 September 2019*

Ismail Z, de Koning CB, Dam J, Penny C and Harmse L. Copper imidazo[1,2-*a*]pyridine complexes induce apoptosis in K562 and HL-60 leukaemic cells. Poster presentation at the LSIF Research Day at St John's College, Johannesburg, South Africa, *24 October 2018*

Ismail Z, de Koning CB, Dam J, Penny C and Harmse L. Apoptotic effects of copper imidazo[1,2-*a*]pyridine complexes on HL-60 and K562 leukaemic cells. Poster presentation at the Faculty Research day 2018 at Wits Medical School, Johannesburg, South Africa, *6 September 2018*

Ismail Z, de Koning CB, Dam J, Penny C and Harmse L. Copper imidazo[1,2-*a*]pyridine complexes induce apoptosis in K562 and HL-60 leukaemic cells. Poster presentation at the 18th World Congress of Basic and Clinical Pharmacology (WCP2018) in Kyoto International Convention Centre, Kyoto, Japan, *01-06 July 2018*

Ismail Z, de Koning CB, Dam J and Harmse L. Imidazo[1,2-*a*]pyridines induce apoptosis in K562 and HL-60 leukaemic cells. Poster presentation at the Wits Cancer Research Symposium at University of the Witwatersrand, Johannesburg, South Africa, *9 February 2017*

Ismail Z, de Koning CB, Dam J and Harmse L. Imidazo[1,2-*a*]pyridines induce apoptosis in K562 and HL-60 leukaemic cells. Poster presentation at the All Africa Congress on Pharmacology and Pharmacy at Misty Hills Hotel and Conference centre, Muldersdrift Gauteng, South Africa, *5 October- 8 October 2016*

Ismail Z, de Koning CB, Dam J and Harmse L. Imidazo[1,2-*a*]pyridines induce apoptosis in K562 and HL-60 leukaemic cells. Oral presentation at the Biennial Research Day and Postgraduate Expo 2016, at The University of the Witwatersrand, Johannesburg, South Africa, *1 September 2016*

Ismail Z, de Koning CB, Dam J and Harmse L. Imidazo-[1, 2-*a*]-pyridine Derivatives Potential as Anti-Leukaemic Agents. Oral presentation at the School of Therapeutic Sciences Research Day 2015 at Khanya Block, Wits Education Campus, Johannesburg, South Africa, *8 September 2015*

Ismail Z, de Koning CB, Dam J and Harmse L. The anti-leukaemic activity of novel imidazo-[1, 2-*a*]-pyridine derivatives. Oral presentation at the SASBCP-TOXSA-WITS Congress 2015 at the Wits Club, University of the Witwatersrand, Johannesburg, South Africa, *31 August- 2 September 2015*

Ismail Z, de Koning CB, Dam J and Harmse L. Novel imidazo[1,2-*a*]pyridines as potential anti-leukaemic agents. Oral presentation at the 6th Cross Faculty Research Symposium 2014 at Senate House, University of the Witwatersrand, Johannesburg, South Africa, *28-29 October 2014*

Ismail Z, de Koning CB, Dam J and Harmse L. *In vitro* anti-leukaemic activity of imidazo[1, 2-*a*]-pyridines. Poster presentation at the Faculty of Health Sciences Biennial Research Day and Postgraduate Expo Research Day at the University of the Witwatersrand Medical School, Johannesburg, South Africa, *16-17 September 2014*

Ismail Z, de Koning CB, Dam J and Harmse L. Novel imidazo-[1, 2-*a*]-pyridines as potential anti-leukaemic agents. Poster presentation at the 17th World Congress of Basic and Clinical Pharmacology (WCP2014) held at the Cape Town International Convention Centre, South Africa, *12 -18 July 2014*

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- 2019** Awarded second place for poster presentation at the School of Therapeutic Sciences Biennial Research day 2019
- 2019** Awarded Best young scientist poster presentation at SASBCP 2019 congress

Publications

Dam, J., Ismail, Z., Kurebwa, T., Gangat, N., Harmse, L., Marques, H., Lemmerer, A., Bode, M.L. and de Koning, C (2017) Synthesis of copper and zinc 2-(pyridin-2-yl)imidazo[1,2-a]pyridine complexes and their potential anticancer activity. *European Journal of Medicinal Chemistry*, 126, 353-368. Doi: <http://dx.doi.org/10.1016/j.ejmech.2016.10.041>

Ismail Z, de Koning CB, Dam J and Harmse L. Novel imidazo-[1, 2-a]-pyridines as anti leukaemic agents. *Journal of Basic and Clinical Pharmacology and Toxicology Volume 115*, 134-135. July 2014

Manuscripts in preparation

Two manuscripts are in preparation and will be submitted for publication in 2020:

Title: Copper complexed imidazo[1,2-a]pyridines induces intrinsic apoptosis in leukaemic HL-60 and K562 cell lines. **Journal:** *Apoptosis*. (Subject to change)

Authors: Ismail Z, de Koning CB, Dam J and Harmse L

Title: Copper-imidazo[1,2-a]pyridines decrease the transcription of NF-κB target genes in leukaemic cells. **Journal:** *Cell death discovery*. (Subject to change)

Authors: Ismail Z, de Koning CB, Dam J, Penny C and Harmse L

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Abbreviations, acronyms and symbols

ABL	Abelson murine leukaemia viral oncogene homolog
AFC	7-amino-4-trifluoromethylcoumarin
AIF	Apoptosis inducing factor
Akt	Serine/ threonine kinase or Protein kinase B
ALL	Acute lymphocytic leukaemia
AML	Acute myelocytic leukaemia
AO	Acridine orange
Apaf-1	Apoptotic protease-activating factor 1
APL	Acute promyelocytic leukaemia
ASK-1	Apoptosis signal-regulated kinase 1
ATM	Ataxia-telangiectasia mutated
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATRA	All- <i>trans</i> retinoic acid
Bad	Bcl-2 associated death promoter protein
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-x	B-cell lymphoma-extra-large protein
BCR	Breakpoint cluster region gene
Bfl-1	Bcl-2-related gene expressed in fetal liver
BH	Bcl-2 homology domain
Bid	BH3 interacting-domain protein
Bik	Bcl-2-interacting killer (apoptosis-inducing)
Bim	Bcl-2-interacting mediator of cell death
BSA	Bovine serum albumin
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
CDK	Cyclin-dependent kinases
c-FLIP	Cellular fllice-like inhibitory protein
ChK	Checkpoint kinase

CLL	Chronic lymphocytic leukaemia
CML	Chronic myelocytic leukaemia
CO ₂	Carbon dioxide
Cu	Copper
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein with low PI
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
DTT	Dithiothreitol
DYRK2	Dual-specificity tyrosine-phosphorylation-regulated kinase 2
E2F	E2 promoter binding transcription factor
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase-1/-2
EtBr	Ethidium bromide
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FoxO	Forkhead family of transcription factor
<i>g</i>	Gravity
g/mol	Grams per mole
G ₀	Quiescence
G ₁	First growth phase of the cell cycle
G ₂	Second growth phase of the cell cycle
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HIF-1 α	Hypoxia inducible factor-1 alpha
HIPK2	Homeodomain interacting protein kinase 2
HO	Hoechst-33342
HRP	Horse radish peroxidase

HSP	Heat shock protein
HTRA2	High temperature requirement protein A2
IAP	Inhibitor of apoptotic protein
IC ₅₀	Half maximal inhibitory concentration
IC ₇₅	75% inhibitory concentration
ICAD	Inhibitor of the caspase-activated deoxyribonuclease
IκB	Inhibitor of Kappa-B
IKKα	IκB kinase α
IL	Interleukin
JAK	Janus kinase
JC-1	5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodine
JNK	c-Jun N-terminal kinases
KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
M	Mitosis
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minutes 2
MMP	Mitochondrial membrane potential
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NF-κB	Nuclear factor kappa for activated B cells
p53	Tumour suppressor protein 53
PARP-1	Poly-ADP-ribose polymerase-1
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
Ph	Philadelphia chromosome
PI	Propidium iodide
PI3K	Phosphoinositol-3 kinase

PKB/Akt	Protein kinase B
<i>PML</i>	Promyelocytic leukaemia gene
PS	Phosphatidylserine
PTEN	Tumour suppressor phosphatase and tensin homolog
PUMA	p53 up-regulated modulator of apoptosis
QTc	Corrected QT interval
<i>RARA</i>	Retinoic acid receptor-alpha
Rb	Retinoblastoma protein
RIP1	Receptor interacting protein-1
RIPK	Receptor interacting protein kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT-qPCR	Real-time quantitative polymerase chain reactions
SD	Standard deviation
SEM	Standard error of the mean
Smac	Second mitochondria-derived activator of caspase
STAT	Signal transducer and activator of transcription
TKI	Tyrosine kinase inhibitor
TNF α	Tumour necrosis factor-alpha
TNF-R	Tumour necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis
α	Alpha
β	Beta
κ	Kappa
λ	Lambda

Units of Measure

µg	Micrograms
µl	Microliter
µM	Micromolar
cm ³	Cubic centimetre
Da	Dalton
kDa	kiloDalton
L	Litre
M	Molar
mg	Milligrams
min	Minutes
hr	Hour
mL	Millilitre
mM	Millimolar
Ng	Nanogram
nM	Nanomolar
Nm	Nanometer
°C	Degrees Celsius
%	Percentage

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CHAPTER ONE: Introduction

1.1 Incidence of Leukaemia

According to GLOBOCAN estimates, leukaemia is the 10th most common cancer worldwide (Ferlay *et al.*, 2019). Seventy eight per cent of leukaemia cases occur in developing countries where the poor are at an increased risk of morbidity and mortality (Global Burden of Disease Cancer, 2015; Vos *et al.*, 2015). Statistics South Africa reported that cancer is the second leading cause of death, after tuberculosis (Singh *et al.*, 2015). In South Africa, leukaemia is the most prevalent cancer among children (Erdmann *et al.*, 2015). According to the National Cancer Registry, for the year 2014, 600 new cases of leukaemia were reported. Currently, the Cancer registry for South Africa is not up to date, and the NCR (2014) is the latest leukaemia statistics available for South Africa. However, these values may be inaccurate due to underreporting in both the private and public sector (Singh *et al.*, 2015).

South Africa, as well as the African continent as a whole, remains under represented in global cancer statistics due to the limitation of reliable population-based incidence data and rural coverage (Crocker-Buque and Pollock, 2015). Research has shown that different population groups (Caucasian vs. African) show varying incidence rates, which may be attributed to different socioeconomic positions and access to healthcare (Erdmann *et al.*, 2015).

A country-wide study on haematological malignancies in South Africa revealed that reporting to the National cancer registry is limited to pathology-confirmed cancer cases, which does not fully capture the incidence of haematological cancers in South Africa (Schonfeld *et al.*, 2016). Many haematological malignancies are regarded as rare cancers, such as acute promyelocytic leukaemia (APL), which disproportionally affects the young and requires advanced diagnostic services for appropriate treatment (Oelofse and Truter, 2018).

1.2 Leukaemia

Leukaemia originates in blood forming tissue such as the bone marrow and immune system cells, particularly leukocytes. Malignant transformation is associated with changes in cell behaviour and growth parameters, thereby affecting the regular function of haematopoietic cells (leukocytes, erythrocytes and thrombocytes) (Boyle, 2008a).

Leukaemia can be classified into myeloid and lymphoid leukaemia, depending on which cell lineage is affected. Leukaemia arising from lymphocytes is classified as lymphocytic leukaemia, whereas abnormal monocytes or granulocytes are classified as myelocytic leukaemia (Kondo, 2010). They are further subdivided into acute and chronic leukaemia based on the stage of malignant cell maturity and its clinical course (Miller and Grodman, 2001). Acute leukaemia has a rapid onset and may be fatal to the patient within days or months following the initial diagnosis. It is characterised by the proliferation of immature immune cells which are non-functional, whereas chronic leukaemia is characterised by its slow progression and defectively-differentiated, but mature cells (Scheinberg *et al.*, 2001). The four main types are chronic lymphocytic leukaemia (CLL), acute lymphocytic leukaemia (ALL), acute myelocytic leukaemia (AML) and chronic myelocytic leukaemia (CML) (Boyle, 2008b).

1.3 Haematopoiesis

Haematopoiesis is the formation of different types of blood cells that arise from pluripotent stem cells in the bone marrow (Figure 1.1) (Pang *et al.*, 2011). Haematopoietic stem cells are defined based on their ability to differentiate into all haematopoietic cell lineages (Kreso *et al.*, 2013; Kreso and Dick, 2014). They evade cell death by promoting the formation of more stem cells or differentiating into myeloid and lymphoid lineages; the two main blood cell lineages (Figure 1.1) (Morrison *et al.*, 2011).

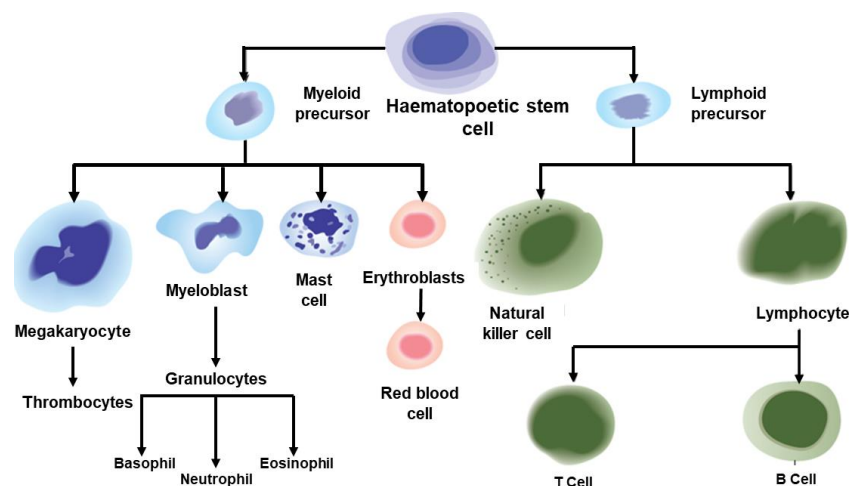


Figure 1.1 Haematopoiesis. The haematopoietic stem cell gives rise to myeloid and lymphoid precursors. The myeloid precursors then differentiate into granulocyte precursors, which produce granulocytes and macrophages, and megakaryocyte and erythrocyte precursors, which produce red blood cells and thrombocytes. Lymphoid precursors differentiate into T and B lymphocytes. Figure adapted from Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e (Fares-Frederickson and David, 2017).

Leukaemia originates either from stem cells which have lost their ability to regulate cellular proliferation or arises from a more differentiated sub-population that may have developed the ability to self-renew (Clarke *et al.*, 2006). The presence of cancer stem cells is significant since their plasticity and dormancy correlate with their resistance to cancer treatment (Plaks *et al.*, 2015), making them the primary drivers of cancer cell growth and survival (Li *et al.*, 2008; Cabarcas *et al.*, 2011; Kreso *et al.*, 2013; Takeishi *et al.*, 2013). This means that even if majority of cancer stem cells are eradicated, the remainder few still have the recurring ability to form tumours and metastasis.

1.4 Factors affecting cell proliferation

The kinetics of tumour formation indicates that a combination of many factors cause cancer (Hanahan and Weinberg, 2011). It takes a long time for the multiple causative mutations to accumulate in a single cell. Cancer cells have many characteristic features that distinguish them from normal cells and are summarised in Figure 1.2 by Hanahan and Weinberg, 2000.

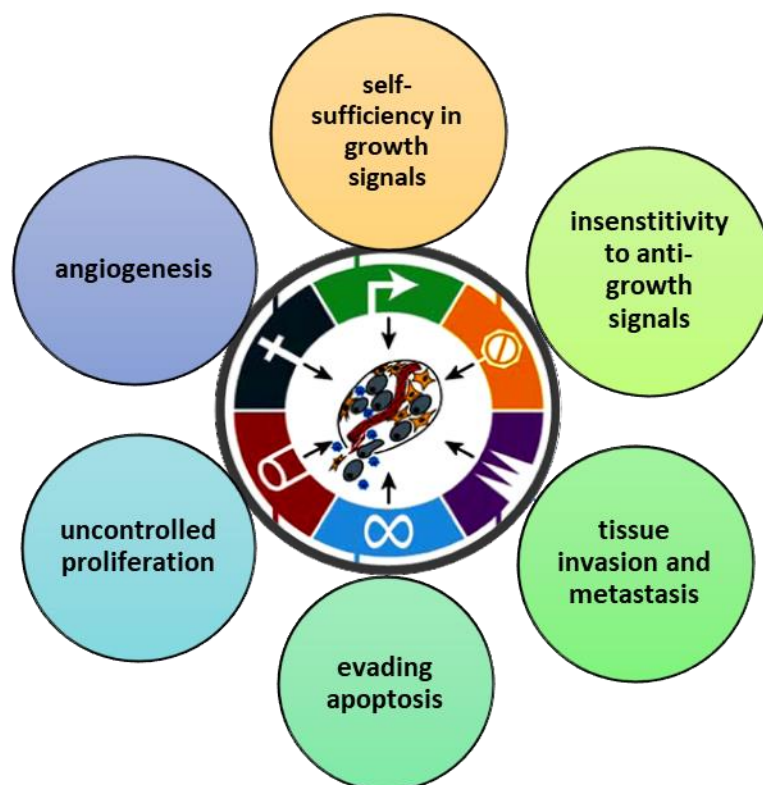


Figure 1.2 The six hallmarks that contribute to the morphological and behavioural properties of a cancer cell, as proposed by Hanahan and Weinberg. Figure adapted from Hanahan and Weinberg, 2000.

As revised by Hanahan and Weinberg in 2011, the biological properties of cancer cells are as follows: i) the acquisition of self-sufficiency of growth signals, leading to uncontrolled proliferation; ii) the loss of sensitivity to growth inhibitory signals, also leading to uncontrolled proliferation; iii) the evasion of apoptosis, in order to allow growth despite genetic errors and external inhibitory growth signals; iv) the uncontrolled proliferative ability, leading to immortality; v) sustained angiogenesis, allowing the tumour to grow beyond the nutrient limitations; vi) the ability to invade neighbouring tissues, the defining property of invasive carcinomas; vii) the ability to cause metastases at distant sites, a typical property of malignant tumours, and viii) the loss of the capacity to repair genetic errors, leading to genomic instability and an increased mutation rate, consequently accelerating all of the other changes (Hanahan and Weinberg, 2011). These changes occurring at a genetic or epigenetic level can lead to an increased resistance to cancer chemotherapy (Adams and Cory, 2007).

1.5 Acute promyelocytic leukaemia

AML is caused by the clonal expansion of blast cells of the myeloid lineage in the bone marrow, peripheral blood, spleen, other organs and tissues such as the skin. APL comprises of 5% to 8% of AML cases (Appelbaum, 2014). APL, is typically characterized by the reciprocal translocation of the promyelocytic leukaemia (*PML*) gene on chromosome 15 with the retinoic acid receptor-alpha (*RARA*) gene on chromosome 17 t(15;17), to form the fusion gene *PML-RARA* (Lo-Coco *et al.*, 2016) causing the expression of the fusion onco-protein PML-RAR α (Redner *et al.*, 1996; Redner, 2002; Lo-Coco and Hasan, 2011). The PML-RAR α onco-protein causes uncontrolled proliferation and inhibition of cellular differentiation (de Thé *et al.*, 1991; Kakizuka *et al.*, 1991). The rapidly proliferating blast cells interfere with normal haematopoiesis and cause anaemia, thrombocytopenia, chronic infections and leukopenia (Shen *et al.*, 2004). Unlike other forms of AML, APL is aggressive and can cause death within months (Lo-Coco and Hasan, 2011).

The majority of patients with APL manifest the t(15;17)(q22;q21) translocation (Yan and Zhang, 2016). In APL, the gain-of-function PML-RAR α fusion protein is thought to impair the pro-apoptotic and growth suppressing function of *PML*, as well as prevent myeloid cell differentiation by repressing retinoic acid target genes (Pandolfi, 2001; Wang and Chen, 2008), thus resulting in constitutive proliferation and inhibition of terminal differentiation (de Thé *et al.*, 1991; Grignani *et al.*, 1993; Mu *et al.*, 1994).

1.5.1 Current treatment of acute promyelocytic leukaemia

Overall, the majority of APL cases can be successfully treated with the retinoid-differentiating agents such as all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO), which induce degradation of the fusion gene *PML-RARA* by binding to the RARA moiety (Lo-Coco *et al.*, 2013). Retinoids induce differentiation and reduce the haemorrhagic complications associated with APL, whereas ATO causes apoptosis and differentiation. The use of ATRA and ATO in combination with anthracycline chemotherapy significantly improves the overall survival compared with chemotherapy alone, with up to a 90% cure rate (Lo-Coco *et al.*, 1998; Advani *et al.*, 1999; Thépot *et al.*, 2011). Anthracyclines intercalate with the DNA of leukaemic cells and inhibit cell replication processes (Lo-Coco *et al.*, 2016). The two most commonly used anthracyclines in APL are daunorubicin and idarubicin (Attar *et al.*, 2013; Howard *et al.*, 2013). APL is associated with an increased risk of disseminated intravascular coagulopathy and death if not treated quickly. However, despite this the prognosis for treated patients to achieve complete remission is better than any other subtype of AML (Barbui and Falanga, 2001).

Treatment with ATRA, ATO and anthracyclines are associated with severe adverse effects. ATRA causes retinoic acid syndrome and hepatotoxicity (Burnett *et al.*, 2015; Lo-Coco *et al.*, 2016) while, ATO can affect electrolyte levels causing cardiac QTc prolongation (Wellstein *et al.*, 2017a). Two major dose limiting adverse effects of anthracyclines include myelosuppression and cardiotoxicity (Wellstein *et al.*, 2017a). Targeted therapy of APL with the immunoconjugate; gemtuzumab ozogamicin which binds to the highly expressed cell surface molecule CD33, is an alternative treatment (Appelbaum and Bernstein, 2017), but not widely used due to its prohibitive cost (Burnett *et al.*, 2015). Despite the various modalities of treatment, the development of drug resistance; both primary and secondary, severe adverse effects and relapse are significant challenges which limit the response to APL treatment (Herbst, 2013).

1.6 Chronic myelocytic leukaemia

Chronic myelocytic leukaemia (CML) is a clonal disease originating from haematopoietic stem cells harbouring the *BCR-ABL* fusion gene (Goldman and Melo, 2003). CML is characterised by this gene; also known as the Philadelphia (Ph) chromosome, which is the reciprocal translocation of the Abelson murine leukaemia viral oncogene homolog (ABL)

gene on chromosome 9 and the breakpoint cluster region (BCR) gene on chromosome 22 (Figure 1.3) (Deininger *et al.*, 2000). The reciprocal translocation designated as t(9;22)(q34;q11), which generates the fusion *BCR-ABL* oncogene, results in the expression of the BCR-ABL tyrosine kinase onco-protein. This BCR-ABL onco-protein is constitutively active and responsible for promoting cancer cell survival and proliferation in CML (Assouline and Lipton, 2011). The Ph chromosome is present in 95% of CML patients, whilst the other 5% of patients have different or more cryptic translocations, which also yield the *BCR-ABL* fusion gene (O'Brien *et al.*, 1997; Ren, 2005). Consequently, every CML patient carries the *BCR-ABL* fusion gene.

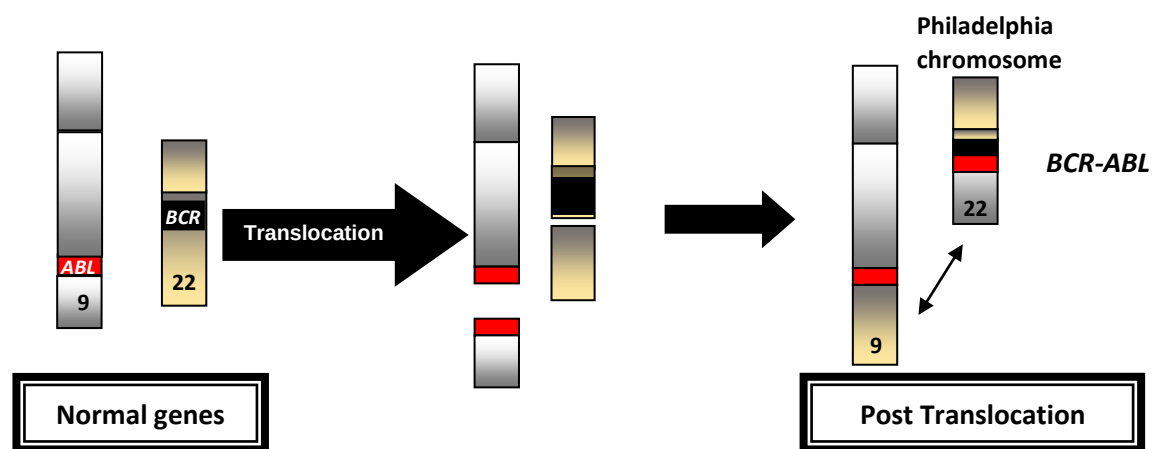


Figure 1.3 The Philadelphia chromosome. The reciprocal translocation between chromosomes 9 and 22 generates the *BCR-ABL* fusion gene on the modified chromosome 22, which is characterised as the Ph chromosome. Figure adapted from Harrison's Principles of Internal Medicine, 20e (Bunz and Vogelstein, 2018).

Wild-type ABL is a non-receptor tyrosine kinase that shuttles between the nucleus and the cytoplasm to transduce signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structures (Colicelli, 2010; Lahiry *et al.*, 2010; Khatri *et al.*, 2016). Its kinase activity is regulated by a myristoyl group located at the N-terminus. This group is not found on the fusion protein causing ABL to be constitutively active. The regulatory tyrosine residue 177 domain on BCR, is phosphorylated by ABL (Deininger *et al.*, 2000). (Ong *et al.*, 2001; Pawson and Nash, 2003; Sugiyama *et al.*, 2019). Subsequently, *BCR-ABL* drives the pathogenesis of CML by activating signalling pathways, such as the mitogen-activated protein kinase (MAPK)/ extracellular signal regulated kinase-1/-2 (ERK1/2), phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) via growth factor receptor-bound protein 2. *BCR-ABL* directly phosphorylates substrates such as chicken tumour virus number 10

regulator of kinase adaptor protein (CRKL), 3-phosphoinositide-dependent protein kinase 1 (PDK1), paxillin (PXN), and E3 ubiquitin ligases (Casitas B-lineage Lymphoma; CBL) (Deininger *et al.*, 2000). This leads to cell growth advantages and resistance to apoptosis, thereby causing an accumulation of myeloid cells in CML patients. There are two *BCR-ABL* variants with different molecular weights; p190 and p210, based on the breakpoints of the BCR gene (Li *et al.*, 1999; Deininger *et al.*, 2000). They differ in length of the BCR sequence but have the same ABL tyrosine kinase.

1.6.1 Current treatment of chronic myelocytic leukaemia

CML is treated by chemotherapy and allogeneic stem cell transplantation. Allogeneic stem cell transplantation is considered a beneficial therapy with complete cytogenetic response of up to 15 years (Jabbour and Kantarjian, 2012). The drawback however, is the mortality and morbidity associated to the procedure, and the scarcity of matched donors. CML was initially treated with busulfan, hydroxyurea and interferon α . However, adverse effects including fever, thrombocytopenia and myalgia reduced the quality of life of many patients and made long-term use intolerable (O'Brien *et al.*, 2003).

In 2001, the discovery of the BCR-ABL fusion protein brought about the very first targeted therapy, a tyrosine kinase inhibitor (TKI), called imatinib mesylate, which selectively inhibits the *BCR-ABL* oncogene (Peggs and Mackinnon, 2003). TKIs compete with adenosine triphosphate (ATP) at the ATP-binding pocket of the kinase domain to prevent BCR-ABL from phosphorylating its target. Imatinib mesylate along with second generation TKIs dasatinib, nilotinib, and bosutinib, and the third generation TKI, ponatinib, have revolutionized the management of CML and demonstrated CML as a typical example for targeted cancer therapy (Kantarjian *et al.*, 2012; Larson *et al.*, 2012; Jabbour *et al.*, 2014; Jabbour *et al.*, 2015).

Despite the major impact that TKIs have had on the treatment of CML, there still remains challenges due to cases of early relapse, drug induced adverse effects, primary acquired TKI resistance and intolerance to imatinib (Mughal *et al.*, 2007; Falchi *et al.*, 2013; Mughal *et al.*, 2016). Most patients harbour residual leukaemic stem cells, which are genetically unstable and less responsive to TKI treatments (Kuczynski *et al.*, 2013). Intolerance to imatinib mesylate can present as gastrointestinal disturbances, inflammatory skin reactions and fluid

retention with the most serious adverse effect being congestive heart failure which leads to the discontinuation of imatinib mesylate (Wellstein *et al.*, 2017b).

The major molecular mechanism of relapse and resistance is due to BCR-ABL kinase point mutations, which affects more than 100 different amino acids (Soverini *et al.*, 2014). Some of the point mutations within the kinase domain, like the gatekeeper threonine-to-isoleucine mutation at position 315 (*T315I*), prevents the binding of TKIs to the ATP-binding pocket. Second generation TKIs such as nilotinib, dasatinib and bosutinib are used as alternative therapies in imatinib failure (Jabbour *et al.*, 2015; Salizzato *et al.*, 2016). Their use requires careful consideration of pre-existing conditions as they may exacerbate and cause serious adverse effects. For example, dasatinib has been associated with pleural effusion, whilst nilotinib causes cardiac arrhythmias due to QTc prolongation (Soverini *et al.*, 2014; Soverini *et al.*, 2018). Despite the fact that the second generation TKIs are more potent and more effective against certain *BCR-ABL* mutations, they still fail to inhibit BCR-ABL harbouring the gatekeeper *T315I* mutation.

The third generation TKI ponatinib is the only TKI that is effective in patients who harbour the *T315I* mutation, and clinically it has been effective in patients who previously failed other TKI therapies (Zhou *et al.*, 2011; Cortes *et al.*, 2013). Arterial thrombosis and hepatotoxicity are major adverse effects (Wellstein *et al.*, 2017b). Some patients develop high blood pressure which may lead to myocardial infarction or ischaemic strokes (Talati *et al.*, 2015; Caldemeyer and Akard, 2016). Therefore, due to its associated risk, ponatinib is only considered in *T315I* mutation cases.

The identification of novel therapeutic targets together with mechanism-based combination strategies are clearly needed to specifically target leukaemic progenitor cells, overcome resistance and achieve longer survival rates. New molecular markers to predict patient response to TKIs are warranted to foresee a poor TKI responder, so that alternative therapies could be offered prior to the development of resistant CML progenitor clones, which are known to decrease responsiveness to alternative treatments (Jabbour *et al.*, 2011; Kuczyński *et al.*, 2013).

1.7 The search for new anti-leukaemic lead compounds

There are two types of approaches used in cancer drug discovery namely; the phenotypic or the target-driven approach (Lage *et al.*, 2018). The phenotypic approach involves the evaluation and identification of compounds which are able to modify a disease phenotype despite not having any knowledge of the specific disease target or mechanism of action. Target-driven evaluation aims at the development of compounds by identifying and targeting specific disease associated proteins and blocking their function. This then translates to a cure for the specific disease. The active site of the target protein is used to design a compound that will bind and inhibits its activity to it in order to induce the desired effect in the specific disease (Lage *et al.*, 2018).

Target-driven drug development has overtaken phenotypic evaluation as an approach to drug discovery in cancer and other diseases, primarily due to the development of bioinformatics that aids the identification of disease-modifying targets (Ramabhadran, 2018). However, target-driven drug development suffers high attrition rates due to safety concerns, thus maintaining the advantages of phenotypic screening (Swinney, 2013; Swinney, 2014). Successful targeted therapy is poorly sustainable for patients due to cost implications. The statistically high success rate of phenotypic evaluation over target-driven drug development maintains it as the better approach to discovering lead compounds in cancer drug discovery, although this is still under intense debate (Swinney and Anthony, 2011).

Against this background, the method of phenotypic evaluation was employed in this research study when assessing the novel imidazo[1,2-*a*]pyridine compounds for their efficacy on HL-60 and K562 leukaemic cell lines. The HL-60 and K562 cell lines were chosen as culture models for myeloid leukaemia. Cell based *in vitro* models are crucial in early drug discovery (Ryan *et al.*, 2016). It is substantially easier to assess sub-cellular events in cell-based models compared to whole organisms with higher levels of biological organization.

1.8 Imidazo[1,2-*a*]pyridines

Imidazopyridines are biologically active nitrogen containing heterocyclic molecules which comprise of an imidazole moiety bound to a pyridine ring (Krause *et al.*, 2017). There are several imidazopyridine derivatives however, the imidazo[1,2-*a*]pyridines moiety is considered the most important in medicinal chemistry. This is owing to their wide range of

biological activities such as anti-inflammatory (Lacerda *et al.*, 2009), antibacterial (Shukla *et al.*, 2012; Abrahams *et al.*, 2013) and antiviral activities (Hamdouchi *et al.*, 1999; Bode *et al.*, 2011; Krause *et al.*, 2017). The imidazo[1,2-*a*]pyridine moiety forms the core structure that makes up several drug classes of which are currently used in clinics. These include zolpidem for insomnia (Chiaro *et al.*, 2018), olprinone for acute heart failure (Mizushige *et al.*, 2002), and alpidem, necopidem and saripidem as anxiolytic agents (Mandlimath and Sathiyarayanan, 2016), GSK812397 is a drug for the treatment of HIV infection, rifaximin as an antibiotic agent and minodronic acid as treatment for osteoporosis (Baumann *et al.*, 2011; Tanishima and Morio, 2013; Li *et al.*, 2015a; Reen *et al.*, 2019). Their medicinal properties make them useful molecules in drug discovery for the development of new chemotherapeutic agents.

The imidazo[1,2-*a*]pyridine scaffold forms the base structure of PI3K inhibitors. PI3K is a key signalling enzyme of the PI3K/Akt signal transduction pathway associated with cancer progression. The discovery of GSK2126458, a highly potent inhibitor of PI3K has been developed with pre-clinical evidence of anti-tumour activity (Knight *et al.*, 2010; Munster *et al.*, 2016). This is the stage of research that occurs before clinical trials can begin and during which feasibility and drug safety data are collected. Additionally, potential PI3K inhibitors like the imidazopyridine analogue HS-173, exhibits anti-cancer activity *in vitro* and *in vivo* (Kim *et al.*, 2011; Lee *et al.*, 2013a), by inducing apoptosis, and blocking the PI3K/Akt pathway in human non-small cell lung cancer (A549, H1299 and NCI-H596) cell lines. In addition, HS-173 is able to suppress cell growth and subsequent angiogenesis in A549 cells at an IC₅₀ concentration of 0.95 μ M (Lee *et al.*, 2013b).

PI3K inhibitors investigated by Han *et al.*, (2016), further confirmed the effectiveness of imidazo[1,2-*a*]pyridine derivatives in an ovarian cancer mouse xenograft model, by suppressing PI3K α and Akt activity at a concentration below 20 nM (Han *et al.*, 2016). However, the data reported of the imidazo[1,2-*a*]pyridines were on the enzyme activity and may not necessarily translate to meaningful IC₅₀ values when tested in cells. A study by Xi *et al.*, (2017), demonstrated the inhibitory effects of imidazo[1,2-*a*]pyridine based derivatives on serine/threonine-protein kinase Nek2 at low nanomolar concentrations *in vitro*, by inducing cell cycle arrest and apoptosis (Xi *et al.*, 2017).

Apart from their postulated inhibitory effects on kinases, imidazo[1,2-*a*]pyridine derivatives attached to curcumin also shows inhibitory effects on cancer cell growth. As demonstrated by

Ramya *et al* (2018), curcumin-based imidazo[1,2-*a*]pyridine derivatives are effective tubulin polymerase inhibitors and inducers of apoptotic cell death in PC-3 (prostrate), HGC-27 (gastric) and HeLa (cervical) cell lines at low micromolar concentrations (IC₅₀ values <3.0µM) (Ramya *et al.*, 2018).

A study by El-Awady *et al* (2016), demonstrated how imidazo[1,2-*a*]pyridine derivatives suppress DNA repair mechanisms in breast (MCF-7) and lung (A549) cancer cells by increasing p21 degradation and, activating tumour suppressor p53 to induce apoptosis (El-Awady *et al.*, 2016). The effect of the imidazo[1,2-*a*]pyridine derivative was investigated in cancer cells originated from breast, cervical, melanoma, liver and lung alone and in combination with doxorubicin. The cells were incubated for 48 hours with the imidazopyridine derivative and cytotoxicity was measured by the Sulphorhodamine-B assay. The imidazo[1,2-*a*]pyridine showed potent activity against the MCF-7 (IC₅₀ = 8.7 µM) and A549 cells (IC₅₀ = 2.4 µM). In addition, synergistic effects were demonstrated by the imidazo[1,2-*a*]pyridine in combination with doxorubicin in the MCF-7, A549 and HepG2 liver cancer cells (El-Awady *et al.*, 2016).

A study evaluating steroidal based imidazo[1,2-*a*]pyridines have shown cytotoxic activity at low IC₅₀ values against five cell lines including leukaemic, breast, colorectal, lung and liver cell lines and induced apoptotic cell death particularly in the A549 lung cells (Dar *et al.*, 2015). Imidazo[1,2-*a*]pyridines are structurally related to the imidazo[1,5-*a*]pyridine, imidazo[4,5-*b*]pyridine and imidazo[4,5-*c*]pyridine scaffold (Krause *et al.*, 2017). However, in comparison to imidazo[1,2-*a*]pyridine derivatives, imidazo[4,5-*b*]pyridines has been found to be less effective in inhibiting cancer cell progression (Marie Kirwen *et al.*, 2017). This supports the importance of the imidazo[1,2-*a*]pyridine moiety in the development of new cancer therapeutic agents.

The basis of investigating imidazo[1,2-*a*]pyridines as anti-cancer agents in this study originated from a previous study in our laboratory which showed the *in vitro* decrease in cell viability of the colorectal cancer cell lines HT-29 and Caco-2, by a small subset of 6-substituted imidazo[1,2-*a*]pyridines with IC₅₀ values ranging between 6 and 22 µM (Dahan-Farkas *et al.*, 2011). These imidazo[1,2-*a*]pyridine derivative were able to activate the intrinsic apoptotic pathway in the colorectal cancer cell lines at a concentration of 100 µM and showed minimal toxicity when tested against normal human peripheral leukocytes

(Dahan-Farkas *et al.*, 2011). Following on from this study these molecules were complexed to copper hoping to improve the cytotoxic effect, and form the basis of this study.

1.9 Copper in cancer

Copper is essential for the function of several enzymes and proteins involved in energy metabolism, respiration, structural functions and DNA synthesis (Tardito and Marchio, 2009; Santini *et al.*, 2014; Stepien *et al.*, 2017). Free copper has the capacity to produce large amounts of reactive oxygen species (ROS) (Iakovidis *et al.*, 2011). Copper-zinc (Cu/Zn) superoxide dismutase 1 and 3 (SOD1 and SOD3) requires copper for free radical detoxification (Che *et al.*, 2016). Whilst in the mitochondria, copper forms part of the mitochondrial enzyme cytochrome c oxidase in ATP production (Stowe and Camara, 2009). Copper is required for the function of other metalloenzymes like ceruloplasmin (ferroxidases), the prime carrier of free copper in haem regulation (Prohaska, 2011) and, lysyl oxidase (LOX) which is a copper-dependent amine oxidase in collagen fibrils formation (Lopez and Greenaway, 2011). Furthermore, copper ions are essential for enzymes like tyrosinase for the production of melanin in mammalian melanocytes (Solano, 2018).

Copper is an essential transitional metal for all living organisms and is a crucial element in oxidation-reduction (redox) chemistry, growth and development (Chang, 2015). Due to its redox properties, unbound copper is highly reactive and may catalyse the Haber-Weiss reaction; and producing free radicals ($\text{OH}^\cdot$) (Liou and Storz, 2010). These reactive oxygen species damage lipids, proteins and, nucleic acids amongst other biomolecules leading to cell death (Bae *et al.*, 2011). Therefore, to prevent the unwanted production of free radicals, copper is always bound to committed proteins and requires a tightly regulated homeostatic mechanism (Reviewed by Nevitt *et al.*, 2012).

Copper is being extensively explored for its role in cancer (Fouani *et al.*, 2017). Published data indicates a paradoxical role of copper in cancer. Elevated levels of copper have been implicated in several human cancers including prostate, brain, breast, colon and lung (Daniel *et al.*, 2005; Chen *et al.*, 2007; Finney *et al.*, 2009; Gupte and Mumper, 2009). This implies the poor regulation of copper levels in cancer cells. An increase of copper appears to be an essential co-factor for angiogenesis (Tisato *et al.*, 2010; Yee *et al.*, 2017). The accumulation of copper in cancer cells makes it a potential anti-cancer strategy. Studies have shown that decreases in copper levels result in a reduction in tumour cell growth, angiogenesis and

metastasis favouring the use of copper chelators (Finney *et al.*, 2009; Wang *et al.*, 2010). A study by Horn and Barrientos, (2008) has shown no significant correlation between cancer development and increased copper levels (Horn and Barrientos, 2008). Moreover, copper modulates proteins involved in cell signalling, cell differentiation and cell death pathways, expanding its role in other processes of human physiology and pathology. Considering its importance, chelation of copper levels may negatively impact many of these cellular processes, and consequently may not be a beneficial or an appropriate cancer therapeutic strategy. In contrast, there is strong evidence for the use of copper as a redox metal for cancer chemotherapy (Baker *et al.*, 2017; Raza *et al.*, 2017).

Copper causes oxidative stress by increasing ROS production (Alvarez *et al.*, 2018). With the recent developments in cancer research the generation of ROS by oxidative stress has shown to be essential in the initiation and execution of apoptosis (Shafagh *et al.*, 2015). The precise role of copper in cancer development is presently unclear. During the process of apoptosis, the elevated ROS levels lead to DNA damage and subsequently damaged cells undergo cell cycle arrest to mediate DNA repair or undergo apoptosis (Chen *et al.*, 2015). A review by McGivern *et al.*, (2018) on recent studies (2014-2018) about oxidized cupric Cu (II) systems and their nuclease activity suggested that apoptotic cell death was caused by ROS production and oxidative damage. Compared to normal cells the increased intracellular copper levels and associated redox disruptions caused by copper complexes are poorly tolerated by cancer cells (Tardito and Marchio, 2009; McGivern *et al.*, 2018).

Free radicals contribute highly to the progression of cancer cells by driving mutations through oxidative damage of DNA. Moreover, cancer cells are already in a state of increased oxidative stress since they have massive energy requirements. For this reason oxidative stress is considered a selective approach in the induction of cell death in cancers (Alvarez *et al.*, 2018).

1.10 Copper complexes as potential cancer chemotherapeutic agents

Platinum metal complexes have been investigated and used in anti-cancer regimens. The current use of platinum is greatly limited by severe dose limiting side effects and intrinsic or acquired drug resistance. Following the success of platinum (II) compounds such as cisplatin, carboplatin and oxaliplatin in cancer chemotherapy (Wheate *et al.*, 2010), a large body of copper complexes have been reviewed for their potential as anti-cancer agents (Santini *et al.*,

2014). With several studies indicating that copper, in its Cu (II) and Cu (I) oxidation states, coordinated to various organic and inorganic molecules, are able to inhibit the growth of cancer cells (Revenko *et al.*, 2010). The success of copper complexes relies on various aspects of the ligand donor as it modulates the lipophilicity, solubility, stability of the complex as well as its ability to permeate the lipid bilayer, all of which may impact the target of the copper complex and, its cellular internalisation (Wadas *et al.*, 2007). Copper complexes are likely to enter the cell through a copper transporter which is essential for maintaining intracellular copper levels (Wang *et al.*, 2011b).

Despite the numerous publications regarding the anti-cancer effects of copper complexes, the molecular targets of copper complexed to ligands are yet to be discovered. However, molecular modelling suggests that they are likely to act by inhibiting topoisomerase and binding and intercalating DNA (Santini *et al.*, 2014; McGivern *et al.*, 2018). A study by Lee *et al.*, (2016), demonstrated the cytotoxic activity of three transitional metal derivatives (copper, zinc and nickel) bound to a Schiff base phenol ligand as potential topoisomerase I inhibitors in A2780, MCF-7, HT-29, HepG2, A549 and PC3 cancer cells. These compounds inhibit topoisomerase I activity, induce DNA cleavage and show DNA binding activity (Beretta *et al.*, 2013). The copper analogue (copper (II) complex 2) was reported to be more cytotoxic than the zinc and nickel analogues against the cancer cell lines (Lee *et al.*, 2016).

Inside the cell, copper can potentially dissociate from the complex it is bound to; and due to its redox nature, cause the accumulation of mitochondrial ROS and subsequently oxidative stress (Liou and Storz, 2010). The accumulation of ROS within a cell is associated with necrotic cell death (Flora, 2009). Several studies support the generation of ROS by copper complexes however, whether ROS is caused by unbound copper or the intact copper complex, remains elusive (McGivern *et al.*, 2018). The fate of copper complexes within the cell is poorly understood, making it more likely that the intact copper complex is responsible for either apoptotic or necrotic cell death.

1.11 Aim and Objectives

Aim:

The broad aim of this study is to identify novel active imidazo[1,2-*a*]pyridine derivatives on leukaemic cell lines: an evaluation of their mode and mechanism of cell death and at a molecular level, elucidate their effect on protein and gene expression.

Objectives:

- Identify active imidazo[1,2-*a*]pyridine compounds
- Identify active compounds causing apoptosis
- Determine the effect of active compounds on a proteome profile
- Determine the effect of active compounds on the gene expression of apoptotic proteins

CHAPTER TWO: Cytotoxicity and Cell Death

2.1 INTRODUCTION

Cell death is a process that is essential for the development and maintenance of normal healthy tissue, and defects in cell death mechanisms underlie many disease states, including cancer (Fuchs and Steller, 2011). Cell death occurs by various mechanisms (Figure 2.1) which are distinguished by different morphological and biochemical features. Cell death can be defined by one of the following:

- i) the loss of cell membrane integrity,
- ii) the disintegration of cells and nuclei into apoptotic bodies and,
- iii) the engulfing of cell contents or remnants by another cell (Kroemer *et al.*, 2009).

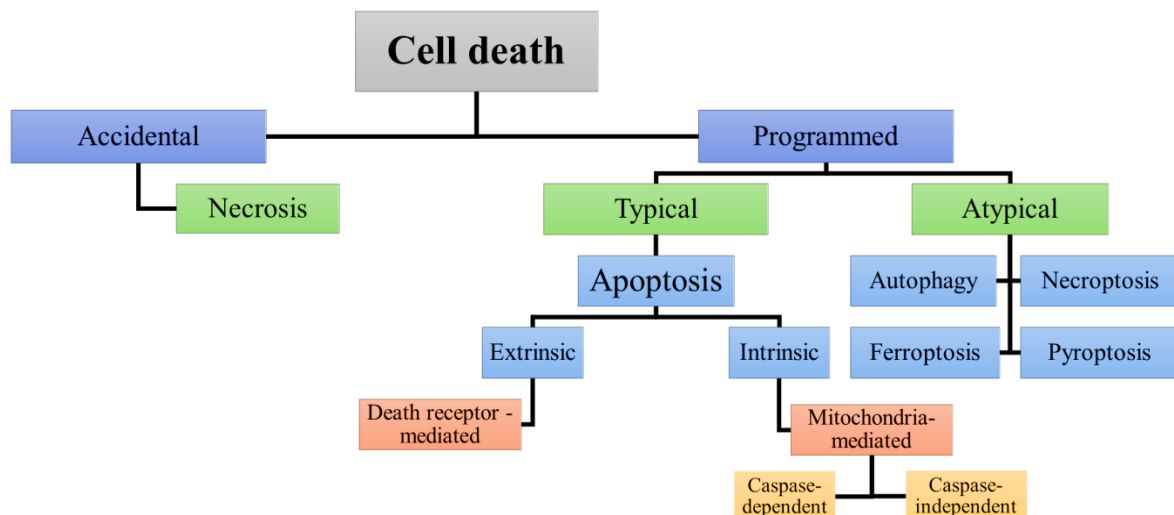


Figure 2.1 Classification of cell death based on the Nomenclature Committee on Cell Death (NCCD) reports, 2018. Cell death is classified as either programmed (PCD) or accidental. Necrosis is a form of accidental cell death, morphologically characterized by organelle swelling and rupture of the cell membrane. PCD is regulated cell death which can be typical or atypical. Apoptosis is a typical form of PCD characterised by distinct morphological features that include membrane blebbing, chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies. Apoptosis is activated via two major pathways; the extrinsic-death receptor and/or the intrinsic-mitochondrial mediated apoptotic pathway. Depending on the proteins involved, the intrinsic pathway can be caspase-dependent or caspase-independent. Atypical forms of PCD include autophagy, necroptosis, pyroptosis and ferroptosis. Autophagy is a pro-survival stress response defined by the activation of the autophagosomic-lysosomal pathway. Necroptosis is a regulated form of necrosis and pyroptosis is characterized by the activation of inflammatory caspases. Ferroptosis is an iron and ROS dependent form of cell death. Figure adapted from NCCD reports, 2018 (Galluzzi *et al.*, 2018).

The two most well defined mechanisms of cell death are apoptosis and necrosis (Kroemer *et al.*, 2009). Aside from these two modes of cell death, there are additional non-apoptotic forms of programmed cell death; autophagy, necroptosis, pyroptosis and ferroptosis (Galluzzi *et al.*, 2018) (Figure 2.1).

2.1.1 What is apoptosis?

Apoptosis is a highly regulated form of programmed cell death that was first reported by Kerr *et al.*, (1972) and is required for the normal growth and development of multicellular organisms. It is characterized by distinct morphological features that include membrane blebbing, chromatin condensation, nuclear fragmentation (karyorrhexis), reduction of cell volume (pyknosis), the formation of apoptotic bodies and phosphatidylserine (PS) exposure on the outer cell membrane (Hassan *et al.*, 2014).

Membrane externalisation of PS allows for the early recognition of apoptotic cells for phagocytosis (Elmore, 2007), causing minimal inflammation (Wong, 2011). Since apoptosis does not cause inflammation, it is considered a desirable form of cell death for cancer patients (Baig *et al.*, 2016). Another feature of apoptotic cell death is the disruption of mitosis, referred to as mitotic catastrophe (Kroemer *et al.*, 2009; Galluzzi *et al.*, 2012). Mitotic catastrophe is morphologically characterised by large cells that contain multiple micronuclei (Mc Gee, 2015).

2.1.2 Apoptosis and cancer

Defects of the normal apoptotic process contributes to the development of cancer (Hassan *et al.*, 2014). Characteristically, cancer cells have the ability to evade apoptosis by altering important apoptotic regulatory proteins, enabling tumour development and metastasis (Wong, 2011). The deregulation of apoptosis and impaired apoptotic signalling contributes to the development of cancer as well as resistance to chemotherapy (Fulda, 2014). The overexpression of anti-apoptotic members of B-cell lymphoma-2 (Bcl-2) family of proteins as well as the loss of function and expression of pro-apoptotic members of apoptosis regulation are examples of apoptotic deregulation in cancer (Labi and Erlacher, 2015).

2.1.3 Why is apoptosis important in cancer treatment?

Apoptosis is an essential mechanism for the removal of unwanted, damaged or dysfunctional cells without triggering the inflammatory response (Wong, 2011; Pistritto *et al.*, 2016).

Apoptotic cell death does not stimulate the inflammatory response, thereby decreasing the risk for severe tumour lysis syndrome. However in cancer, defects of apoptosis allows for cells to evade cell death resulting in that uncontrolled proliferation and the survival of damaged cells (Fulda, 2014).

The manipulation of apoptotic cell death signalling networks is a therapeutic strategy to effectively manage cancer. Treatment strategies or drug combinations that increase apoptosis are beneficial for patients and is one of the desired endpoints when investigating new treatments. There is a growing body of evidence describing the ability of chemotherapeutic drugs to induce apoptosis in cancer cells as well as to find combinations of chemotherapeutic drugs that induce apoptosis. Examples of some compounds that activate apoptosis in cancer include doxorubicin (Sharifi *et al.*, 2015), cisplatin (Singh *et al.*, 2013), 5-fluorouracil (Dart *et al.*, 2004) and arsenic trioxide (Kumar *et al.*, 2014; Sun *et al.*, 2018).

A drug that is able to induce apoptosis via the intrinsic or extrinsic apoptotic pathway, can be used to reverse most cancerous phenotypes (Labi and Erlacher, 2015). In haematological malignancies, the aim is to eliminate leukaemic cells by reactivating cell death pathways by direct targeting of anti-apoptotic proteins such as those of the Bcl-2 or the inhibitor of apoptotic proteins (IAP) families, or by reactivating the p53 response (Samudio *et al.*, 2010). An example is the mouse double minute 2 protein (MDM2) inhibitor idasanutlin ([NCT02545283](#)), currently in phase 3 clinical trials for AML (Khurana and Shafer, 2019). Nutlins are the first small molecule inhibitors that bind to MDM2 and target its interaction with p53. The ubiquitin ligase MDM2 is a negative regulator of p53 and its expression (Zhang and Lozano, 2017). The inhibition of MDM2 is postulated to reactivate p53 and its tumour suppressor functions.

Drugs which target apoptosis regulation are currently under clinical evaluation. Therapies targeting apoptosis currently in clinical trials include MDM2 inhibitors (Ravandi *et al.*, 2016; Reis *et al.*, 2016; Khurana and Shafer, 2019), Bcl-2 inhibitors (Schimmer *et al.*, 2014; Konopleva *et al.*, 2016), IAP inhibitors (Prabhu *et al.*, 2017; Zhou *et al.*, 2018) and Smac mimetics (DiPersio *et al.*, 2015; Donnellan *et al.*, 2016). Molecular targeted compounds

aiming at restoring apoptosis in leukaemic cells have shown promising activity in clinical trials (Reviewed in Cassier *et al.*, 2017 and Khuran and Shafer., 2019).

2.1.4 Apoptotic pathways

Based on the origin of death signals, apoptosis is further divided into the extrinsic and intrinsic apoptotic pathways. These two main pathways and their convergence are shown in Figure 2.2.

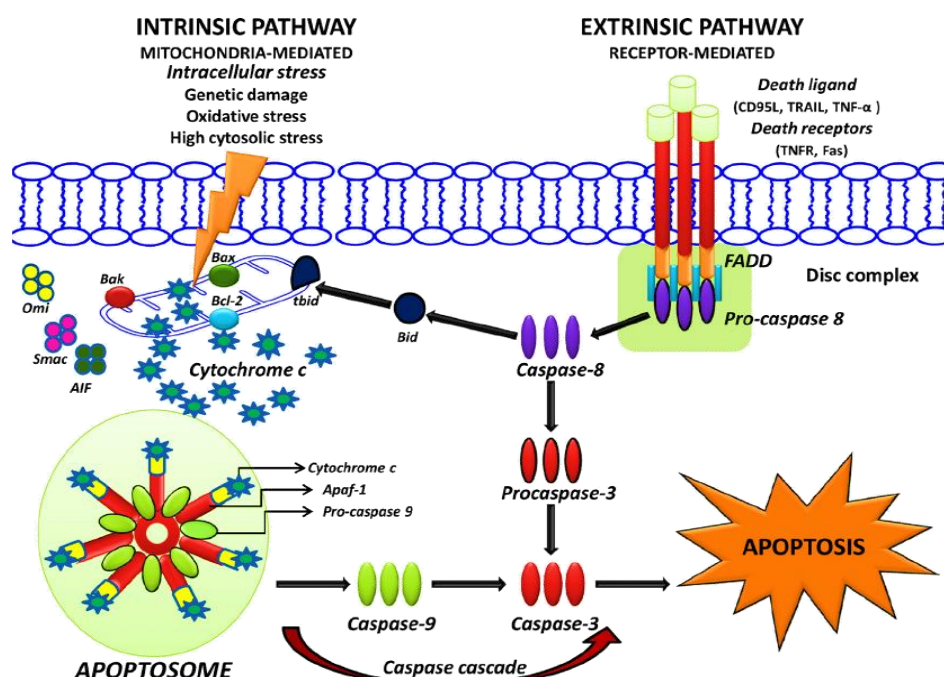


Figure 2.2 The two main apoptotic pathways (Beesoo *et al.*, 2014) The intrinsic/mitochondria-mediated pathway is activated in response to intracellular signals caused by stress, DNA damage, ROS and chemotherapeutic agents. Activation of Bcl-2 family of proteins causes increase in mitochondrial membrane permeability and subsequently loss in mitochondrial membrane potential (MMP). Cytochrome c is released to the cytosol where it recruits apoptotic protease activating factor-1 (Apaf-1), ATP and pro-caspase-9 to form an apoptosome complex, which induces the proteolytic cleavage and activation of caspase-9. Activated caspase-9 cleaves pro-caspase-3/7 to active caspase-3/7. Anti-apoptotic protein Bcl-2 prevents cytochrome c release by blocking oligomerization of Bcl-2 antagonist/killer (Bak) and Bcl-2 associated X protein (Bax). The extrinsic/death receptor-mediated apoptotic pathway is initiated by the binding of death ligands (Fas/CD95, TRAIL, TNF-α) to their respective death receptors (Fas, TRAIL-R, TNF-R). This is followed by receptor trimerization and subsequent formation of the death inducing signalling complex (DISC) which binds the adaptor protein, Fas-associated death domain (FADD). DISC-FADD complex associates with pro-caspase-8, causing autocatalytic cleavage to form active caspase-8 which then activates effector caspase-3, -6 and -7 which cleave proteins responsible for the morphological and biochemical changes characteristic of apoptotic cells, like cell membrane blebbing, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies (Fulda, 2014; Crowley *et al.*, 2016b).

2.1.4.1 The extrinsic/ death receptor-mediated apoptotic pathway

The death receptor facilitated apoptotic pathway is initiated in a sequential manner as shown in Figure 2.2. The extrinsic pathway comprises death receptors TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and TRAIL-R2, first apoptotic signal (Fas) as well as tumour necrosis factor receptor 1 (TNF-R1), which are members of the TNF-receptor family, and share the common characteristic of having a cysteine-rich death domain (DD) (Park *et al.*, 2016). The DD is a highly conserved region of approximately 80 amino acids that forms a hexa-helical bundle (Park *et al.*, 2007). Death domains are the key components of death receptors and direct cells to apoptotic cell death (Pistritto *et al.*, 2016).

The four receptors which interact with TRAIL are death receptor 4 (DR4/TRAIL-R1), death receptor 5 (DR5/TRAIL-R2), decoy receptor 1 (DcR1/TRAIL-R3) and decoy receptor 2 (DcR2/TRAIL-R4) (Micheau, 2018). Decoy receptors compete with death receptors for ligand binding (Naimi *et al.*, 2018). The interaction of TRAIL with TRAIL-R1 and TRAIL-R2 leads to the induction of apoptosis whereas the interaction of TRAIL with decoy receptors results in deregulated apoptosis signalling (Riccioni *et al.*, 2005).

On the cell surface, ligands such as Fas/CD95 and TRAIL interact with the DD domains of FADD and/or TNF receptor-associated death domain (TRADD). They allow for the formation of a DISC complex in the cytoplasmic compartment by recruiting FADD and pro-caspase-8, cause the proteolytic cleavage and activation of caspase-8, which in turn activates effector caspase-3 and -7 (Hassan *et al.*, 2014). The effector caspases cleave and activate enzymes like endonuclease and caspase-activated deoxyribonuclease (CAD) which degrades DNA and cause chromatin condensation (Wong, 2011; Fulda, 2014; Crowley *et al.*, 2016b). Recruitment of FADD triggers pro-apoptotic pathways, while TRADD induces and modulates pro-survival signals such as those mediated by nuclear factor kappa for activated B cells (NF- κ B), c-Jun N-terminal Kinase (JNK) and p38 enzymes (Azijli *et al.*, 2013).

TRADD binds to and forms complex I with receptor interacting protein-1 (RIP1), TNF receptor-associated factor-2 (TRAF-2), TRAF-5 and the cellular inhibitor of apoptosis protein-1 and -2 (cIAP-1/ cIAP-2) (Gentle *et al.*, 2011). This complex modulates pro-survival signals, however, under certain circumstances, RIP1 is de-ubiquitinated which leads to the dissociation of RIP1 and TRADD from complex I (Bertrand *et al.*, 2008; Witt and Vucic, 2017). Once caspases-8 and -10 are activated, they amplify the death signal, either through

direct activation of the effector caspases-3, -6 and -7, or by promoting Bcl-2 homology domain 3 (BH3) interacting-domain death agonist (Bid) engagements of Bax and Bak to activate the intrinsic apoptotic pathway. RIP1 and TRADD then bind to FADD and caspases-8 and -10 to form complex II (Wang *et al.*, 2008b).

2.1.4.2 Intrinsic/ mitochondria-mediated apoptotic pathway

The intrinsic apoptotic pathway is triggered by changes in the mitochondrion. This pathway is activated by cell stress and is initiated by the translocation of the pro-apoptotic Bcl-2 family member; Bax, to the mitochondrion (Pistritto *et al.*, 2016). Under normal conditions, Bax and Bak shuttle between the cytosol and the mitochondrial outer membrane (MOM). In apoptotic cells, active Bax and Bak accumulate at the MOM, where they oligomerize and form pores in the MOM causing the release of cytochrome c and other pro-apoptotic proteins (Hata *et al.*, 2015). Bax undergoes a conformational change, which allows it to insert into the MOM, and exposes the BH3 domain. This facilitates dimerization via interaction with another Bax molecule (O'Neill *et al.*, 2016). Further oligomerization and pore formation is based on the assembly of dimer units. Bcl-2 disrupts this process by forming a heterodimer with the pro-apoptotic protein Bax thereby preventing mitochondrial pore formation.

The loss of MMP in response to apoptotic stimuli causes changes in the permeability of the mitochondrial outer membrane leading to the release of pro-apoptotic protein cytochrome c (Tait and Green, 2010; Estaquier *et al.*, 2012; Lopez and Tait, 2015). The release of cytochrome c to the cytoplasm triggers the formation of an apoptosome complex in the presence of ATP by binding and activating initiator caspase-9 and adaptor molecule Apaf-1 which in turn, activates effector caspases-3, -6 and -7 (Pistritto *et al.*, 2016; Bao *et al.*, 2018). Effector caspases cleave structural proteins resulting in morphological changes associated with apoptosis (Gray *et al.*, 2010; Hanahan and Weinberg, 2011).

The mitochondrial permeability transition pore that allows the release of cytochrome c is regulated by Bax whilst Bcl-2 inhibits cytochrome c release (Peña-Blanco and García-Sáez, 2018). Other mitochondrial proteins released into the cytosol are apoptosis inducing factor (AIF), endonuclease-G, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis binding protein with low pI (Smac/DIABLO) and the serine protease; high temperature requirement protein A2 (HTRA2/Omi) (Wong, 2011).

Smac/DIABLO and HTRA2/Omi prevent the action of IAPs thus promoting caspase activation so that apoptosis proceeds (Pistritto *et al.*, 2016). Endonuclease-G is responsible for the inter-nucleosomal cleavage of chromatin; as it inhibits the DNA fragmentation factor subunit also known as inhibitor of CAD (ICAD) (Julien *et al.*, 2016).

2.1.5 Crosstalk between the extrinsic and intrinsic apoptotic pathways

Activation of the extrinsic pathway can lead to the initiation of the intrinsic apoptotic pathway via the activation of Bid. Activated caspase-8 causes the cleavage and activation of the BH3-only protein; Bid (Huang *et al.*, 2016). Truncated and active Bid in turn cause pro-apoptotic proteins Bax and Bak to assemble into oligomers in the MOM, forming pores and allowing the release of pro-apoptotic protein cytochrome c to the cytoplasm thereby activating the intrinsic apoptotic pathway (Pfeffer and Singh, 2018).

2.1.6 Caspases

Caspases are central proteins during apoptosis induction (McIlwain *et al.*, 2013). Caspase activation is the end result of cell death signalling which is either intrinsic or extrinsic (Shalini *et al.*, 2015). Caspases are synthesized as inactive zymogens that are activated by proteolytic cleavage (Li and Yuan, 2008). Caspases are classified as either pro-inflammatory or pro-apoptotic. Pro-inflammatory caspases include caspase-1, -4, -5, -11 and -12 which regulate cytokine maturation during inflammation (Li and Yuan, 2008). Caspase-1, caspase-4 and caspase-5 induces death by pyroptosis by activating inflammatory cells such as interleukins (IL-1 β and IL-18) (Vande Walle and Lamkanfi, 2016). Pro-apoptotic caspases include caspase-2, -3, -6, -7, -8, -9 and -10 which are primarily involved in mediating cell death signalling transduction. These are further divided into initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, -7) (McIlwain *et al.*, 2013). Initiator caspases are further divided into caspases involved in the extrinsic (caspase-8 and caspase-10) and intrinsic (caspase-9) apoptotic pathway. Caspase-2 is difficult to classify as it displays combined features of both apoptotic pathways.

Initiator caspases are monomers that activate pro-domain mediated dimerization (Pop and Salvesen, 2009). Following apoptotic signalling, dimerization is facilitated by the assembly of caspases to oligomeric activation platforms. Adaptor molecules from the activation platforms specifically bind caspase pro-domains such as death effector domains (DEDs) of

caspase-8 and caspase-10 and caspase recruitment domains (CARDs) of caspase-1, caspase-2 and caspase-9 (Park *et al.*, 2016). Initiator caspases have their own activation platform. The DISC recruits and activates caspase-8 and caspase-10 while the apoptosome complex activates caspase-9. In some cases, specific adaptor proteins within the activation complex are able to direct signalling toward different pathways. For instance, caspase-2 and caspase-8 can trigger either cell death or the NF- κ B survival pathway (Wang *et al.*, 2008b). Effector caspases are inactive dimers that require cleavage of inter-subunit linkers that separates the large and small subunits of the catalytic domain to become active (Pop and Salvesen, 2009). Following activation, auto-proteolytic cleavage matures the caspases to signal downstream regulatory events or generate caspase stability.

Once initiator caspases are activated through apoptotic signalling, they cleave and activate downstream executioner caspases-3, -6 and -7 (Olsen and Zhivotovsky, 2011). In turn, executioner caspases cleave key substrates essential for the characteristic features of apoptosis which include chromatin condensation, DNA fragmentation, cleavage of structural proteins and the formation of apoptotic bodies (Crowley *et al.*, 2016a; Pistritto *et al.*, 2016).

2.1.7 Caspase-dependent apoptosis

Caspase-dependent cell death results from the activation of executioner caspases (Kroemer *et al.*, 2009). Upon an apoptotic signal, changes in the inner mitochondrial membrane results in the mitochondrial release of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Lopez and Tait, 2015). Cytochrome c, Smac/DIABLO, and the serine protease HTRA2/Omi are released into the cytosol where they activate the caspase-dependent mitochondrial pathway (Tait and Green, 2010). Cytochrome c binds and activates Apaf-1 as well as pro-caspase-9, forming the apoptosome complex leading to caspase-9 activation and subsequently caspase-3 activation (Li and Yuan, 2008). During apoptosis and once cells have committed to die, the pro-apoptotic proteins, AIF, endonuclease-G and CAD, are released from the mitochondria (Wong, 2011).

2.1.8 Caspase-independent apoptosis

The apoptotic initiation factor, AIF, which is released from the mitochondria into the cytosol can cause caspase-dependent or caspase-independent cell death (Kroemer *et al.*, 2007). In response to apoptotic stimuli and the loss of mitochondrial membrane potential, AIF

translocates from the mitochondria to the nucleus (Muñoz-Pinedo *et al.*, 2006); via nuclear transporters, where it interacts with endonuclease-G to cause chromatin condensation and DNA fragmentation in the absence of caspase activity (Li *et al.*, 2001; Tait and Green, 2008).

The exact mechanism underlying AIF translocation to the nucleus remains unknown, however the enzyme poly-ADP-ribose polymerase-1 (PARP-1) which has DNA repair activity is associated with mitochondrial AIF release (Hong *et al.*, 2004; Yu *et al.*, 2006). In addition, heat shock protein-70 (HSP70) has been reported to antagonize AIF-mediated apoptosis by binding to AIF in the cytosol and sequestering it to the cytoplasm (Gurbuxani *et al.*, 2003).

In AML cell lines, treatment with traditional chemotherapeutic agents like doxorubicin, vincristine, and paclitaxel induce caspase activation and apoptosis (Carter *et al.*, 2003; Samudio *et al.*, 2010). In contrast, Tait and Green, (2008) showed that the inhibition of caspase activity in the presence of the chemical caspase inhibitor zVAD-fmk does not prevent cell death induced by any of chemotherapeutic agents. This implies the loss of mitochondrial membrane potential and the release of AIF from mitochondria (Tait and Green, 2008).

In lung adenocarcinoma cell lines treated with staurosporine (Zhang *et al.*, 2004) in the presence of a broad spectrum caspase inhibitor, Q-VD-OPH (a carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate) (Caserta *et al.*, 2003; Chauvier *et al.*, 2007), AIF was translocated to the nucleus (Sudhakar and Chow, 2014). This implies that the nuclear translocation of AIF during cell death was caspase-independent (Sudhakar and Chow, 2014). Therefore, apoptosis can occur in the absence of caspase activation and can be described as caspase-independent cell death.

2.1.9 Tumour suppressor p53 and apoptosis

The tumour suppressor protein p53 plays a major role in the regulation of genomic stability, apoptosis and the cell cycle (Kojima *et al.*, 2016). The p53 protein is mutated in approximately 50% of all cancers (Yogosawa and Yoshida, 2018). In the event of DNA damage, the tumour suppressor p53 functions as a gatekeeper in the cell cycle to prevent the progression of damaged cells through the G1/S phase by causing cell cycle arrest. Once DNA repair proteins fix the damage, the cell is able to progress through the cell cycle (Kojima *et*

al., 2016). When DNA damage is irreparable, p53 initiates apoptosis by stimulating the transcription of pro-apoptotic proteins such as Bax, Bid and Apaf-1 that initiate the intrinsic pathway as well as Fas and TRAIL receptors that induce the extrinsic pathway of apoptosis (Zhang and Lozano, 2017). Therefore, given the crucial nature of p53 in cell death, the effects associated with it being mutated are central to cancer pathogenesis and resistance to chemotherapy (Hientz *et al.*, 2017).

2.1.10 Regulation of apoptosis

The apoptotic pathways are regulated by IAPs which prevent apoptosis at different levels. IAPs such as X-linked inhibitor of apoptosis (XIAP), cIAP-1, cIAP-2, survivin and livin inhibit apoptosis by various mechanisms including binding to caspases and preventing their activation (Garg *et al.*, 2016; Rathore *et al.*, 2017; Saraei *et al.*, 2018). IAPs are frequently overexpressed in cancer, preventing apoptotic cell death.

The expression of several of some apoptotic regulatory proteins Bcl-2, cIAP-1 and cIAP-2 are regulated by the transcription factor, NF- κ B (Fulda, 2014). In turn, NF- κ B activity is controlled by its cellular location, and is only active when located in the nucleus.

Translocation to the nucleus is brought about by the proteosomal degradation of inhibitor of kappa B (I κ B α) subunit which is responsible for localizing NF- κ B to the cytoplasm (Morotti *et al.*, 2017). cIAP-1 and 2 which possess E3 ubiquitin ligase activity, activate the canonical NF- κ B pathway by causing ubiquitination of RIP1. This activates I- kappa B kinase (IKK) to phosphorylate and cause the dissociation of I κ B α from NF- κ B (Vrábel *et al.*, 2019). Pro-inflammatory cytokines, LPS, growth factors, and antigen receptors activate an IKK complex (IKK β , IKK α , and IKK γ), which phosphorylates I κ B proteins. Phosphorylation of I κ B α results in I κ B α degradation which in turn leads to the translocation of NF- κ B to the nucleus (Wan and Lenardo, 2009). Once NF- κ B translocates to the nucleus, it is able to induce the transcription of anti-apoptotic, pro-inflammatory and IAP genes (Park and Hong, 2016; Morotti *et al.*, 2017). Effector caspases may interact with RIP and stimulate signal transduction kinase pathways that activate NF- κ B, causing the inhibition of apoptosis (Xia *et al.*, 2014; Park and Hong, 2016).

XIAP inhibits both intrinsic and extrinsic apoptotic pathways. This occurs by the characteristic BIR2 and BIR3 domains of XIAP directly binding to and inhibiting caspase-9, caspase-3 and caspase-7 (Wong, 2011). Caspase-3 is targeted for ubiquitin-mediated

proteasomal degradation by cIAP-1 and cIAP-2. Inhibitors of IAPs have been described, such as Smac/DIABLO and HTRA2/Omi, with Smac/DIABLO identified as a potent IAP binding inhibitor (Obexer and Ausserlechner, 2014). Similarly, HTRA2/Omi directly binds IAP proteins in the cytosol.

2.1.11 Autophagic cell death

Autophagy *per se* is not a form of cell death but rather a cell survival pathway. Autophagy occurs to provide energy in response to cell stress caused by nutrient deprivation, ensuring cell survival (Nencioni *et al.*, 2013; Dong *et al.*, 2016). During this process, proteins are degraded to provide amino acids for protein synthesis or energy for ATP production via the gluconeogenic pathway. Macroautophagy, the primary pathway of autophagy, occurs when a small membrane cistern, called the phagophore, forms around a portion of cytoplasm or organelle in the cytosol. This phagophore seals itself to form a double-membrane vesicular structure termed an auto-phagosome. The auto-phagosome then fuses with the endocytic system via microtubules to deliver its contents to the lysosome for degradation (Yang and Klionsky, 2010). However, under persistent nutrient deprivation, autophagy may initiate cell death (Galluzzi *et al.*, 2018).

Autophagic cell death is characterized by prominent cytoplasmic vacuolization where cells are destroyed by the degradation of cellular components via an autophagosomic-lysosomal pathway (Nakamura and Yoshimori, 2017). Autophagic cell death differs from apoptosis based on the presence of auto-phagosomes and auto-phagolysosomes in dying cells (Mishima *et al.*, 2008). Another prominent morphological feature of autophagy is the lack of chromatin condensation and nuclear fragmentation (Helgason *et al.*, 2013; Crowley *et al.*, 2016b).

2.1.12 Necrosis and necroptosis

2.1.12.1 Necrosis

Necrotic cell death is characterized by the swelling of cells, a rapid loss of membrane integrity and lysis of the cells. Swelling of the cytoplasmic organelles, especially the mitochondria is followed by plasma membrane rupture and the release of cytosolic and nuclear contents. In cancer patients, the release of cellular contents into the surrounding tissues and blood stream initiates a severe inflammatory response causing the tumour lysis syndrome (Moffitt *et al.*, 2010; Baig *et al.*, 2016). The subsequent increase of pro-

inflammatory molecules, as well as cytokines like interleukin-1 alpha (IL-1 α) can promote cancer cell proliferation and cancer progression (Sonnenschein and Soto, 2013; Chan *et al.*, 2015).

2.1.12.2 Necroptosis

Recently, a regulated form of necrosis called necroptosis has been described. Necroptosis is controlled via activation of specific receptors and mediators like RIP1, and RIP3 and the mixed lineage kinase domain like pseudokinase (MLKL) (Cai *et al.*, 2014). The morphological changes of necrosis and necroptosis are very similar since they both result in organelle swelling and membrane rupture (Galluzzi *et al.*, 2018).

Necroptosis is dependent on the activation of RIP3 and MLKL pseudo-kinase (Moriwaki and Chan, 2013; Zhang *et al.*, 2016). The primary mechanism for necroptosis is via the activation of cell death receptors, primarily TNF-R1 and to a lesser extent Fas/CD95, TRAIL-R1 or TRAIL-R2, upon ligand binding. These receptors are also involved in pro-survival pathways and in extrinsic apoptosis. The fate of a cell is dependent on the components of the complex that forms (Figure 2.3).

The binding and activation of TNF α to TNF-R1 signals the recruitment of TRADD, kinase RIP1 and several ubiquitin E3 ligases to form the pro-survival complex I (Dasgupta *et al.*, 2017). In complex I, RIP1 is poly-ubiquitinated by cIAP-1 and cIAP-2. When RIP1 is de-ubiquitinated it dissociates from complex I and can form either complex IIa or IIb. Complex IIa mediates the activation of caspase-8 and initiates apoptosis. When caspase-8 cannot be activated, complex IIb assembles to form the necrosome and triggers necroptosis. In complex IIb, RIP1 and RIP3 are phosphorylated causing oligomerisation (Chan *et al.*, 2015). RIP3 phosphorylates MLKL which in turn translocates to the plasma membrane where it facilitates Na⁺ and Ca²⁺ influx across the plasma membrane causing cell death (Chan *et al.*, 2015; Galluzzi *et al.*, 2018). Active caspase-8 cleaves and inactivates RIP1 and prevents necrosome formation, and ultimately necroptosis (Zhou and Yuan, 2014).

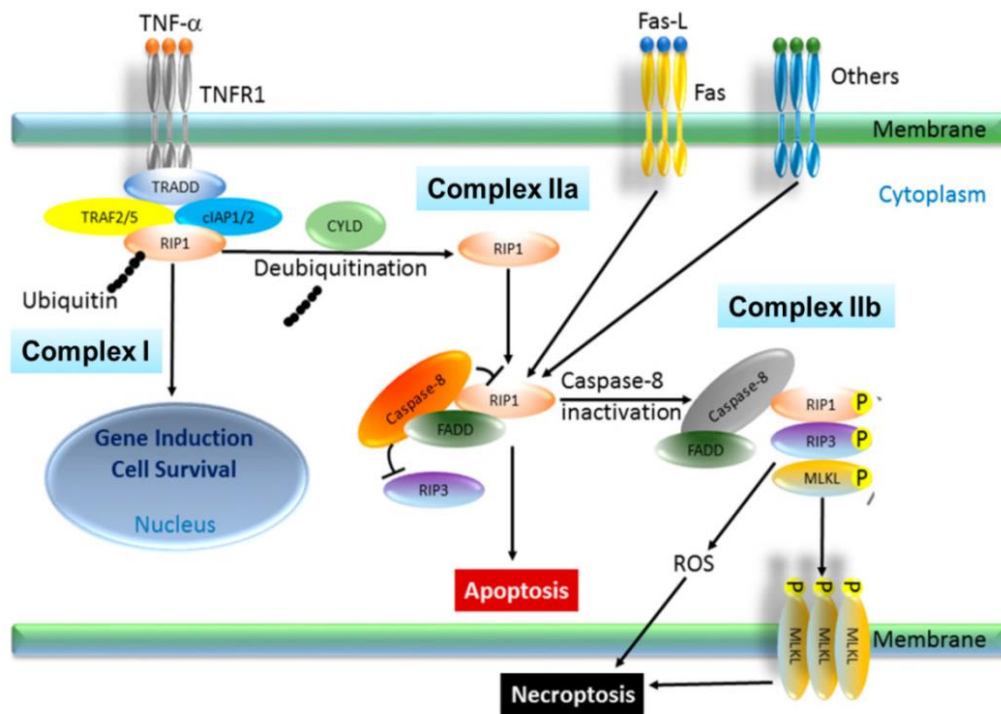


Figure 2.3 Necroptosis signalling pathway (Dasgupta *et al.*, 2017). RIP1 protein can initiate both apoptosis and necroptosis. The activation of complex I occurs as a result of poly-ubiquitination of RIP1 by cIAP-1 and cIAP-2 leading to cell survival via the NF- κ B pathway. De-ubiquitination of RIP1 leads to the formation of one of two complexes; complex IIa and complex IIb. Complex IIa activates caspase-8 and initiates apoptosis. Complex IIb is formed when caspase-8 is inactive and cause the insertion of MLKL into the plasma membrane causing necroptosis.

2.1.13 Other atypical forms of cell death

Apart from necroptosis, there are other atypical subtypes of cell death including ferroptosis, pyroptosis, parthanatos, mitochondrial permeability transition-dependent necrosis, and cell death associated with the release of neutrophil extracellular traps (netosis or etosis) (Galluzzi *et al.*, 2018). However, with the exception of pyroptosis and ferroptosis these pathways are not well described.

2.1.13.1 Pyroptosis

Pyroptosis is a pro-inflammatory form of programmed cell death that is characterized by the activation of inflammatory caspases (caspase-1, caspase-4, caspase-5) (Vande Walle and Lamkanfi, 2016). The activation of pyroptosis occurs by the activation inflammasomes, multiprotein intracellular like complexes, which activate pro-inflammatory cytokines. The canonical pathway is a caspase-1-dependent pathway whereby sensor proteins such as the Nod-like receptor; NLRC4, activate inflammasomes and causes the downstream activation of

pyroptosis (Galluzzi *et al.*, 2018). In the non-canonical pathway, liposaccharides from bacteria bind and activate caspase-4 and caspase-5 causing gasdermin D cleavage and pyroptosis. The morphological characteristics of pyroptosis include cell swelling, phosphatidylserine exposure and plasma membrane rupture, releasing the cytoplasmic contents including pro-inflammatory molecules (Crowley *et al.*, 2016b; Vande Walle and Lamkanfi, 2016).

2.1.13.2 Ferroptosis

An unusual form of cell death, ferroptosis, is iron and ROS-dependent (Yang and Stockwell, 2016). The morphological features of ferroptosis include a decrease in the size of mitochondria, an increase in mitochondrial membrane density and outer mitochondrial membrane rupture whilst the nucleus retains structural integrity (Galluzzi *et al.*, 2018). The absence of chromatin condensation in ferroptotic cells makes it easy to distinguish this type of cell death from apoptosis.

2.1.14 Aim and Objectives

Therapeutic failure as a result of drug resistance and severe drug induced toxicity warrants the search for alternative chemotherapeutic agents able to induce apoptotic cell death at concentrations below 10 μ M.

Aim:

To evaluate the growth inhibitory effects of a library of imidazo[1,2-*a*]pyridines in leukaemic HL-60 and K562 cells and to determine if active compounds induce apoptotic cell death.

Objectives:

- Identify test compounds that inhibit leukaemic cell growth by 80% when tested at a screening concentration of 100 μ M using the MTT cytotoxicity assay.
- Determine the 50% inhibitory concentration (IC₅₀ concentration) of the active test compounds identified in the first objective.
- Evaluate changes to cell morphology when exposed to the active test compounds to exclude compounds causing necrotic cell death.

- Determine if apoptosis is induced by the active test compounds by measuring apoptotic indicators such as phosphatidylserine externalisation using Annexin-V binding and the activation of caspase-3/7
- Determine intrinsic or extrinsic apoptosis by measuring mitochondrial membrane potential, caspase-8 and caspase-9 activity
- Assess the toxicity of active test compounds on normal peripheral leukocytes

2.2 METHODS

2.2.1 Cell culture

The HL-60 cell line (acute promyelocytic leukaemia) and the K562 cell line (chronic myelocytic leukaemia) was purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. The cell lines were authenticated at the start of the study by short tandem repeat (STR) analysis performed by Inqaba Biotechnical Industries, Pretoria, South Africa. An ethics waiver for the use of these cell lines was obtained from the Human Research Ethics Committee, see Appendix A.

2.2.2 Cell maintenance

Both HL-60 and K562 cells were maintained in suspension, in 75 cm² tissue culture flasks (Corning®, Sigma-Aldrich®/ Merck, South Africa) at 37°C, in a humidified, 5% CO₂ atmosphere. They were cultured in a 1:1 mixture of DMEM and Ham's F-12 nutrient mixture (Sigma-Aldrich®/ Merck, South Africa) supplemented with; 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Gibco®, Life technologies, South Africa), 1% (v/v) solution of 200 mM L-glutamine, 1% of 10 mM sodium pyruvate (Sigma-Aldrich®), 50 µg/ml of gentamicin and 100 µg/ml of carbenicillin mixture (Sigma-Aldrich®/ Merck, South Africa). All solutions, buffers and media were prepared in Milli-Q water.

2.2.3 Sub-culturing of cell lines

Cells were sub-cultured by removing 70% of the suspension culture and replacing it with fresh complete media. This was repeated every third day or when they reached 80% confluency at a cell density of 1×10^6 cells/ml for both HL-60 and K562 cells. Cell cultures were monitored daily using the Olympus CKX41 (Tokyo, Japan) inverted phase-contrast

microscope. In order to maintain sterility, all procedures were conducted under aseptic conditions in Class II biosafety units.

2.2.4 Cryopreservation of cells

Cells were cryopreserved when they were in logarithmic growth phase. Freezing media consisted of a 1:1 mixture of DMEM and Ham's F-12 nutrient mixture (Sigma-Aldrich®/ Merck, South Africa) supplemented with 20% FBS and 5% (v/v) cell culture grade dimethyl sulfoxide (DMSO) (Monitoring and Control Laboratories). Cell cultures were aspirated and transferred into 50 ml conical tubes for harvesting at $250 \times g$ for 5 minutes using the Sorvall® T-6000 centrifuge. Cell pellets were resuspended in 20 ml of freezing media, and 1.0 ml cell suspension volumes were transferred to labelled cryovials (Greiner Bio-One). The cryovials were placed in a Corning® CoolCell® freezing container (Thermo Scientific™, South Africa) to facilitate a controlled freezing process for at least 4 days at -70°C and thereafter, transferred to liquid nitrogen for long term storage.

Cryopreserved cells were quick thawed in a 37°C water bath, transferred to 25 cm^2 tissue culture flasks (Corning®, Sigma-Aldrich®/ Merck, South Africa) and maintained in cell culture media supplemented with 20% FBS (Sigma-Aldrich®/ Merck, South Africa). Once the cells were proliferating, the FBS in the culture media was decreased to the standard 10%.

2.2.5 Test compounds

The School of Chemistry from the University of the Witwatersrand supplied 43 test compounds whose structures were based on the imidazo[1,2-*a*]pyridine scaffold (Figure 2.4). The compounds were synthesized by the Groebke-Blackburn-Bienaymé multicomponent coupling reaction by Dr Jean Dam of the School of Chemistry, University of the Witwatersrand. The test compounds were arbitrarily called JD-compounds after the initials of Dr. Dam. Their structures and purity were confirmed by x-ray diffraction crystallography and elemental analysis by Dr. Dam. A 10 mM stock solution of each test compound was prepared in either 100% ethanol or DMSO and stored at 4°C . All experiments were performed with freshly diluted test compounds.

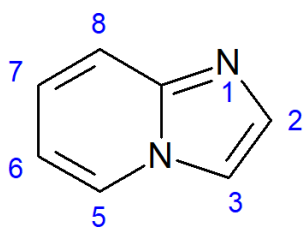


Figure 2.4 Imidazo[1,2-*a*]pyridine scaffold used for the synthesis of test compounds

2.2.6 Controls

All experiments included a negative control and vehicle only control to evaluate the effect of the solute used to solubilize the compounds on cell growth. The effects of the test compounds were compared to camptothecin, an inhibitor of topoisomerase I and a known inducer of apoptosis (Chu *et al.*, 2014). Camptothecin is the parent compound of topotecan and irinotecan which are anti-cancer agents used in the treatment of metastatic colorectal and ovarian cancers (Pierzynska-Mach *et al.*, 2014). Camptothecin was used throughout this study as a positive control.

2.2.7 Determination of cell numbers and viability

The trypan blue exclusion assay was used to determine the cell density and viability of cultured cells as well as leukocytes prior to each experiment. A 1:1 ratio of cell suspension to trypan blue dye solution (0.2% w/v) (Sigma Aldrich®/ Merck, South Africa) was mixed and 10 µl of the mixture was loaded onto the Neubauer haemocytometer chamber and viewed using the Nikon Optiphot light microscope at 100X magnification.

2.2.8 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was used to construct calibration curves for both cell lines to determine the appropriate cell density to be used in subsequent experiments (Appendix B). This assay was also used to identify active compounds and to determine IC₅₀ values. The mitochondrial enzyme, succinate dehydrogenase, reduces MTT to an insoluble formazan product (solubilized by DMSO) and is an indicator of cell viability (Mosmann, 1983).

Cells were seeded in 96-well plates at a density of 10 000 (HL-60) and 8 000 (K562) cells per well in a volume of 180 µl and exposed to the test compounds at 37 °C and 5% CO₂ for 48

hours. Controls included (i) untreated cells, (ii) media and (iii) camptothecin. Following incubation, a volume of 40 μ l of MTT (5 mg/ml) was added to each well and the plates were incubated for a further 2 hours at 37°C. After incubation, the plates were centrifuged at 250 $\times$ *g* for 5 minutes using the Sorvall® T-6000 centrifuge, and 190 μ l supernatant was removed with caution to avoid aspiration of the MTT formazan crystals. A volume of 200 μ l of DMSO was used to dissolve the purple formazan crystals with agitation at 1000 rpm using an orbital microplate shaker (Thermo-Shaker™ MB100-4P) for 2 minutes. Absorbance was measured with the Lab systems i-EMS microplate reader at a wavelength of 540 nm with reference wavelength of 690 nm and the percentage cell viability was calculated.

IC₅₀ values were calculated after construction of sigmoidal dose response curves using non-linear regression analysis with the Graph-Pad Prism® version 8.0 software package. All assays were performed at least four times and all data-points were obtained in quadruplicate.

2.2.9 Isolation of peripheral leukocytes

Human peripheral leukocytes were used as control cells to determine toxicity and changes to the proteomic profile of apoptotic related proteins induced by the active test compounds. Drug-free, human peripheral blood was obtained from volunteers with informed written consent in accordance with the guidelines of the University of the Witwatersrand Human Ethics Committee (Ethics clearance number: M170509), see Appendix C.

Whole blood was collected from healthy volunteers by venepuncture into EDTA-treated blood collection tubes. An equal volume of the anti-coagulated venous blood was layered onto 15 ml of HISTOPAQUE®-1077 (Sigma Aldrich®/ Merck, South Africa) and centrifuged at 250 $\times$ *g* for 30 minutes at room temperature (Gupta *et al.*, 2010). During centrifugation, erythrocytes and granulocytes sediment to the bottom of the tube due to the density of the histopaque, and the lymphocytes and mononuclear cells are found at the plasma-Histopaque interface. This layer was harvested and washed thrice in cell culture media by centrifugation at 250 $\times$ *g* for 10 minutes each. The cell pellets containing the leukocytes were resuspended in 50 ml of RPMI-1640 containing 2 mM L-glutamine, and supplemented with 10% heat-inactivated FBS (Gibco®, Life technologies, South Africa) and 50 μ g/ml gentamicin. The effect of the test compounds on leukocytes was determined as described in section 2.2.8.

2.2.10 Cell morphology determined by Hoechst-33342, acridine orange and ethidium bromide staining

The effects of the active test compounds on cell morphology was determined by staining treated and control cells with the fluorochromes Hoechst-33342 (HO) (Sigma Aldrich®/Merck, South Africa), acridine orange (AO) (Thermo Fisher, Johannesburg, RSA) and ethidium bromide (EtBr) (Sigma Aldrich®/Merck, South Africa) according to the methods described by Ribble and Mpoke (Mpoke and Wolfe, 1997; Ribble *et al.*, 2005).

HO is a nucleic acid stain which crosses the cell membrane and stains the nuclei of living cells a homogenous blue colour. The nuclei of apoptotic cells have a globular punctate staining pattern with increased fluorescence intensity as illustrated in Figure 2.5(A), (D) and (E). AO crosses the cell membrane and stains nucleic acids green, acidic compartments bright orange/red (Figure 2.5(B)) and, is a preliminary indicator for autophagic lysosome formation and acidification of the cell (Pierzynska-Mach *et al.*, 2014). EtBr penetrates cells with damaged membranes and intercalates into DNA with a 10 times increase in red/orange fluorescence intensity and is an indicator for necrosis as shown in Figure 2.5 (F). Therefore, a combination of HO, AO and EtBr were used to evaluate the morphology of the treated leukaemic cell lines.

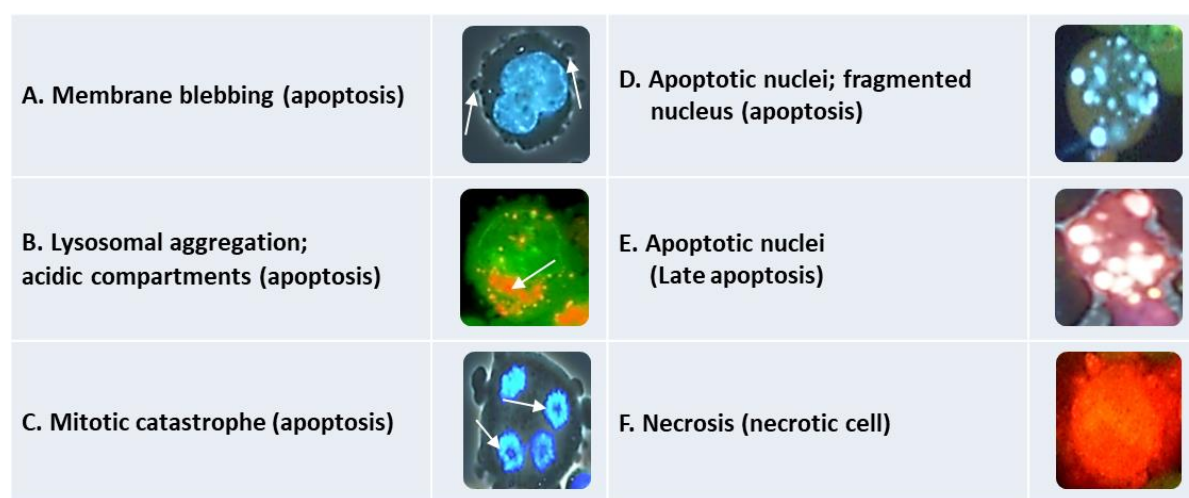


Figure 2.5 Fluorescent micrographs showing apoptotic nuclei and necrotic cells as stained by vital dyes, HO, AO and EtBr. Images were viewed on an Olympus BX41 epifluorescence microscope and captured on an Olympus DP72 camera.

Cells were seeded in 24-well plates at a density of 1×10^6 cells/ml in a volume of 1 ml and treated with active compounds and camptothecin at IC_{75} concentrations for 24 hours. The cells were viewed under phase contrast microscopy (Olympus CKX41 inverted microscope)

followed by harvesting by centrifugation at $250 \times g$ for the staining with the combined fluorochromes. The cells were washed twice with phosphate buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4 , 2.7 mM KCl and 1.8 mM KH_2PO_4 , pH 7.4) before the addition of the staining solution which contained HO (5 $\mu\text{g/ml}$), AO (10 $\mu\text{g/ml}$) and EtBr (10 $\mu\text{g/ml}$) in PBS. Staining was performed for 30 minutes in the dark (Ribble *et al.*, 2005). Following incubation, the stained, unfixed cells were centrifuged at $250 \times g$ and resuspended in 30 μl of PBS and placed on a microscope slide under a coverslip. The slides were viewed with the Olympus BX41 epifluorescence microscope (Tokyo, Japan) at 400X magnification using the following filter cubes: (i) U-MWU2 long pass filter (λ_{ex} =330-385 nm, λ_{em} =420 nm) for Hoechst-33342 (ii) U-MNIBA3 narrow bandwidth filter (λ_{ex} =470-490 nm, λ_{em} =510-550 nm) for acridine orange (iii) U-MWIG2 long pass filter (λ_{ex} =520-550 nm, λ_{em} =580 nm) for ethidium bromide. Images were captured using the Olympus DP72 digital camera and analysed with the Olympus CellSens software package (Olympus, Tokyo, Japan).

2.2.11 Detection of apoptosis by Annexin-V/PI

The detection of PS on the outer surface of the cell membrane was determined using the ThermoFisher Alexa Fluor® 488 Annexin-V/ Dead Cell Apoptosis Kit (ThermoFisher Scientific, Johannesburg, South Africa). The externalization of PS is an early event in apoptosis (Lee *et al.*, 2013c). Annexin-V (conjugated with the fluorophore, Alexa Fluor® 488) binds to PS on the cell membrane. Propidium iodide (PI) dye is included to detect the cellular DNA in necrotic/late apoptotic cells since it does not penetrate the membranes of viable cells. Thus, viable non-apoptotic cells are Annexin-V and PI negative, early apoptotic cells are Annexin-V positive and PI negative, late apoptotic cells are both Annexin-V and PI positive, and necrotic cells are Annexin-V negative and PI positive.

Cells were seeded in 12-well plates at a density of 1×10^6 cells/ml in a volume of 2 ml and treated with the active test compounds and camptothecin at IC_{75} concentrations. HL-60 cells were treated for 14 hours and K562 cells for 24 hours. Following treatment, cells were harvested by centrifugation at $250 \times g$ for 5 minutes and washed with PBS. Cell pellets were resuspended and incubated with 100 μl Annexin-V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl_2 , pH 7.4) containing Alexa Fluor® 488 (6% v/v) and PI (1% v/v) for 15 minutes in the dark at room temperature. The suspension of stained cells was further diluted to 500 μl with annexin binding buffer. The samples were then analyzed using the

LSRFortessa™ flow cytometer (BD Biosciences) measuring the fluorescence emission at 530 nm (FL1) and 620 nm (FL3) for Alexa Fluor® 488 detection and PI detection, respectively.

Size was assessed using forward and side light scattering in forward scatter (FSC) and side scatter (SSC) channels, respectively. Since Annexin-V/PI staining patterns may vary for different cells and environmental conditions, gating boundaries were adjusted accordingly for each cell line. Positive and negative controls including unstained and untreated viable cells, necrotic cells staining positive for PI only and apoptotic cells staining positive for Annexin-V only were included to perform compensation. For PI positive control, cells were fixed and permeabilised with BD Cytfix/Cytoperm™ solution (BD Biosciences) and for the Annexin-V positive control; cells were treated with doxorubicin (Sigms-Aldrich®) for 4 hours at 10 µM. Data was analysed using FlowJo (Tree Star) software. Quadrant analysis was performed on at least 10 000 cells to determine the percentage of viable, early apoptotic, late apoptotic and necrotic cells.

For detection by fluorescence microscopy, 30 µl of the stained cell suspension was transferred to a microscope slide and covered by a coverslip. Cells were viewed with an Olympus BX41 epifluorescence microscope using the U-MNIB3 and the U-MWIG2 filter cubes and images were captured as described in section 2.2.10.

2.2.12 Determination of caspase-3/7 activity

Caspase-3/7 activity was measured using the Abcam® Caspase-3/7 fluorometric assay kit (ab39383). Caspase-3 and -7 are both executioner caspases which when activated initiates apoptotic cell death. Caspase-3/7 cleaves proteins with the amino acid sequence Asp-Glu-Val-Asp (DEVD). The assay is based on the detection of the cleavage of substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). Since both caspase-3 and caspase-7 contributes to the cleavage of the DEVD sequence in proteins, one cannot distinguish if the signal generated is by caspase-3 or caspase-7 activity. Substrate DEVD-AFC emits blue light at a wavelength of 405 nm, whereas the cleaved substrate AFC emits a yellow-green fluorescence which is detected at a wavelength of 535 nm.

Cells were seeded in 12-well plates at a density of 1×10^6 cells/ml in a volume of 2 ml and treated with active test compounds at IC₇₅ concentrations for various time periods. Untreated cells were included as a negative control and camptothecin treated cells as a positive control

for each experiment. Following treatment, cells were resuspended in 50 µl of cold cell lysis buffer and incubated on ice for 10 minutes. Fifty microliters of two times reaction buffer containing 10 mM dithiothreitol (DTT) (Abcam) was added to each sample and the protein concentration of each was determined using the Bradford assay. Thereafter, 5 µl of the 1 mM DEVD-AFC substrate was added and the samples were incubated at 37°C for 2 hours in the dark. The samples were transferred to black 96-well plates and fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm on the FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices) equipped with the SoftMax Pro Microplate Data Acquisition and Analysis 6.4 software package (Molecular Devices, California, USA). The specific activity of caspase-3/7 was calculated for at least three independent experiments and results were expressed as a fold increase compared to the untreated control.

2.2.13 Determination of protein concentration

The Bradford protein assay (BIO-RAD, Life Science) as described by Bradford, (1976) was used to determine the protein concentration in cell lysates. The BIO-RAD assay is based on the formation of a complex between protein in solution and the Coomassie Brilliant Blue G-250 dye which is determined spectrophotometrically at 595 nm. A standard curve was constructed using standard bovine serum albumin (1.0 mg/ml) (Fluka™). Lysates were diluted according to manufacturer's protocols to prevent interference with the assay. Protein concentrations of lysate were needed for caspase-3/7, caspase-9 and proteomic profile assays. The bicinchoninic acid assay was an unsuitable option since copper interferes with the assay.

The protein assay was carried out in 96-well microtiter plates. The dye reagent was prepared in a 1:4 dilution of the concentrated dye reagent with Milli-Q water. In each well, 10 µl of each standard and test sample was added, followed by 200 µl of the diluted dye reagent. Thereafter, the plates were shaken using the orbital Thermo Scientific™ Multiskan™ GO microplate shaker at 1000 rpm for 5 minutes. The absorbance of each sample was read at 595 nm using the Thermo Scientific™ Multiskan™ GO microplate spectrophotometer with SkanIt software. Data points were collected in triplicate. A standard curve was constructed for each protein determination and is shown in Appendix D, Figures D1-7.

2.2.14 Assessment of mitochondrial membrane potential

The mitochondrial specific cationic 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) dye (Sigma Aldrich®/Merck, South Africa) was used to detect changes in the potential ($\Delta\psi_m$) across mitochondrial membranes (Estaquier *et al.*, 2012). In healthy cells, with intact mitochondrial membranes, the JC-1 dye forms J-aggregates/dimers indicated by yellow-orange/red fluorescence. Upon the loss of the mitochondrial membrane potential, the JC-1 dye remains in its monomeric form which is indicated by the presence of only green fluorescence and the absence of orange fluorescence (Chen *et al.*, 2017).

HL-60 and K562 cells were seeded in 6-well plates at 1×10^6 cells/ well in a 2 ml volume and treated with active compounds at IC_{75} concentrations and incubated at 37°C for 14 and 18 hours, respectively. Cells were harvested and washed in 1 ml of PBS (pH 7.4). Cell pellets were then resuspended in 500 μ l of a 2 μ M solution of JC-1 dye and incubated at 37°C in a CO₂ incubator for 15 minutes. Thereafter, the cells were washed twice in PBS solution (pH 7.4) by centrifuging at $400 \times g$ for 5 minutes. Each cell pellet was resuspended in 100 μ l of assay buffer and 20 μ l of cell suspension mix was transferred onto a microscope slide and covered with a coverslip. Cells were observed at a 400X magnification for changes in MMP by fluorescence microscopy with the BX41 Olympus epifluorescence microscope (Tokyo, Japan) using the U-MNIBA3 filter cube (λ_{ex} =470-490 nm, λ_{em} =510-550nm). Images were captured using the Olympus DP72 digital camera and analyzed with Olympus CellSens software package. Experiments were performed three times. Three independent fields were counted for each experiment using the CellSens program to analyze the data.

2.2.15 Determination of caspase-9 activity

Caspase-9 activity was measured using the Abcam® Caspase-9 (active) FITC staining kit (ab65615) according to the manufacturer's protocol and quantified by spectrofluorimetry. The caspase-9 selective inhibitor LEHD- fluoromethylketone (FMK) conjugated to FITC (FITC-LEHD-FMK) irreversibly binds to active but not to inactive caspase-9 in apoptotic cells. HL-60 and K562 cells were seeded in 6-well plates at a density of 1×10^6 cells/well in a 2 ml volume and treated with active test compounds at IC_{75} concentrations for 14 and 24 hours, respectively. Untreated cells were included as a negative control and camptothecin as a positive control.

Following treatment, the cells from each well were transferred to micro-centrifuge tubes and the protein concentration of each was determined using the Bradford assay (section 2.2.12). Thereafter, 1 μ l of FITC-LEHD-FMK reagent was added to 300 μ l of each tube and incubated in the dark for one hour at 37°C in a humidified incubator. Cells were then centrifuged at $400 \times g$ for 5 minutes and resuspended in 500 μ l of wash buffer. After three wash steps, the cell pellets were resuspended in 100 μ l of wash buffer and transferred to black 96-well fluorescent plates. Fluorescence was measured by the FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices) equipped with the SoftMax Pro Microplate Data Acquisition and Analysis 6.4 software package (Molecular Devices, California, USA). Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Specific activity was calculated by the ratio of enzyme activity divided by the protein concentration of the assay and presented as fold increase relative to the untreated control.

These results were confirmed by fluorescence microscopy. Cell suspensions were transferred to coverslips and mounted on microscope slides for cells to be viewed with an Olympus BX41epifluorescence microscope using the Olympus U-MNIB3 filter cube. Images were captured and analysed as described previously (section 2.2.10).

2.2.16 Determination of caspase-8 activity

Caspase-8 activity was measured using the Abcam® Caspase-8 (active) Red staining kit (ab65618) (Biocom Africa, Pretoria, South Africa) according to the manufacturer's protocol and measured by fluorescence microscopy. The activated caspase-8 selective inhibitor IETD-FMK conjugated to sulfo-rhodamine (Red-IETD-FMK) is used as a fluorescent marker that penetrates cells and irreversibly binds to active but not to inactive caspase-8. Untreated cells were included as a negative control and camptothecin as a positive control.

Cells were treated with active test compounds and camptothecin at IC_{75} concentrations for 6, 14, 18 and 24 hour time periods. Following treatment, cells were centrifuged at $400 \times g$ for 5 minutes and cell pellets were resuspended in 50 μ l PBS containing 1 μ l Red-IETD-FMK reagent and incubated for one hour at 37°C. Cells were then centrifuged at $400 \times g$ for 5 minutes and resuspended in 100 μ l of wash buffer. For fluorescence microscopic analysis, 20 μ l of the cell suspension was transferred onto a microscope slide and covered with a coverslip. The presence of active caspase-8 was observed with the BX41 Olympus

epifluorescence microscope at 400X magnification using the U-MWG2 filter cube (λ_{ex} = 520-550 nm, λ_{em} = 580 nm). Images were captured and analysed as described previously (section 2.2.9).

2.2.17 Statistical analysis

Data was analysed using GraphPad Prism® version 8.0 software package (GraphPad Software, San Diego, California, USA, www.graphpad.com) to determine if the difference between the untreated and treated cells were statistically significant ($p < 0.05$). The one-way analysis of variance (ANOVA) was performed followed by Dunnett's *post-hoc* comparison test where $p < 0.05$ was considered significant. The IC_{50} values were determined by sigmoidal dose response curves using non-linear regression analysis. Statistical analysis of IC_{50} values was performed using the two-sample, unpaired Mann-Whitney U-test ($p < 0.05$). All experiments were repeated at least three times and all data points were obtained in triplicate or as otherwise stated. The results were reported as mean $\pm$ standard deviation (SD) of at least three independent experiments.

2.3 RESULTS

2.3.1 Cytotoxic effects of imidazo[1,2-*a*]pyridines on HL-60 and K562 cells

A series of forty-three imidazo[1,2-*a*]pyridine compounds were evaluated against HL-60 and K562 cells at a concentration of 100 μ M. Cells were treated with test compounds for 48 hours (Figure 2.6 and Figure 2.7) to identify active compounds. Test compounds which caused at least 80% inhibition of cell proliferation at 100 μ M concentration were considered to be cytotoxic. Twenty seven imidazo[1,2-*a*]pyridine compounds were active against HL-60 cells (Figure 2.6), whereas twenty-three were active against K562 cells (Figure 2.7). These compounds were further evaluated to determine their half maximal inhibitory concentrations (IC_{50} values) in the two myeloid cell lines. Appendix E; Table E1 indicates which compounds were zinc, platinum or copper coordination complexes with their differences in chemical structure. Pyridyl imidazo[1,2-*a*]pyridines were used to assemble the zinc, platinum and copper complexes by combining the relevant imidazo[1,2-*a*]pyridine in an equimolar ratio with the appropriate metal salts. The compounds differ in their R groups which included cyclohexyl, benzyl, adamantly and ethyl glycinate substituents.

HL-60 cells

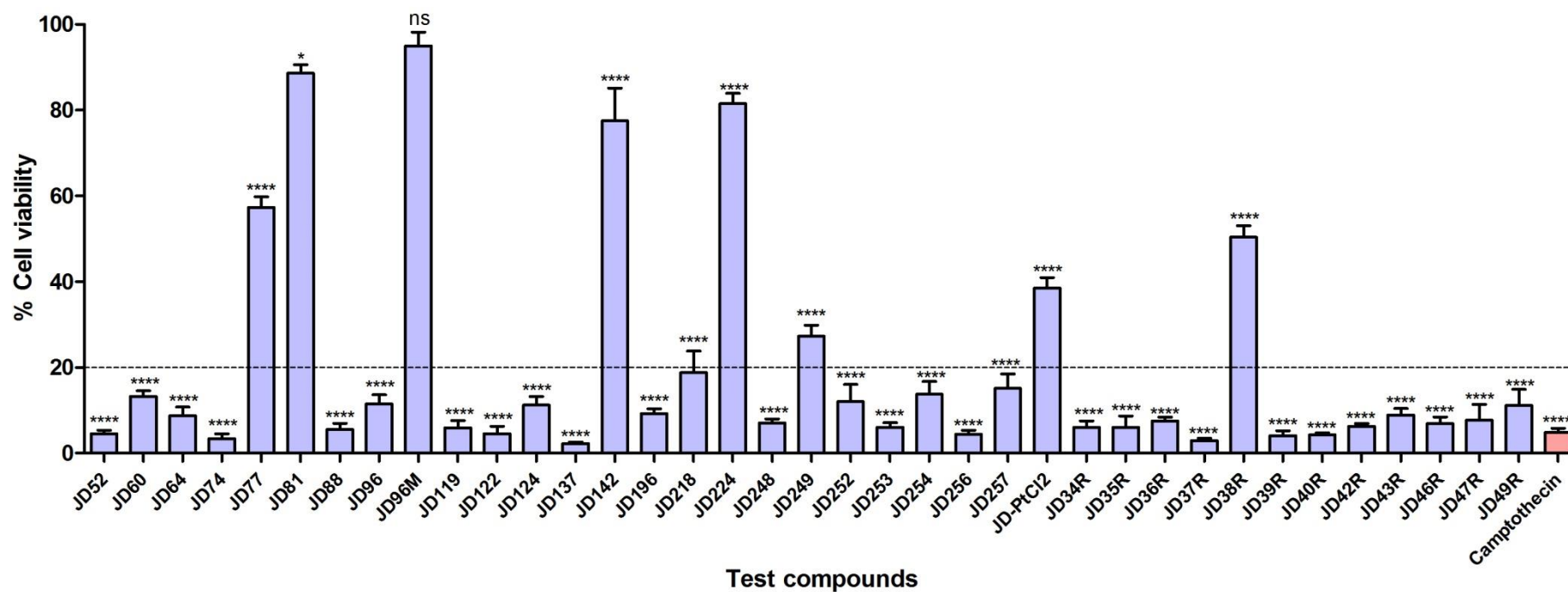


Figure 2.6 The effect of imidazo[1,2-*a*]pyridines at a concentration of 100 μ M on the viability of HL-60 cells. HL-60 cells were incubated with imidazo[1,2-*a*]pyridines for 48 hours. Camptothecin was used as a positive control. The bar graph shows 27 compounds inhibited HL-60 cell viability by more than 80%. Results were expressed as a mean $\pm$ standard error of mean (SEM) of at least three independent experiments. The broken line indicates the 20% cell survival threshold.

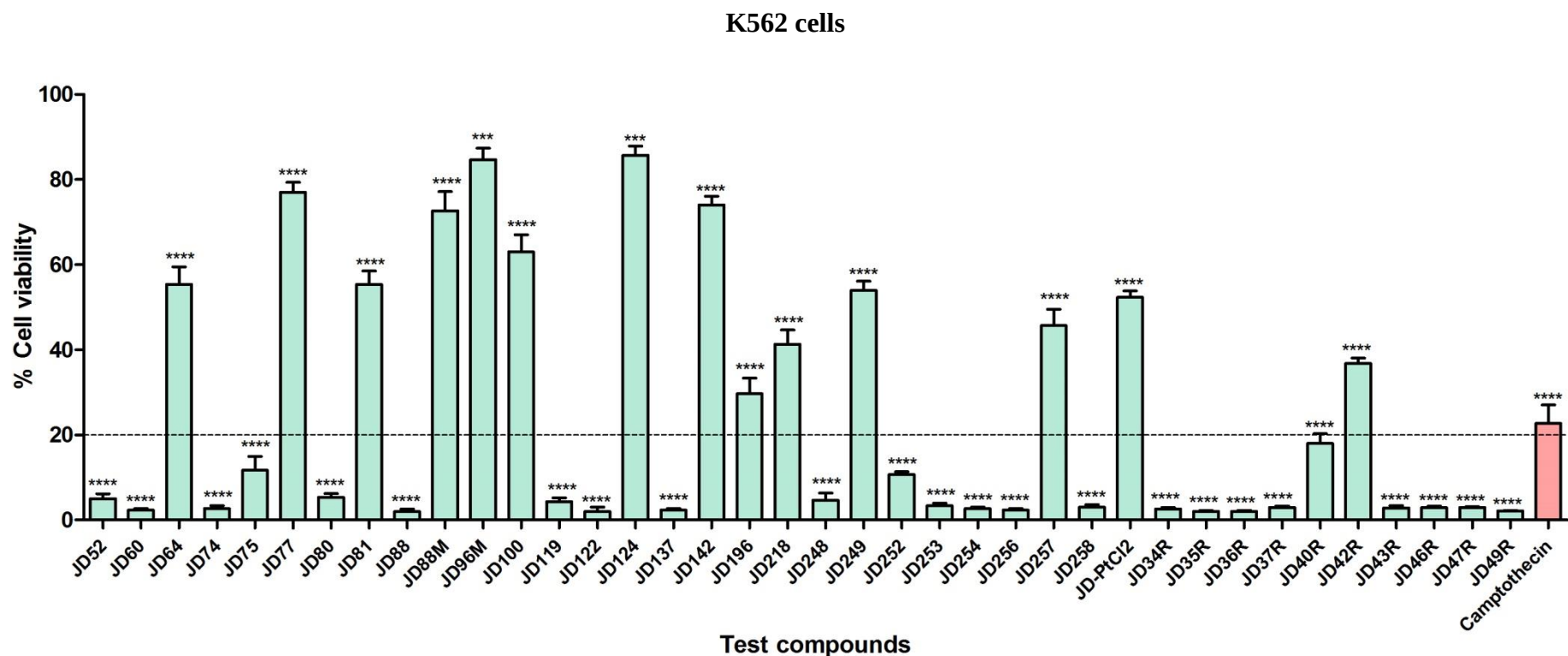


Figure 2.7 The effect of imidazo[1,2-*a*]pyridines at a concentration of 100 μ M on the viability of K562 cells. K562 cells were incubated with imidazo[1,2-*a*]pyridines for 48 hours. Camptothecin was used as a positive control. The bar graph shows 23 compounds inhibited K562 cell viability by more than 80%. Results were expressed as a mean $\pm$ SEM of at least three independent experiments. The broken line indicates the 20% cell survival threshold.

2.3.2 IC₅₀ values of active imidazo[1,2-*a*]pyridines in leukaemic cell lines

The active test compounds identified in the previous section were further evaluated to obtain IC₅₀ values. Cells were exposed to a range of concentrations between 0.01 μ M and 100 μ M for 48 hours and sigmoidal dose response curves were constructed. IC₅₀ values were calculated from at least four independent experiments, and all data points were obtained in quadruplicate. The data was obtained from the MTT cytotoxicity assay.

Despite their promising activity at 100.0 μ M (Figure 2.6 and Figure 2.7), some compounds were found to be unstable in an aqueous environment and displayed inconsistent and high IC₅₀ values. IC₅₀ values were obtained for twenty of the screened test compounds in HL-60 cells and eighteen in K562 cells (Table 2.1). The HL-60 cell line was more sensitive to the positive control camptothecin than the K562 cell line with an IC₅₀ value of 7 nM. According to NCI, IC₅₀ values below 10.0 μ M are considered as good lead candidates (Kumar *et al.*, 2016).

The results indicated that zinc and platinum imidazo[1,2-*a*]pyridine complexes had poor activity against the leukaemic cell lines while copper-imidazo[1,2-*a*]pyridine complexes were active and showed promising cytotoxic activity with IC₅₀ values below 10.0 μ M. Among the most active copper-imidazo[1,2-*a*]pyridines were JD88, JD253, JD256, JD46R, JD34R, JD47R and JD49R. The IC₅₀ values of the active compounds are shown in Table 2.1.

The IC₅₀ values reported in Table 2.1 indicated that compounds JD34R, JD256 and JD47R showed a preferential selectivity to the HL-60 cell line, whilst JD88, JD37R, JD46R and JD49R showed selectivity to the K562 cell line. Despite the low IC₅₀ values of JD34R and JD256, the structures of these compounds could not be verified by x-ray crystallography therefore they were not used in subsequent investigations. Even though JD37R displayed a low IC₅₀ value against K562 cells, its cytotoxic potential in HL-60 cells was poor; as indicated by an IC₅₀ value of above 10 μ M.

Table 2.1 The IC₅₀ values of the active imidazo[1,2-*a*]pyridines and camptothecin in leukaemic cells after 48 hours

TREATMENT	IC ₅₀ values (μM)		TREATMENT	IC ₅₀ values (μM)	
	HL-60	K562		HL-60	K562
JD52	30.2±0.6	37.3±3.3	JD257	31.3±3.3	Not active
JD60	Not active	34.0±3.9	JD34R	4.0±0.4	10.2±0.6
JD64	20.5±3.3	Not active	JD35R	12.1±0.8	9.1±1.8
JD74	14.6±1.4	66.6±4.4	JD36R	13.6±0.9	12.1±1.4
JD75	Not active	20.9±1.0	JD37R	13.7±0.9	3.5±0.9
JD88	2.2±0.5	1.7±0.3	JD40R	16.0±1.9	12.0±3.5
JD119	26.7±3.6	57.8±1.7	JD43R	32.0±2.8	12.0±3.5
JD122	20.4±3.3	22.7±1.1	JD46R	8.6±0.5	7.0±1.4
JD137	12.0±1.3	31.1±4.3	JD47R	2.1±0.3	5.4±0.3
JD253	6.4±0.9	6.9±0.4	JD49R	5.1±1.1	3.4±0.6
JD256	5.4±0.1	7.4±0.5	Camptothecin	0.007	2.8±0.3

*All data are expressed as the mean ± SD.

2.3.3 Selection of copper complexes for further evaluation

The three most active imidazo[1,2-*a*]pyridines; JD88, JD47R and JD49R, were copper coordination complexes with IC₅₀ values ranging between 1.0 and 6.0 μM. For this reason they were further investigated to determine mechanisms by which they induce their cytotoxic effects on HL-60 and K562 cells. Cells were exposed to concentrations equal to the IC₅₀ or IC₇₅ value; listed in Table 2.2, of the copper-imidazo[1,2-*a*]pyridine complexes, to assess modes of cell death. The IC₇₅ concentration represents the concentration at which 75% of the cell population is inhibited and was used to ensure an apoptotic effect was induced at exposure periods less than 24 hours.

Table 2.2 The IC₅₀ and IC₇₅ concentrations of the active copper-imidazo[1,2-*a*]pyridine complexes in leukaemic cells after 48 hours of exposure

TREATMENT	HL-60		K562	
	IC ₅₀ values (μM)	IC ₇₅ values (μM)	IC ₅₀ values (μM)	IC ₇₅ values (μM)
JD88	2.2±0.5	7.8±0.4	1.7±0.3	7.1±1.0
JD47R	2.1±0.3	4.3±1.5	5.4±0.3	7.7±1.1
JD49R	5.1±1.1	8.5±2.7	3.4±0.6	5.8±1.4
Camptothecin	0.007	0.02±0.01	2.8±0.3	6.2±0.1

*All data are expressed as the mean ± SD.

The active test compounds reduced the viability of both leukaemic cell lines in a dose-dependent manner after 48 hours as shown in Figure 2.8. Representative dose response curves for both cell lines are shown in Figure 2.8 with their corresponding IC₅₀ values for that particular experiment.

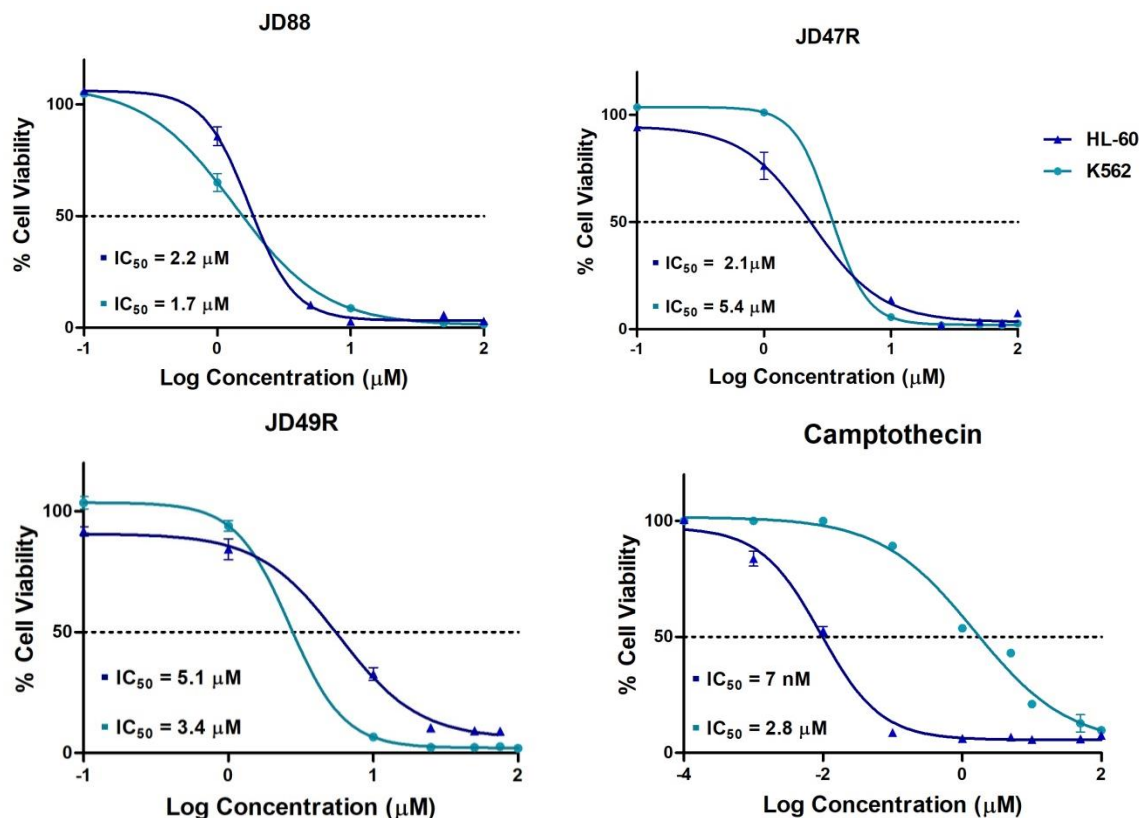


Figure 2.8 Representative sigmoidal dose-response curves of active copper-imidazo[1,2-*a*]pyridine complexes and camptothecin for the HL-60 and K562 cell lines. Cells were treated with a range of concentrations between 0.01 and 100 μM for 48 hours. The dose response curves shown here is a representative curve. Each data point represents the mean cell viability at each concentration and the error bars indicate the SD.

The IC₅₀ values of the test compounds were compared between the two cell lines. Statistical analysis of IC₅₀ values was performed using the two-sample, unpaired Mann-Whitney U-test with significance set at $p < 0.05$ and a 95% confidence interval (Table 2.3).

Table 2.3 Statistical analysis showing significant differences of IC₅₀ values between HL-60 and K562 cells

TREATMENT	<i>p</i> -value	Significance (<i>p</i> <0,05)	One or two-tailed <i>p</i> -value
JD88	0,20	ns	Two-tailed
JD47R	0,10	ns	Two-tailed
JD49R	0,34	ns	Two-tailed
Camptothecin	0,03	significant	Two-tailed

*ns= non-significant

2.3.4 Evaluation of active imidazo[1,2-*a*]pyridines on leukocyte viability

Freshly isolated peripheral leukocytes, seeded at a density of 40 000 cells/well, were exposed to the three most active test compounds and camptothecin at a 20 μ M concentration for 24 hours. This high concentration was selected as it caused a 100% cell death of leukaemic cells. Exposure of leukocytes to the copper-imidazo[1,2-*a*]pyridine complexes indicated that JD88 was the most toxic compound causing around 60% of leukocyte death after 24 hours (Figure 2.9A). The other test compounds displayed a more favourable effect with leukocyte viability ranging between 55% and 70%. Considering the low IC₅₀ of JD88, it was then evaluated at various concentrations against leukocytes as shown in Figure 2.9B. The data indicated that lower concentrations of JD88 resulted in higher leukocyte viability.

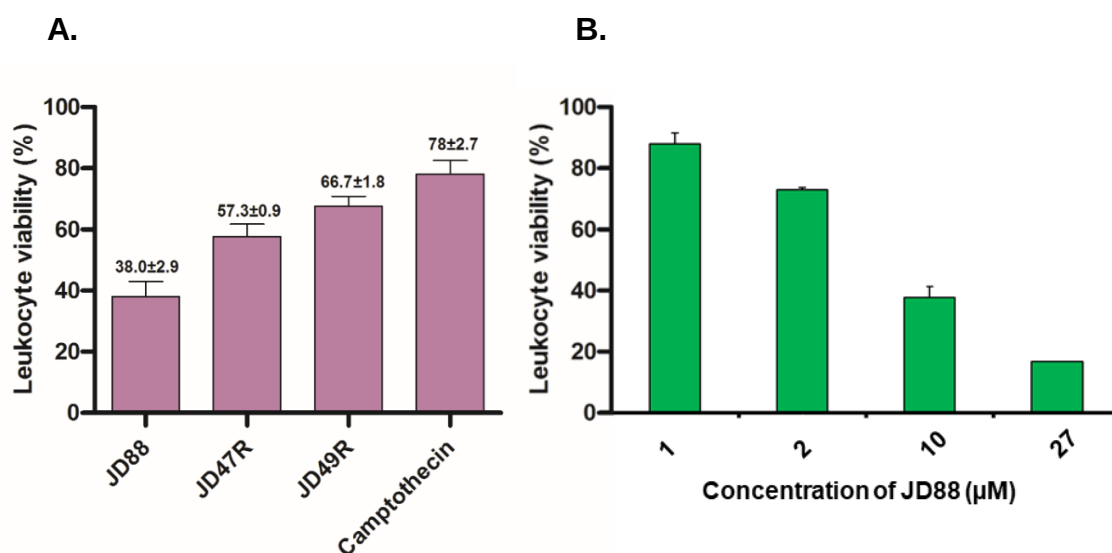


Figure 2.9 The effect of active copper-imidazo[1,2-*a*]pyridine complexes on leukocyte viability. **A** shows leukocyte viability after exposure to 20 μ M of active test compounds for 24 hrs. **B** shows leukocyte viability when treated with JD88 at varying concentrations for 24 hrs. Data obtained is an average of at least three independent experiments. Each bar represents the mean cell viability at 20 μ M of each test compound and the error bars indicate the SD.

2.3.5 Evaluation of copper-imidazo[1,2-*a*]pyridines induced morphological changes

HL-60 and K562 cells were treated with copper-imidazo[1,2-*a*]pyridines; JD88, JD47R and JD49R and camptothecin for 24 hours, at a concentration equivalent to their IC₇₅ values. Cells were observed with an Olympus BX41 microscope using the U-MWU2 filter cube for HO, the U-MNIBA3 filter cube for AO and the U-MWIG2 for EtBr detection. Fluorescent images were captured using an Olympus DP72 camera.

2.3.5.1 Effect of copper-imidazo[1,2-*a*]pyridines on cell morphology in HL-60 cells

HL-60 cells were treated for 24 hours at a concentration equivalent to the IC₇₅ value of JD88, JD47R and JD49R, and images are shown in Figure 2.10. Camptothecin was used as a positive control. At a 24 hour exposure, camptothecin treated cells showed cell membrane blebbing and fragmented nuclei, characteristic of late apoptotic cells (Figure 2.10; panels e-h). All treated cells showed features of membrane blebbing as seen by phase contrast in panels e, i, m and q (Figure 2.10). Morphological changes characteristic of apoptosis includes membrane blebbing, chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies. These results indicate that compared to the untreated control, all copper-imidazo[1,2-*a*]pyridines were able to induce morphological changes consistent with apoptosis. At their IC₇₅ concentration, a minimal percentage of cells were necrotic.

HO staining of camptothecin treated cells showed the presence of apoptotic nuclei and an increase in fluorescence intensity (Figure 2.10; panel f). The copper-imidazo[1,2-*a*]pyridines JD88, JD47R and JD49R treated cells showed fragmented and condensed nuclei staining blue with increased fluorescence intensity characteristic of apoptotic nuclei in early and late apoptosis (Figure 2.10; panels j, n and r). Comparable to camptothecin (panels f and g), JD88 (panels j and k) and JD49R (panels r and s) showed that some cells were in a late apoptotic stage as evidenced by the globular nature of the HO and/or AO stained nuclei. In contrast to treated cells, panels a, b, c, and d of untreated HL-60 control cells showed the typical staining pattern and morphology (Figure 2.10). HO staining of the control cells showed nuclei with a characteristic round shape and homogenous blue fluorescence as indicated in panels b and d of Figure 2.10.

In Figure 2.10, panels c, g, k, o and s demonstrated cells stained with AO/EtBr. AO/EtBr staining of camptothecin treated cells showed intense orange-red fluorescence with and without globular nuclei, indicative of late apoptosis (Figure 2.10; panel g). A few cells had evenly stained non-globular nuclei indicating necrotic cell death (Figure 2.10; panel g and o). In contrast, AO stained the healthy nuclei and cell cytoplasm of viable cells green (Figure 2.10; panel c). AO emits green fluorescence in both apoptotic and viable cells. Hence, this explains that due to AO staining, panels f and n show apoptotic nuclei (An) and panels g and o; the corresponding cells, are also stained green. Furthermore, apoptotic nuclei can be visible in live cells.

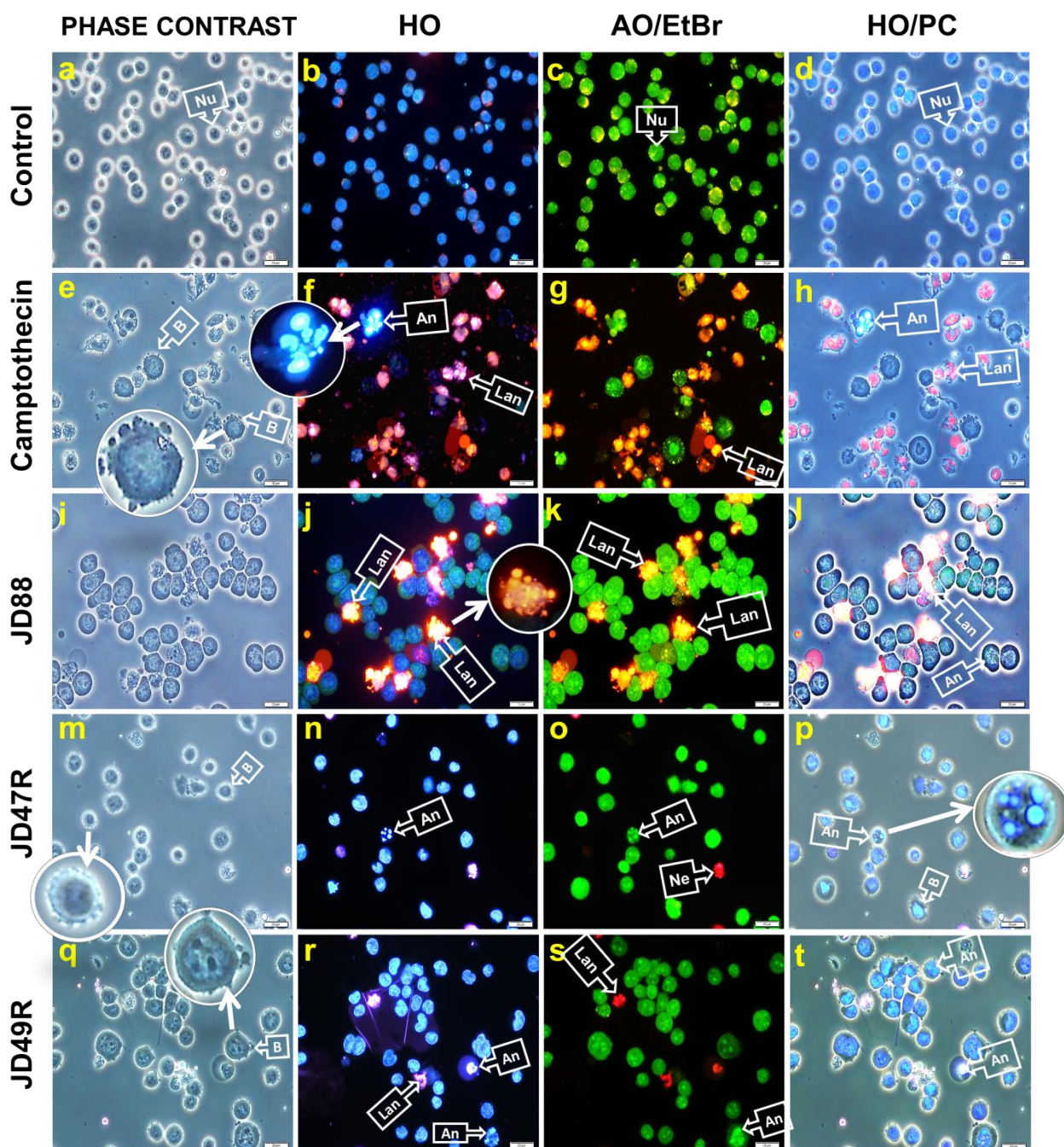


Figure 2.10 Fluorescent micrographs of HL-60 cells showing changes to cell morphology after treatment with active test compounds and camptothecin. Cells were exposed to the copper-imidazo[1,2-*a*]pyridines for 24 hrs at IC₇₅ concentrations and stained with HO/AO/EtBr. Experiments were repeated at least three times and the images shown are representative of a single experiment. Cells were observed with the Olympus BX41 epifluorescence microscope using the appropriate filter cubes and images were captured at a 400X magnification with the Olympus DP72 digital camera. ISO: 400, Scale bar: 20 μ m. Legend: B= membrane blebbing; Nu=normal nucleus; An= apoptotic nucleus; Lan= late apoptotic nucleus and Ne= necrotic nucleus. Blue fluorescence represents HO, green fluorescence represents AO and orange/red fluorescence represents EtBr.

2.3.5.2 Effect of copper-imidazo[1,2-*a*]pyridines on cell morphology in K562 cells

K562 cells were treated for 24 hours at a concentration equivalent to the IC₇₅ value of copper-imidazo[1,2-*a*]pyridine complexes; JD88, JD47R and JD49R and camptothecin. Membrane blebbing was a prominent feature in all treated K562 cells as indicated by phase contrast images in panels e, i, m and q (Figure 2.11).

Hoechst-33342 staining of camptothecin treated cells showed fragmented nuclei with increased fluorescence intensity characteristic of apoptotic nuclei (Figure 2.11; panels f and h). JD88, JD47R, and JD49R treated K562 cells showed apoptotic nuclei in early and late apoptosis with increased fluorescence intensity as indicated by HO staining (Figure 2.11; panel f, j, n and r). The K562 cells treated with JD88 showed that some cells were in a late apoptotic stage as evidenced by the globular nature of the HO and/or AO stained nuclei (Figure 2.11; panels j and k). In contrast, untreated K562 cells were evenly stained blue with oval shaped nuclei as indicated by HO staining (Figure 2.11; panels b and d).

Treatment of K562 cells with camptothecin (Figure 2.11; panel g) caused perinuclear lysosomal aggregation and acidic granules as evidenced by AO staining. Similarly, treatment of K562 cells with JD47R caused perinuclear lysosomal aggregation which appeared orange when stained with AO and red when stained with HO (Figure 2.11; panels n and o). K562 cells treated with JD49R showed chromatin condensation (Figure 2.11; panels r and s). Necrotic cells were also observed in JD49R treated K562 cells as depicted by homogenous EtBr staining (Figure 2.11; panel s). In contrast to treated cells, AO stained healthy nuclei and cell cytoplasm an emerald green colour (Figure 2.11; panel c).

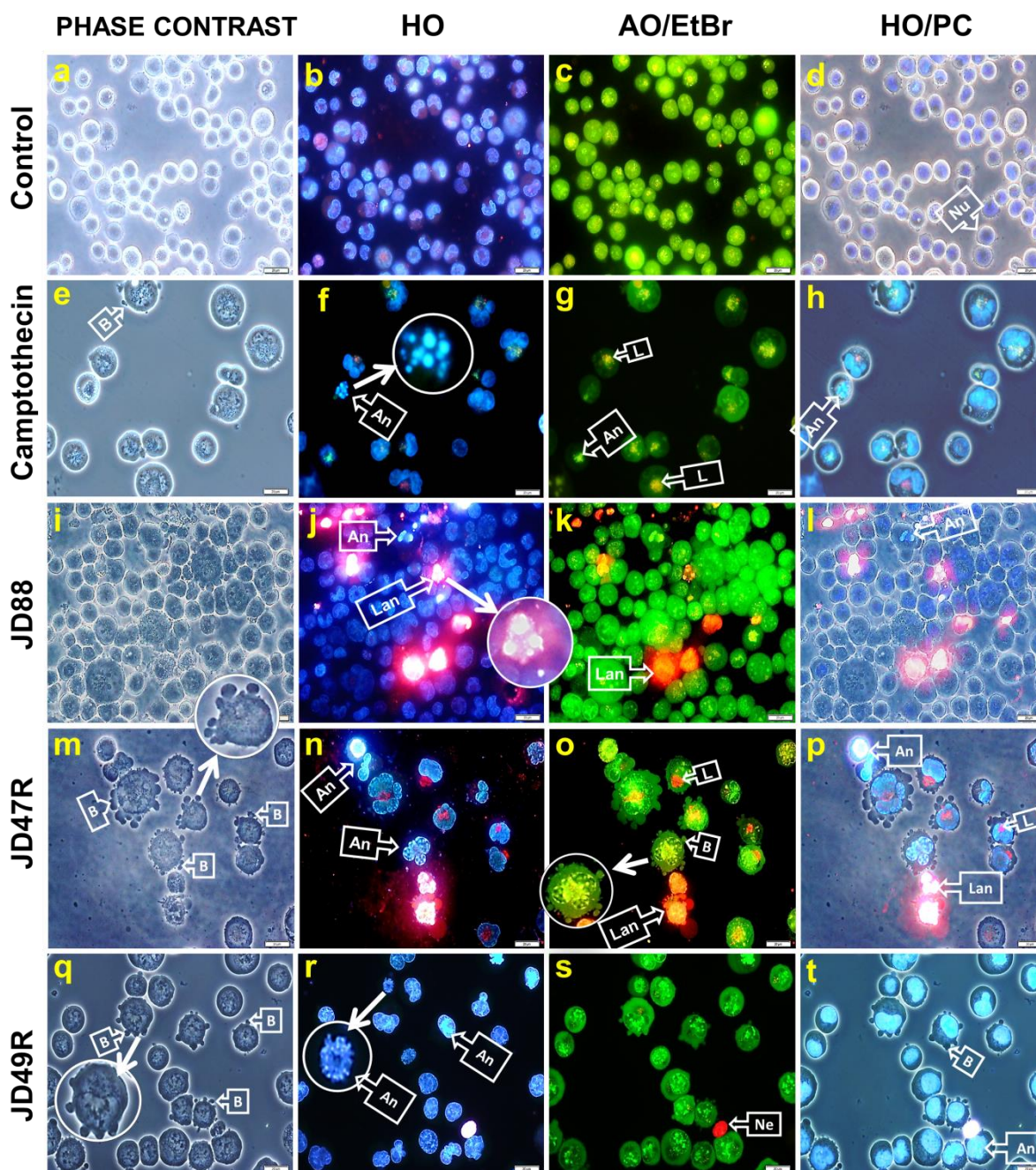


Figure 2.11 Fluorescent micrographs of K562 cells showing changes to cell morphology after treatment with active test compounds and camptothecin. Cells were exposed to the copper-imidazo[1,2-*a*]pyridines for 24 hrs at IC_{75} concentrations and stained with HO/AO/EtBr. Experiments were repeated at least three times and the images shown are representative of a single experiment. Cells were observed with the Olympus BX41 epifluorescence microscope using the appropriate filter cubes and images were captured at a 400X magnification with the Olympus DP72 digital camera. ISO: 400, Scale bar: 20 μ m. Legend: L=lysosomes; B= membrane blebbing; Nu=normal nucleus; An= apoptotic nucleus; Lan= late apoptotic nucleus and Ne= necrotic nucleus. Blue fluorescence represents HO, green fluorescence represents AO and orange/red fluorescence represents EtBr.

2.3.6 Detection of phosphatidylserine by Annexin-V/PI staining

Phosphatidylserine presence on the outer plasma membrane was detected with Annexin-V conjugated to Alexa Fluor 488. HL-60 and K562 cells were seeded at a density of 1×10^6 cells/ml and treated with the active test compounds and camptothecin at IC_{75} concentrations, for 14 and 24 hours, respectively.

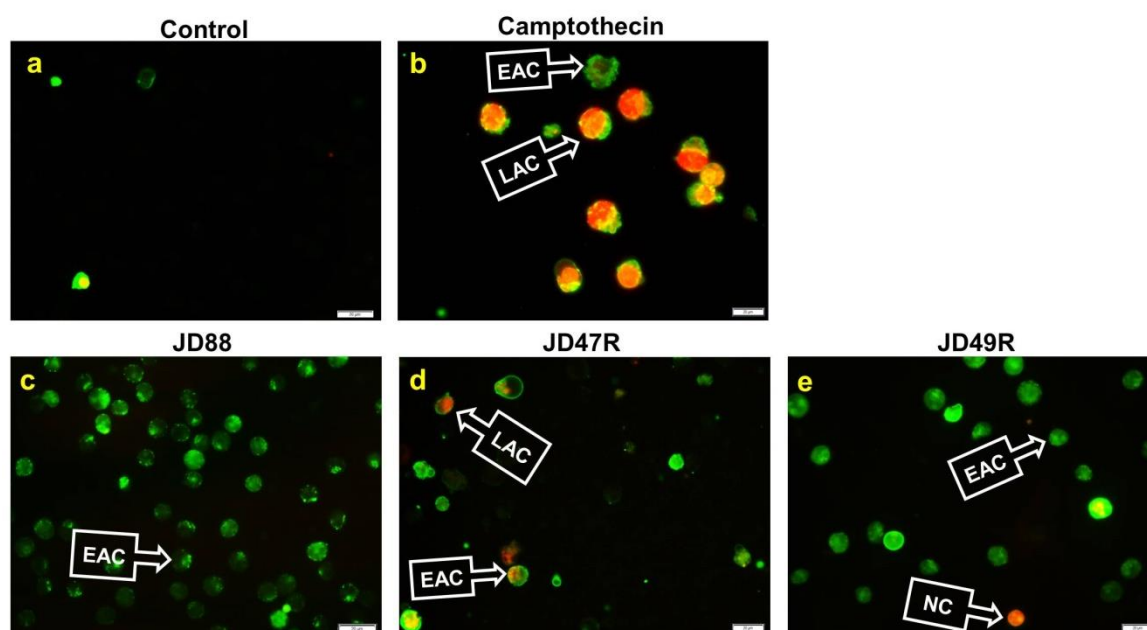
2.3.6.1 The effect of copper-imidazo[1,2-*a*]pyridines on phosphatidylserine exposure in HL-60 cells

Annexin-V binding to PS in HL-60 cells was evaluated after a 14 hour exposure period which was determined empirically. The Annexin-V assay was performed at several time periods at an IC_{75} concentration to determine the optimum treatment period. JD88, JD47R and JD49R treated HL-60 cells showed an increase in the early apoptotic and late apoptotic cell population with minimal necrosis as determined by fluorescence microscopy (Figure 2.12A).

After incubating cells with Annexin-V/PI, untreated cells showed no fluorescence (panel a), early apoptotic cells stained green (panels c, d and e), late apoptotic cells stained green and red/orange (panels b and d), and necrotic cells stained red (panel b). The positive control camptothecin showed an increase in Annexin-V and PI binding indicating late apoptosis and early necrosis (Figure 2.12A; panel b). In comparison to the camptothecin, the copper-imidazo[1,2-*a*]pyridines JD88, JD47R and JD49R stained positive for PS indicated by green fluorescence (Figure 2.12A; panels c, d and e). In untreated control cells (Figure 2.12A; panel a), no specific fluorescence binding was observed which indicated the absence of PS on the cell membrane.

This data was confirmed by flow cytometry (Figure 2.12B). Camptothecin treated cells showed a 62% increase in cells in late apoptosis/early necrosis (Figure 2.12B; panel b). The copper-imidazo[1,2-*a*]pyridines showed a significant increase in Annexin-V fluorescence, without an increase in PI fluorescence (Figure 2.12A; panels c, d and e; Table 2.4), in contrast to untreated control cells which showed a high percentage (97%) of viable cells (Figure 2.12B; panel a) with no specific fluorescence. The flow cytometry data (Figure 2.12B; panels c, d and e; Table 2.4) confirmed the data obtained by fluorescence microscopy.

A.



B.

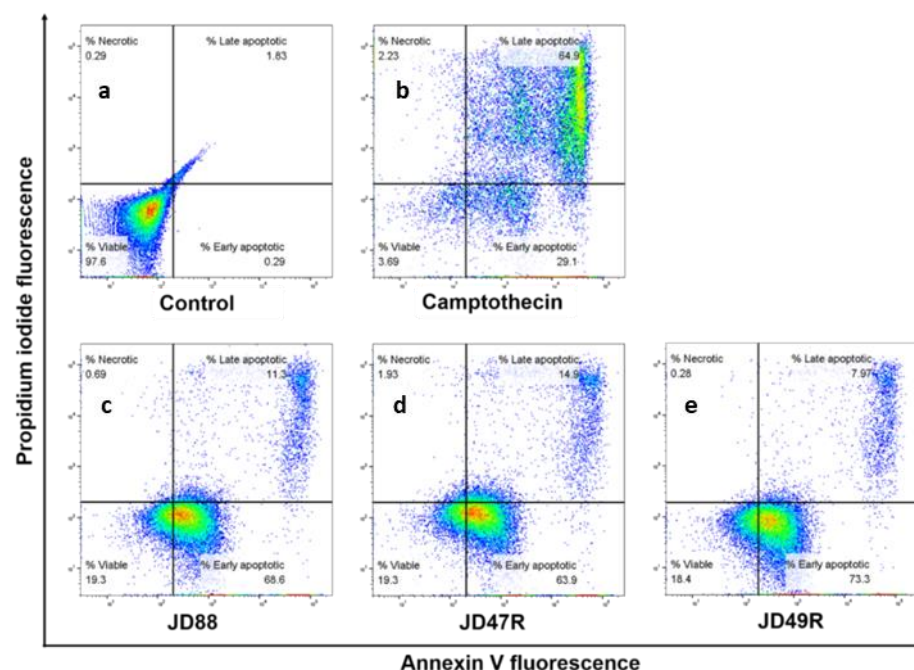


Figure 2.12 Copper-imidazo[1,2-*a*]pyridines increase Annexin-V binding in HL-60 cells. HL-60 cells were treated for 14 hrs at IC₇₅ concentrations. **A.** Fluorescence microscopy of treated cells. Cells were observed with the Olympus BX41 epifluorescence microscope s and images were captured with the Olympus DP72 digital camera. Fluorescent images are representative of three experiments. Exposure time: 140 ms, magnification: 400X, ISO: 400, Scale bar: 20 μ m. Legend: EAC=early apoptotic cell; LAC= late apoptotic cell and NC= necrotic cell. **B.** Representative scatter plots obtained by flow cytometric analysis to detect early and late apoptotic cells. The subpopulations were analysed and expressed as percentages of the total acquired events. Experiments were repeated three times and the scatter plots shown are representative of one experiment.

The Annexin-V data from the flow cytometric analysis of three independent experiments are presented in Table 2.4. The average percentages of different cell populations (quadrants) were reported following exposure to the copper-imidazo[1,2-*a*]pyridines. Significant differences were observed when comparing treated cells to the untreated control.

Camptothecin treated cells showed a highly significant 61.9% increase in late apoptotic cells ($p < 0.001$) compared to the copper-imidazo[1,2-*a*]pyridines (Figure 2.12B; panel b; Table 2.4).

Compared to the untreated control, the number of viable cells in treated cultures dropped significantly ($p < 0.001$). Flow cytometric analysis showed a highly significant ($p < 0.001$) increase in the percentage of early apoptotic cells with JD88, JD47R and JD49R showing 66.1%, a 66.0% and a 55.7% increase in Annexin-V fluorescing cells, respectively (Table 2.4). This was followed by an increase in double positively (late apoptotic) stained JD47R and JD49R treated HL-60 cells of 15.7 and 20.8%, respectively, indicating that treated cells had progressed to late apoptosis.

Table 2.4 Quantitative analysis of single Annexin-V or PI positive and double positive stained HL-60 cells.

HL-60 cells	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
Control	97.0 ± 0.7	0.19	2.2 ± 0.5	0.7 ± 0.3
Camptothecin	6.1 ± 1.3 ****	30.3 ± 1.8 ***	61.9 ± 2.1 ****	2.0 ± 0.2 **
JD88	16.8 ± 3.1 ****	66.1 ± 1.7 ****	14.0 ± 2.2 ^{NS}	0.35 ± 0.2 ^{NS}
JD47R	18.0 ± 0.5 ****	66.0 ± 4.4 ****	15.7 ± 4.8 *	0.25 ^{NS}
JD49R	22.2 ± 1.7 ****	55.7 ± 5.1 ****	20.8 ± 3.7 **	1.4 ± 0.3 ^{NS}

Data is expressed as the mean percentage of total cell populations. Results are representative of three independent experiments. Data was analyzed by one-way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.6.2 The effect of copper-imidazo[1,2-*a*]pyridines on Annexin-V binding in K562 cells

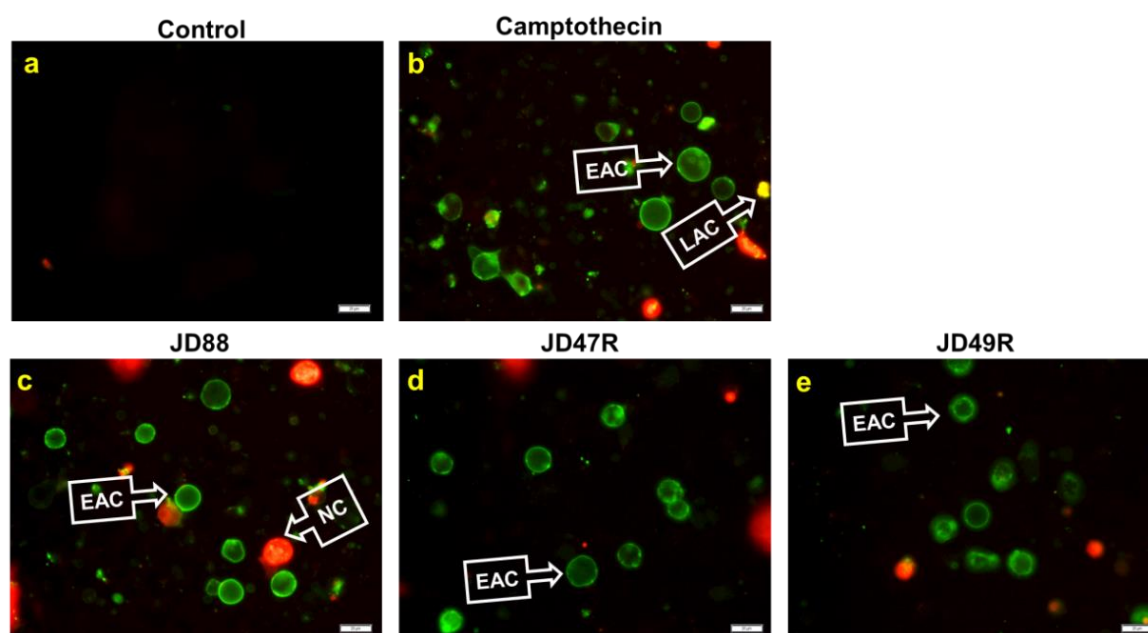
In K562 cells, the binding of Annexin-V to PS was evaluated after an 18 hour exposure period. After incubation with Annexin-V/PI, the positive control camptothecin showed an increase in Annexin-V binding indicating early apoptosis (Figure 2.13A; panel b). Similarly, the copper complexes JD88, JD47R and JD49R showed an increase in Annexin-V binding indicated by green fluorescence (Figure 2.13A, panels c, d and e). A small increase in PI

binding was observed in JD88 treated cells (panel c) and JD49R treated cells (panel e). There was a complete absence of fluorescence in the untreated control indicating the absence of PS on the outer cell membrane of viable cells (Figure 2.13A; panel a).

Flow cytometric analysis of treated K562 cells showed an increase in the early apoptotic and late apoptotic cell population with some necrotic cells (Figure 2.13B). Compared to the untreated control, the number of viable cells showed a highly significant decrease in the cell population of all treatments ($p < 0.001$) (Table 2.5). Camptothecin treated cells were positive for Annexin-V, showing an increase of about 60% indicative of apoptotic cell death.

Compared to the untreated control, cells treated with the copper-imidazo[1,2-a]pyridines showed a non-significant increase in PI staining (Table 2.5), despite Figure 2.13A showing several cells stained red with PI, these cells were late apoptotic/necrotic cells since exclusive necrosis does not show any Annexin-V binding (green fluorescence). In addition, a significant increase in Annexin-V binding was observed with JD88, JD47R and JD49R showing a 51.9%, 53.7% and 57.2% increases, respectively (Figure 2.13B; panels c, d and e; Table 2.5). These observations were comparable to camptothecin (Figure 2.13B; panel b; Table 2.5).

A.



B.

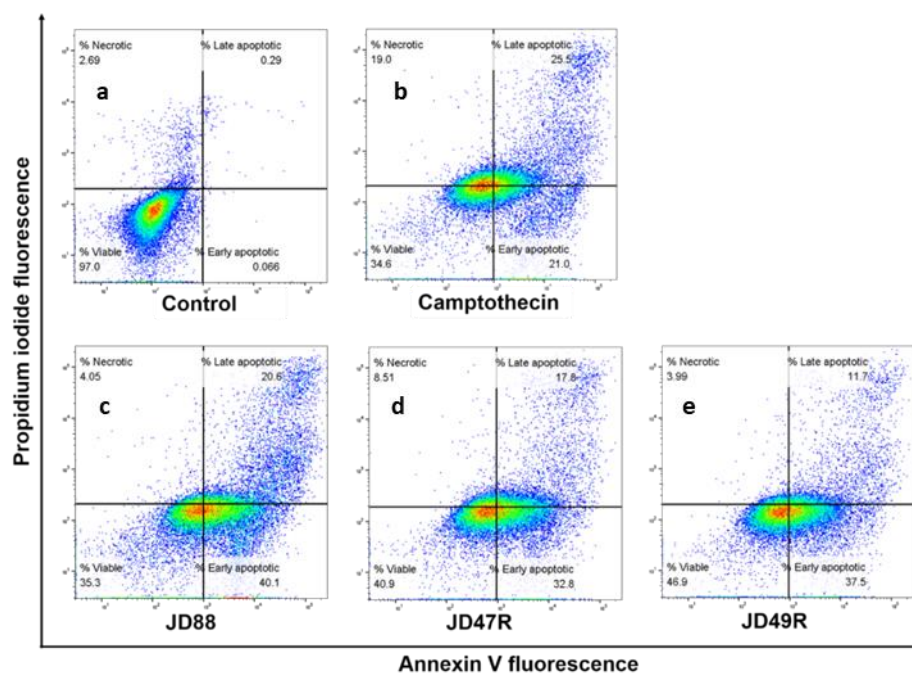


Figure 2.13 Copper-imidazo[1,2-*a*]pyridines increase Annexin-V binding in K562 cells. K562 cells were treated for 18 hrs at IC_{75} concentrations. **A.** Fluorescence microscopy of treated cells. Cells were observed with the Olympus BX41 epifluorescence microscope s and images were captured with the Olympus DP72 digital camera. Fluorescent images are representative of three experiments. Exposure time: 140 ms, magnification: 400X, ISO: 400, Scale bar: 20 μ m. Legend: EAC=early apoptotic cell; LAC= late apoptotic cell and NC= necrotic cell. **B.** Representative scatter plots obtained by flow cytometric analysis to detect early and late apoptotic cells. The subpopulations were analysed and expressed as percentages of the total acquired events. Experiments were repeated three times and the scatter plots shown are representative of one experiment.

Table 2.5 Quantitative analysis of single Annexin-V or PI positive and double positive stained K562 cells

K562 cells	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
Control	99.0 ± 0.8	0.59 ± 0.3	0.3 ± 0.2	0.5 ± 0.3
Camptothecin	33.3 ± 3.5 ****	59.4 ± 3.1 ****	5.4 ± 0.1 **	0.3 ± 0.1 ^{NS}
JD88	40.6 ± 2.2 ****	51.9 ± 0.2 ****	10.5 ± 0.1 ****	0.2 ^{NS}
JD47R	27.5 ± 1.4 ****	53.7 ± 0.8 ****	11.2 ± 2.0 ****	0.3 ± 0.1 ^{NS}
JD49R	32.3 ± 4.6 ****	57.2 ± 2.0 ****	5.1 ± 0.4 *	0.2 ^{NS}

Data is expressed as the mean percentage of total cell populations. Results are representative of three independent experiments. Data was analyzed by one-way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.7 Activation of caspase-3/7 in leukaemic cells by copper-imidazo[1,2-*a*]pyridines

Caspase-3/7 activity was determined based on the detection of the DEVD-AFC (7-amino-4-trifluoromethylcoumarin) substrate cleavage and was quantified using a spectrofluorimeter. Caspase-3/7 activity was measured in order to further confirm apoptotic cell death caused by the copper-imidazo[1,2-*a*]pyridine complexes using a concentration equivalent to their IC₇₅ values. Exploratory experiments at different time periods were performed to determine the exposure period required for each copper-imidazo[1,2-*a*]pyridine at IC₇₅ on HL-60 and K562 cells on caspase-3/7 activity. In HL-60 cells, at a treatment period of 18 hours caspase-3/7 activity was at its peak whereas in K562 cells at a time period of 24 hours, caspase-3/7 was at its highest.

2.3.7.1 Induction of caspase-3/7 activity by copper-imidazo[1,2-*a*]pyridines in HL-60 cells

HL-60 cells were exposed for 18 hours to observe the maximal increase in caspase activity. The copper-imidazo[1,2-*a*]pyridines induced caspase-3/7 activity with JD88, JD47R and JD49R showing an 9-fold, 11-fold and 8-fold, increase, respectively (Figure 2.14A). Statistical analysis indicated that the increases were highly significant ($p < 0.001$). At this time period, camptothecin was a potent inducer of caspase-3/7 activity with an approximate 11-fold increase which was highly significant ($p < 0.001$) (Figure 2.14A).

2.3.7.2 Induction of caspase-3/7 activity by copper-imidazo[1,2-*a*]pyridines in K562 cells

In K562 cells, caspase-3/7 detection required a 24 hour treatment period with the copper-imidazo[1,2-*a*]pyridines. In K562 cells, the copper-imidazo[1,2-*a*]pyridines induced caspase-3/7 activity (Figure 2.14B) with JD88, JD47R and JD49R showing a 2.8-fold, 1.9-fold and 2.6-fold, respectively (Figure 2.14B). Statistical analysis indicated that JD88 caused a significant increase ($p < 0.01$) and was the most potent inducer in K562 cells. In contrast, JD47R was the least potent inducer of caspase-3/7 at this time period. This was comparable to camptothecin, which caused an equally poor increase in caspase-3 activity (2.2-fold).

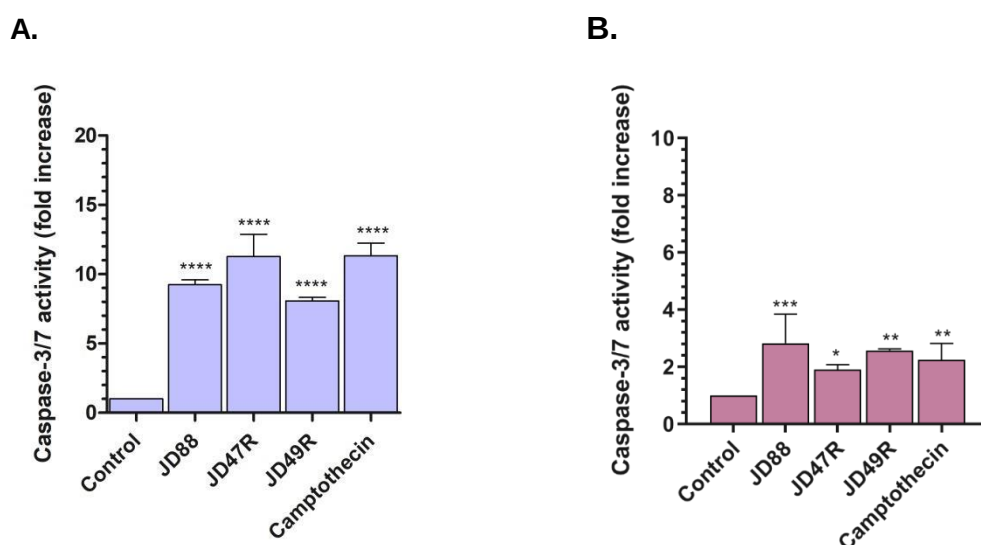


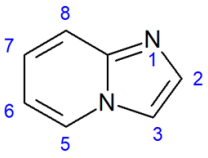
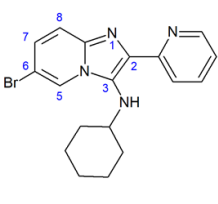
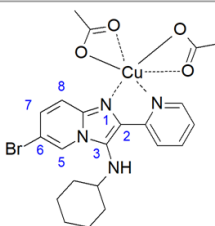
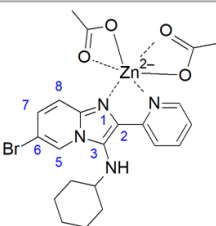
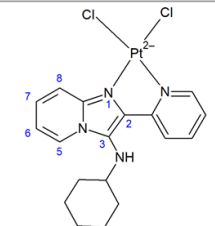
Figure 2.14 Copper-imidazo[1,2-*a*]pyridines induce caspase-3/7 activity in A. HL-60 and B. K562 leukaemic cells. A. HL-60 cells were treated for 18 hrs and B. K562 cells were treated for 24 hrs, with test compounds and camptothecin at their IC_{75} concentration. Specific activity of caspase-3/7 was calculated and results were presented as the mean fold increase above the untreated control. Each bar represents the mean $\pm$ SD of three independent experiments. Data was analyzed by one way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.8 Analysis of structure-activity relationships of imidazo[1,2-*a*]pyridines

The test compounds in this study were based on an imidazo[1,2-*a*]pyridine scaffold and were synthesised by Dr Jean Dam of the School of Chemistry, University of the Witwatersrand. Different substitutions and metals were added to this scaffold in order to generate a library of test compounds. To assess the effect of the substituents on biological activity, a qualitative structure activity relationship (SAR) analysis was performed to compare the effects of various substitutions to the core scaffold on cancer activity. Compounds with similar

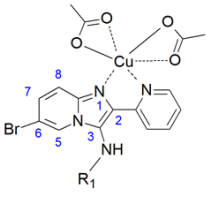
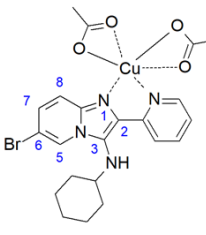
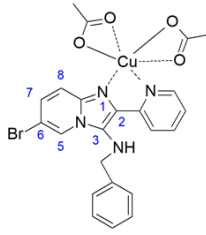
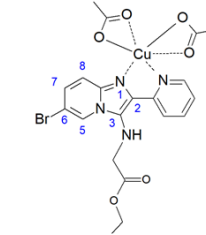
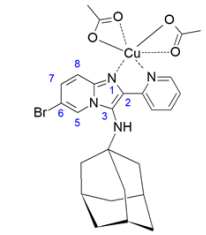
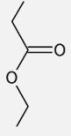
structures are presented in Table 2.6. The test compounds were compared according to their basic scaffold and substitution points.

Table 2.6 Comparison of imidazo[1,2-*a*]pyridine base structure to structures with the addition of transition metal coordination complexes

Imidazo[1,2- <i>a</i>]pyridine base structure				
Compounds	JD60	JD88	JD96	JD-PtCl ₂
Structures				
Transition metal coordination complex	No coordination complex	Copper coordination complex	Zinc coordination complex	Platinum coordination complex
HL-60	Inactive	IC ₅₀ = 2.2±0.5 μM	Inactive	Inactive
K562	IC ₅₀ =36.2±4.1 μM	IC ₅₀ = 1.7±0.3 μM	Inactive	Inactive
Comments	- The imidazo[1,2-<i>a</i>]pyridine amine is inactive against HL-60 cells and shows poor activity against K562 cells. - The addition of a copper coordination complex increases the cytotoxic activity; as indicated by the decreased IC₅₀ values against both cell lines to less than 3.0 μM - Zinc and platinum molecules do not increase activity in either cell line			

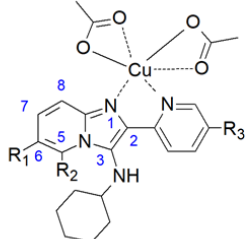
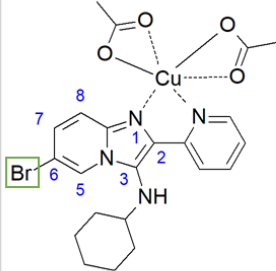
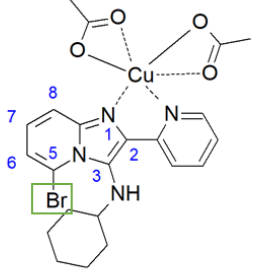
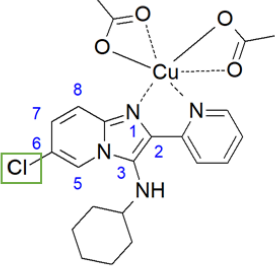
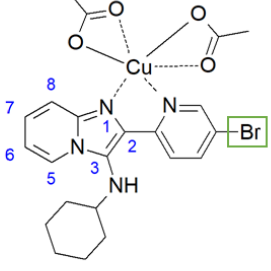
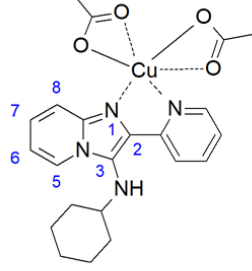
A qualitative structure activity analysis was conducted to evaluate the apoptotic inducing effect between the various substituent groups on 1-position of the amine arm of the active copper-imidazo[1,2-*a*]pyridine coordination complex; JD88, which was identified in Table 2.6. Several analogues that contain the copper complex were synthesised and an analysis was performed to determine the effect on activity of the different substitutions when compared in Table 2.7. It was found that the cyclohexyl amine arm significantly increased the cytotoxic activity in HL-60 and K562 cells. When this group was substituted with ethyl glycinate or benzyl, activity was decreased in both cell lines, whereas the amantadine group rendered the compound inactive. Three compounds showed IC₅₀ values below 7.0 μM for HL-60 cells and below 8.0 μM for K562 cells. A student's t-test was used to compare IC₅₀ values assuming unequal variances (Table 2.7). The statistical analysis showed that the IC₅₀ values of JD88 and JD253 were statistically equivalent when comparing HL-60 and K562 cells. In contrast, the IC₅₀ values of JD256 were significantly different when compared between HL-60 and K562 cells.

Table 2.7 Comparisons between copper complexed structures with substitutions on the amine arm

Copper coordination complex structure with substitutions at the amine arm (R_1)				
Compounds	JD88	JD253	JD256	JD258
Structures				
R_1	 cyclohexyl	 benzyl	 ethyl glycinate	 amantadine
HL-60	$IC_{50} = 2.2 \pm 0.5 \mu M$	$IC_{50} = 6.5 \pm 1.0 \mu M$	$IC_{50} = 4.1 \pm 0.6 \mu M$	Inactive
K562	$IC_{50} = 1.7 \pm 0.3 \mu M$	$IC_{50} = 7.7 \pm 0.5 \mu M$	$IC_{50} = 7.0 \pm 0.07 \mu M$	Inactive
Comparison of HL-60 & K562 IC_{50} values where $p < 0.05$ was statistically significant	Non-significant difference, $p = 0.2$	Non-significant difference, $p = 0.18$	Significant, $p = 0.013$	Not determined
Comments	- The addition of the cyclohexyl amine arm with the addition of the copper complex increases activity in both HL-60 and K562 cells. - The addition of the ethyl glycinate to the amine arm with the addition of the copper complex decreases the activity of the compound in both cell lines, whereas the addition of an amantadine group to the amine arm rendered the compound inactive.			

A comparison of the relationship between structure and activity of JD88, the most active test copper-complex, and its analogues are defined in Table 2.8. JD88 and its analogues contained copper diacetate complexes and a cyclohexylamine arm. The addition of halogens (bromine or chlorine) on the 2-pyridyl ring instead of the imidazo[1,2-*a*]pyridine core caused decreases in activity. The addition of bromine at 6-position increased the activity of JD88 against HL-60 cells, while a substitution of chlorine at the same position reduced the activity of JD49R in HL-60 and K562 cells. A substitution at only 5-position with bromine, as seen in JD47R, showed a non-significant increase of activity in HL-60 cells and a significant reduction of activity in K562 cells. The substitution with bromine at R_3 in JD37R reduces the activity of test compounds in HL-60 and K562 cells (Table 2.8). An un-substituted analogue such as JD46R (R_1 , R_2 and R_3 = H) had an observed reduction in efficacy in the myeloid cell lines.

Table 2.8 Structure activity relationships of copper-imidazo[1,2-*a*]pyridine coordination complexes

Base structure of copper coordination complexes					
Compounds	JD88; Copper 6-bromo-N-cyclohexyl-2-(pyridin-2-yl)imidazo[1,2- <i>a</i>]pyridin-3-amine acetate	JD47R; Copper 5-bromo-N-cyclohexyl-2-(pyridin-2-yl)imidazo[1,2- <i>a</i>]pyridin-3-amine acetate	JD49R; Copper 6-chloro-N-cyclohexyl-2-(pyridin-2-yl)imidazo[1,2- <i>a</i>]pyridin-3-amine acetate	JD37R; Copper 2-(5-bromopyridin-2-yl)-N-cyclohexylimidazo[1,2- <i>a</i>]pyridin-3-amine acetate	JD46R; Copper N-cyclohexyl-2-(pyridin-2-yl)imidazo[1,2- <i>a</i>]pyridin-3-amine acetate
Structures					
Amidogen substituent	Cyclohexyl amine				
R ₁	Br	-	Cl	-	-
R ₂	-	Br	-	-	-
R ₃	-	-	-	Br	-
HL-60	IC ₅₀ = 2.2±0.5 μM	IC ₅₀ = 2.1±0.3 μM	IC ₅₀ = 5.1±1.1 μM	IC ₅₀ = 13.7±0.9 μM	IC ₅₀ = 8.6±0.5 μM
K562	IC ₅₀ = 1.7±0.3 μM	IC ₅₀ = 5.4±0.3 μM	IC ₅₀ = 3.4±0.6 μM	IC ₅₀ = 3.5±0.9 μM	IC ₅₀ = 7.0±1.4 μM
Comments	• The addition of a bromine at R₁ increased the activity in K562 cells, indicating JD88 was the most potent copper complex in these cells • A substitution at only R₂ with bromine in JD47R increased the activity in HL-60 cells, indicating JD47R was the most potent copper complex in these cells				

2.3.9 The effect of copper-imidazo[1,2-*a*]pyridines on mitochondrial membrane potential in leukaemic cells

Mitochondria are essential for the maintenance of cellular metabolism. Disruption of mitochondrial function leads to a loss in MMP, a feature of early apoptosis (Estaquier *et al.*, 2012). The cationic dye JC-1 was used to measure changes to the MMP which may lead to the activation of the intrinsic apoptotic pathway. Healthy cells with high MMP cause JC-1 to cross the mitochondrial membrane and form J-aggregates which fluoresce red/orange. The JC-1 dye remains in its green monomeric form in apoptotic cells with a low MMP.

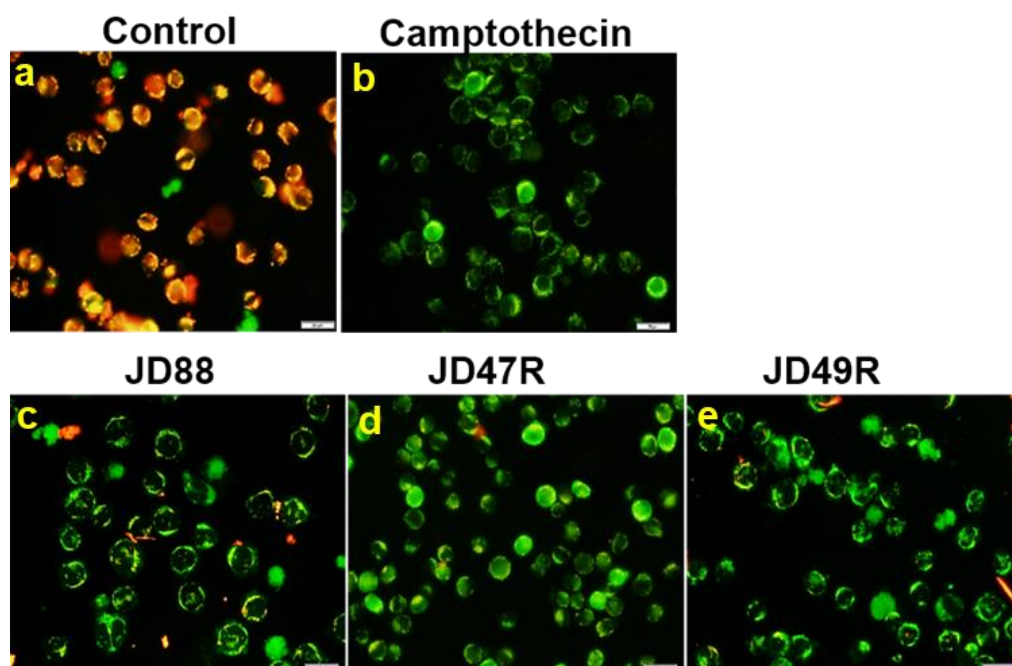
Exploratory experiments at various treatment periods were performed to determine the effect of the copper-imidazo[1,2-*a*]pyridines on MMP. For HL-60 a treatment period of 14 hours was chosen and for K562 cells a period of 18 hours was selected.

2.3.9.1 The effects of copper-imidazo[1,2-*a*]pyridines on the mitochondrial membrane potential of HL-60 cells

At a treatment period of 14 hours, a near complete loss of MMP was observed in HL-60 cells for all test compounds and camptothecin as illustrated by the presence of green fluorescence only and the absence of yellow-orange/red fluorescence (Figure 2.15; panels b, c, d and e). HL-60 cells treated with camptothecin showed only green fluorescence with JC-1 indicating a loss of MMP (Figure 2.15A; panel b). Similar to camptothecin, JD88, JD47R and JD49R treated HL-60 cells (Figure 2.15A; panels c, d and e) showed mostly green fluorescence indicating that JC-1 crossed the mitochondrial membrane poorly. In contrast, untreated HL-60 control cells displayed orange/red punctate fluorescence in the perinuclear area indicative of the formation of J-aggregates in the mitochondrial matrix and implying an intact mitochondrial membrane (Figure 2.15A; panel a).

The data presented in Figure 2.15B, is the average for each treatment of three independent experiments measuring the average percentage of cell population staining positive for JC-1 monomers and J-aggregate formation. In each experiment three fields were counted and analysed. The treated cells showed a low percentage of cells with J-aggregates indicating a near complete loss in MMP after 14 hours of treatment.

A.



B.

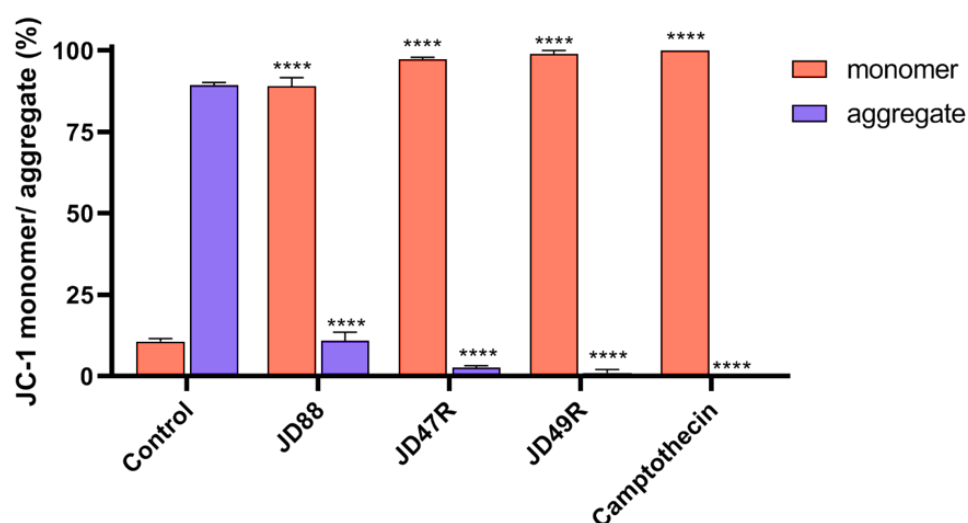


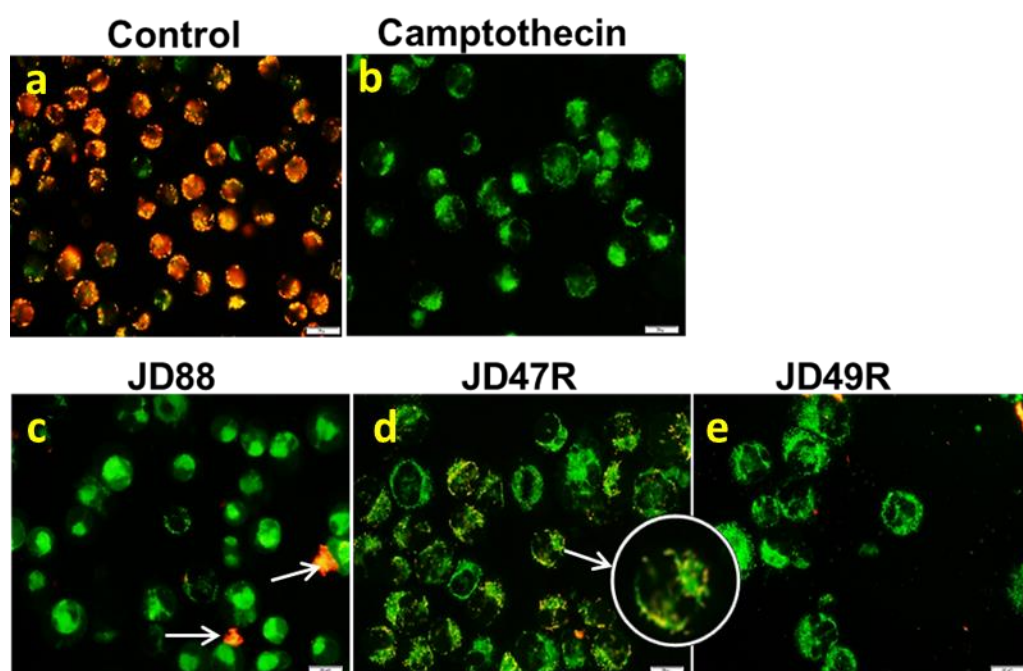
Figure 2.15 Copper-imidazo[1,2-*a*]pyridines cause a loss of mitochondrial membrane potential in HL-60 cells. Cells were treated at IC₇₅ concentrations for 14 hrs, stained with JC-1 dye and observed with the Olympus BX41 epifluorescence microscope. **A.** Images were captured with the Olympus DP72 digital camera. Magnification: 400X, Exposure: 121 ms; ISO: 400; Scale bar: 20 μ m. In **B**, the percentage of cells staining positive for both J-aggregates and JC-1 monomers from three independent experiments are shown. Three separate fields were analysed for each slide. Error bars represent the standard deviation. Data was analyzed by one way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.9.2 The effects of copper-imidazo[1,2-*a*]pyridines on the mitochondrial membrane potential of K562 cells

K562 cells were treated with copper-imidazo[1,2-*a*]pyridines JD88, JD47R and JD49R at their IC₇₅ concentration for 18 hours. In Figure 2.16A, all treated cells, including camptothecin, showed green fluorescence indicative of a complete loss of MMP (panels b, c, d and e), whereas the mitochondria of untreated K562 control cells displayed typical orange staining of healthy mitochondria indicative of JC-1 aggregate formation (panel a). In JD88 treated cells, the presence of red blobs are the result of JC-1 insoluble aggregates (Figure 2.16A; panel c). Low levels of yellow/orange aggregates in JD47R treated cells can be seen in Figure 2.16A; panel e, which indicates aggregate formation.

The data presented in Figure 2.16B, is the average for each treatment of three independent experiments measuring the average percentage of cell population staining positive for JC-1 monomers and J- aggregate formation. In each experiment three fields were counted. As seen, the treated cells showed a low percentage of cells with J-aggregates indicating a near complete loss in MMP after 18 hours of treatment.

A.



B.

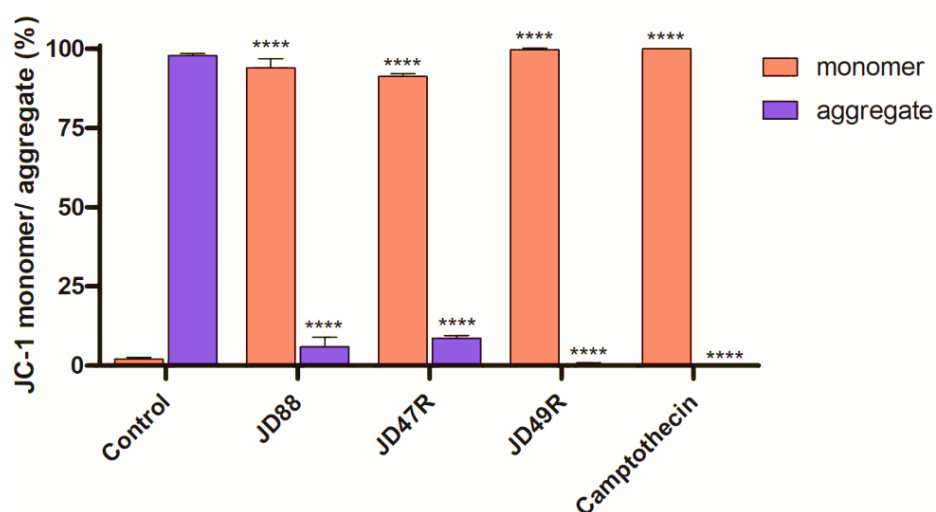


Figure 2.16 Copper-imidazo[1,2-*a*]pyridines cause a loss of mitochondrial membrane potential in K562 cells. Cells were treated at IC₇₅ concentrations for 18 hrs, stained with JC-1 dye and observed with the Olympus BX41 epifluorescence microscope. **A.** Images were captured with the Olympus DP72 digital camera. Magnification: 400X, Exposure: 121 ms; ISO: 400; Scale bar: 20 μ m. Arrows indicate insoluble JC-1 aggregates. In **B**, the percentage of cells staining positive for both J-aggregates and JC-1 monomers from three independent experiments are shown. Three separate fields were analysed for each slide. Error bars represent the standard deviation. Data was analyzed by one way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.10 Activation of caspase-9 in leukaemic cells by copper-imidazo[1,2-*a*]pyridines

After confirming the changes in MMP by the copper-imidazo[1,2-*a*]pyridines, the activation of caspase-9 was determined in order to confirm the activation of the intrinsic apoptotic pathway. HL-60 and K562 cells were treated with the copper-imidazo[1,2-*a*]pyridine complexes at concentrations equal to their IC₇₅ values for 14 and 24 hours, respectively. The data represented was obtained from three independent experiments. Specific caspase-9 activity was calculated as enzyme activity per unit of protein and expressed as a fold increase of enzyme activity compared to the untreated control.

2.3.10.1 Induction of caspase-9 activity by copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 cells

The ability of the copper-imidazo[1,2-*a*]pyridines to induce caspase-9 activity in HL-60 cells was assessed 14 hours after treatment with test compounds and camptothecin at their IC₇₅ concentration. The bar graph in Figure 2.17A represents caspase-9 activity in HL-60 cells. The copper-imidazo[1,2-*a*]pyridines were potent inducers of caspase-9 activity with highly significant increases ranging between 5- and 7-fold above the untreated control ($p < 0.001$) (Figure 2.17A). JD88 in particular, showed an increase in caspase-9 activity of 6.9-fold that was comparable to the positive control camptothecin of 6.4-fold.

In K562 cells (Figure 2.17B), the copper-imidazo[1,2-*a*]pyridine, JD88 was the most potent inducer of caspase-9 activity and caused a 5.3-fold increase ($p < 0.001$) above the control, which was comparable to camptothecin (6.5-fold). Copper-imidazo[1,2-*a*]pyridines JD47R and JD49R caused fold increases in caspase-9 activity of 4.1 and 3.8-fold, respectively. These results were confirmed by fluorescence microscopy as shown in Appendix F, Figure F1 and F2.

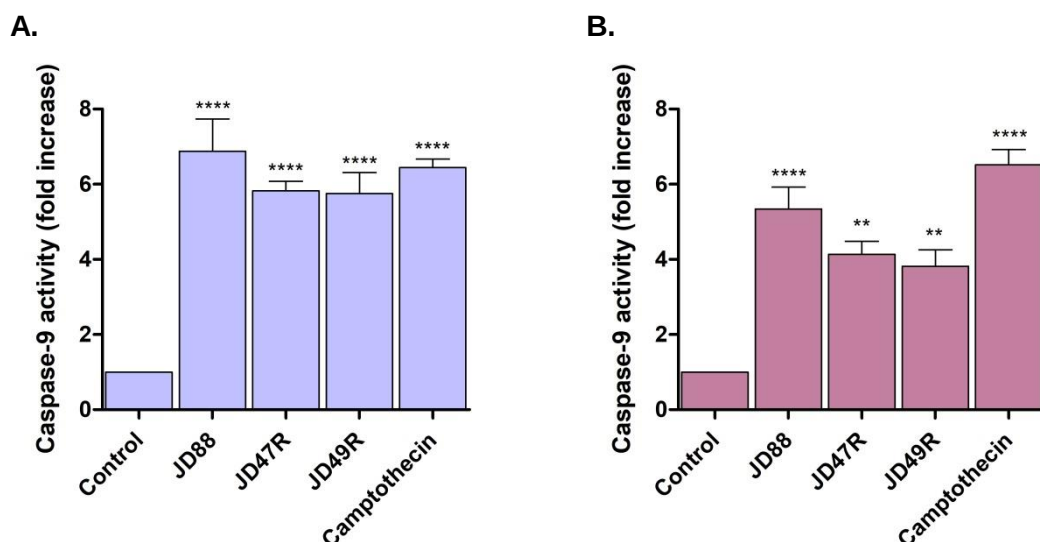


Figure 2.17 Copper-imidazo[1,2-*a*]pyridines induce caspase-9 activity in leukaemic cells. A. Fold increase above the control of caspase-9 activity in HL-60 cells exposed to copper-imidazo[1,2-*a*]pyridines and camptothecin for 14 hrs at their IC₇₅ concentrations. **B.** Fold increase above the control of caspase-9 activity in K562 cells exposed to copper-imidazo[1,2-*a*]pyridines and camptothecin for 24 hrs at their IC₇₅ concentrations. Specific activity of caspase-9 was calculated and results were expressed as the mean fold increase $\pm$ SD above the untreated control. Data was analyzed by one way ANOVA ($p < 0.05$) followed by Dunnett's *post-hoc* comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

2.3.11 Activation of caspase-8 in leukaemic cells by copper-imidazo[1,2-*a*]pyridines

The caspase-8 assay was performed in order to determine if the extrinsic apoptotic pathway was activated in response to the copper-imidazo[1,2-*a*]pyridine complexes. HL-60 and K562 cells were treated with the copper-imidazo[1,2-*a*]pyridines at concentrations equal to their IC₇₅ values for 6, 14, 18 and 24 hour time periods. The data was obtained from three independent experiments. In this assay an increase in red fluorescence is indicative of caspase-8 activation. Results obtained are shown in Figure 2.18 and Figure 2.19 for HL-60 and K562 cells, respectively.

2.3.11.1 Induction of caspase-8 by copper-imidazo[1,2-*a*]pyridines in HL-60 cells

Following treatment with the copper-imidazo[1,2-*a*]pyridines, an increase in red fluorescence was observed for HL-60 cells at all three time periods in comparison to the untreated control (Figure 2.19). Quantification of the number of cells showing caspase-8 activity indicated that it increased in a time-dependent manner. Camptothecin treated cells also showed an increase in caspase-8 activity over time (Figure 2.18). However, the percentage of treated cells

staining positive for active caspase-8 did not exceed 20% after a 24 hour treatment period (Figure 2.18).

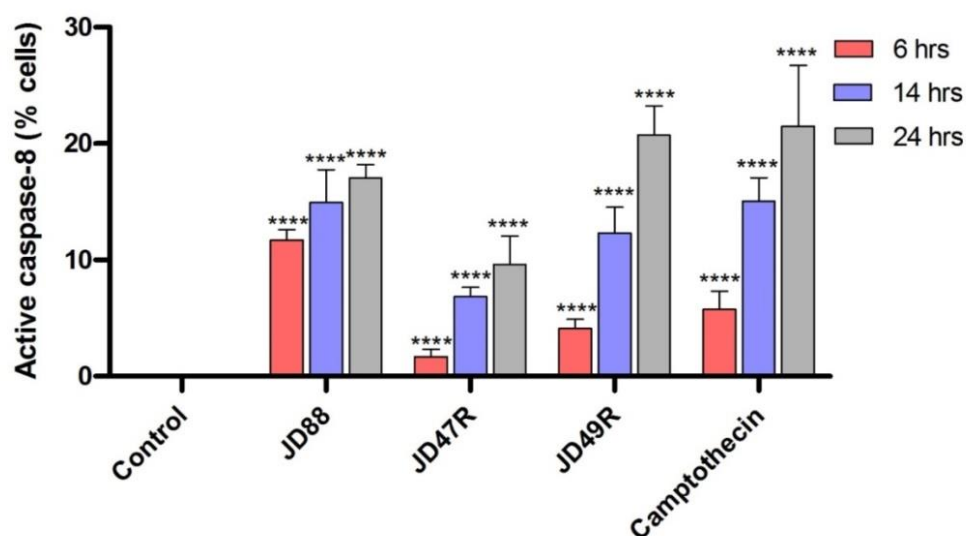


Figure 2.18 Induction of caspase-8 activity in HL-60 cells. HL-60 cells were treated with the copper-imidazo[1,2-*a*]pyridines and camptothecin at IC₇₅ concentrations for 6, 14 and 24 hrs. Each bar represents the mean $\pm$ SD of values obtained from three independent experiments. Data was analyzed by one way ANOVA ($p < 0.05$) followed by Dunnett's post-hoc comparison test. (NS; non-significant; * $p < 0.1$; is non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant).

Treated cells showed a loss of membrane integrity and shape, as indicated by phase contrast images, and were positively stained for caspase-8 activity, as displayed by the red fluorescence in Figure 2.19; panels m-d'. Cells treated with JD88 (panels m, p and r) and JD49R (panels z, b' and d') showed greater increases in caspase-8 activity compared to JD47R (panels t, v and x).

2.3.11.2 Induction of caspase-8 activity by copper-imidazo[1,2-*a*]pyridines in K562 cells

Following treatment with the copper-imidazo[1,2-*a*]pyridine at 6, 18 and 24 hour time periods, there was no indication of caspase-8 activity in K562 cells (Figure 2.20). This implies that the response to the test compounds are cell line dependent and not compound dependent.

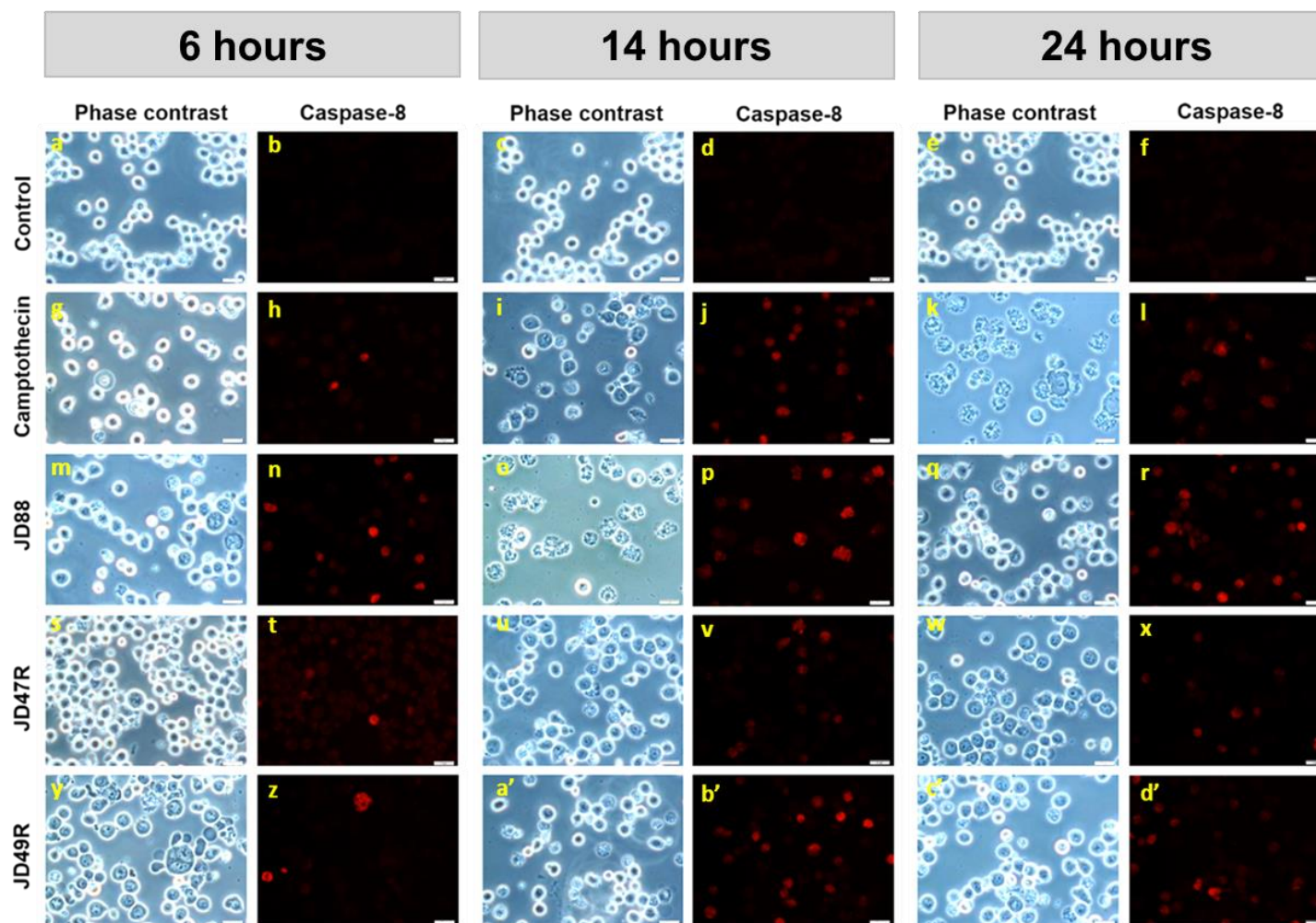


Figure 2.19 Copper-imidazo[1,2-*a*]pyridines induce caspase-8 activity in HL-60 cells. HL-60 cells were treated with the copper-imidazo[1,2-*a*]pyridines and camptothecin at IC₇₅ concentrations for 6, 14 and 24 hrs. Cells were viewed for caspase-8 fluorescence with an Olympus BX41 epifluorescence microscope using the Olympus UMWG2 filter cube and images were captured with the Olympus DP72 digital camera. Magnification: 400X; exposure time: 140 ms; ISO: 400; Scale bar: 20 μ m

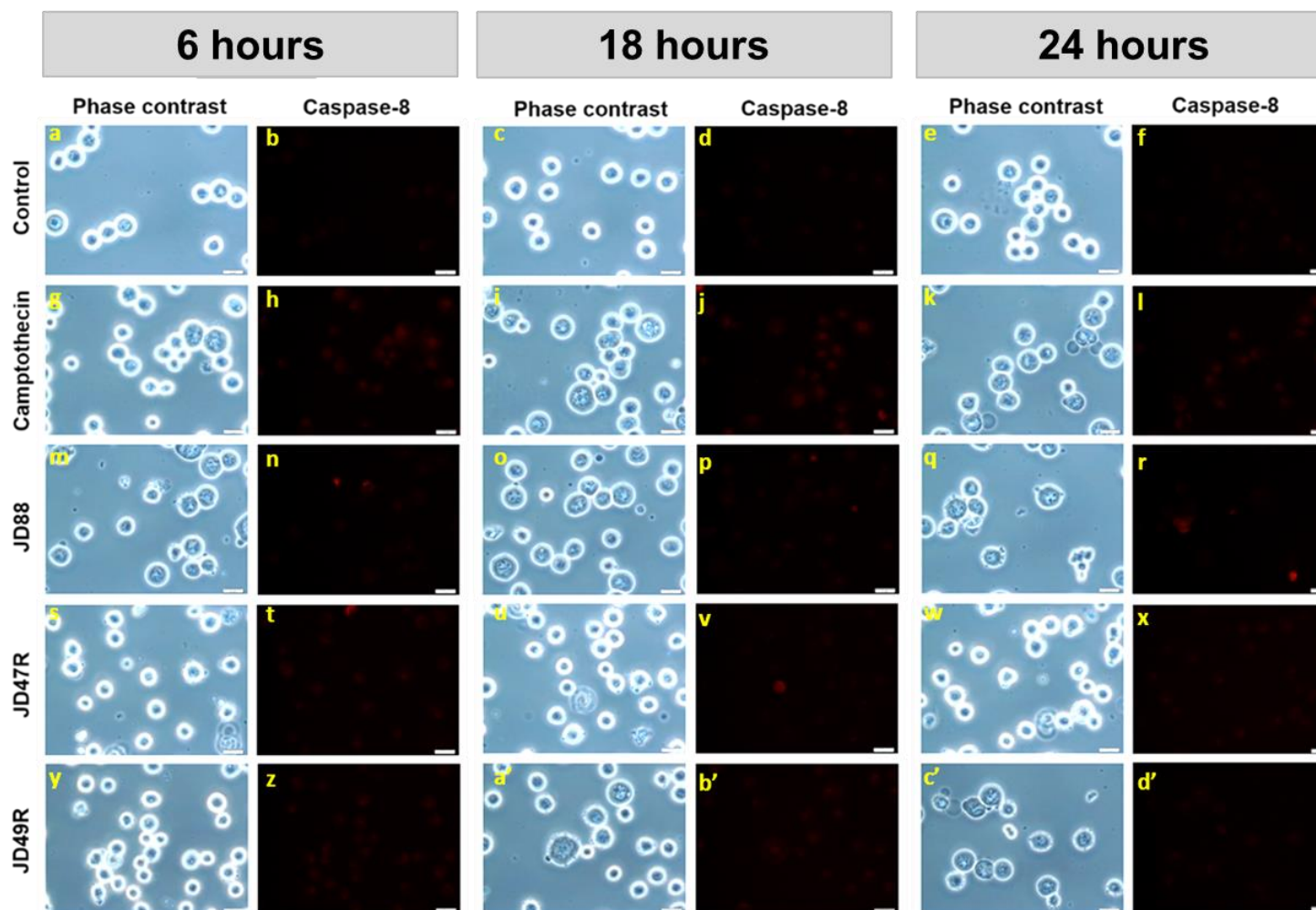


Figure 2.20 Copper-imidazo[1,2-*a*]pyridines fail to induce caspase-8 activity in K562 cells. K562 cells were treated with the copper-imidazo[1,2-*a*]pyridines and camptothecin at IC₇₅ concentrations for 6, 18 and 24 hours. Cells were viewed for caspase-8 activity with an Olympus BX41 epifluorescence microscope using the Olympus UMWG2 filter cube and images were captured with the Olympus DP72 digital camera. Magnification: 400X; exposure time: 140 ms; ISO: 400; Scale bar: 20 μm

2.4 DISCUSSION

In this study, the cytotoxic effects of novel test compounds on the cell viability of HL-60 and K562 leukaemic cells were evaluated. A total of forty-three compounds belonging to the class of imidazo[1,2-*a*]pyridines were screened against both cell lines. The IC₅₀ values were obtained for compounds displaying more than an 80% inhibition of cell growth at a concentration of 100 µM. The IC₅₀ values indicated that zinc and platinum-imidazo[1, 2-*a*]pyridine complexes had poor anti-cancer activity, while copper-imidazo[1,2-*a*]pyridine complexes were identified as active by showing anti-cancer activity with IC₅₀ values in the low micromolar range (<10.0 µM). Using this data, the three most potent copper-imidazo[1,2-*a*]pyridine complexes were selected for further investigation due to their high potency against both cell lines. Copper complexes show promising anticancer activity and are currently under various phases of pre-clinical evaluation as reviewed by Santini *et al.*, (2014) and McGivern *et al.*, (2018). An evaluation of the mechanism of cell death provides important pre-clinical information in the development of new anti-cancer agents. Therefore, the mechanism of cell death and a set of specific apoptotic markers were investigated following exposure of cells to the copper-imidazo[1,2-*a*]pyridines.

2.4.1 The cytotoxicity of copper-imidazo[1,2-*a*]pyridines on HL-60 and K562 cells

The library of imidazo[1,2-*a*]pyridines evaluated at the start of this study included uncoordinated ligands which had poor cytotoxic activity compared to the metallo-imidazo[1,2-*a*]pyridines. In particular, the results obtained supported the enhanced activity of copper, but not zinc or platinum, complexed to the imidazo[1,2-*a*]pyridines. No further work was performed on the uncoordinated imidazo[1,2-*a*]pyridines.

Based on their low IC₅₀ values three of the copper-imidazo[1,2-*a*]pyridines, JD88, JD47R and JD49R were selected for further investigations with HL-60 and K562 cells. The three active copper-imidazo[1,2-*a*]pyridines showed IC₅₀ values ranging between 1.0 to 6.0 µM for both cell lines. JD47R was the most effective compound against the HL-60 cell line with an IC₅₀ value of 2.1±0.3 µM and JD88 was the most effective compound against the K562 cell line with an IC₅₀ value of 1.7±0.3 µM.

Camptothecin was selected as a positive control for its ability to induce apoptosis (Chu *et al.*, 2014). Camptothecin showed an IC₅₀ value of 7 nM for HL-60 cells and an IC₅₀ value of 2.8

μM for K562 cells. A study by Zi *et al.*, (2019) investigated the anti-tumour activity of camptothecin conjugates using camptothecin, etoposide and cisplatin as positive controls. They found that the IC₅₀ values of camptothecin (<0.064 μM) was several fold more active than both etoposide (0.31±0.2 μM) and cisplatin (1.67±0.4 μM) in HL-60 cells (Zi *et al.*, 2019). This supports the low IC₅₀ value that was obtained in this study. Despite the use of camptothecin derivatives like irinotecan for colorectal and ovarian cancers, drug resistance remains a critical clinical problem (Gershenson, 2002; Beretta *et al.*, 2013; Hammond *et al.*, 2016; Shibutani *et al.*, 2017). In spite of the copper complexes not yielding effects within the same concentration range as camptothecin in HL-60 cells, the test compounds yielding IC₅₀ values below the recommended NCI range of <10 μM, was considered promising. Furthermore, these copper-imidazo[1,2-*a*]pyridines were of significant interest as they showed low toxicity towards unstimulated normal human peripheral leukocytes.

IC₅₀ values of the test compounds and camptothecin were compared between the two cell lines to determine significant differences. Statistical analysis of IC₅₀ values was performed using the two-sample, unpaired Mann-Whitney U-test with significance set at $p < 0.05$ and a 95% confidence interval. The results are presented in Table 2.3 and indicates that HL-60 cells were significantly more sensitive to camptothecin (IC₅₀: 7 nM) than K562 cells (IC₅₀: 2.8 ±0.3 μM) with a significant difference of $p = 0.03$ ($p < 0.05$). There were no significant differences in the IC₅₀ values of the copper-imidazo[1,2-*a*]pyridines tested against each cell line; JD88 ($p > 0.05$, $p = 0.63$), JD47R ($p > 0.05$; $p = 0.1$) and JD49R ($p > 0.05$; $p = 0.34$) indicating similar efficacy in each of the two cell lines.

The difference in the IC₅₀ values of camptothecin between HL-60 and K562 cells may be attributed to the differences in the molecular characteristics of the two cell lines. HL-60 cells harbour the *PML-RAR* oncogene while K562 cells harbour the *BCR/ABL* oncogene. The characteristic *BCR/ABL* oncogene makes K562 cells resistant to chemotherapy induced-apoptosis (Helgason *et al.*, 2011; Crivellaro *et al.*, 2015).

Novel copper thiosemicarbazone complexes caused cell growth inhibition in HL-60 human promyelocytic leukaemic, NCI-H1573 lung adenocarcinoma, SKBR-3 human breast, MCF-7 human breast and A375 human melanoma cell lines (Pahonțu *et al.*, 2016). Using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium) assay the most active complex, Cu(HL)(H₂O)₂(SO₄), had an IC₅₀ value of 1.6 μM in the HL-60 cell line, after a 72 hour incubation. This copper complex was more active than palladium

and platinum complexes in HL-60 cells with IC₅₀ values of 6.5 μ M and 6.4 μ M, respectively. Another study by Zhou *et al.*, (2016), showed that copper(II) complexes based on chiral Schiff-base ligands exhibited potent cytotoxic effects against MDA-MB231 breast cancer cells with IC₅₀ values in the micromole range (<6 μ M) (Zhou *et al.*, 2016).

A study carried out by Paixão *et al.*, (2017) investigated the effects of copper (II) complexes on K562 cells. Two copper (II) complexes with the heterocyclic base 1, 10-phenanthroline showed cytotoxic effects with IC₅₀ values between 1.6-1.8 μ M after 72 hour incubation (Paixão *et al.*, 2017). The two copper (II) complexes displayed higher cytotoxic effects than their free ligands and carboplatin. A similar study was conducted by Correia *et al.*, (2015) who found that Schiff copper (II) complexes containing phenanthroline exhibited cytotoxic activity below 5 μ M that was 10-fold higher than the corresponding vanadium complexes, and positive control cisplatin in human MCF-7 breast and A2780 ovarian cancer cells (Correia *et al.*, 2015). In HL-60 promyelocytic leukaemia cells, the phen-containing copper compounds were more active than the oxidovanadium (IV) complexes however, when compared to cisplatin they did not vary that much.

Imidazo[1,2-*a*]pyridine derivatives in this study showed cytotoxic effects however, when coordinated to copper their cytotoxic effects were enhanced in both leukaemic cell lines with lower IC₅₀ values. It is important to highlight that the published data discussed earlier refers to studies where copper compounds were incubated for 72 hours as opposed to the 48 hour incubation period of this current study. This suggests that the copper-imidazo[1,2-*a*]pyridines were able to exhibit similar cytotoxic effects (low micromolar concentrations) at shorter treatment/exposure periods which may prove to be beneficial to patients.

2.4.2 Toxicity of copper-imidazo[1,2-*a*]pyridines towards normal peripheral leukocytes

The primary challenge in cancer chemotherapy is the need to selectively kill cancer cells, while sparing normal cells. Neutropenia, leukopenia and bone marrow toxicity are among the common adverse effects observed in leukaemia treatment (Cicconi and Lo-Coco, 2016) and hinders patient compliance (Burnett *et al.*, 2015; Caldemeyer and Akard, 2016). In this study, the cytotoxic effects of the copper-imidazo[1,2-*a*]pyridines were evaluated against normal peripheral leukocytes to determine their toxicity.

Normal peripheral leukocytes were treated with active copper-imidazo[1,2-*a*]pyridines at a 20 μM concentration for 24 hours. With the exception of JD88, the compounds showed lower toxicity to normal peripheral leukocytes compared to the leukaemic cell lines at a concentration of 20 μM (Figure 2.9A). Leukocytes have a very short survival period once harvested which may have contributed to the cell death observed with the copper complexes.

At a concentration of 20 μM , there was 100% cell death for the two leukaemic cells lines indicating a potential sparing effect on leukocytes. This provides evidence for some selectivity of the compounds for leukaemic cells over normal cells. Since the 20 μM concentration of JD88 was almost 10 times that of its IC_{50} value, JD88 was then tested at various concentrations guided by the IC_{50} values obtained against the other cancer cell lines. Leukocyte viability improved with lower concentrations of JD88 (Figure 2.9B). At a 20 μM concentration, leukocyte viability was at approximately 38% and this improved to about 85% when leukocytes were treated at a 1.0 μM concentration.

The cytotoxicity of organic copper complexes is dependent on the ligand that is coordinated to the metal. A study by Lewis *et al.*, (2016) demonstrated the differences in cytotoxic effects in a group of synthetic copper complexes coordinated to imidazole and thiazole moieties on benzene. The most active copper complex had an imidazole ligand in its structure and displayed greater cytotoxicity towards K562 and Hela cells than the thiazoles (Lewis *et al.*, 2016). In the same way, the structure of the imidazo[1,2-*a*]pyridine is a fused bicyclic 5-6 nitrogen containing heterocyclic compound that consists of an imidazole moiety fused to a pyridine ring. The development of imidazo[1,2-*a*]pyridine derivatives similar in structure to the most active compounds shows that structure plays a crucial role in the cytotoxic effect of the test compounds.

In a study by Hajrezaie *et al.*, (2014), the copper (II) complex, derived from N,N'-dimethyl ethylene diamine and 2-hydroxyacetophenone Schiff base ligand, $\text{Cu}(\text{BrHAP})_2$ showed cytotoxic effects against HT-29 cells with an IC_{50} value of 2.9 μM after a 24 hour treatment. Cytotoxicity data obtained by this study showed that copper complexes show promising potential as anti-cancer agents. The Casiopéinas family of copper compounds which have been actively studied have a general formula $[\text{Cu}(\text{N}-\text{N})(\text{N}-\text{O})]\text{NO}_3$ and $[\text{Cu}(\text{N}-\text{N})(\text{O}-\text{O})]\text{NO}_3$, where the N-N ligand denotes an aromatic ligand either 2,2'-bipyridine or 1,10-phenanthroline, N-O represent an essential amino acid or peptides and the O-O represents a non-aromatic ligand either acetylacetonate or salicylaldehyde (Galindo-

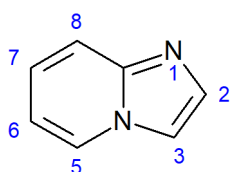
Murillo *et al.*, 2015; García-Ramos *et al.*, 2016) The Casiopeínas have shown promising cytotoxic activity to cancer cells both *in vitro* and *in vivo* and as a result phase I clinical trials were carried out (Galindo-Murillo *et al.*, 2015). *In vitro* studies like that conducted by Galindo-Murillo., (2015) on various cancer cell lines showing a 10-fold enhanced effect when compared to well-known cisplatin (Galindo-Murillo *et al.*, 2015).

The panel of leukaemic cells in a study by Bortolozzi *et al.*, (2014) were treated for 72 hours with a phosphine copper complex and evaluated by the MTT assay. This complex inhibited leukaemia cell growth with IC₅₀ values ranging from 0.9 μ M to 23 μ M with B-lymphoblastic cell lines showing the greatest sensitivity (IC₅₀ values 0.9 μ M to 4.2 μ M). The IC₅₀ values obtained for HL-60 and K562 cells were 12.1 $\pm$ 2.8 and 23.2 $\pm$ 1.0 μ M, respectively. In comparison with the cytotoxic results (< 6 μ M) presented in this study, copper coordinated to imidazo[1,2-*a*]pyridines show more promising anti-cancer activity than the phosphine copper (I) complexes do in myeloid cells. Thereby suggesting that the phosphine copper (I) complex does not have the potential to be therapeutically significant in myelocytic leukamias.

2.4.3 Structure activity relationship of imidazo[1,2-*a*]pyridines

Structure activity relationship analysis showed that the coordination of copper to the imidazo[1,2-*a*]pyridine core increased its activity significantly. This is supported by studies which reported that the coordination of transitional metals copper and zinc to pyridine scaffolds significantly increases their cytotoxic activity against cancer cells (Ma *et al.*, 2015; Fei *et al.*, 2016; Zhou *et al.*, 2016).

A.



B.

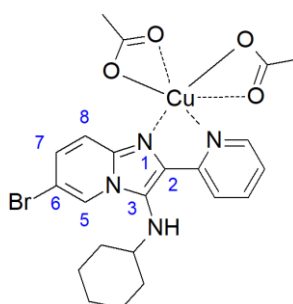


Figure 2.21 Chemical structures. A. Scaffold of imidazo[1,2-*a*]pyridine. **B.** Structure of the most active copper-imidazo[1,2-*a*]pyridine complex; JD88

The modification of the core imidazo[1,2-*a*]pyridine structure to synthesise the copper-imidazo[1,2-*a*]pyridine JD88 impacted its potency substantially. The structure and activities

of the active test compounds were compared in Table 2.8. The addition of a bromine group on 6-position of JD88 showed an increase in cytotoxicity in both cell lines. The addition of a chlorine group at the same position (C6) of the JD49R molecule showed comparable activity ($3.4 \pm 0.6 \mu\text{M}$) to that of JD88 in K562 cells ($1.7 \pm 0.3 \mu\text{M}$) but not in HL-60 cells ($5.1 \pm 0.9 \mu\text{M}$). In contrast, JD47R had no substitution on 6-position but instead at 5-position of the imidazole ring. Of interest is the loss of potency in both cell lines caused by the absence of a halogen on the imidazole ring as shown by JD46R. This observation was in contrast to that reported by Harmse *et al.*, (2019) who found that JD46R was more potent than JD49R in HT-29 colorectal cancer cells. This emphasises the potential selectivity of the various copper imidazo [1,2-*a*] pyridines for specific cancers. Placement of a halogen molecule (bromine/ chlorine) on the 2-pyridyl ring as in JD37R showed no benefit. This suggests the importance of the substitutions on position 6 for cytotoxic effects.

Based on JD88 (Figure 2.21A) being the superior test compound in both leukaemic cell lines; the following was deduced for the cell lines tested in this study. The copper coordinated complexes which contained a cyclohexyl amine showed greater anti-cancer activity than an adamantyl, a benzyl or an ester substituent. The addition of a bromine atom at 6- position of the imidazo[1,2-*a*]pyridine core increased potency in both cell lines. For these reasons, these active copper coordination complexes were selected to further explore the underlying mechanisms of their anti-leukaemic effects in HL-60 and K562 cells.

2.4.4 The effect of copper-imidazo[1,2-*a*]pyridines on the morphology of HL-60 and K562 cells

Following on from their low IC_{50} values, the three most active copper-imidazo[1,2-*a*]pyridines JD88, JD47R and JD49R were further evaluated. Morphological studies on both cell lines (Figure 2.10 and Figure 2.11) showed the presence of typical features of apoptosis. Preliminary studies observed changes to cell morphology using the vital stains HO, AO and EtBr. Changes in morphology such as chromatin condensation, nuclear fragmentation, lysosome accumulation and membrane blebbing are indicative of apoptosis (Mpoke and Wolfe, 1997; Ribble *et al.*, 2005; Galluzzi *et al.*, 2018).

Fluorescence microscopy indicated that necrosis was not a prominent feature of cell death with a low percentage (<5%) of cells staining positive for necrosis in both the HL-60 and K562 cell lines (Figure 2.10 and Figure 2.11, respectively). Necrosis is characterized by the

swelling of cells and the leakage of cytoplasm containing inflammatory cellular contents. In apoptosis, inflammation is avoided, which is beneficial to cancer patients since apoptotic cell death does not contribute to tumour lysis syndrome which is associated with necrotic cell death (Howard *et al.*, 2011).

In both cell lines, JD88, JD47R and JD49R induced morphological changes consistent with apoptosis which included membrane blebbing, chromatin condensation and fragmented nuclei being prominent features when staining live cells with the vital dyes (Figure 2.10 and Figure 2.11). Fluorescence microscopy of HL-60 cells treated with the positive control camptothecin, showed extensive fragmentation of nuclei observed with HO and EtBr co-staining of nuclei (Figure 2.10; panels f and g). Such cells were considered to be late apoptotic due to the co-staining of HO and EtBr combined with the globular appearance of the nuclei. This is in contrast to necrotic nuclei which display a homogenous red colour. The globular staining pattern of apoptotic cells suggest that the membranes have already started to pinch off just prior to the cells breaking apart into apoptotic bodies. This observation was common in JD88 treated cells of both cell lines (Figure 2.10 and Figure 2.11; panel j).

Apoptotic cells show an increase in HO fluorescence intensity and the formation of apoptotic nuclei and chromatin condensation. Chromatin condensation is another morphological feature of early apoptotic cells and can be seen in Figure 2.10 and Figure 2.11 of treated HL-60 and K562 cells, respectively. Morphological analysis of K562 cells showed similar changes to that of HL-60 cells when treated with the copper-imidazo[1,2-*a*]pyridines. Camptothecin treated cells showed distinct nuclear fragmentation, this being more obvious in HL-60 cells (Figure 2.10; panel f) than in K562 cells (Figure 2.11; panel f). This was most possibly caused by camptothecin showing greater potency (IC₅₀ values) against HL-60 cells than it did against K562 cells (section 2.3.2).

Membrane blebbing is a characteristic feature associated with the execution phase of apoptosis which is due to the proteolytic cleavage of cytoskeletal proteins by effector caspases (caspase-3/7 and caspase-6) (Galluzzi *et al.*, 2018). Membrane blebbing was a common feature in the copper-imidazo[1,2-*a*]pyridines treated cells in both cell lines (Figure 2.10; panels e, m and q and, Figure 2.11; panels e, i, m and q), providing further support for apoptosis as a mechanism of cell death.

2.4.5 Annexin-V binding to phosphatidylserine indicating the induction of apoptosis

The exposure of PS on the outer cell membrane is a characteristic feature of early apoptosis (Lee *et al.*, 2013c) and is measured by the binding of Annexin-V to PS. Copper-imidazo[1,2-*a*]pyridines were able to induce the exposure of PS on the outer membrane in both HL-60 and K562 cells, albeit to different extents, providing evidence for cell death via apoptosis.

At a concentration equivalent to their IC₇₅ value, copper-imidazo[1,2-*a*]pyridines were able to induce PS exposure in HL-60 and K562 cells. Necrotic cell death as determined with PI staining was not common in both cell lines treated with copper-imidazo[1,2-*a*]pyridines with less than 2% of cells staining positive for PI alone (Table 2.4 and Table 2.5).

After a 14 hour treatment period, HL-60 cells treated with JD88, JD47R and JD49R showed an increase in Annexin-V binding indicative of early apoptosis. In contrast to the copper-imidazo[1,2-*a*]pyridines, camptothecin caused late apoptosis/ necrosis in HL-60 cells as indicated by the high percentage of cells that were positive for both Annexin-V and PI staining (61.9%) as reported in Table 2.4. The flow cytometric data mirrored the fluorescence microscopy data as seen with camptothecin in panel b of Figure 2.12. The increase in dual staining indicates that apoptosis occurred faster in camptothecin treated cells than other treated cells at this time period.

Fluorescence and flow cytometric analysis in K562 cells (Table 2.5) showed similar changes to that of HL-60 cells when treated with the copper-imidazo[1,2-*a*]pyridines. However, in K562 cells the exposure period was 18 hours, four hours longer than in HL-60 cells. This observation indicates that the induction of apoptosis is time-dependent and that there are differences in the responses of the two cell lines to apoptotic stimuli.

Several studies have reported on the increase in Annexin-V binding in response to copper (II) complexes on various cancer cell lines. Apoptosis was induced in MCF-7 breast cancer cells in response to polypyridyl-based copper (II) complexes (Salimi *et al.*, 2015), in Hela cervical cancer cells in response to copper complexed to a N-(pyridin-2-ylmethylene) dehydroabietylamine ligand (Fei *et al.*, 2016) and in A549 lung cancer cells in response to copper complexed to a Schiff base ligand (Ma *et al.*, 2015). Two chiral enantiomers induced apoptosis as indicated by the increase in Annexin-V binding in a dose-dependent manner after treatment at 5, 10 and 15 μ M (Zhou *et al.*, 2016).

In a study by Zhu *et al.*, (2015), copper (II) complexed with coumarin derivative were able to induce apoptosis in human hepatic (HepG2), human promyelocytic leukaemia (HL-60), and human prostate (PC3) cell lines (Zhu *et al.*, 2015b). This was in correlation with the results obtained in this study and may serve as evidence for copper complexes and their apoptotic inducing effects in APL. The exposure of PS on the outer cell membrane alone is not a conclusive measure of apoptosis and requires confirmation by further apoptotic assays such as an increase in caspase-3/7 activity (Tait and Green, 2010).

2.4.6 Copper-imidazo[1,2-*a*]pyridines increase caspase-3/7 activity in HL-60 and K562 cells

In this study, caspase-3/7 activity provides further evidence for the induction of apoptosis by copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 leukaemic cells. Caspase-3/7 are effector caspases that cause the cleavage of cytoskeletal proteins (Samudio *et al.*, 2010), disruption in the nuclear membrane and the release of CAD from its protein inhibitor ICAD to allow DNA fragmentation and apoptosis (McIlwain *et al.*, 2013). In addition to caspase-3 causing cell disassembly, during apoptosis caspase-3 plays an important role in releasing anti-inflammatory cytokines and suppressing the inflammatory response (Martin *et al.*, 2012).

The induction of caspase-3/7 activity is a late apoptotic event and was measured after an 18 and 24 hour treatment period in HL-60 and K562 cells, respectively. All the active copper-imidazo[1,2-*a*]pyridines were able to induce caspase-3/7 activity in both cells lines (section 2.3.8; Figure 2.14). However, the increase in caspase-3/7 activity was more pronounced in HL-60 cells than in K562 cells, which was consistent with their increased potency towards the HL-60 cells (section 2.2; Table 2.2). These results are consistent with that of Zhou *et al.*, (2016) regarding copper complexes inducing apoptosis by the activation of caspase-3. MDA-MB-231 breast cancer cells were treated for 16 hours and caspase-3 activation was measured using a caspase-3 colorimetric assay kit (Zhou *et al.*, 2016).

At an IC₇₅ concentration, camptothecin caused an 11-fold increase in caspase-3/7 activity in HL-60 cells, similar to JD47R, while causing a 2.2-fold increase in K562 cells. In HL-60 cells, JD47R caused the greatest fold increase (11-fold) in caspase-3/7 activity among the copper-imidazo[1,2-*a*]pyridines, whereas in K562 cells a 1.9-fold increase was observed. In K562 cells, JD88 caused the highest fold increase (2.8-fold) whereas JD47R was the most

potent inducer of caspase-3/7 in HL60 cells. These differences may be attributed to the molecular characteristics of each cell line.

In a study by Bortolozzi *et al.*, (2014), caspase-3 activity was measured in twelve different human leukaemia cell lines, which included acute myelocytic and chronic myelocytic cells, to evaluate growth inhibition and apoptosis by a copper (I) complex $[\text{Cu}(\text{thp})_4][\text{PF}_6]$ bearing a tris (hydroxymethyl) phosphine ligand. It was found that this phosphine copper (I) complex induced an increase in caspase-3 activity particularly in the SEM and RS4;11 cell lines originated from B-cell acute lymphoblastic leukaemia cells at a 2.5 μM concentration (Bortolozzi *et al.*, 2014). Caspase-3 activation was measured by western blot analysis on protein cell lysates after a 12 hour treatment period.

2.4.7 Indicators for apoptosis via the intrinsic mitochondrial apoptotic pathway

Having confirmed that apoptosis is the mechanism of cell death, the next step was to determine whether the intrinsic or extrinsic apoptotic pathway was activated in order to obtain a better understanding of the cellular effects of the copper-imidazo[1,2-*a*]pyridines on HL-60 and K562 cells. Caspase-3/7 can be activated by either the intrinsic or extrinsic pathway. Activation of intrinsic apoptotic pathway would confirm that the copper-imidazo[1,2-*a*]pyridines caused changes to the mitochondria. Therefore, mitochondrial membrane potential and the activation of caspase-9 were measured whilst caspase-8 activity provided an indication of activation of the extrinsic apoptotic pathway.

The change in mitochondrial outer membrane permeability (Figure 2.22) occurs as a result of changes in an electrical gradient across the mitochondrial membrane also known as the MMP (Estaquier *et al.*, 2012). The loss of MMP and release of pro-apoptotic molecules like cytochrome c, AIF and Smac/DIABLO are associated with apoptosis induced by chemotherapeutic drugs (Lopez and Tait, 2015).

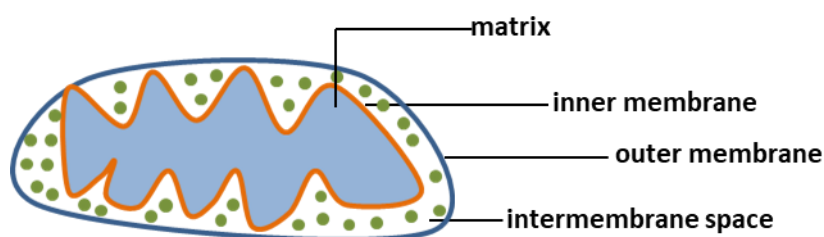


Figure 2.22 The sub-compartments of mitochondria. Figure adapted from Molecular Biology of the Cell (Alberts *et al.*, 2015).

The decrease of MMP occurs in mitochondria-mediated apoptosis, and this loss increases mitochondrial outer membrane permeability, which facilitates the release of the pro-apoptotic protein cytochrome c and the apoptogenic factors, Smac/DIABLO and HTRA2/Omi (Hassan *et al.*, 2014). Cytochrome c is released from the inner mitochondrial membrane into the cytosol where it interacts with Apaf-1 to activate pro-caspase-9 and subsequently, caspase-9 (Wong, 2011).

2.4.8 Copper-imidazo[1,2-*a*]pyridines cause a loss of mitochondrial membrane potential in HL-60 and K562 cells

The ability of copper-imidazo[1,2-*a*]pyridines to cause apoptosis through activation of the intrinsic pathway was determined by evaluating the effect of copper-imidazo[1,2-*a*]pyridines on MMP and on caspase-9 activity. An apoptotic signal such as internal stress caused by DNA damage or hypoxia changes the permeability of the outer mitochondrial membrane allowing the release of pro-apoptotic proteins into the cytosol (Lopez and Tait, 2015). The increase in mitochondrial membrane permeability occurs as a result of the formation of Bak/Bax and Bax/Bax protein oligomers which form the transition pore. To determine whether caspase-3 is activated as a result of induction of the mitochondrial pathway, changes in MMP were assessed by use of the membrane permeable dye; JC-1.

The JC-1 dye is a positively charged fluorescent dye that accumulates in the mitochondria, driven by the negative charge on the inside of the inner membrane. At low concentrations (due to a low MMP), the dye does not cross the membrane and remains in its monomeric form, displaying a green fluorescence. At high concentrations (due to a high MMP), in healthy cells the dye accumulates in the mitochondria forming J-aggregates, causing a shift in the fluorescence spectrum from green to red-orange (Tait and Green, 2010). The JC-1 dye is useful in determining the status of the MMP.

The positive control camptothecin caused a complete loss of MMP in HL-60 (Figure 2.15A; panel b) and K562 cells (Figure 2.16A; panel b), respectively, as indicated by the presence of green fluorescence alone and the absence of red fluorescence. Similarly to the positive control, the copper-imidazo[1,2-*a*]pyridines induced a loss of MMP in HL-60 (Figure 2.15) and K562 cells (Figure 2.16) suggesting that JD88, JD47R and JD49R cause a loss of MMP. In both cell lines, JD49R was the most potent regarding the loss of MMP showing complete disruption in MMP. In HL-60 cells, JD88, compared to JD47R and JD49R, was the least

potent with more cells (11%) forming J-aggregates that fluoresce yellow-orange, indicative of an intact membrane potential (Figure 2.15). Likewise, K562 cells treated with JD88 (6%) and JD47R (8.7%) caused less of an effect on MMP than JD49R indicating cells with an intact membrane potential (Figure 2.16).

The loss of MMP facilitates the release of the pro-apoptotic protein cytochrome c and the apoptotic factors, Smac/DIABLO and HTRA2/Omi. Once released into the cytosol, cytochrome c interacts with Apaf-1, resulting in the activation of pro-caspase-9, a characteristic of the intrinsic apoptotic pathway, which in turn cleaves and activates effector caspase-3, -6 and -7, resulting in apoptosis.

Cancer cells exhibit greater oxidative stress than normal cells due to excessive energy requirements (Gupta *et al.*, 2011; Carmen *et al.*, 2012; Raza *et al.*, 2017). Mitochondrial membrane permeability is a measure of mitochondrial function and in cancer ROS targets mitochondrial DNA (mtDNA) which is prone to oxidative damage. This in turn, disrupts the function of the respiratory chain, further increasing ROS production. This state of oxidative stress makes cancer cells susceptible to chemotherapeutic agents which may initiate apoptosis (Hajrezaie *et al.*, 2015). A study by García-Ramos *et al.*, (2016) demonstrated the anti-proliferative activity of copper (II) coordination compounds Casiopeínas® on neuroblastoma cells by ROS production and mitochondria dysfunction. The Casiopeínas® caused a disruption in MMP indicating apoptosis was induced via the intrinsic pathway (García-Ramos *et al.*, 2016). Similarly, the loss in MMP caused by the copper-imidazo[1,2-*a*]pyridines serves as evidence for the induction of the intrinsic apoptotic pathway in leukaemic HL-60 and K562 cells.

2.4.9 Copper-imidazo[1,2-*a*]pyridines increase caspase-9 activity in HL-60 and K562 cells

Since the copper-imidazo[1,2-*a*]pyridines caused a loss of MMP, the activation of caspase-9 by the copper-imidazo[1,2-*a*]pyridines was investigated in the HL-60 and K562 cell lines. Leukaemic cells were treated at two different time periods as the copper-imidazo[1,2-*a*]pyridines induced apoptotic effects in the cell lines at different times. Caspase-9 activity was measured in HL-60 and K562 cells, which were treated at an IC₇₅ concentration for 14 and 24 hours, respectively. The data showed that all the test compounds induced caspase-9 activity and thus confirms the activation of the intrinsic apoptotic pathway.

In HL-60 cells, JD88 was the most potent inducer and after 14 hours of exposure caused a 6.9-fold increase in caspase-9 activity that was higher than camptothecin which showed a 6.4-fold increase. JD49R showed the lowest activation of caspase-9 at this exposure time (Figure 2.17A). Similarly, in K562 cells, JD88 was also the most potent inducer of caspase-9 activity among the copper-imidazo[1,2-*a*]pyridines with a 5.3-fold increase while JD49R was the least active compound in inducing caspase-9 activity (3.8-fold) at this time period. Camptothecin showed the greatest activity in K562 cells with an increase of 6.5-fold in caspase-9 activity. The caspase-9 data indicates that the active copper-imidazo[1,2-*a*]pyridines activates the intrinsic apoptotic pathway in both cell lines.

The loss of MMP leads to the formation of the apoptosome complex and activation of caspase-9 (Lopez and Tait, 2015; Peña-Blanco and García-Sáez, 2018). Considered together, the JC-1 and caspase-9 results indicate the activation of the intrinsic mitochondrial pathway. The current findings are consistent with that observed in the literature for related copper complexes and their ability to cause mitochondrial toxicity (García-Ramos *et al.*, 2016).

Since cancer cells have an increased mitochondrial biogenesis which includes increased mitochondrial number, ATP production and mitochondrial DNA copy numbers, these changes provide a possibility to preferentially target cells with increased mitochondrial function to improve therapeutic selectivity (Vyas *et al.*, 2016). Cardiac myocytes have abundant mitochondria and clinical use of the copper-imidazo[1,2-*a*]pyridines may cause cardiotoxicity (Bernard *et al.*, 2008; Fernández-Vizarra *et al.*, 2011). However, this has not stopped the use of other existing chemotherapeutic agents like doxorubicin despite it being cardiotoxic (Krischer *et al.*, 1997). The standard protocol is to administer a cardioprotective agent such as dexrazoxane to patients (Choi *et al.*, 2010; Emanuel and Wilson, 2018). The clinical use of the copper based Casiopeínas® are limited by the development of dose-dependent cardiotoxicity as reported by Silva-Platas., (2016).

2.4.10 Effect of copper-imidazo[1,2-*a*]pyridines on caspase-8 activity in HL-60 and K562 cells

The copper-imidazo[1,2-*a*]pyridines and camptothecin were poor activators of caspase-8 in HL-60 cells. They caused an increase in caspase-8 activity in a time-dependent manner of close to 20% of cells (Figure 2.18), which was more than that observed in K562 cells (Figure 2.20). Neither the copper-imidazo[1,2-*a*]pyridines nor camptothecin induced meaningful

caspase-8 activity in K562 cells, suggesting that the copper-imidazo[1,2-*a*]pyridines do not activate the extrinsic apoptotic pathway. This data is consistent with a previous study on colorectal cancer cells which showed no activation of caspase-8 by copper-imidazo[1,2-*a*]pyridines JD88, JD46R and JD47R at a 10 μ M concentration (Harmse *et al.*, 2019).

2.4.11 In Summary:

Copper-imidazo[1,2-*a*]pyridines were selectively active against HL-60 and K562 leukaemic cells, with three complexes, JD88, JD47R and JD49R, showing IC₅₀ values between 1.0 and 6.0 μ M. Treatment with the copper complexes resulted in fragmented apoptotic nuclei suggestive of apoptotic cell death, which was confirmed by increased Annexin-V binding and caspase-3/7 activation. The disruption of the mitochondrial membrane potential, the activation of caspase-9 while showing an absence in caspase-8 activity in both leukaemic cell lines provided evidence for cell death by intrinsic apoptosis. The copper-imidazo[1,2-*a*]pyridines were thus effective inducers of apoptotic cell death at low micromolar concentrations.

Having collected evidence that cell death occurred via the intrinsic apoptotic pathway, an apoptotic regulatory protein array was used to further investigate the cellular response to the copper-imidazo[1,2-*a*]pyridines.

CHAPTER THREE: Apoptotic proteome array

3.1 INTRODUCTION

Deregulation of apoptosis has been associated with many cancers including haematological malignancies (Zaman *et al.*, 2014; Green and Llambi, 2015; Lopez and Tait, 2015; Pistritto *et al.*, 2016; Bao *et al.*, 2018)(Reviewed in Pfeffer and Singh., 2018). Cancer cells are able to evade apoptosis due to the decreased expression (down-regulation) or the increased expression (up-regulation) of pro-apoptotic proteins and the up-regulation of anti-apoptotic proteins, causing mutant cells to survive and proliferate (Hassan *et al.*, 2014). These imbalances in the apoptotic regulatory networks help damaged cells to evade apoptosis, and provide novel therapeutic targets and strategies.

3.1.1 Regulation of caspases

The activation of caspases is central to apoptotic cell death. Caspases cleave a variety of proteins at aspartic acid residues resulting in regulated cell death (Wong, 2011). Since proteolysis is irreversible, activation of caspases in cells are tightly regulated by caspase inhibition, caspase degradation and decoy inhibitors (Pop and Salvesen, 2009). Decoy proteins are proteins structurally related to caspase pro-domains which compete for the same adaptor proteins found within activation complexes (Pop and Salvesen, 2009). An example is , cFLIP, with a non-functional catalytic domain prevents caspase-8 recruitment to the DISC complex (Kong *et al.*, 2017).

Another method by which caspase regulation occurs is by degradation via the proteasome complex (Senft *et al.*, 2018). Activated caspases have a much greater turnover than their inactive forms and it has been reported that the proteins responsible for their rapid removal are IAPs. IAPs contain RING and ubiquitin associated domains that promotes ubiquitin ligation, targeting proteins for proteosomal degradation (Blankenship *et al.*, 2009). An example of a caspase regulator and inhibitor is XIAP (Saleem *et al.*, 2013). For instance, the proteolytic processing of the pro-caspase-9 inter-subunit linker promotes caspase-9 regulation by XIAP by exposing new sites/epitomes necessary for XIAP binding (Li *et al.*, 2017).

Increased levels of IAPs are therefore responsible for the removal of active caspases before they reach an apoptotic threshold. This is confirmed by Schile *et al.*, (2008) who

demonstrated that mice without a XIAP RING domain showed elevated caspase activity implying the necessary XIAP ubiquitin ligase activity for caspase removal.

Since some anti-apoptotic proteins are overexpressed and/or overactive they can be exploited as cancer selective targets. Therefore, the evaluation of the effects of novel lead compounds on the activity or expression levels of apoptotic regulatory proteins are an important part of the evaluation of potential anti-cancer agents.

3.1.2 Regulation by Bcl-2 family of proteins

The Bcl-2 family of proteins are important regulators of apoptosis. These proteins share one or more of the four BH domains (BH1, BH2, BH3 or BH4). The anti-apoptotic proteins Bcl-2 and Bcl-x conserve all four BH domains, whereas the pro-apoptotic proteins are subdivided into those with multiple BH domains (Bax and Bak) and BH3-only domains (Bim and Bid) (Hata *et al.*, 2015) (Figure 3.1). A second group of proteins (Bad, Bik, Bmf, Hrk, Noxa, and Puma), facilitate the effects of Bax and Bak by neutralizing anti-apoptotic proteins (Singh *et al.*, 2018).

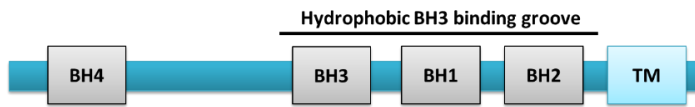
This family includes anti-apoptotic proteins, pro-apoptotic proteins and proteins that cause changes in mitochondrial membrane permeability (Pfeffer and Singh, 2018). Anti-apoptotic family members include Bcl-2 and Bcl-x whilst Bcl-2 associated death promoter protein (Bad) and Bax are examples of pro-apoptotic Bcl-2 family members whose overexpression promotes apoptotic cell death (Ashkenazi *et al.*, 2017). The susceptibility of cells to apoptosis is determined partly by the relative ratios of pro-apoptotic to anti-apoptotic Bcl-2 family members.

The anti-apoptotic Bcl-2 family member Bcl-2 protein is an important regulatory protein in apoptosis (Hata *et al.*, 2015). It inhibits intrinsic apoptosis by preventing the release and oligomerisation of Bax and Bak which facilitates mitochondrial permeabilisation (Tzifi *et al.*, 2012; Lopez and Tait, 2015). This occurs when the conserved BH1, BH2 and BH3 domains of anti-apoptotic protein Bcl-2 form a hydrophobic cleft that provides a binding site for the dimerization of BH3 domains of pro-apoptotic family members Bax and Bak (Shamas-Din *et al.*, 2011).

A. Pro-survival/ anti-apoptotic proteins

BH1-4 domain

- Bcl-2, Bcl-xl, Mcl-1, Bfl1/A1 and Bcl-w



Pro-apoptotic proteins

i) BH3-only domain

- Activators: tBid, Bim, Noxa and Puma
- Sensitizers: Bad, Bik, Bmf and Hrk



ii) Multiple BH domain

- Effectors: Bax, Bak and Bok



B.

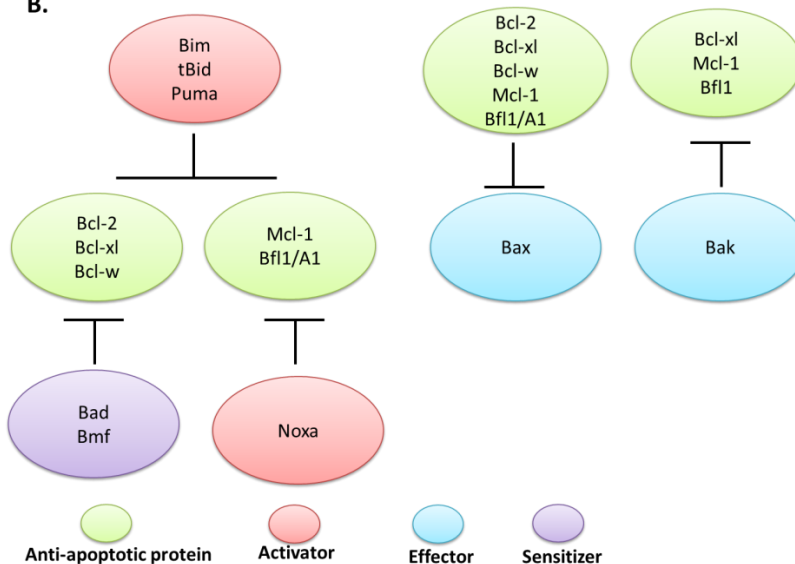


Figure 3.1 The Bcl-2 family consists of pro-survival and pro-apoptotic proteins. A. The pro-survival/ anti-apoptotic members conserve all four BH domains. The pro-apoptotic Bcl-2 family members can be further divided into the multi-BH-domain effector proteins (containing BH1, BH2 and BH3 domains) and BH3-only proteins (only region of homology to Bcl-2 is BH3). The pro-apoptotic proteins are divided into activators, sensitizers and effectors according to their functions in the apoptotic pathway. Membrane insertion occurs via the trans-membrane (TM) domain for anchoring to organelles. TM domains are found in pro-survival, effector and some BH3 only proteins (Bik, Bim and Hrk). Figure adapted from Campbell and Tait (2018). **B.** Interactions between the Bcl-2 family proteins. All anti-apoptotic proteins interact with Bax, Bim, Puma and tBid. Only Mcl-1 and A1 interact with Noxa. Bcl-2, Bcl-x and Bcl-w inhibit Bad and Bmf, while Bak is targeted by Bcl-x, Mcl-1 and A1. Figure adapted from Hata *et al.*, (2015).

When protein levels of Bcl-2 or Bcl-x are high, they can form heterodimers with the pro-apoptotic protein Bax and prevent apoptosis. Whereas, when the protein levels of Bcl-2 and

Bcl-x are low, Bax homodimerisation is favoured which drives the cell toward apoptosis (Dewson *et al.*, 2008). Bax homodimers cause the formation of a transition permeability pore complex in mitochondria causing the loss of MMP (O'Neill *et al.*, 2016).

Pro-apoptotic Bad can heterodimerise with anti-apoptotic molecules like Bcl-2 preventing both Bcl-2 and Bcl-x from heterodimerising with Bax thereby favouring Bax homodimerisation (Hata *et al.*, 2015). The antagonistic actions of these two groups of proteins have been attributed to their ability to form heterodimers (Fulda, 2014). Bcl-x has been reported to directly bind to the voltage-dependent ion channel (VDAC) protein which is a constituent of the transition permeability pore (Huang *et al.*, 2013). The interaction of Bcl-x with VDAC inhibits the release of cytochrome c suggesting that Bcl-x can also mediate its effects directly at the transition permeability pore (Lauterwasser *et al.*, 2016).

The expression levels of pro-survival Bcl-2 proteins are regulated by protein turnover. Bcl-2 and Bcl-x are relatively stable proteins as reflected by the long half-life of Bcl-2 of approximately 20 hours (Rooswinkel *et al.*, 2014). In contrast, protein turnover of Mcl-1 and A1 occurs constitutively through poly-ubiquitination and proteasomal degradation as shown by their half-lives of approximately 30 and 15 minutes, respectively (Adams and Cooper, 2007).

Activation of Bcl-2 proteins can be altered based on cellular localization. Bid is located in the cytosol but upon cleavage by caspase-8, truncated Bid is formed (tBID), which translocates to the mitochondria and activates apoptosis. Both Bax and Bak harbour mitochondrial membrane anchoring C-terminal tails. In healthy cells, Bak is constitutively bound to the outer mitochondrial membrane and Bax is localised to the cytosol and endoplasmic reticulum (Todt *et al.*, 2015). However, Bax and Bak are in a dynamic equilibrium between cytosol and membranes (Schellenberg *et al.*, 2013), and are constitutively translocated from the endoplasmic reticulum to the cytosol by pro-survival Bcl-2 proteins (Todt *et al.*, 2015). In the absence of other Bcl-2 proteins Bax becomes localised to the mitochondrial membrane.

The up-regulation of Bcl-2 pro-survival proteins can occur through chromosomal translocation, gene amplification, and increased gene expression/translation and protein stability. Increases in gene copy number of pro-survival proteins are more common than translocation (Beroukhi *et al.*, 2010). The elevation of Bcl-2, Bcl-x and Mcl-1 predisposes to leukaemia and lymphoma development. Pro-survival Bcl-2 expression is only considerably

oncogenic and additional gene alterations are clearly required for tumour formation. The combined gene amplification of *MYC* with *MCL1* or *BCLX* is common in cancer, and in cell culture and mouse models increased Bcl-2-like proteins, and *MYC* are considered as a potent oncogenic combination (Campbell *et al.*, 2010). This demonstrates the sum effect of all pro-survival proteins present may be more important for survival than expression levels of an individual protein (Carrington *et al.*, 2017). This is an important consideration for therapeutic targeting of pro-survival Bcl-2 proteins and minimization of damage to normal tissues.

3.1.3 Cell signalling pathways that regulate Bcl-2 family of proteins

The PI3K pathway and the ERK1/2 signalling pathway are two essential regulators of apoptosis and influence the expression and/or activity of Bcl-2 family proteins (Cook *et al.*, 2017). Transcription factors forkhead box O3 (FoxO3), FoxO4, NF- κ B and p53 regulate the transcription of Bcl-2 family proteins (Zhang *et al.*, 2011).

PI3K regulates cytokine-mediated cell survival and proliferation (Thomas *et al.*, 2013). The PI3K pathway can be activated by growth factors such as epidermal growth factor and platelet-derived growth factor, cytokines such as interleukin -2 and -3 and some hormones like insulin. PI3K activates Akt leading to the transcription of anti-apoptotic genes by activating canonical NF- κ B (Reviewed by Ghoneum and Said, 2019). Akt phosphorylates the forkhead superfamily of transcription factors; FoxO3 and FoxO4, preventing apoptosis (Roy *et al.*, 2010). Forkhead family of transcription factors are characterised based on their distinct fork head DNA binding domain (Tzivion *et al.*, 2011). These transcription factors are responsible for increasing the cyclin dependent kinase (CDK) inhibitor p21^{Cip1} and p27^{Kip1}, which inhibits cell cycle progression causing cell cycle arrest of cells at G₀/G₁ (Dijkers *et al.*, 2000; Cappellini *et al.*, 2003). Simultaneously, Akt phosphorylates and inactivates Bad and inhibits its pro-apoptotic function (Datta *et al.*, 1997). Akt survival signals are counteracted by the tumour suppressor phosphatase and tensin homolog (PTEN) which inhibits the action of PI3K (Arcaro and Guerreiro, 2007).

The ERK1/2 signalling pathway stimulates cell survival by increasing the expression of pro-survival Bcl-2 proteins and repressing pro-apoptotic Bcl-2 proteins. ERK1/2 phosphorylation promotes the transcription and expression of Bcl-2, Bcl-x and Mcl-1 marks Bad, Bik and Bim for degradation (Cook *et al.*, 2017). Bim can be transcriptionally regulated by FoxO3, which itself is a target of ERK1/2 (Dijkers *et al.*, 2000). ERK1/2-mediated FoxO3 phosphorylation

promotes MDM2-dependent ubiquitination and degradation, thereby repressing Bim transcription (Yang *et al.*, 2008) .

3.1.4 The regulation of apoptosis by tumour suppressor p53

The tumour suppressor protein p53 is a critical regulator of apoptosis (Hanahan and Weinberg, 2011). This nuclear phosphoprotein binds to DNA and alters the expression of genes associated with cell proliferation and apoptosis (Zhang and Lozano, 2017). In response to DNA damage and cellular stress, phosphorylation of p53 by the protein ataxia telangiectasia mutated (ATM) leads to its stabilization (Vassilev, 2007; Chen, 2012), causing a rapid increase in p53 protein levels within the cell (Lavin and Gueven, 2006). Under these conditions, p53 behaves as a transcription factor by stimulating the expression of pro-apoptotic Puma, Bid, Bax, Fas-L and TRAIL in the nucleus (Zilfou and Lowe, 2009; Jamil *et al.*, 2015). P53 induces the transcription of the CDK inhibitor p21 as well, which blocks cell cycle progression at G₁. As a result, cells with damaged DNA are arrested in G₁ until the damage is repaired and the levels of p53 and p21 decrease so that cells can progress into S phase (Lehman-McKeeman, 2019). With more extensive damage of DNA, p53 sets in motion the process of apoptosis to prevent the proliferation of damaged cells with mutations (Green and Kroemer, 2009).

E3 ubiquitin ligases are fundamental elements of the ubiquitin-proteasome pathway and have been reported to play a key role in cancer development (Qi and Ronai, 2015) by regulating the expression and stability of tumour suppressor proteins like p53 (Senft *et al.*, 2018). MDM2 is overexpressed in several cancers and contributes to tumour development by reducing the availability of functional p53 protein (Hientz *et al.*, 2017). p53 degradation is increased by MDM2 in normal cells. It is not measureable and has a short half-life. In cancer cells, MDM2 levels are increased resulting in decreased functional p53 (Klaunig and Wang, 2019).

3.1.5 Regulation of Inhibitor of apoptosis proteins (IAPs)

Both initiator and effector caspases are negatively regulated by members of the IAP family which include XIAP, cIAP-1, cIAP-2, livin and survivin (Pfeffer and Singh, 2018). Baculovirus IAP repeat (BIR) domains bind to the active site of caspases and inhibit their proteolytic function, as XIAP inhibits both apoptotic pathways by binding and inhibiting

caspase-9, caspase-3 and caspase-7 (Wong, 2011). XIAP inhibits caspase-9 via its BIR3 domain and caspase-3/7 via its BIR2 domain. These domains interact with their target caspases to achieve inhibition by a two-site mechanism. The other IAPs (cIAP-1, cIAP-2, survivin) do not directly inhibit caspases (Eckelman *et al.*, 2006). Some IAPs may target effector caspases for ubiquitination and proteasomal degradation, thus limiting their executioner potential. Lastly, cIAP-1 and cIAP-2 contribute to the activation of anti-apoptotic signals by regulating NF- κ B activation during TNF signalling (Fulda, 2014).

The increased expression of members of the IAP family is commonly associated with the inhibition of apoptosis and manifestation of cancers (Saleem *et al.*, 2013). In leukaemia patients, the overexpression of survivin and cIAPs in leukaemia correlates with poor prognosis and higher rates of treatment failure and relapse (Hess *et al.*, 2007; Carter *et al.*, 2012). The increased expression of XIAP has been detected in AML (Cassier *et al.*, 2017) and CML patients, and is associated with poor prognosis (Cai *et al.*, 2011; Ibrahim *et al.*, 2012). The activation of NF- κ B and PI3K/Akt pathways is responsible for increased levels of XIAP (Zhang and Lozano, 2017; Yogosawa and Yoshida, 2018).

Molecules that bind IAPs decrease the activation threshold of caspase-3 and sensitize malignant cells to chemotherapeutic agents (Saraei *et al.*, 2018; Zhou *et al.*, 2018). Since the overexpression of XIAP is associated with the pathogenesis of AML, exploiting it as a therapeutic target has been the interest of many researchers (Carter *et al.*, 2005; Fakler *et al.*, 2009; Zhou *et al.*, 2018). The development of a selective small molecule XIAP inhibitor that targets the BIR2 domain of XIAP was shown to antagonize the inhibitory effects of XIAP on caspase-3 and caspase-7 thereby activating the intrinsic apoptotic pathway (Zhou *et al.*, 2018). This novel drug, RO6867520 (RO-BIR2), developed by Roche targeting XIAP to reactivate blocked apoptosis, has also shown promising clinical outcomes in AML patients (Zhou *et al.*, 2018).

3.1.6 Smac/DIABLO and HTRA2/Omi mitochondrial proteins

The loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins Smac/DIABLO and HTRA2/Omi normally sequestered from the intermembrane space into the cytosol (Bose and Grant, 2015). They target IAP members and facilitate IAP inhibition. These proteins are able to bind to the characteristic BIR domain of IAPs and prevent their interaction with caspases (Rathore *et al.*, 2017), thereby restoring the normal function of

caspases. Smac/DIABLO binds to survivin, cIAP-1/2 but mainly XIAP, at its BIR3 domain and inhibits and restores the function of initiator caspase-9 and effector caspase-3 (Fulda, 2014). In response to mitochondrial damage, the serine protease HTRA2/Omi recruits XIAP and cIAP-1 (Suzuki *et al.*, 2001). HTRA2/Omi binds and cleaves XIAP and cIAP-1, preventing the inhibition of caspase activation (Rathore *et al.*, 2017).

3.1.7 Regulation of TRAIL receptors

Defects in the extrinsic death receptor pathway cause cancer cells to evade apoptotic cell death. A number of mechanisms of TRAIL resistance have been described. One of which includes the abnormal decreased expression of TRAIL-specific surface receptors (Naimi *et al.*, 2018). Particularly, the impairment of death receptor expression and the aberrant overexpression of decoy receptors prevent TRAIL-mediated apoptosis. Studies have shown the correlation of resistance of TRAIL with poor expression of TRAIL-R1 and TRAIL-R2, and the increased expression of cFLIP (Chiewchengchol *et al.*, 2016). The overexpression of decoy receptors also contributes to cancer cell survival against TRAIL-mediated cytotoxic responses (Naimi *et al.*, 2018).

3.1.8 Caspase-8 inhibition: cFLIP

The majority of signals that inhibit caspase-8 do so by affecting the recruitment of caspase-8 to the DISC complex. For example, anti-apoptotic cellular FLICE-inhibitory protein (cFLIP), which shares significant structural similarities with caspases-8 and -10, allows it to compete for binding sites and thus prevents the activation of caspase-8/10 by displacing them from DISC (Kong *et al.*, 2017). The inhibitor of NF- κ B called the A20 binding and inhibitor 1 (ABIN1) exerts its anti-apoptotic effect by affecting the interaction of RIP1 and FADD with caspase-8.

Other mechanisms for the negative regulation of caspase-8 involve the induction of survival signalling pathways that later inhibit caspase-8 activation. CIAP-1 and cIAP-2 contain the BIR repeat, a CARD and RING E3 ligases, which help to recruit TRAF-1 and 2 and inhibit TNF- α apoptotic signalling (Lopez *et al.*, 2011). They also execute their inhibitory potential by inducing pro-survival signals through the NF- κ B pathway (Fulda, 2014).

3.1.9 Aim and Objectives

A better understanding of the deregulation of apoptosis in cancer will aid the development of alternative approaches to cancer therapy. In this chapter, the expression levels of apoptotic regulatory proteins will be explored in leukaemic cells.

Aim:

Evaluate the effect of active test compounds on the expression of apoptotic-regulatory proteins by use of an apoptotic proteome array.

Objectives:

- Evaluate the effect of the imidazo[1,2-*a*]pyridines on the expression levels of apoptosis associated proteins
- Identify potential cellular targets of the test compounds within cell death signaling pathways by interrogating the dataset

3.2 METHODS

Effect of the copper complexes on protein expression was determined using an apoptotic regulatory proteome array from R&D Biosystems. The Proteome Profiler™ Array kit (ARY009, R&D Biosystems) is a membrane based antibody array used for the parallel determination of the expression levels of apoptosis regulatory proteins and is validated for protein detection in cell lysates. This kit is beneficial as it simultaneously detects expression levels of 35 human apoptosis regulatory proteins.

The exposure period and concentration of test compounds used in the array required careful consideration. Upon cell death, proteins are rapidly degraded and this would not prove an accurate assessment of the effects of the test compounds on the apoptotic regulatory proteins. Since apoptotic markers were increased after 18 hours of treatment, this treatment period was selected for the proteome profile analysis. A concentration equivalent to the IC₇₅ value was chosen since phase contrast microscopy showed no obvious changes to cell morphology at this concentration after 18 hours, in contrast to a 24 hour treatment period, when cells were stressed and irregular shaped.

3.2.1 Human apoptosis proteome array

The apoptosis array kit contained four nitrocellulose membranes with each having 35 different antibodies printed in duplicate. Four samples were prepared for each cell line which included: the two treated samples with the most active copper-imidazo[1,2-*a*]pyridines, an untreated control and the positive control camptothecin.

3.2.2 Treatment of cells

A total of 10 million cells at a density of 0.5×10^6 cells/ ml were treated with active test compounds at concentrations equivalent to their IC₇₅ value for 18 hours. Treated cells were harvested and solubilized in lysis buffer 17 (which contained a Complete™, mini, EDTA free protease inhibitor cocktail tablet (Millipore Sigma)). Cell lysate were diluted 1:5 before the protein concentration was determined with the Bradford protein assay as described in section 2.2.13 (Bradford, 1976).

3.2.3 Membrane array preparation

The membranes were first blocked in array buffer 1 (a buffered protein solution with preservatives) in a four well multi-dish for 1 hour at 4°C on a rocking platform shaker. After blocking, cell lysates were diluted to a volume containing 400 µg of protein for each lysate with array buffer 1, and this was incubated with the membranes overnight at 4°C on a rocking platform shaker in a final volume of 2 ml. This step allowed for specific target proteins in the cell lysates to bind to the captured antibodies on the nitrocellulose membranes. On the following day, each array was washed thrice with 20 ml 1× washing buffer for 10 minutes on a rocking platform shaker to remove unbound proteins. This step was followed by incubation with the detection biotinylated antibody cocktail (15 µl) which was diluted to 1.5 ml of 1× array buffer 2/3 for 1 hour on a rocking platform. The signals for captured proteins were amplified with these biotinylated antibodies. Thereafter, the arrays were washed as previously with 1× wash buffer. The membranes were then incubated with a 1:2000 dilution of streptavidin-horseradish peroxidase (HRP) conjugate in 1× array buffer 2/3 for 30 minutes on a rocking platform shaker. Streptavidin binds with high affinity to biotin and biotinylated molecules, and coupled with HRP, this conjugate allows for the detection of the protein-antibody complex. Thereafter, the membranes were washed as described above and carefully placed in a plastic sheet protector before detection.

For each cell line the four array membranes were incubated simultaneously to ensure that there are no artefactual changes due to small changes in the incubation periods. The membranes were incubated simultaneously for 1 minute with 1 ml of chemiluminescent detection reagent mix per membrane. Chemiluminescent substrates are luminol based and in the presence of HRP, luminol oxidises and forms a light emitting product (Khan *et al.*, 2014). Light emission occurs only during the enzyme-substrate reaction. Therefore, chemiluminescence is a useful and sensitive method for detecting very low levels of proteins in a single sample. A chemiluminescent signal corresponding to the amount of bound protein was produced at each capture spot. The membranes were viewed on the Bio-Rad ChemiDoc system (Bio-Rad, USA) using the ChemiDoc MP Imager and Image Lab™ software version 4.1 (BioTechniques, NY, USA) for quantification.

Data obtained from the different treatments was analyzed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post hoc* comparison test. The p -values compared to the untreated control are illustrated in all figures. The p -values of 0.01 and 0.001 were considered to be highly significant, while a p -value of 0.05 was considered to be significant and a p -value of 0.1 was considered to be poorly significant. Data analysis and construction of graphs was done using GraphPad Prism v.8.0. In order to improve the stringency of the data from the proteomics, only comparisons with a p -value of 0.001 was considered to be truly meaningful.

The average signal (pixel density) was determined from the pair of duplicate spots representing each apoptosis-related protein. The pixel density from the chemiluminescent signals was quantified using the Image Lab Software version 6.0. The level of protein expression was categorised based on pixel density as shown in Table 3.1. Figure 3.2 is the template of the array for protein identification using the coordinates referenced in Table 3.2.

Table 3.1 Grading scale used to categorise protein expression levels based on pixel density

Pixel density	Expression level
0 - 50 000	Very low
50 000 - 200 000	Low
200 000 - 400 000	Moderate
400 000 - 600 000	Moderately high
600 000 - 900 000	High
900 000 >	Very high

Table 3.2 Human apoptosis array coordinates which correspond to apoptotic regulatory proteins

Coordinates	Target/control	Coordinates	Targets/control
A1, A2	Reference spots	C13, C14	HO-2/HMOX2
A23, A24	Reference spots	C15, C16	HSP27
B1, B2	Bad	C17, C18	HSP60
B3, B4	Bax	C19, C20	HSP70
B5, B6	Bcl-2	C21, C22	HTRA2/Omi
B7, B8	Bcl-x	C23, C24	Livin
B9, B10	Pro-caspase-3	D1, D2	PON2
B11, B12	Cleaved caspase-3	D3, D4	p21 ^{CIP1} /CDKN1A
B13, B14	Catalase	D5, D6	p27 ^{Kip1}
B15, B16	cIAP-1	D7, D8	Phospho-p53 (S15)
B17, B18	cIAP-2	D9, D10	Phospho-p53 (S46)
B19, B20	Claspin	D11, D12	Phospho-p53 (S392)
B21, B22	Clusterin	D13, D14	Phospho-Rad17 (S635)
B23, B24	Cytochrome c	D15, D16	SMAC/Diablo
C1, C2	TRAIL-R1/DR4	D17, D18	Survivin
C3, C4	TRAIL-R2-DR5	D19, D20	TNF-R1/TNFRSF1A
C5, C6	FADD	D21, D22	XIAP
C7, C8	Fas/TNFRSF6/CD95	D23, D24	PBS (negative control)
C9, C10	HIF-1 α	E1, E2	Reference spots
C11, C12	HO-1/HMOX1/HSP32		

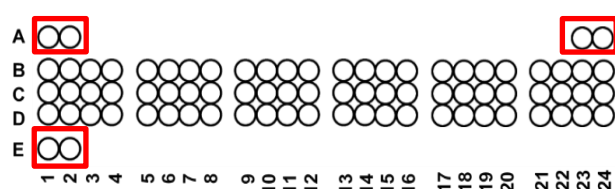


Figure 3.2 Human apoptosis array coordinates of the spotted proteins. A pair of duplicate spots represents each apoptosis-related protein.

3.3 RESULTS

Changes in the expression levels of the apoptotic regulatory proteins in response to the active copper-imidazo[1,2-*a*]pyridines was performed on HL-60 and K562 cells as well as normal peripheral leukocytes. For each cell line the two most active copper-imidazo[1,2-*a*]pyridines were selected. Cells were treated at a concentration equivalent to their IC₇₅ value for 18 hours. This treatment period and treatment concentrations show no obvious morphology changes associated with cell death like the disintegration of cell membranes as opposed to when they were exposed to 24 hours of treatment showing visibly stressed and irregular shaped cells.

HL-60 cells were treated with JD88 at 7.8 µM, JD47R at 4.3 µM and camptothecin at 0.02 µM. K562 cells were treated with JD88 at 7.1 µM, JD49R at 5.8 µM and camptothecin at 6.2 µM. Normal peripheral leukocytes were treated with JD88 and JD47R at concentrations equal to their IC₇₅ values in HL-60 cells. JD47R was selected for the leukocytes since it was more potent than JD49R.

The protein expression levels were calculated by measuring the pixel density of each spot on the membrane. The percentage change in protein expression was calculated by comparing the pixel density of each protein of the treated cells to that of the untreated control cells. This is reported in Table 3.3 (HL-60 cells), Table 3.4 (K562 cells) and Table 3.5 (leukocytes), along with the level of significant difference between treated cells and untreated control cells.

3.3.1 The effect of active copper-imidazo[1,2-*a*]pyridines on apoptotic regulatory proteins in HL-60 cells

The effects of the compounds on the expression of apoptosis-related proteins by JD88, JD47R and camptothecin are shown in Figure 3.3.

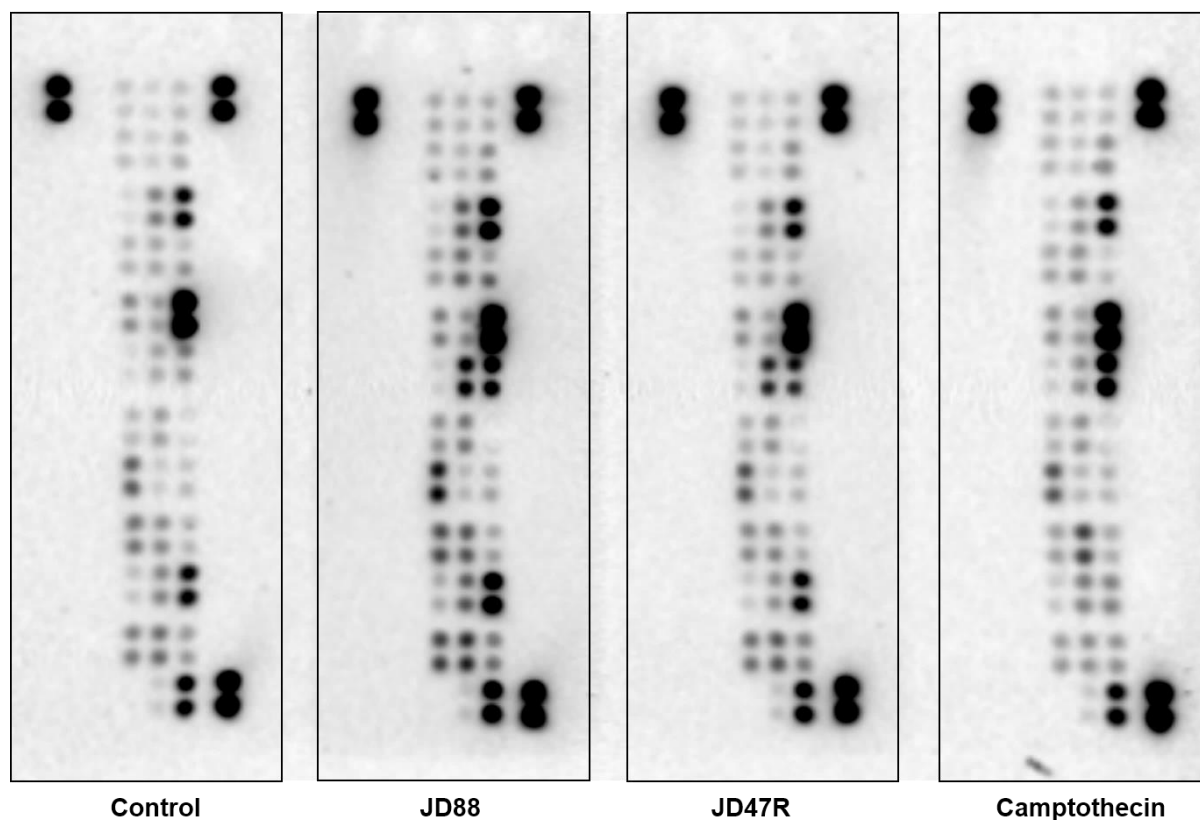


Figure 3.3 Chemiluminescent signals on nitrocellulose membrane arrays showing expression levels of apoptosis-related proteins in HL-60 control and treated cells. The chemiluminescent signals generated represent the variation in expression levels of proteins. HL-60 cells were treated with JD88, JD47R and camptothecin at a concentration equivalent to their IC₇₅ value for 18 hrs, and compared to an untreated control. Proteome array membranes were incubated with 400 µg of protein from each cell lysate. Chemiluminescent images were captured using the BIO-RAD ChemiDoc™ MP Imager.

Table 3.3 Expression levels of apoptotic regulatory proteins of **HL-60** cells, the percentage change of expression after treatment.

Apoptotic protein	Baseline expression level	JD88		JD47R		Camptothecin	
		% Change	Sign	% Change	Sign	% Change	Sign
Bad	Low	+3	ns	+4	ns	+4	ns
Bax	Low	+42	****	+44	****	+98	****
Bcl-2	High	+91	****	+9	**	+23	***
Bcl-x	Low	-6	ns	-8	ns	-13	ns
cIAP-1	Low	+1	ns	+7	ns	+13	**
cIAP-2	Low	+7	ns	+4	ns	+12	ns
Livin	Low	-5	ns	-12	ns	-14	ns
Survivin	Moderate	+33	****	-26	***	-32	****
XIAP	Moderate	+29	****	-8	ns	-28	****
HTRA2/Omi	Moderate	+80	****	+59	****	-15	ns
Smac/DIABLO	Moderate	+86	****	+19	**	+17	**
Cytochrome c	Very high	+48	****	+27	**	+6	ns
Pro-caspase-3	Very high	+3	ns	+10	*	+1	ns
Cleaved caspase-3	Moderate	+359	****	+143	****	+615	****
TRAIL-R1/DR4	Low	+16	*	+19	*	+18	*
TRAIL-R2/DR5	Low	+18	**	+2	ns	+14	*
FADD	Moderate	+55	****	+4	*	+2	ns
Fas/TNFRSF6/CD95	Low	+120	****	+10	ns	+55	***
TNF-R1/TNFRSF1A	Low	+14	***	+1	ns	+10	**
Clusterin	Low	+93	****	+61	****	+46	***
HIF-1α	Low	+1	ns	+9	ns	+17	ns
HO-1/HMOX1/HSP32	Low	+267	****	+232	****	+32	ns
HO-2/HMOX2	Low	+27	*	+6	ns	+11	ns
HSP27	Low	+6	ns	+8	ns	+11	ns
HSP60	Low	+57	****	+33	**	+153	****
HSP70	Low	+47	****	0	ns	+37	****
Phospho-p53 (S15)	Low	+52	****	0	ns	+23	**
Phospho-p53 (S46)	Moderate	+4	ns	+4	ns	0	ns
Phospho-p53 (S392)	Low	+12	*	+1	ns	+4	ns
Phospho-Rad17 (S635)	Low	+69	****	+25	*	+12	ns
p21^{Cip1/CDKN1A}	Low	+6	ns	0	ns	+17	***
p27^{Kip1}	Very low	+19	*	+1	ns	+9	ns
Catalase	Low	-12	ns	-3	ns	+5	ns
Claspin	High	+81	****	+14	*	-67	****
PON2	Low	+19	*	+13	ns	+20	*

The positive (+) sign indicates an increase in protein expression, whilst the negative (–) sign indicates a decrease. The one-way ANOVA ($p < 0.05$) followed by the Dunnet's *post-hoc* comparisons test was performed using the pixel density of treated and untreated cells. Legend: ns = non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant.

3.3.1.1 Expression of effector caspase-3

Pro-caspase-3 was highly expressed in HL-60 control cells (Figure 3.4A) and following treatment with the copper-imidazo[1,2-*a*]pyridines and camptothecin, no significant changes were observed (Figure 3.4B; Table 3.3). Cleaved caspase-3 was moderately expressed in HL-60 control cells (Figure 3.4A; Table 3.3). The copper-imidazo[1,2-*a*]pyridines JD88, JD47R and camptothecin significantly increased the expression of cleaved caspase-3 by 359% ($p<0.001$), 143% ($p<0.001$) and 615% ($p<0.001$), respectively (Figure 3.4C).

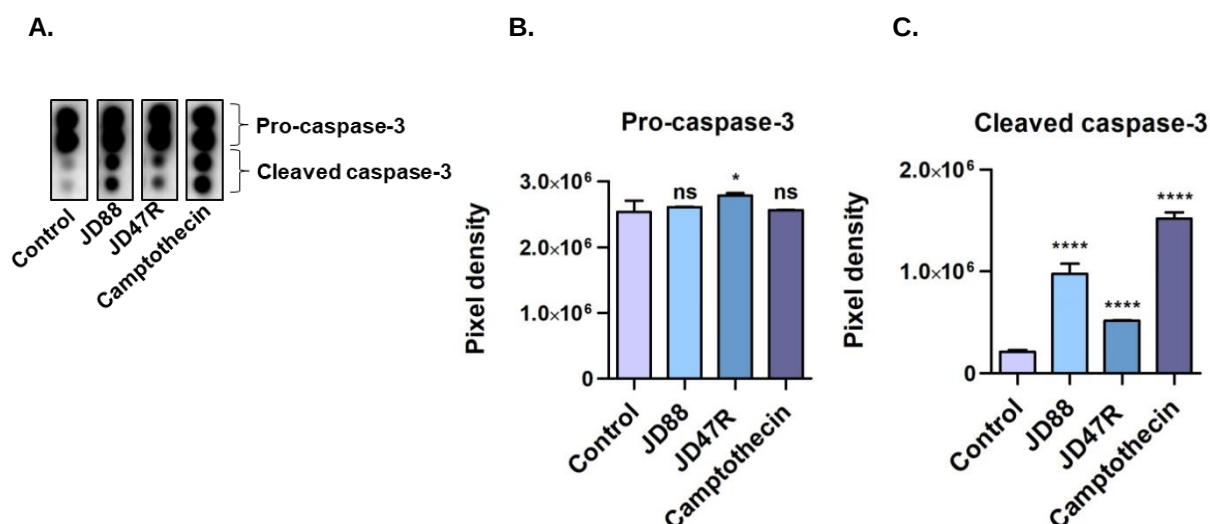


Figure 3.4 The expression of caspase-3 in HL-60 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** is the section of the membrane array for pro-caspase-3 and cleaved caspase-3 expression; **B** shows the effect by the copper complexes on pro-caspase-3 expression and **C** shows the effect by the copper complexes on cleaved caspase-3 expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.1.2 Expression of haem-oxygenase, heat shock proteins and clusterin

The cytoprotective haem-oxygenase proteins (HO-1/HMOX1/HSP32 and HO-2/HMOX2) were poorly expressed in HL-60 control cells (Figure 3.5A). The copper-imidazo[1,2-*a*]pyridines JD88 and JD47R caused a 267% and 232% increase ($p<0.001$) in HO-1/HMOX1/HSP32 while JD88 increased HO-2/HMOX2 by 27% which was considered less meaningful (Figure 3.5B; Table 3.3). In contrast, camptothecin showed no significant change in the expression of either protein. Hypoxia-inducible factor, HIF-1 α , a known positive regulator of HO-1/HMOX1/HSP32 expression (Toth and Warfel, 2017) was poorly expressed in HL-60 control cells and no significant change in expression was induced by the copper-imidazo[1,2-*a*]pyridines or camptothecin (Table 3.3).

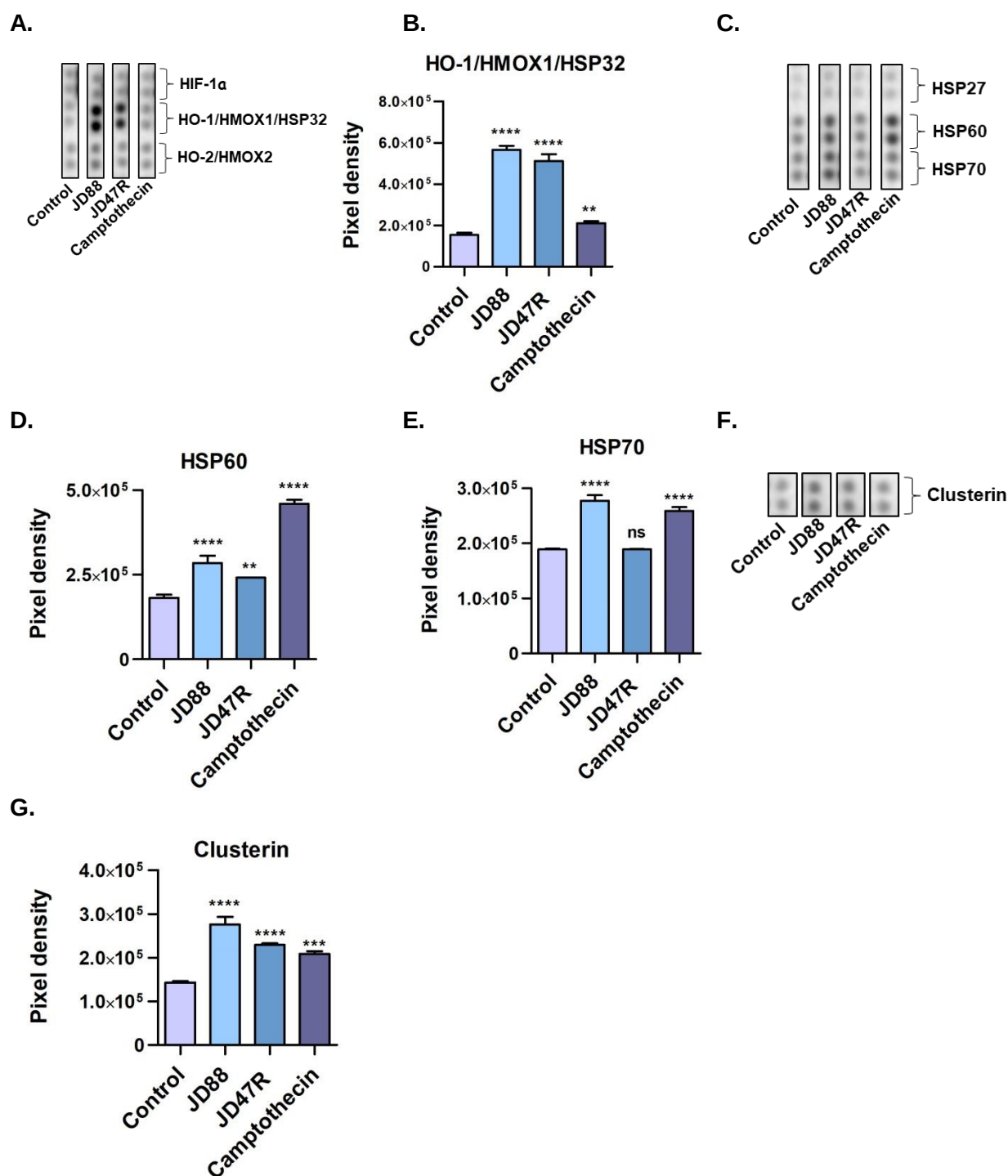


Figure 3.5 The expression of heat shock proteins and clusterin in HL-60 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for HIF-1 α , HO-1/HMOX/HSP32 and HO-2/HMOX2 expression; **B** shows the effect by the copper complexes and camptothecin on HO-1/HMOX1/HSP32 expression; **C** is the section of the membrane array for HSP27, HSP60 and HSP70 expression; **D** and **E** shows the effect by the copper complexes on HSP60 and HSP70 expression, respectively. **F** shows is the section of the membrane array for clusterin and **G** shows the effect by the copper complexes and camptothecin on clusterin expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

The other heat shock proteins; HSP27, HSP60 and HSP70 were poorly expressed in HL-60 control cells, with HSP27 being the lowest (Figure 3.5C). There was no meaningful change in the expression of HSP27 (Table 3.3). However, copper-imidazo[1,2-*a*]pyridines JD88, JD47R and camptothecin were able to significantly increase the expression of HSP60 by 57% ($p<0.001$), 33% ($p<0.05$) and 153% ($p<0.001$), respectively (Figure 3.5D). JD88 and camptothecin increased the expression of HSP70 by 47% ($p<0.001$) and 37% ($p<0.001$), respectively, with JD47R causing no significant increase (Figure 3.5E).

Similar to heat shock proteins, clusterin is a cytoprotective protein involved in decreasing cell stress (Zoubeidi *et al.*, 2010). Clusterin was poorly expressed in HL-60 control cells and in response to JD88 its expression was increased by 93% ($p<0.001$) while JD47R was increased by 61% ($p<0.001$) and camptothecin by 46% ($p<0.01$) (Figure 3.5F; Table 3.3).

3.3.1.3 Expression of Bcl-2 family of proteins

The pro-apoptotic proteins Bad and Bax were poorly expressed in HL-60 control cells (Figure 3.6A; Table 3.3). Compared to the control, the copper-imidazo[1,2-*a*]pyridines JD88 and JD47R as well as camptothecin caused a highly significant increase in the expression levels of Bax, by 42%, 44% and 98% ($p<0.001$), respectively (Figure 3.6B). There was no significant change observed in Bad expression as reported in Table 3.3.

The expression of anti-apoptotic Bcl-2 was higher than that of Bcl-x in HL-60 control cells (Figure 3.6C; Table 3.3). The copper-imidazo[1,2-*a*]pyridines and camptothecin caused no meaningful change in the expression of Bcl-2 and Bcl-x with the exception of JD88 causing a highly significant increase of 91% ($p<0.001$) in Bcl-2 expression (Figure 3.6D, Table 3.3).

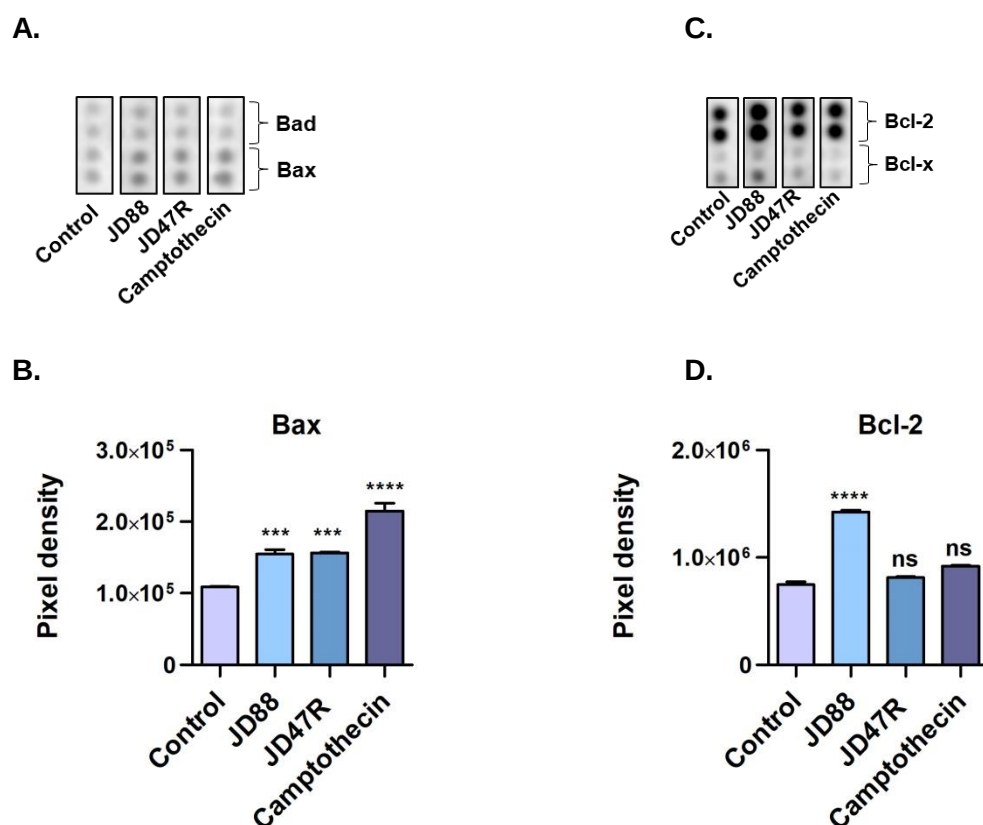


Figure 3.6 The expression of Bcl-2 family of proteins in HL-60 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for Bad and Bax expression; **B** shows the effect by the copper complexes and camptothecin on Bax expression; **C** shows the section of the membrane array for Bcl-2 and Bcl-x expression and **D** shows the effect by the copper complexes and camptothecin on Bcl-2 expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.1.4 Expression of Smac/DIABLO and HTRA2/Omi proteins

Smac/DIABLO and HTRA2/Omi are pro-apoptotic mitochondrial proteins. Smac/DIABLO was moderately expressed in HL-60 control cells (Figure 3.7A; Table 3.3). Compared to the control, the expression levels of Smac/DIABLO was significantly increased to 86% by JD88, changing the expression level from moderate to moderately high (Figure 3.7B). JD47R and camptothecin caused increases of 17% ($p < 0.05$) and 19% ($p < 0.05$), respectively (Figure 3.7B). HTRA2/Omi was low-moderately expressed in HL-60 control cells (Figure 3.7A; Table 3.3). Treatment with JD88 and JD47R caused a change in HTRA2/Omi expression from moderate to moderately high with significant increases of 80% ($p < 0.001$) and 59% ($p < 0.001$), respectively (Figure 3.7C). In contrast, camptothecin caused no meaningful change in the expression of HTRA2/Omi (Figure 3.7C).

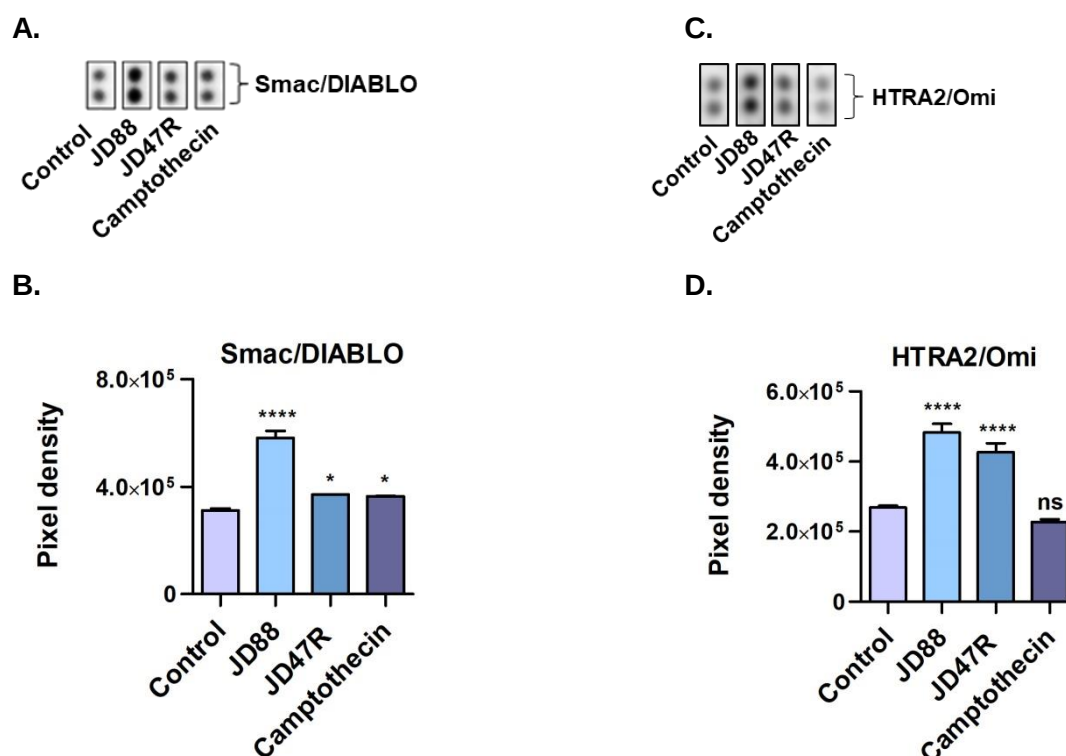


Figure 3.7 The expression of Smac/DIABLO and HTRA2/Omi in HL-60 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for Smac/DIABLO expression; **B** shows the effect by the copper complexes and camptothecin on Smac/DIABLO expression; **C** shows the section of the membrane array for HTRA2/Omi expression and **D** shows the effect by the copper complexes and camptothecin on HTRA2/Omi expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.1.5 Expression of apoptotic inhibitory proteins (IAPs)

The most potent member of the IAP family; XIAP, was moderately expressed in HL-60 control cells (Figure 3.8A; Table 3.3). JD88 caused a significant increase in the expression of XIAP ($p < 0.001$) while JD47R showed no significant effect (Figure 3.8B). In contrast, camptothecin caused a 28% decrease in XIAP expression ($p < 0.001$) as shown in Figure 3.7B. The cellular inhibitor of apoptosis proteins; cIAP-1 and cIAP-2, were poorly expressed in HL-60 control cells and the copper-imidazo[1,2-*a*]pyridines and camptothecin showed no significant effect on their protein expression (Table 3.3). Survivin belongs to the group of IAPs and was moderately expressed in HL-60 control cells (Figure 3.8C). JD88 caused a significant 33% increase in survivin expression ($p < 0.01$), whilst both JD47R and camptothecin showed a contrasting effect by significantly decreasing survivin expression ($p < 0.01$) (Figure 3.8D).

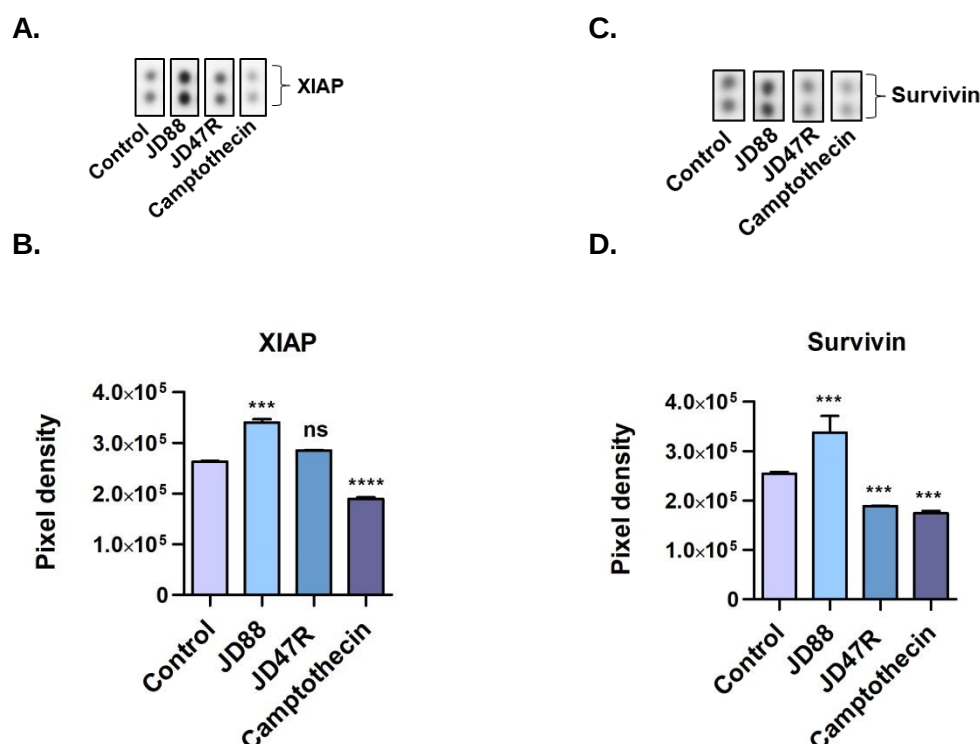


Figure 3.8 The expression of anti-apoptotic proteins XIAP and survivin in HL-60 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for XIAP expression; **B** shows the effect by the copper complexes and camptothecin on XIAP expression; **C** shows the section of the membrane array for survivin expression and **D** shows the effect by the copper complexes and camptothecin on survivin expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.1.6 Expression of death receptor proteins

The proteome array included the death receptor proteins, TRAIL-R1, TRAIL-R2, FADD, Fas and TNF-R1. Proteome data showed that all death receptor proteins were poorly expressed in HL-60 control cells with the exception of FADD which was moderately expressed (Table 3.3, Figure 3.9A). Copper-imidazo[1,2-*a*]pyridines and camptothecin caused poorly significant increases ($p < 0.1$) in TRAIL-R1 and TRAIL-R2 expression (Table 3.3). JD88 caused a significant increase in the expression of FADD ($p < 0.001$), whilst JD47R and camptothecin caused no meaningful changes (Figure 3.9B). The proteome array showed changes in Fas expression with JD88 and camptothecin causing significant increases of 120% ($p < 0.001$) and 55% ($p < 0.01$), respectively, while JD47R caused no significant change (Figure 3.9C). The copper complexes and camptothecin caused no meaningful changes in the expression of death receptor TNF-R1 (Table 3.3).

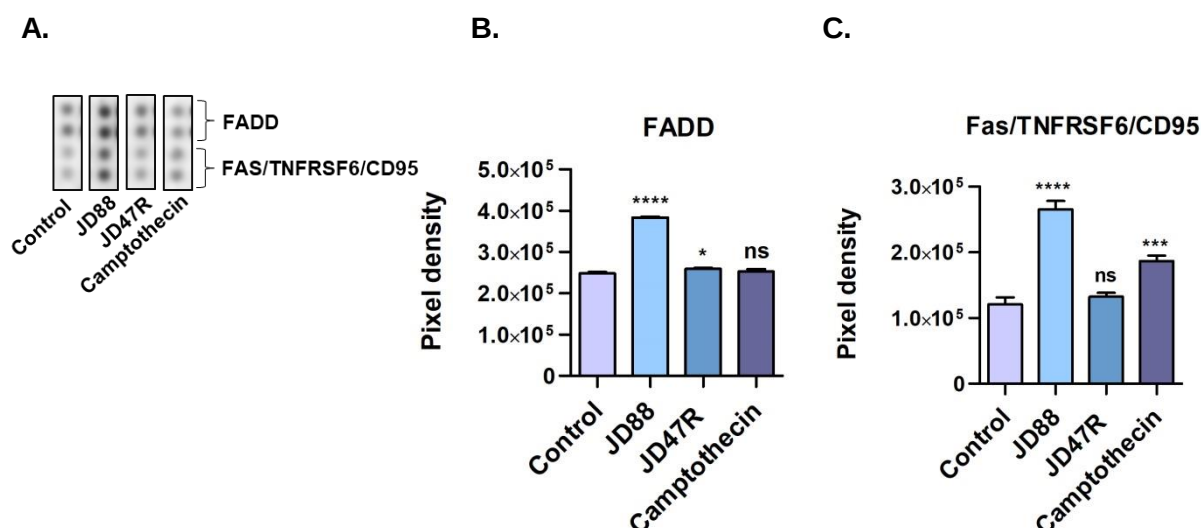


Figure 3.9 The expression of death receptor proteins in HL-60 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for FADD and Fas expression; **B** shows the effect by the copper complexes and camptothecin on FADD expression and **C** shows the effect by the copper complexes and camptothecin on Fas expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.1.7 Expression of other apoptotic regulatory proteins

Phosphorylated tumour suppressor protein p53 (at serine sites 15, 46 and 392) (Yogosawa and Yoshida, 2018) were poorly expressed in HL-60 cells, and treatment showed no meaningful changes to their expression with the exception of JD88 (Table 3.3), which increased phospho-p53 (S15) expression by 52% ($p < 0.001$). Similarly to p53, in response to DNA damage, the protein kinase ATR phosphorylates Rad17 at two amino acid residues; serine site 635 and 645 (Wang *et al.*, 2006). Phospho-Rad17 (S635) was included in the array but was poorly expressed in HL-60 cells. Phospho-rad17 expression was significantly increased by 69% ($p < 0.001$) by JD88 (Table 3.3).

Tumour suppressor proteins p21^{Cip1} and p27^{Kip1}, which are cyclin-dependent kinase inhibitors involved in p53-dependent cell cycle arrest (Muñoz-Alonso *et al.*, 2005; McCubrey *et al.*, 2017), were poorly expressed in HL-60 control cells. Expression of these proteins showed no meaningful changes in response to the copper-imidazo[1,2-*a*]pyridines and camptothecin, (Table 3.3). Similarly, the expression of catalase and PON2 in HL-60 cells were low and in response to the copper-imidazo[1,2-*a*]pyridines and camptothecin, they showed no meaningful changes (Table 3.3).

Cytochrome c was highly expressed in HL-60 cells (Figure 3.10A). JD88 caused a 48% increase ($p<0.001$) and JD47R caused a 27% increase ($p<0.05$), while camptothecin caused no significant change (Figure 3.10B; Table 3.3). The expression of claspin was high in HL-60 cells (Figure 3.10C) and JD88 further increased its expression by 81% ($p<0.001$), while JD47R caused no meaningful change and camptothecin significantly decreased its expression by 67% ($p<0.001$) (Figure 3.10D; Table 3.3).

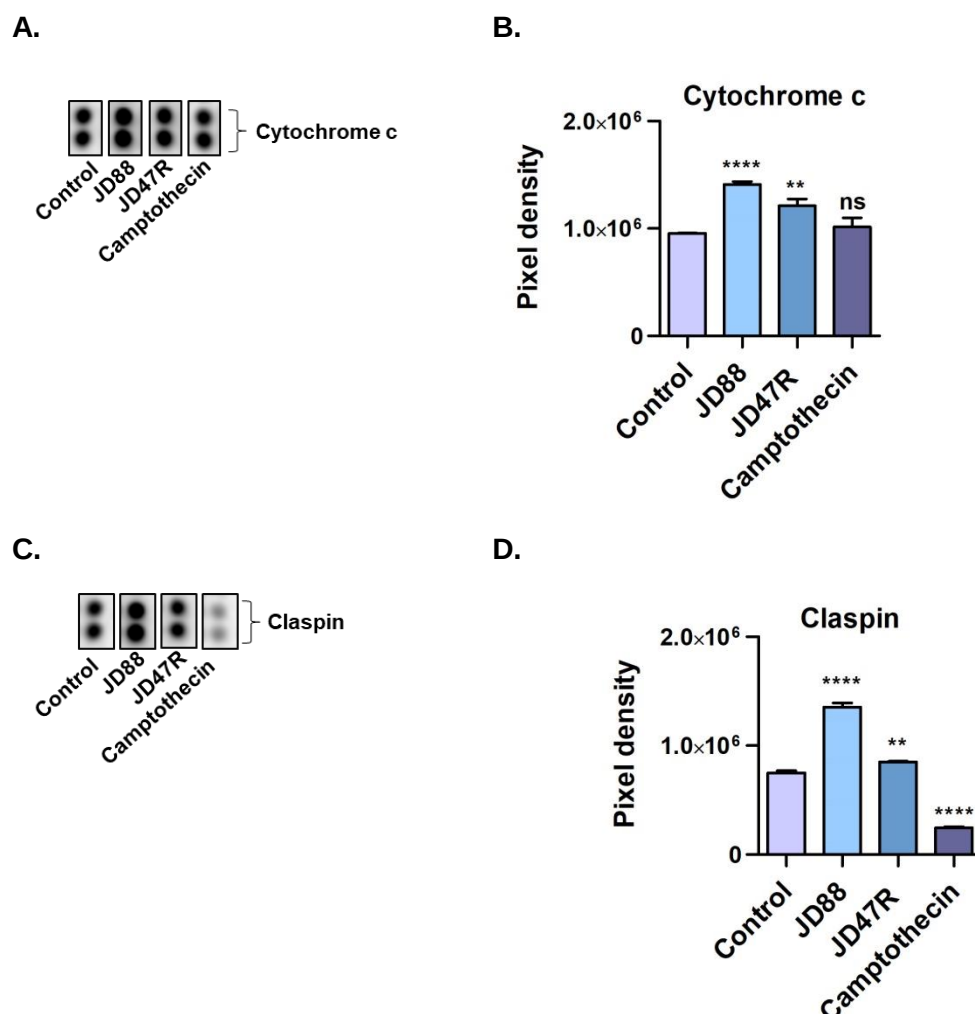


Figure 3.10 The expression of cytochrome c and claspin in HL-60 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for cytochrome c expression; **B** shows the effect by the copper complexes and camptothecin on cytochrome c expression; **C** shows the section of the membrane array for claspin expression and **D** shows the effect by the copper complexes and camptothecin on claspin expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2 The effect of active copper-imidazo[1,2-*a*]pyridines on apoptotic regulatory proteins in K562 cells

The copper-imidazo[1,2-*a*]pyridine complexes altered the expression of apoptosis regulatory proteins in K562 cells after 18 hours of treatment at an IC₇₅ concentration. The effects on expression of the apoptosis-related proteins by JD88, JD49R and camptothecin are shown in Figure 3.11 and their corresponding levels of expression are shown in Table 3.4.

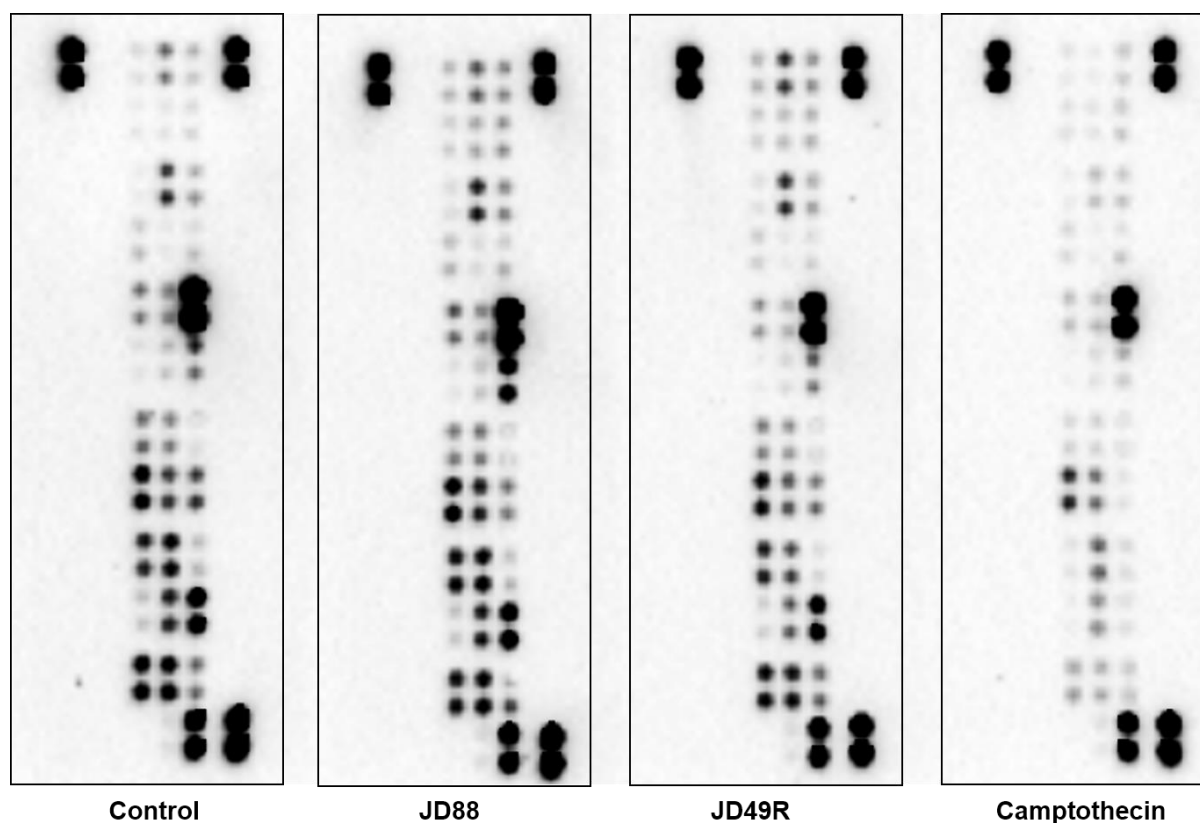


Figure 3.11 Chemiluminescent signals on nitrocellulose membrane arrays showing expression levels of apoptosis-related proteins in K562 control and treated cells. The chemiluminescent signals generated represent the variation in expression levels of proteins. K562 cells were treated with JD88, JD49R and camptothecin at a concentration equivalent to their IC₇₅ value for 18 hrs, and compared to an untreated control. Proteome array membranes were incubated with 400 µg of protein from each cell lysate. Chemiluminescent images were captured using the BIO-RAD ChemiDoc™ MP Imager.

Table 3.4 Expression levels of apoptotic regulatory proteins of **K562** cells, the percentage change of expression after treatment.

Apoptotic protein	Baseline expression level	JD88		JD49R		Camptothecin	
		% Change	Sign	% Change	Sign	% Change	Sign
Bad	Low	+81	****	+79	****	+12	ns
Bax	Very low	+161	****	+116	****	+92	****
Bcl-2	Low	+19	ns	-5	ns	-39	**
Bcl-x	Low	+3	ns	-19	ns	-35	*
cIAP-1	Low	-41	****	-30	****	-90	****
cIAP-2	Low	-13	ns	-7	ns	-15	ns
Livin	Very low	-1	ns	-4	ns	-12	ns
Survivin	Moderate	+28	****	+9	ns	-87	****
XIAP	Moderate	-26	****	-26	****	-73	****
HTRA2/Omi	High	-6	ns	-6	ns	-64	****
Smac/DIABLO	Mod high	+31	****	+4	ns	-9	ns
Cytochrome c	Mod high	+14	ns	+7	ns	+15	*
Pro-caspase-3	Very high	+14	*	-8	ns	-9	ns
Cleaved caspase-3	Low	+255	****	+135	****	-1	ns
TRAIL-R1/DR4	Low	+11	ns	+58	****	-86	****
TRAIL-R2/DR5	Very Low	+295	****	+176	****	+17	ns
FADD	Low	+15	**	+8	ns	-68	****
Fas/TNFRSF6/CD95	Very low	+9	ns	+6	ns	-10	ns
TNF-R1/TNFRSF1A	Very low	-3	ns	-8	ns	-54	ns
Clusterin	Low	+12	ns	+18	*	-15	*
HIF-1α	Moderate	-8	ns	-29	***	-24	***
HO-1/HMOX1/HSP32	Very low	-2	ns	-11	ns	-18	ns
HO-2/HMOX2	Low	-5	ns	-18	ns	-50	****
HSP27	Low	+23	****	-15	*	-26	****
HSP60	Moderate	-1	ns	-32	****	-41	****
HSP70	Low	-2	ns	-44	****	-52	****
Phospho-p53 (S15)	Very low	-7	ns	-5	ns	-14	ns
Phospho-p53 (S46)	Low	+14	ns	-25	****	-45	****
Phospho-p53 (S392)	Very low	-5	ns	+18	ns	0	ns
Phospho-rad17(S635)	Low	+20	**	+43	****	-35	****
p21^{Cip1/CDKN1A}	Very low	+104	****	+94	****	+36	****
p27^{Kip1}	Very low	+10	ns	+12	ns	-15	ns
Catalase	Very low	+19	ns	+6	ns	+12	ns
Claspain	Mod high	-9	ns	-15	*	-93	****
PON2	Low	+54	**	+57	**	-4	ns

The positive (+) sign indicates an increase in protein expression, whilst the negative (–) sign indicates a decrease. The one-way ANOVA ($p < 0.05$) followed by the Dunnet's *post-hoc* comparisons test was performed using the pixel density of treated and untreated cells. Legend: ns = non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant.

3.3.2.1 Expression of effector caspase-3

Pro-caspase-3 was highly expressed in K562 control cells (Figure 3.12A) and the copper-imidazo[1,2-*a*]pyridines and camptothecin caused no meaningful change in its expression (Figure 3.12B, Table 3.4). Cleaved caspase-3 expression was low in K562 control cells (Figure 3.12A). The copper-imidazo[1,2-*a*]pyridines; JD88 and JD49R, significantly increased the expression of cleaved caspase-3 by 255% ($p<0.001$) and 135% ($p<0.001$), respectively (Figure 3.12C). In contrast, camptothecin caused no meaningful change in cleaved caspase-3 expression.

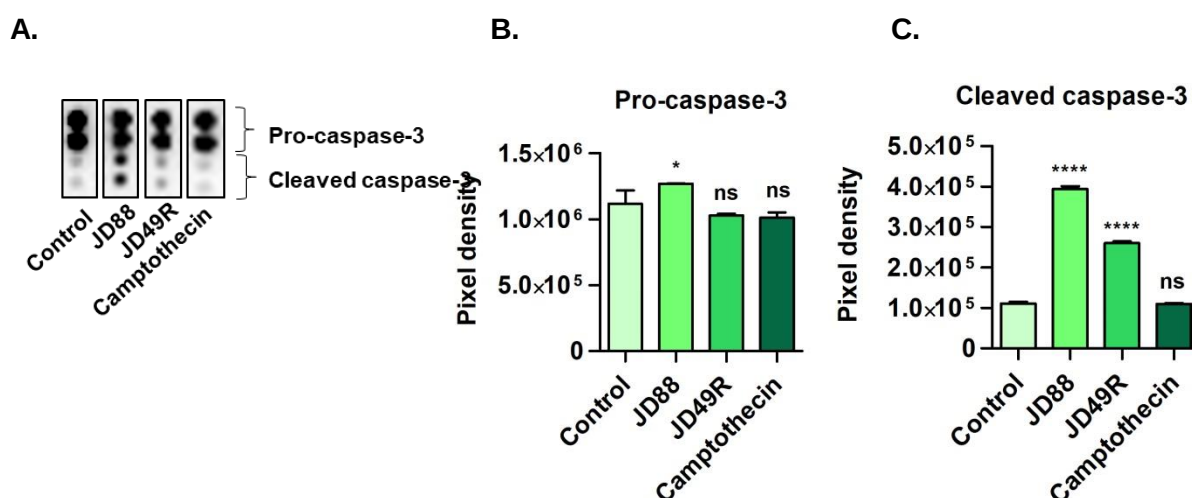


Figure 3.12 The expression of caspase-3 in K562 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** is the section of the membrane array for pro-caspase-3 and cleaved caspase-3 expression; **B** shows the effect by the copper complexes on pro-caspase-3 expression and **C** shows the effect by the copper complexes on cleaved caspase-3 expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2.2 Haem-oxygenase, Heat shock protein and clusterin expression

The haem-oxygenase proteins, HO-1/HMOX1/HSP32 and HO-2/HMOX2 were poorly expressed in K562 control cells and showed no meaningful changes following treatment with the copper-imidazo[1,2-*a*]pyridines (Table 3.4). The hypoxia-inducible factor, HIF-1 α was moderately expressed in K562 control cells (Figure 3.13A). The expression of HIF-1 α was decreased significantly ($p<0.001$) by JD49R and camptothecin while JD88 caused no meaningful change (Figure 3.13B; Table 3.4).

Heat shock proteins; HSP27, HSP60 and HSP70, expression were poor to moderate in K562 control cells, with HSP27 being the lowest (Figure 3.13A; Table 3.4). The expression of HSP27 was significantly increased by JD88 ($p<0.001$). In contrast, JD49R and camptothecin caused a decrease of 15% ($p<0.1$) and 26% ($p<0.001$), respectively (Figure 3.13D). HSP60 and HSP70 were both significantly decreased by JD49R and camptothecin with no effect shown by JD88 on their expression levels (Figure 3.13E and F). The cytoprotective protein clusterin was poorly expressed in K562 cells and in response to copper-imidazo[1,2-*a*]pyridines and camptothecin no meaningful changes were induced (Table 3.4).

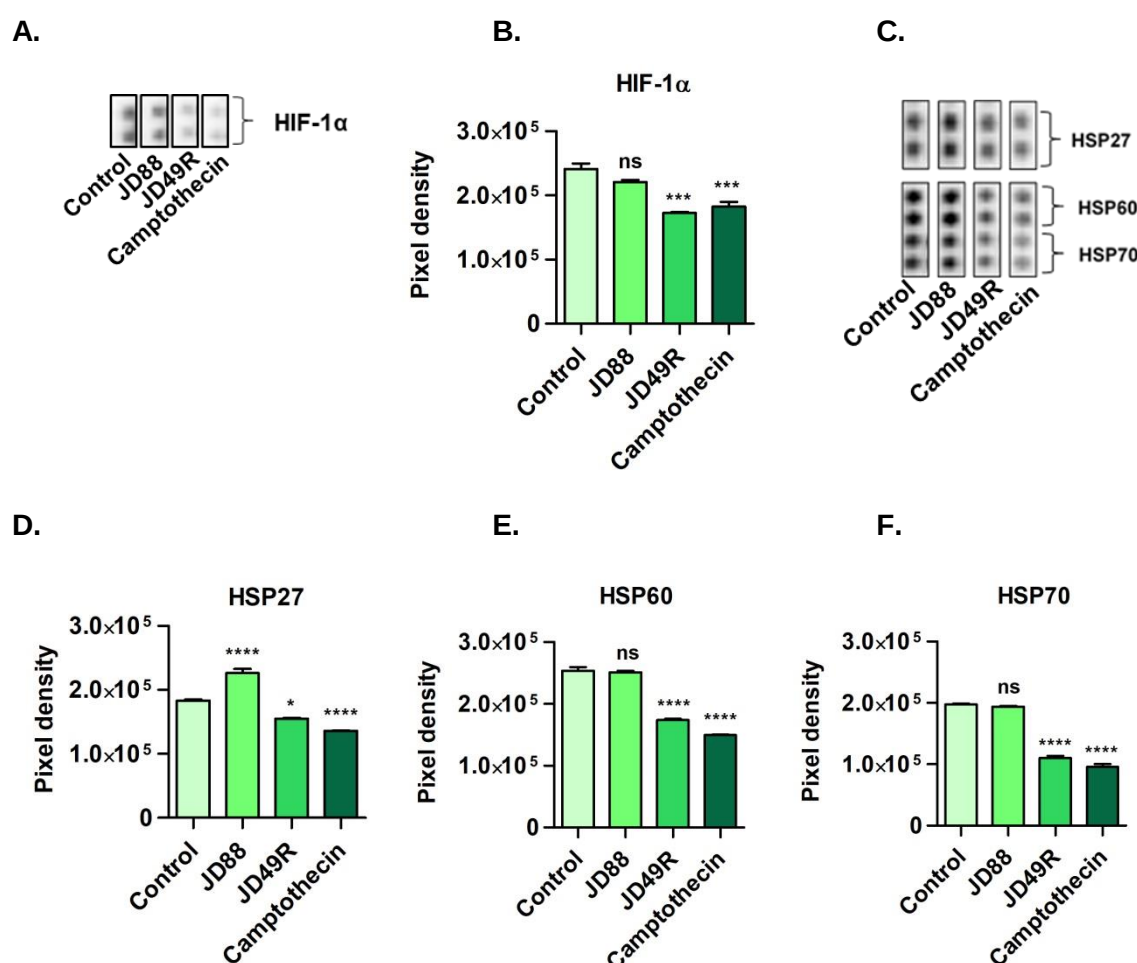


Figure 3.13 The expression of heat shock proteins in K562 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for HIF-1 α expression; **B** shows the effect by the copper complexes and camptothecin on HIF-1 α expression; **C** is the section of the membrane array for HSP27, HSP60 and HSP70 expression; **D**, **E** and **F** shows the effect by the copper complexes on HSP27, HSP60 and HSP70 expression, respectively. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2.3 Expression of the Bcl-2 family of proteins

The pro-apoptotic proteins Bad and Bax were poorly expressed in K562 control cells (Figure 3.14A; Table 3.4). Compared to the control, the copper-imidazo[1,2-*a*]pyridines JD88 and JD49R caused a highly significant increase in the expression levels of Bad by 81% ($p<0.001$) and 79% ($p<0.001$), respectively, while camptothecin caused no meaningful change in Bad expression (Figure 3.14A and B). Similarly, Bax expression was significantly increased by JD88, JD49R and camptothecin ($p<0.001$) as shown in Figure 3.14A and C.

The anti-apoptotic proteins Bcl-2 and Bcl-x were poorly expressed in K562 control cells (Table 3.4). The copper complexes caused no meaningful changes in the expression of either protein whilst camptothecin caused decreases in Bcl-2 ($p<0.05$) and Bcl-x expression ($p<0.1$) that were considered not meaningful (Table 3.4).

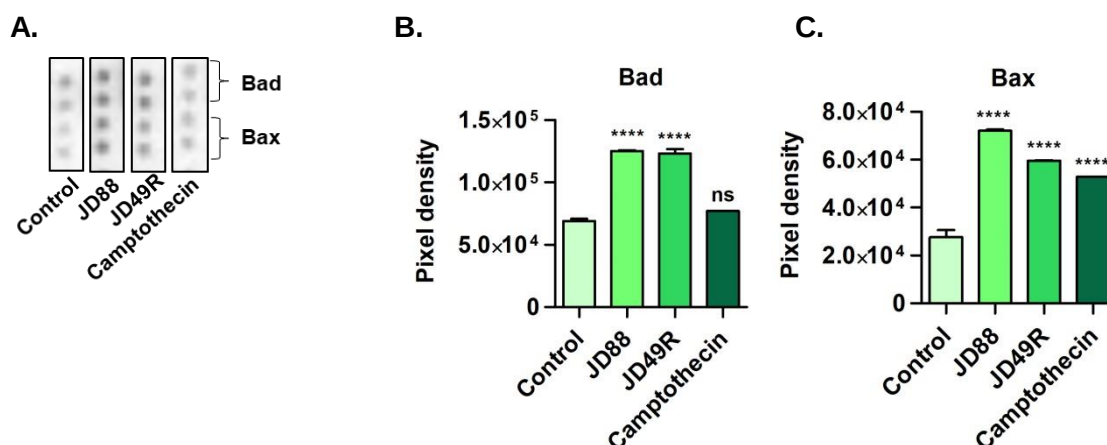


Figure 3.14 The expression of pro-apoptotic Bcl-2 proteins in K562 cells. Cells were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for Bad and Bax expression; **B** shows the effect by the copper complexes and camptothecin on Bad expression and **C** shows the effect by the copper complexes and camptothecin on Bax expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2.4 Expression of Smac/DIABLO and HTRA2/Omi proteins

Pro-apoptotic Smac/DIABLO was expressed moderately in K562 control cells (Figure 3.15A; Table 3.4). Compared to the control, the expression level of Smac/DIABLO was significantly increased by JD88 (31%) whilst no meaningful changes were induced by JD49R or camptothecin (Figure 3.15B; Table 3.4). Mitochondrial protein HTRA2/Omi was moderately expressed (high end of moderate) in K562 control cells (Table 3.4; Figure 3.15C). Treatment

with JD88 and JD49R caused no meaningful changes in expression whilst camptothecin caused a 64% decrease ($p < 0.001$) as reported in Table 3.4; Figure 3.15D.

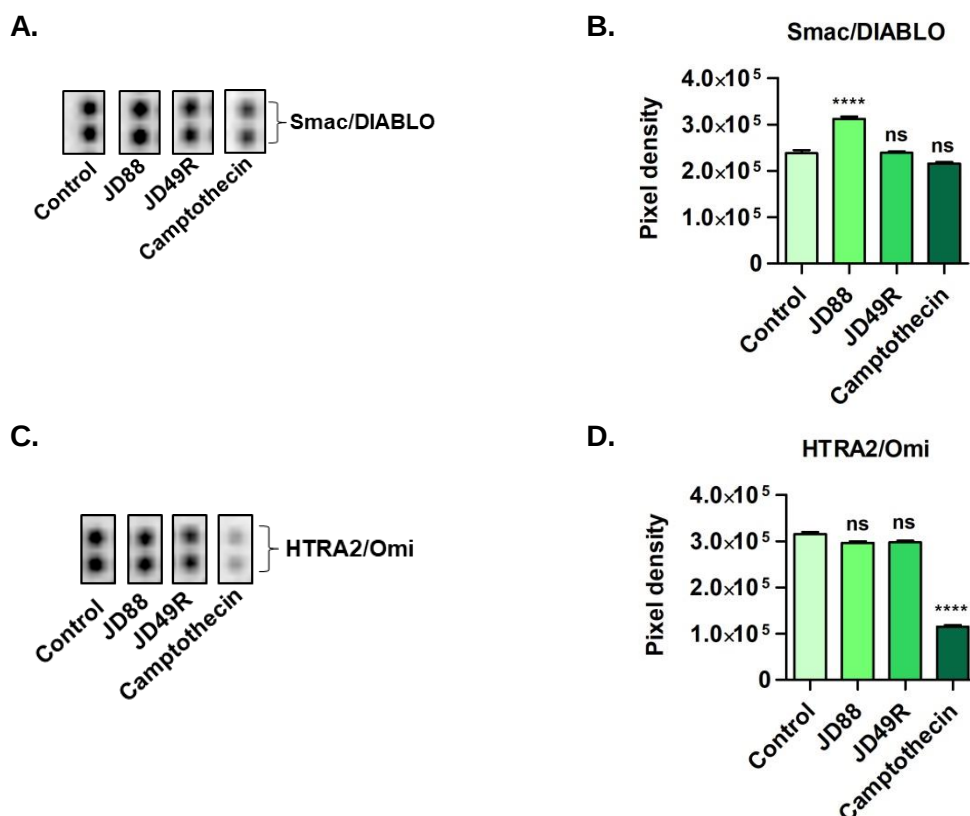


Figure 3.15 The expression of Smac/DIABLO and HTRA2/Omi in K562 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for Smac/DIABLO expression; **B** shows the effect by the copper complexes and camptothecin on Smac/DIABLO expression. **C** shows the section of the membrane array for HTRA2/Omi expression and **D** shows the effect by the copper complexes and camptothecin on HTRA2/Omi expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.2.5 Expression of inhibitor of apoptotic proteins, XIAP, cIAP-1, cIAP-2 and survivin

XIAP was highly expressed in K562 control cells (Figure 3.16A; Table 3.4). Copper-imidazo[1,2-*a*]pyridines JD88 and JD49R as well as camptothecin caused significant decreases in XIAP expression of 26% ($p < 0.001$), 26% ($p < 0.001$) and 73% ($p < 0.001$), respectively (Figure 3.16B). cIAP-1 was moderately expressed while cIAP-2 was poorly expressed in K562 cells (Figure 3.16C). Copper-imidazo[1,2-*a*]pyridines and camptothecin decreased cIAP-1 expression while no meaningful changes were observed in the expression of cIAP-2 (Figure 3.16D). Survivin was expressed moderately in K562 control cells (Figure

3.16E). JD88 caused a 33% increase in survivin expression ($p<0.001$), whilst JD49R caused no meaningful change. Camptothecin significantly decreased the expression of survivin by 87% ($p<0.001$) (Figure 3.16F; Table 3.4).

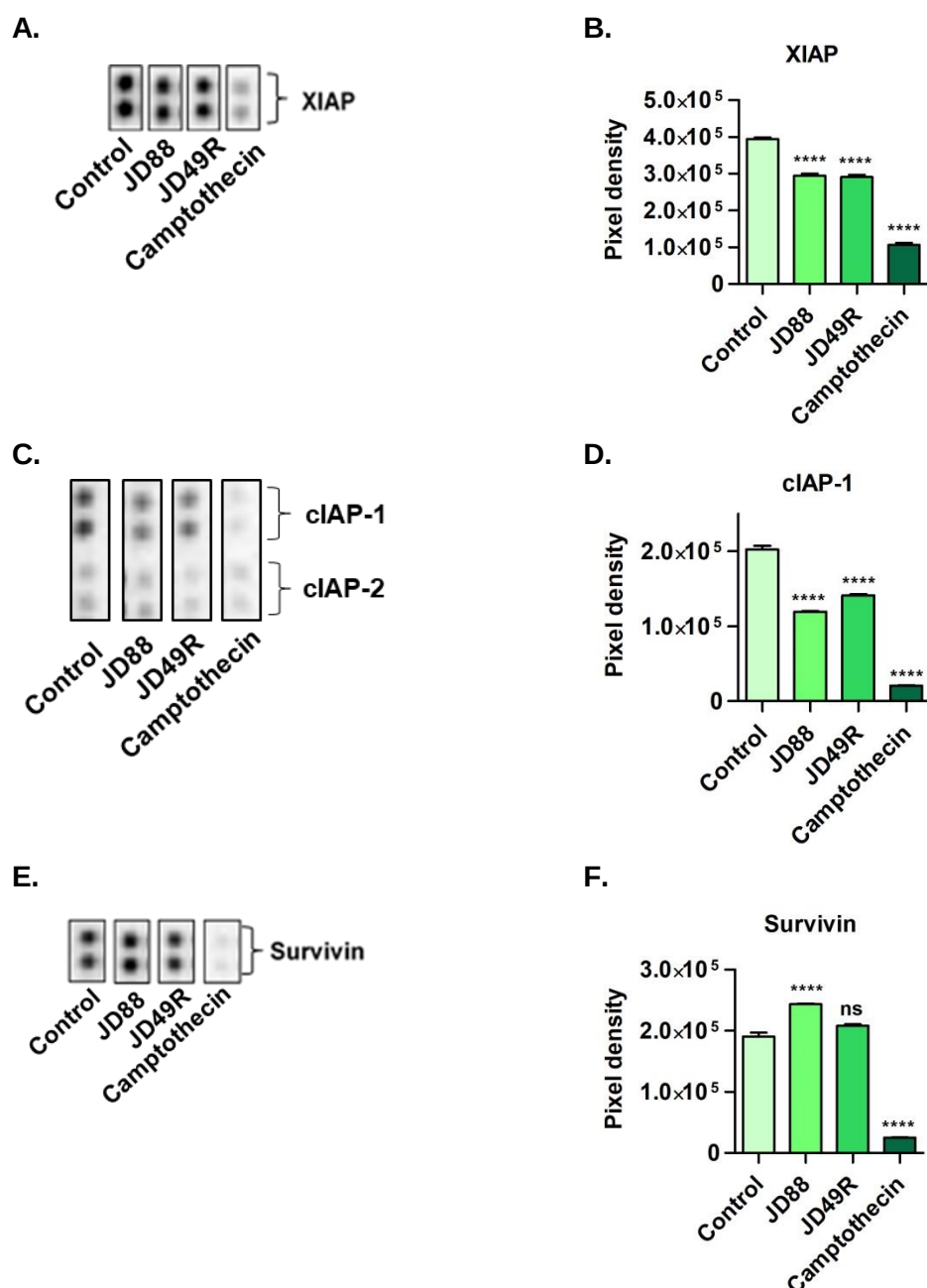


Figure 3.16 The expression of XIAP, cIAPs and survivin in K562 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for XIAP expression; **B** shows the effect by the copper complexes and camptothecin on XIAP expression; **C** shows the section of the membrane array for cIAP-1/2 expression; **D** shows the effect by the copper complexes and camptothecin on cIAP-1 expression; **E** shows the section of the membrane array for survivin expression and **F** shows the effect by the copper complexes and camptothecin on survivin expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnett's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2.6 Expression of death receptor proteins

The proteome array data showed that death receptor proteins, TRAIL-R1, TRAIL-R2, FADD, Fas and TNF-R1 were poorly expressed in K562 control cells (Table 3.4). Amongst the five proteins, only TRAIL receptor expression was altered. JD49R increased the expression of TRAIL-R1 by 58% ($p<0.001$) as shown in Figure 3.17A and B. JD88 caused no meaningful change in expression whilst camptothecin significantly suppressed TRAIL-R1 by 87% ($p<0.001$) (Figure 3.17B). JD88 and JD49R significantly increased TRAIL-R2 by 295% and 176%, respectively. Despite the significant increase ($p<0.001$) in TRAIL-R2 expression, the level of expression was considerably lower than that of TRAIL-R1 (Figure 3.17A and C).

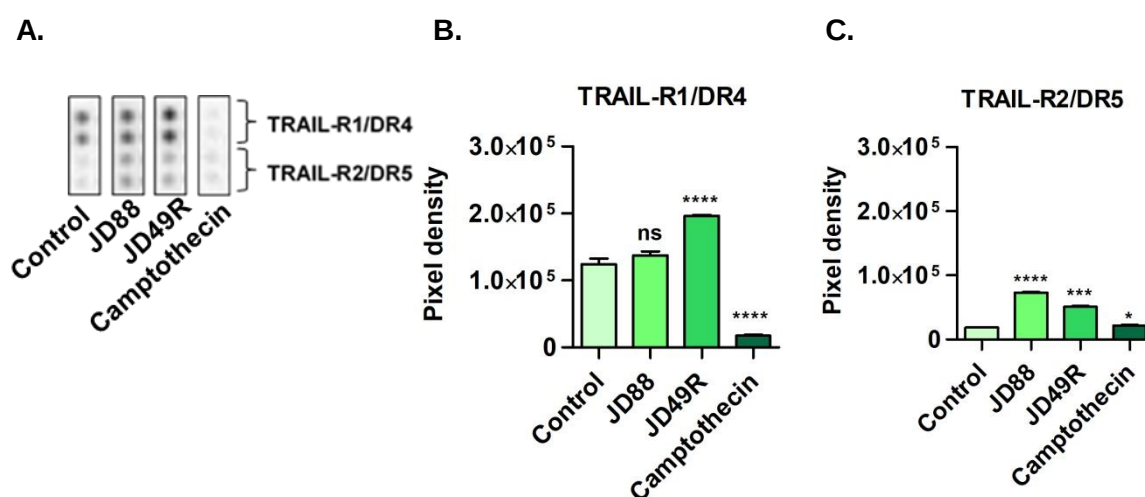


Figure 3.17 The expression of death receptor proteins in K562 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for TRAIL-R1 and TRAIL-R2 expression; **B** and **C** shows the effect by the copper complexes on TRAIL-R1 and TRAIL-R2 expression, respectively. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.2.7 Expression of other regulatory proteins

The three phosphorylated p53 proteins were poorly expressed in K562 control cells (Figure 3.18A; Table 3.4). The copper complexes and camptothecin showed no significant changes in their expression with the exception of phospho-p53 (S46). Differential changes in phospho-p53 (S46) was observed with JD88 causing a 14% increase ($p<0.05$) and JD49R causing a 25% decrease ($p<0.01$) in expression (Figure 3.18B). In comparison, camptothecin caused a more meaningful decrease of 45% ($p<0.001$) in phospho-p53 (S46) expression.

The expression of p21^{Cip1} was poor in K562 control cells (Figure 3.18C). In response to the JD88, JD49R and camptothecin, the expression of p21^{Cip1} was increased by 80%, 71% and 20%, respectively. However, since the expression levels were very poor these increases were not considered to be meaningful (Figure 3.18D, Table 3.4).

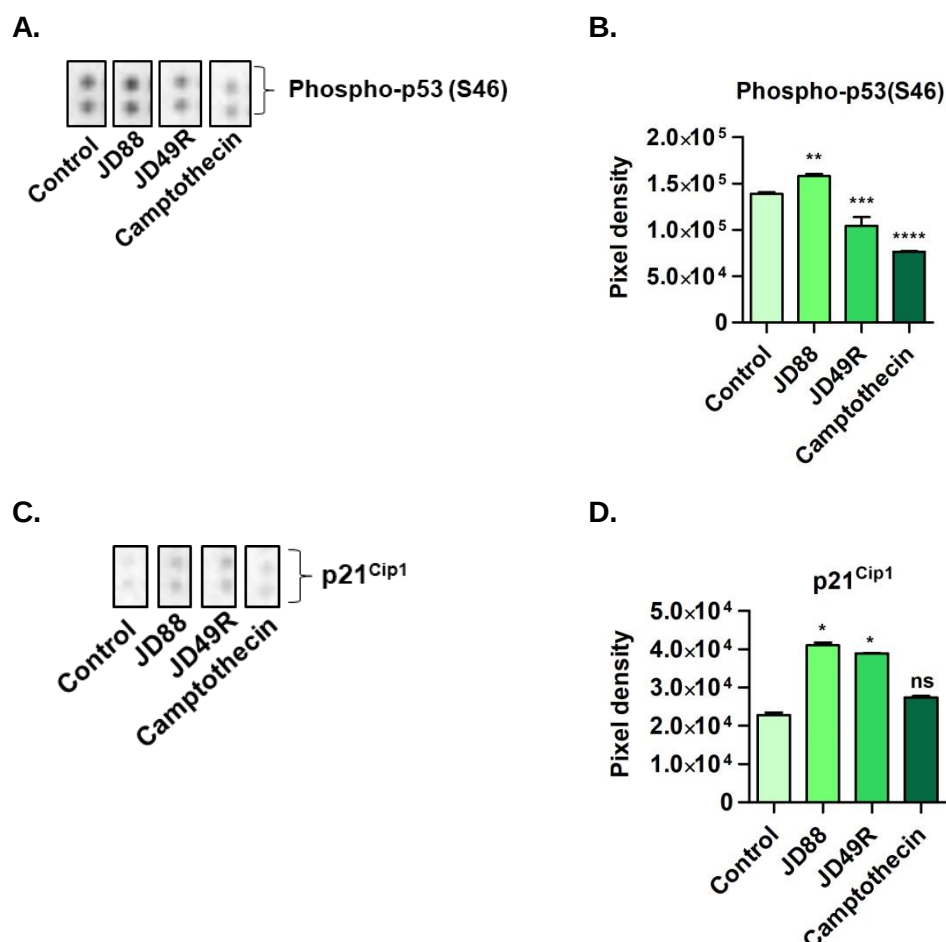


Figure 3.18 The expression of phosphorylated p53 and regulatory protein p21^{Cip1} in K562 cells. Cells were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for phospho-p53 (S46) expression; **B** shows the effect by the copper complexes on phospho-p53 (S46) expression; **C** shows the section of the membrane array for p21^{Cip1} expression and **D** shows the effect by the copper complexes on p21^{Cip1} expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.3 The effect of active copper-imidazo[1,2-*a*]pyridines on apoptotic regulatory proteins in leukocytes

The copper-imidazo[1,2-*a*]pyridine complexes changed the expression of apoptosis regulatory proteins in leukocytes after 18 hours of treatment at an IC₇₅ concentration. The effects of the compounds on the expression of apoptosis-related proteins by JD88 and JD47R are shown in Figure 3.19. The percentage change in expression levels of the tested proteins are summarized in Table 3.5. The positive control camptothecin was not tested against leukocytes because the leukocytes obtained for this study was from a single donor, and not enough cells could be harvested from a single donor to perform an experiment including camptothecin.

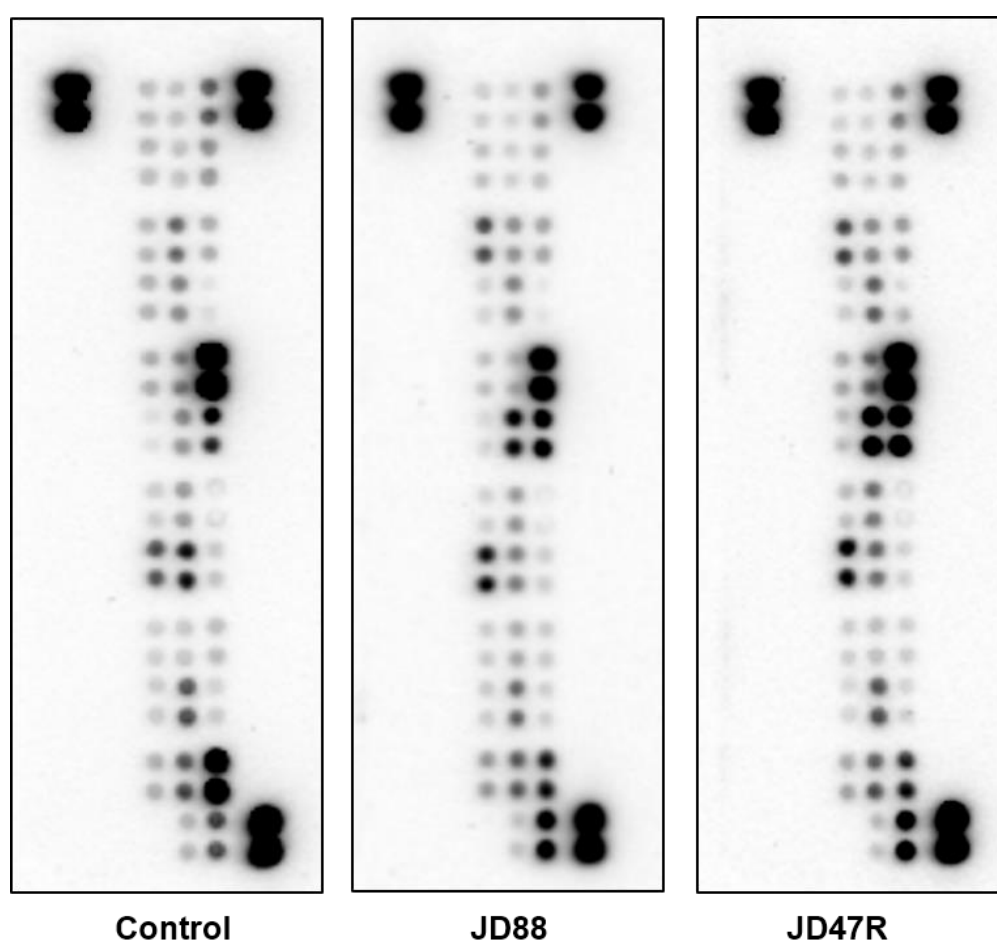


Figure 3.19 Chemiluminescent signals on nitrocellulose membrane arrays showing expression levels of apoptosis-related proteins in control and treated peripheral leukocyte. The chemiluminescent signals generated represent the variation in expression levels of proteins. Leukocytes were treated with JD88 and JD47R for 18 hrs at a concentration equivalent to the IC₇₅ value obtained for HL-60 cells, and compared to an untreated control. Proteome array membranes were incubated with 400 µg of protein from each cell lysate. Chemiluminescent images were captured using the BIO-RAD ChemiDoc™ MP Imager.

Table 3.5 Expression levels of apoptotic regulatory proteins in **peripheral leukocytes**, the percentage change of expression after treatment.

Apoptotic protein	Baseline expression level	JD88		JD47R	
		% Change	Sign	% Change	Sign
Bad	Moderately high	-47	****	-43	****
Bax	Moderately high	-55	****	-53	****
Bcl-2	Moderate	-14	ns	-6	ns
Bcl-x	Very low	+3	ns	+6	ns
cIAP-1	Low	+7	ns	-15	ns
cIAP-2	Low	-10	ns	-23	**
Livin	Low	-15	ns	-10	ns
Survivin	Low	-21	*	-29	**
XIAP	Moderate	+1	ns	-13	ns
HTRA2/Omi	High	-8	ns	-24	**
Smac/DIABLO	High	+30	****	+22	****
Cytochrome c	Moderately high	+211	****	+111	****
Pro-caspase-3	Moderately high	-37	****	-14	**
Cleaved caspase-3	Very high	+39	****	+96	****
TRAIL-R1/DR4	Low	-44	****	-50	****
TRAIL-R2/DR5	Low	-43	****	-31	****
FADD	Moderate	-28	****	-40	****
Fas/TNFRSF6/CD95	Moderate	-17	ns	+2	ns
TNF-R1/TNFRSF1A	Low	+2	ns	-10	ns
Clusterin	Very high	-66	****	-73	****
HIF-1α	Moderately high	-56	****	-4	ns
HO-1/HMOX1/HSP32	Moderate	+121	****	+242	****
HO-2/HMOX2	Moderate	-17	**	+19	**
HSP27	High	-51	****	-49	****
HSP60	Moderate	+18	*	-6	ns
HSP70	Moderately high	-29	***	-21	**
Phospho-p53 (S15)	Moderate	-48	****	-51	****
Phospho-p53 (S46)	Moderate	-48	****	-27	****
Phospho-p53 (S392)	Low	-10	ns	+17	*
Phospho-Rad17 (S635)	Moderate	-14	ns	+6	ns
p21^{Cip1/CDKN1A}	Moderate	+1	ns	+17	ns
p27^{Kip1}	Moderate	+137	****	+112	****
Catalase	Low	-17	ns	-10	ns
Claspin	Low	-12	ns	-2	ns
PON2	Low	-21	ns	-27	*

The positive (+) sign indicates an increase in protein expression, whilst the negative (–) sign indicates a decrease. The one-way ANOVA ($p < 0.05$) followed by the Dunnet's *post-hoc* comparisons test was performed using the pixel density of treated and untreated cells. Legend: ns = non-significant; ** $p < 0.05$ = significant; *** $p < 0.01$ = highly significant and **** $p < 0.001$ = highly significant.

3.3.3.1 Expression of caspase-3

Compared to the untreated control, the highly expressed pro-caspase-3 was significantly decreased by JD88 ($p<0.001$) (Figure 3.20A and B, Table 3.5). In contrast, cleaved caspase-3 expression in leukocytes was increased in response to JD88 and JD47R by 39% ($p<0.001$) and 96% ($p<0.001$), respectively (Figure 3.20C).

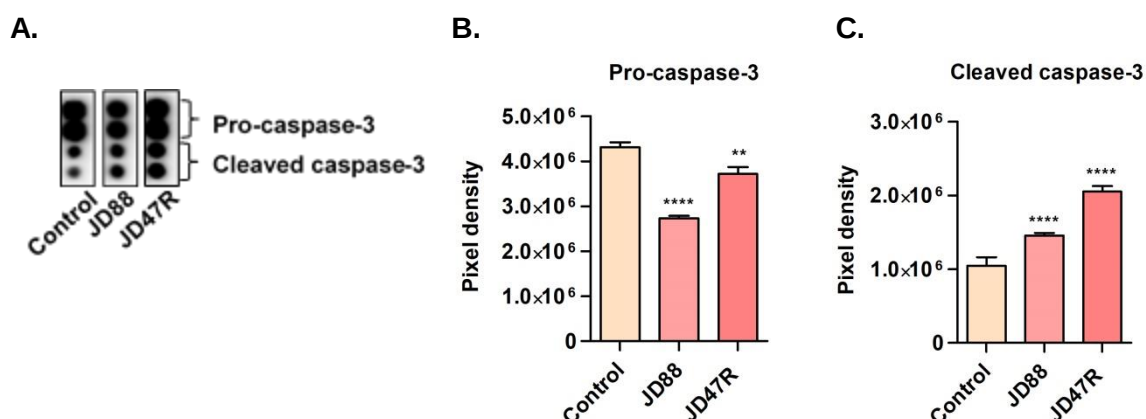


Figure 3.20 The expression of pro-caspase-3 and cleaved caspase-3 in leukocytes. Leukocytes were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** is the section of the membrane array for pro-caspase-3 and cleaved caspase-3 expression; **B** shows the effect by the copper complexes on pro-caspase-3 expression and **C** shows the effect by the copper complexes on cleaved caspase-3 expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.3.2 Haem-oxygenase, Heat shock protein and clusterin expression

The proteins; HO-1/HMOX1/HSP32 and HO-2/HMOX2, were moderately expressed in leukocytes (Figure 3.21A). The copper-imidazo[1,2-*a*]pyridines JD88 and JD47R significantly increased HO-1/HMOX1/HSP32 expression ($p<0.001$) but not HO-2/HMOX2 expression ($p<0.05$) as shown in Figure 3.21B and C. HIF-1 α protein was moderately expressed in control leukocytes (Figure 3.21A). JD88 decreased the expression of HIF-1 α whilst JD47R caused no significant change in its expression (Figure 3.21D; Table 3.5).

Heat shock protein, HSP27 was highly expressed in leukocytes, whilst HSP60 was poorly expressed and HSP70 was moderately high as shown in Figure 3.21E. JD88 and JD47R decreased the expression of HSP27 significantly by 51% ($p<0.001$) and 49% ($p<0.001$), respectively (Figure 3.21E and F). The copper complexes decreased HSP70 expression as well, however these changes were less meaningful (Figure 3.21G; Table 3.5), whilst no meaningful changes were induced in HSP60 expression (Table 3.5). The cytoprotective clusterin was highly expressed in leukocyte controls (Figure 3.21H; Table 3.5) and its

expression was significantly decreased by JD88 and JD47R by 66% ($p<0.001$) and 73% ($p<0.001$), respectively (Figure 3.21I).

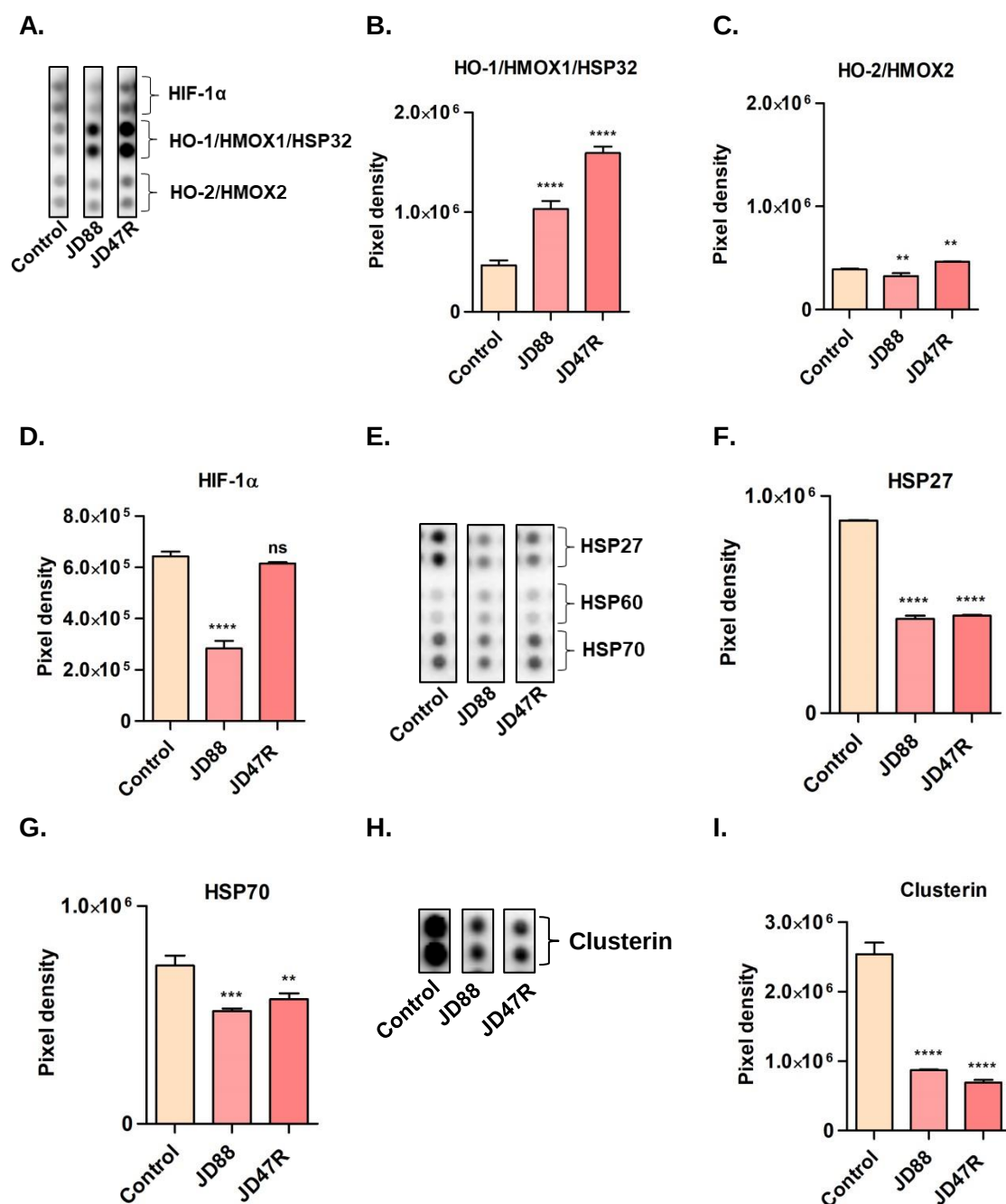


Figure 3.21 The expression of HO, HSPs and clusterin in leukocytes. Leukocytes were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for HIF-1 α , HO-1/HMOX1/HSP32 and HO-2/HMOX2. **B**, **C** and **D** shows the effect by the copper complexes on HO-1/HMOX1/HSP32, HO-2/HMOX2 and HIF-1 α expression, respectively; **E** is the section of the membrane array for HSP27, HSP60 and HSP70 expression and **F** and **G** shows the effect by the copper complexes on HSP27 and HSP70 expression, respectively. **H** shows the section of the membrane array for clusterin expression and **I** shows the effect by the copper complexes on clusterin expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.3.3 Expression of Bcl-2 family of proteins

The pro-apoptotic protein Bad and Bax were moderately expressed in the leukocytes control (Figure 3.22A). In comparison to the untreated control, JD88 and JD47R caused significant decreases in the expression levels of Bad by 47% and 43% (Figure 3.22B), respectively. JD88 and JD47R decreased Bax by 55% and 53%, respectively ($p < 0.001$) (Figure 3.22C). The anti-apoptotic proteins Bcl-2 and Bcl-x were low-moderately expressed where both copper-imidazo[1,2-*a*]pyridines caused no meaningful changes in their expression (Table 3.5).

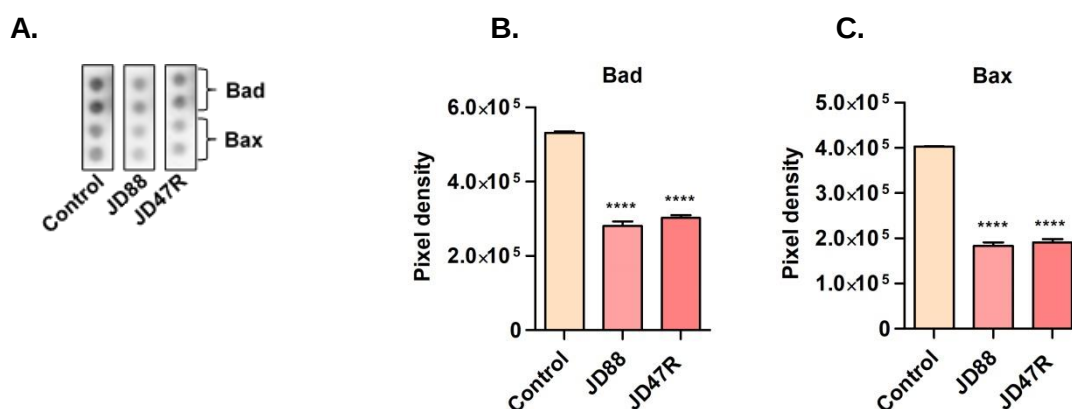


Figure 3.22 The expression of pro-apoptotic Bcl-2 family of proteins in leukocytes. Leukocytes were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** is the section of the membrane array for Bad and Bax expression; **B** and **C** shows the effect by the copper complexes on Bad and Bax expression, respectively. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.3.3.4 Expression of Smac/DIABLO and HTRA2/Omi proteins

Smac/DIABLO was moderately expressed in control leukocytes (Figure 3.23A; Table 3.5). The copper complexes JD88 and JD47R significantly increased Smac expression by 30% and 22% ($p < 0.001$), respectively, as shown in Figure 3.23B. HTRA2/Omi was highly expressed in control leukocytes (Figure 3.23C) however, the copper-imidazo[1,2-*a*]pyridines didn't cause a meaningful change in its expression (Figure 3.23D; Table 3.5).

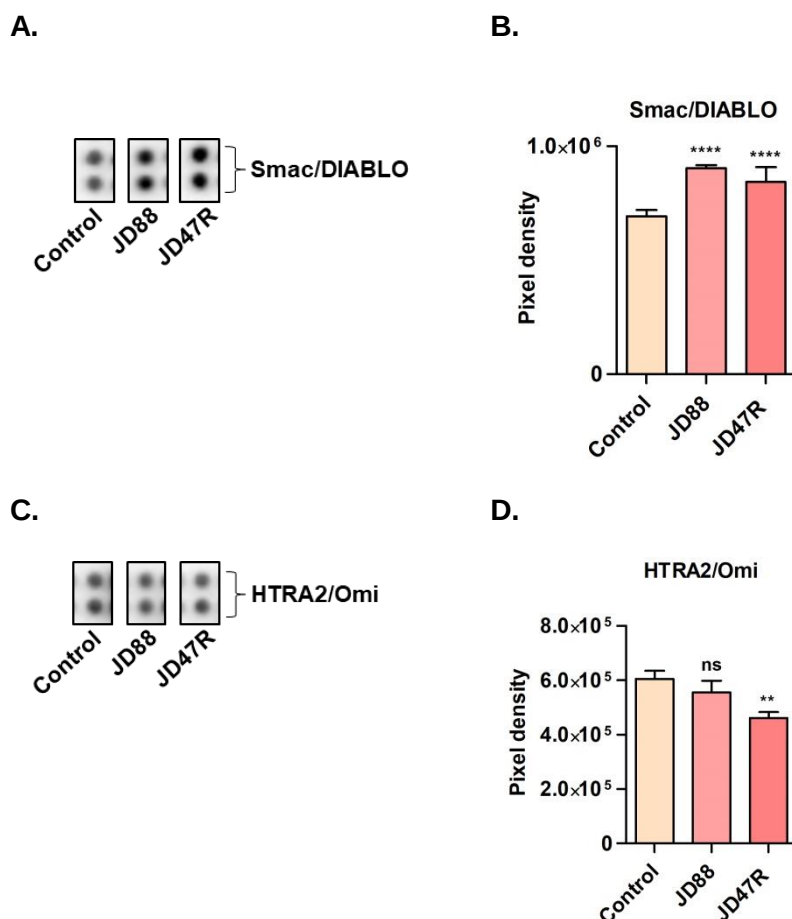


Figure 3.23 The expression of Smac/DIABLO and HTRA2/Omi in leukocytes. Leukocytes were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** is the section of the membrane array for Smac expression; **B** shows the effect by the copper complexes and on Smac expression. **C** shows the section of the membrane array for HTRA2/Omi expression and **D** shows the effect by the copper complexes on HTRA2/Omi expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$; **** $p < 0.001$.

3.3.3.5 Expression of inhibitor of apoptotic proteins

Analysis of the proteome array demonstrated that the IAPs including XIAP, cIAPs as well as survivin and livin were poorly expressed in the leukocytes control (Table 3.5). The copper-imidazo[1,2-*a*]pyridines caused no meaningful changes to their protein expression (Table 3.5).

3.3.3.6 Expression of death receptor proteins

The death receptors TRAIL-R1 and TRAIL-R2 were poorly expressed in control leukocytes (Figure 3.24A; Table 3.5). The copper-imidazo[1,2-*a*]pyridines significantly decreased the expression of both TRAIL receptor proteins as shown in Figure 3.24B and C. Death receptors

FADD and Fas were moderately expressed (Figure 3.24D) while TNF-R1 was poorly expressed in control leukocytes (Table 3.5). Both copper-imidazo[1,2-*a*]pyridines JD88 and JD47R decreased FADD expression by 28% ($p<0.001$) and 40% ($p<0.001$), respectively (Figure 3.24D). Fas protein was differentially expressed with JD88 and JD47R causing changes of 17% and 2%, respectively, which were considered to be insignificant (Figure 3.24F; Table 3.5). TNF-R1 expression was similarly left unchanged by the copper-imidazo[1,2-*a*]pyridines (Table 3.5).

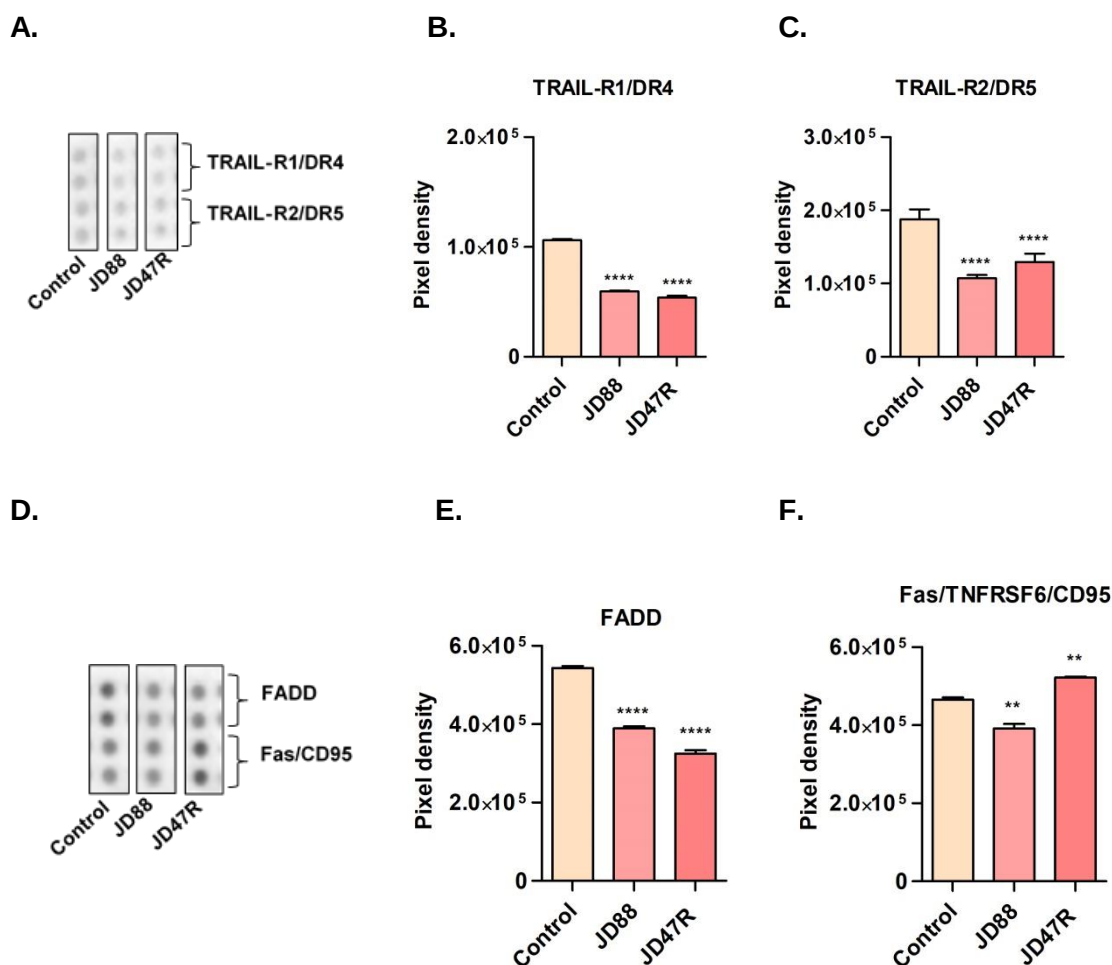


Figure 3.24 The expression of death receptor proteins in leukocytes. Leukocytes were exposed to IC_{75} concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for TRAIL-R1 and TRAIL-R2; **B** and **C** shows the effect by the copper complexes on TRAIL-R1 and TRAIL-R2 expression; **D** shows the section of the membrane array for FADD and Fas expression; **E** and **F** shows the effect by the copper complexes on FADD and Fas expression. Data was analysed by one-way ANOVA ($p<0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p<0.1$; ** $p<0.05$; *** $p<0.01$, **** $p<0.001$.

3.3.3.7 Expression of other regulatory proteins

The three phosphorylated p53 proteins were poorly expressed in control leukocytes (Figure 3.25A; Table 3.5) and the copper-imidazo[1,2-*a*]pyridines caused significant decreases in phospho-p53 (S15) and phospho-p53 (S46) expression ($p < 0.001$) but no meaningful changes in phospho-p53 (S392) expression (Figure 3.25B; Table 3.5). Cell cycle regulatory proteins p21^{Cip1} and p27^{Kip1} (Figure 3.25C) were poorly expressed in control leukocytes (Table 3.5). In response to JD88 and JD47R, p27^{Kip1} was significantly increased by 137% and 112% ($p < 0.001$), respectively (Figure 3.25D). The test compounds caused no meaningful changes to p21^{Cip1} expression (Table 3.5).

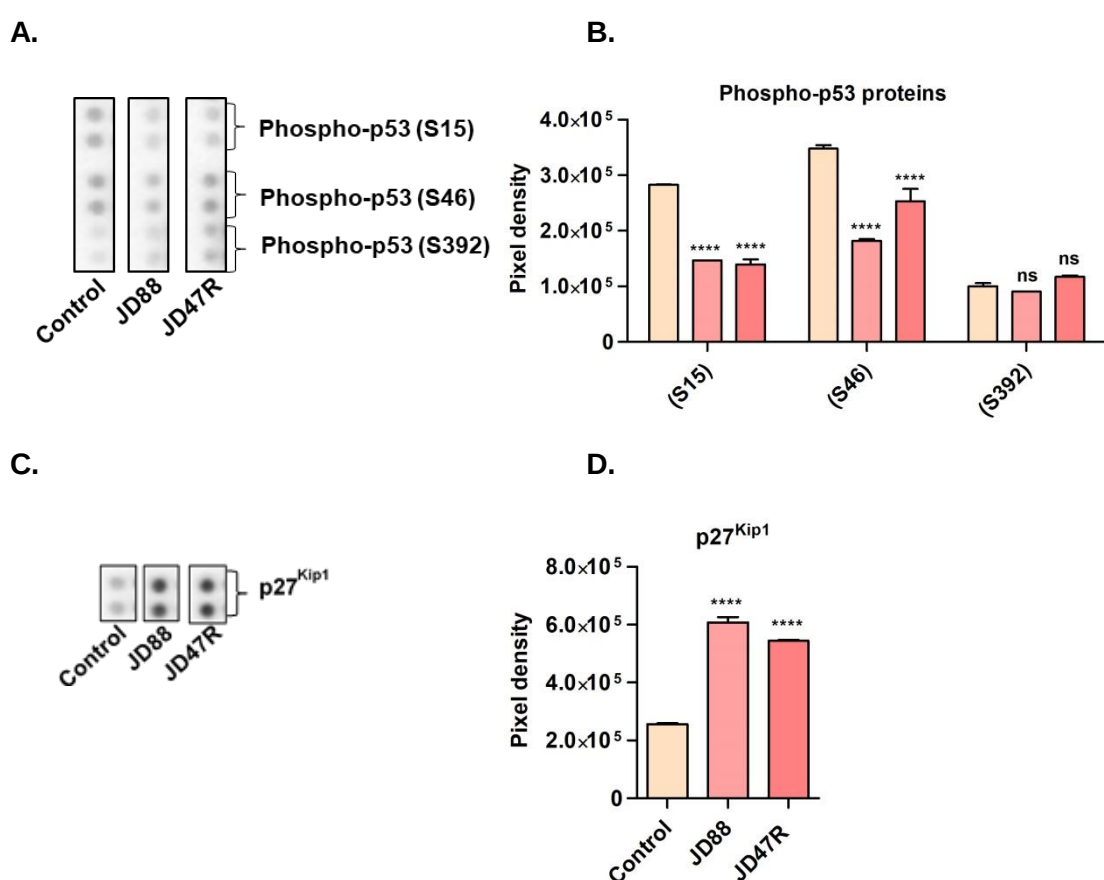


Figure 3.25 The expression of phosphorylated p53 proteins and the regulatory protein p27^{Kip1} in leukocytes. Leukocytes were exposed to IC₇₅ concentrations of each copper complex for 18 hrs. **A** shows the section of the membrane array for phospho-p53 (S15), (S46) and (S392); **B** shows the effect by the copper complexes on phospho-p53 proteins expression; **C** shows the section of the membrane array for p27^{Kip1} expression and **D** shows the effect by the copper complexes on p27^{Kip1} expression. Data was analysed by one-way ANOVA ($p < 0.05$) followed by Dunnet's *post-hoc* comparative test to the control. The error bars represent the mean $\pm$ SD. *P*-values: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$, **** $p < 0.001$.

3.4 DISCUSSION

The proteome array data provides information on the expression levels of apoptosis associated regulatory proteins. Having collected evidence that cell death occurs by apoptosis, the proteome arrays provided a more comprehensive analysis of the effects of the copper-imidazo[1,2-*a*]pyridines on cell death pathways. Changes in expression that is highly significant ($p < 0.001$) analysed with Dunnet's *post hoc* comparative test was considered to be meaningful (highlighted yellow) in this study. The proteome array showed some common effects in the two leukaemic cell lines whilst peripheral leukocytes had a different response as reflected in its protein expression profile.

3.4.1 Copper-imidazo[1,2-*a*]pyridines increases expression levels of cleaved caspase-3

The baseline expression of cleaved caspase-3 was low in HL-60 and K562 control cells, whereas in peripheral leukocytes its expression was high. Poor expression of caspase-3 is associated with the evasion of apoptosis and poor prognosis in leukaemia (Montiel-Cervantes *et al.*, 2018). The high level of caspase-3 in normal leukocytes is evident because deregulation of the apoptotic process is less likely to occur in these cells. Proteome array data showed an increase in cleaved caspase-3 confirming the activation of executioner caspase-3 and the induction of apoptotic cell death by the active copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 cells. In addition it indicates that the compounds are likely to cause apoptosis in normal peripheral leukocytes.

Table 3.6 Comparison of changes in the expression of cleaved caspase-3 in HL-60, K562 and leukocytes relative to untreated controls

Cleaved caspase-3	HL-60	K562	leukocytes	Comments
JD88	359%	255%	39%	Copper complexes were effective in increasing levels of cleaved caspase-3 in all cells.
JD47R	143%	Nt	96%	
JD49R	Nt	135%	Nt	
Camptothecin	615%	-1%	Nt	

*Nt-compound not tested in these cells

As shown in Table 3.6, the active copper-imidazo[1,2-*a*]pyridines significantly increased cleaved caspase-3 in HL-60 cells. Compared to the copper-imidazo[1,2-*a*]pyridines, camptothecin was a more potent inducer of caspase-3 cleavage in HL-60 cells but were unable to cleave caspase-3 in K562 cells. Caspase-3/7 activity was much lower in K562 cells

and although proteome analysis showed no change in cleavage there was some signal. This could probably be due to a time effect or even a general suppressive effect on enzymatic activity caused by camptothecin. These observations were consistent with data from apoptotic enzyme assays (Chapter 2; section 2.3.7) where the copper-imidazo[1,2-*a*]pyridines showed a significant increase in caspase-3/7 activity which was more prominent in HL-60 cells. The effect of JD88 on cleaved caspase-3 reflects that of the caspase-3/7 assay in HL-60 cells where JD88 was shown to be more active than JD47R. Since activation of caspase-3 would be an unwanted effect in normal leukocytes, JD88 would be preferred since it caused a smaller increase in cleaved caspase-3 in leukocytes (39%) compared to JD47.

Caspase-3 is a terminal effector caspase (McIlwain *et al.*, 2013). Following cleavage of pro-caspase-3 by either initiator caspase-8, caspase-9 or both, caspase-3 is activated (Pfeffer and Singh, 2018). Activated caspase-3 is responsible for the morphological features associated with apoptosis (Crowley *et al.*, 2016a), as it causes the proteolytic cleavage of cytoskeletal proteins and poly (ADP-ribose) polymerase (PARP) leading to cell disassembly, DNA fragmentation and apoptosis (Pal *et al.*, 2010). Caspase-3/7 releases and activates the endonuclease CAD; which is also responsible for nuclear fragmentation, by cleaving and inactivating its inhibitor ICAD (Hanus *et al.*, 2008).

The increased expression of cleaved caspase-3 serves as further validation for the ability of copper-imidazo[1,2-*a*]pyridines to induce apoptosis. Other copper complexes have been reported to cause apoptosis by inducing caspase-3 activity *in vitro*. Salimi *et al.*, (2015) demonstrated how copper (II)-polypyridyl complexes in MCF-7 breast cancer cells induced caspase-3 activity by measuring cleaved caspase-3 levels using western blot analysis. However, several papers indicate that MCF-7 cells do not express caspase-3 (Jänicke, 2009; Foo *et al.*, 2014). Another group demonstrated that their copper complex caused an increase in caspase-3 gene transcription in MDA-MB-231 breast cancer cells together with an increase in caspase-3 activity as analysed by colorimetric analysis (Zhou *et al.*, 2016).

Due to their highly conserved peptide sequences, effector caspases-3 and -7 share the same substrate specificities (Julien *et al.*, 2016). Therefore, the substrate (Ac-DEVD-AFC) used to measure caspase activity (Chapter 2; section 2.3.7) was not able to distinguish caspase-3 from caspase-7 activity. The proteome array data provides evidence that caspase-3 was cleaved to its active form. Previous *in vitro* studies have demonstrated the induction of apoptosis by

causing caspase-3 activation specifically in HL-60 and K562 cells (Martins *et al.*, 1997; Pal *et al.*, 2010; Niu *et al.*, 2011; Prabhu *et al.*, 2017).

3.4.2 Test compounds increases HO-1/HMOX1/HSP32 in HL-60 cells and leukocytes but not in K562 cells.

In the proteome array, the baseline expression levels of HO-1/HMOX1/HSP32 in both HL-60 and K562 control cells were low, whereas in peripheral leukocytes it was moderate. This difference could have been because of the leukocytes being stressed as they were outside of their normal environment. In HL-60 cells and leukocytes, the active copper-imidazo[1,2-*a*]pyridines and camptothecin significantly increased HO-1/HMOX1/HSP32 expression (Table 3.7). In K562 cells, HO-1/HMOX1/HSP32 expression was poor and not affected by test compounds.

HO-1/HMOX1/HSP32 is an inducible gene that is stimulated in response to oxidative stress whereby the cell protects itself from free radical damage (Chiang *et al.*, 2018). Additionally, it regulates cell proliferation, modulates inflammatory responses and facilitates angiogenesis (Loboda *et al.*, 2015). HO-1/HMOX1/HSP32 expression is often up-regulated in resistant leukaemias (CML) compared to sensitive leukaemias (AML) (Liu *et al.*, 2017a). This was not reflected in the proteome arrays of HL-60 and the K562 cells.

Evidence suggests that HO-1/HMOX1/HSP32 may play a role in tumour induction and growth (Was *et al.*, 2010). During cell stress, HO-1/HMOX1/HSP32 could be further elevated in response to treatment with radiation or chemotherapy thus promoting cancer cell survival and diminishing the effects of potential chemotherapeutic agents (Sorrenti *et al.*, 2018). For example, in response to imatinib, HO-1/HMOX1/HSP32 expression is increased, thereby inhibiting apoptosis and promoting cancer cell survival by activating the JNK signalling pathway (Lin *et al.*, 2015; Cao *et al.*, 2016). JNKs are a family of protein kinases that play a central role in stress signalling pathways activated by cytokines, heat shock as well as growth factors.

Table 3.7 Comparison of changes in the expression of HO-1/HMOX1/HSP32 and HIF-1 α in HL-60, K562 and leukocytes relative to untreated controls

HO-1/HMOX1/HSP32	HL-60	K562	leukocytes	Comments
JD88	267%	-2%	121%	Copper complexes stimulated HO-1/HMOX1/HSP32 expression in HL-60 cells and leukocytes.
JD47R	232%	Nt	242%	
JD49R	Nt	-11%	Nt	
Camptothecin	32%	-18%	Nt	
HO-2/HMOX2	HL-60	K562	leukocytes	Comments
JD88	13%	-5%	-17%	No meaningful changes caused by the copper complexes, however camptothecin decreased HO-2/HMOX2 in K562 cells
JD47R	6%	Nt	12%	
JD49R	Nt	-18%	Nt	
Camptothecin	11%	-50%	Nt	
HIF-1 α	HL-60	K562	leukocytes	Comments
JD88	1%	-8%	-56%	JD49R and camptothecin decreased HIF-1 α expression in K562 cells, while JD88 caused a meaningful decrease in leukocytes.
JD47R	9%	Nt	-4%	
JD-49R	Nt	-29%	Nt	
Camptothecin	17%	-24%	Nt	

*Nt-compound not tested in these cells

The increased expression of HO-1/HMOX1/HSP32 in HL-60 cells may be due to its cytoprotective response against the oxidative stress that the copper-imidazo[1,2-*a*]pyridines may have caused; displaying chemo-resistance and corroborating with published literature. Studies by Vyas *et al.*, (2015) and Mendo *et al.*, (2015), reported on the ability of copper complexes to induce DNA damage and changes to structural proteins as a result of ROS generation. However, it can be hypothesized that the increase in HO-1/HMOX1/HSP32 is a result of copper-mediated ROS production which is known to stimulate apoptosis. A recent study showed the induction of HO-1/HMOX1/HSP32 expression in HT-29 cells in response to the copper-imidazo[1,2-*a*]pyridines JD46R, JD47R and JD88 (Harmse *et al.*, 2019). The study demonstrated a burst of ROS levels at 6 hours following treatment with all three copper-imidazo[1,2-*a*]pyridines which was sufficient to initiate HO-1/HMOX1/HSP32 gene transcription and protein synthesis. The burst of ROS would correspond with the increase of HO-1/HMOX1/HSP32 expression levels observed in this study. The ability of copper-imidazo[1,2-*a*]pyridines to cause ROS information requires further investigation.

Imidazole derived HO-1/HMOX1/HSP32 inhibitors have shown their ability to inhibit HO-1/HMOX1/HSP32, HO-2/HMOX2 and tyrosine kinase activities and effectively eradicate imatinib-resistant CML cell lines (Sorrenti *et al.*, 2018). Primary cells and cell lines derived

from patients with ALL express HO-1/HMOX1/HSP32 in a constitutive manner (Cerny-Reiterer *et al.*, 2014). Drug combinations consisting of HO-1/HMOX1/HSP32 inhibitors and either a TKI or alkylating agent (bendamustine) act synergistically to decrease the growth of imatinib-resistant ALL cells and induce apoptosis (Cerny-Reiterer *et al.*, 2014), providing evidence for its potential as a drug target (Was *et al.*, 2010; Podkalicka *et al.*, 2018). These findings suggest that the inhibition of HO-1/HMOX1/HSP32 activity or the suppression of HO-1/HMOX1/HSP32 expression can be beneficial as it may facilitate apoptotic cell death. HO-1/HMOX1/HSP32 is considered a potential drug target in AML, CML and other cancers (Salerno *et al.*, 2017; Chiang *et al.*, 2018).

The baseline expression of HIF-1 α was low to moderate in HL-60 control cells, K562 control cells and peripheral leukocytes (Table 3.7). Increased levels of HIF-1 α is usually associated with leukaemias like AML and CML (Salerno *et al.*, 2017). Treatment with the copper-imidazo[1,2-*a*]pyridines caused inconsistent changes in its expression that were considered insignificant. HIF-1 α regulates the transcription of HO-1/HMOX1/HSP32 (Toth and Warfel, 2017). The expression of HIF-1 α did not correlate with the increase of HO-1/HMOX1/HSP32 expression in HL-60 cells and leukocytes implying that HIF-1 α was not responsible for the induction of HO-1/HMOX1/HSP32 gene transcription and accumulation in this study. HIF-1 α is not the only transcription factor to regulate HO-1/HMOX1/HSP32, additionally it is controlled by transcription factors like NF- κ B and nuclear factor erythroid 2 p45-related factor 2 (Nrf2) and activator protein-1 (AP-1) (Alam and Cook, 2007; Wardyn *et al.*, 2015). Nrf2 is frequently overexpressed in leukaemias, together with HO-1/HMOX1/HSP32, whilst in normal cells HO-1/HMOX1/HSP32 is a target gene of Nrf2 responding to oxidative stress (Furfaro *et al.*, 2016). Although HO-2/HMOX2 gene expression does not respond to the same stimuli as HO-1/HMOX1/HSP32 (due to the absence of regulatory elements in its promoter), its expression is influenced by hypoxic conditions (Muñoz-Sánchez and Cháñez-Cárdenas, 2014).

HO-2/HMOX2 is the constitutive isoform of the HO system and is highly expressed in neuronal populations of the brain where its expression has been shown to protect against apoptotic cell death (Muñoz-Sánchez and Cháñez-Cárdenas, 2014). Basal levels of HO-2/HMOX2 maintain haem homeostasis and protect cells against oxidative stress by regulating protein kinase B (PKB)/Akt, extracellular superoxide dismutase and apoptotic signalling kinase-1, changing the rate of heme degradation (Muñoz-Sánchez and Cháñez-Cárdenas,

2014). After silencing HO-2/HMOX2, a decrease in extracellular superoxide dismutase and phosphorylated Akt (at serine site 473 and threonine site 308) was observed affecting Bad and Bax regulation and increasing the pro-apoptotic protein; apoptotic signalling kinase-1 in mice, suggesting that the control of these enzymes by HO-2/HMOX2 is critical in the regulation of apoptosis (Turkseven *et al.*, 2007). The lack of induction of HO-2/HMOX2 expression by the copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 favoured the induction of apoptosis. However, camptothecin caused a 50% decrease in HO-2/HMOX2 expression indicating that HO-2/HMOX2 is an important factor in camptothecin induced apoptosis.

3.4.3 Test compounds change the expression of the Bcl-2 family of proteins and cytochrome c promoting apoptotic cell death

The baseline expression of pro-apoptotic Bad and Bax was low in HL-60 and K562 cells while in peripheral leukocytes it was moderately expressed. Low levels of Bad and Bax are frequently observed in leukaemias and higher levels are associated with tumour suppressor activity in normal haematopoietic cells (Bekhet *et al.*, 2016; Reichenbach *et al.*, 2017).

Bcl-2 family of proteins regulate the intrinsic mitochondrial apoptotic pathway through cytochrome c release and caspase-9 activation (Fulda, 2014). In peripheral leukocytes, the copper-imidazo[1,2-*a*]pyridines suppressed the expression of both Bax and Bad without inducing meaningful changes in Bcl-2 anti-apoptotic proteins (Table 3.8). These findings suggest that the copper-imidazo[1,2-*a*]pyridines did not have a significant effect on leukocyte cell death mediated by the Bcl-2 family of proteins.

Table 3.8 Comparison of the changes in the expression of pro- and anti-apoptotic Bcl-2 proteins in HL-60, K562 and leukocytes relative to untreated controls

Bad	HL-60	K562	leukocytes	Comments
JD88	3%	81%	-47%	Copper complexes increased Bad in K562 cells and decreased it in leukocytes while causing no meaningful changes in HL-60 cells
JD47R	4%	Nt	-43%	
JD49R	Nt	79%	Nt	
Camptothecin	4%	12%	Nt	
Bax	HL-60	K562	leukocytes	Comments
JD88	42%	161%	-55%	Copper complexes and camptothecin significantly increased Bax expression in HL-60 and K562 cells but decreased it in leukocytes
JD47R	44%	Nt	-53%	
JD49R	Nt	116%	Nt	
Camptothecin	98%	92%	Nt	
Cytochrome c	HL-60	K562	leukocytes	Comments
JD88	48%	14%	211%	Copper complex JD88 increased cytochrome c in HL-60 cells. JD88 and JD47R significantly increased cytochrome c in leukocytes.
JD47R	27%	Nt	111%	
JD49R	Nt	7%	Nt	
Camptothecin	6%	15%	Nt	
Bcl-2	HL-60	K562	leukocytes	Comments
JD88	91%	19%	-14%	In HL-60 cells, Bcl-2 was overexpressed in response to JD88. Based on the expression level of Bcl-2, the changes induced by camptothecin were not considered meaningful
JD47R	9%	Nt	-6%	
JD49R	Nt	-5%	Nt	
Camptothecin	23%	-39%	Nt	
Bcl-x	HL-60	K562	leukocytes	Comments
JD88	-6%	3%	3%	JD49R and camptothecin caused meaningful decreases in K562 cells contributing to apoptosis induction
JD47R	-8%	Nt	6%	
JD49R	Nt	-19%	Nt	
Camptothecin	-13%	-35%	Nt	

*Nt-compound not tested in these cells

The copper-imidazo[1,2-*a*]pyridines significantly increased Bad protein expression in K562 cells while no meaningful changes were observed in HL-60 cells and a significant decrease ($p < 0.001$) was observed in peripheral leukocytes (Table 3.8). In comparison to the controls, camptothecin caused no meaningful change in Bad expression. Bad is phosphorylated by Akt and phosphorylated Bad promotes cell survival by allowing Bcl-2 to bind and inhibit Bax activity (Datta *et al.*, 2000). At low levels, Bad is phosphorylated by Akt which results in the evasion of apoptosis. However, dephosphorylated Bad inactivated Bcl-2/Bcl-x by forming a heterodimer thereby facilitating Bax mediated apoptosis (Datta *et al.*, 2002). Therefore, an increase of Bad levels will bind either Bcl-2 or Bcl-x and favour apoptosis. Jointly the

increase in Bax and Bad expression overcome the increase of Bcl-2. Furthermore, high levels of Bad protein expression have been associated with improved survival rates in breast and colorectal cancers as a result of apoptosis induction (Cannings *et al.*, 2007; Sinicrope *et al.*, 2008).

The copper-imidazo[1,2-*a*]pyridines significantly increased the expression of Bax in HL-60 and K562 cells, whereas its expression in peripheral leukocytes was decreased (Table 3.8). Camptothecin caused significant increases of Bax protein levels in both HL-60 and K562 cells. The increase in pro-apoptotic Bax expression suggests promotion of apoptosis by the copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 cells (Table 3.8). Bax is predominantly located in the cytosol (Youle and Strasser, 2008) and in normal cells, form heterodimers with Bcl-2. This prevent Bax relocalisation to the mitochondria and protects mitochondria from damage and apoptosis (Popgeorgiev *et al.*, 2018). During apoptosis, Bax is translocated from the cytosol to the mitochondria where it undergoes a conformational change to form a mitochondrial membrane pore after homodimerisation (Luna-Vargas and Chipuk, 2016). The increased expression of Bax in this study favours the homodimerisation of Bax leading to the increased permeability of the outer mitochondrial membrane, and allows for the release of cytochrome c from the mitochondria (Reichenbach *et al.*, 2017). Data obtained from the proteome array showed that the increased expression of Bax (Table 3.8) is consistent with the loss of MMP as a result of mitochondrial pore formation, and activation of caspase-9 in HL-60 and K562 cells (Chapter 2; section 2.3.9 and 2.3.10).

These findings are consistent with a previous study by Kumar *et al.*, (2014) who demonstrated the activation of the intrinsic apoptotic pathway in HL-60 cells evidenced by Bax up-regulation, Bcl-2 down-regulation, caspase-3 activation and MMP depolarisation (Kumar *et al.*, 2014). Another study reported on the induction of apoptosis in K562 cells via the intrinsic apoptotic pathway due to the increase in Bax: Bcl-2 ratio (Mallick *et al.*, 2012). Other copper complexes have been shown to increase Bax expression and induce apoptosis in various cancer cell lines (Ma *et al.*, 2015; Salimi *et al.*, 2015).

Cytoplasmic cytochrome c has an essential function in the intrinsic apoptotic pathway, facilitating the activation of downstream caspases through the activation of pro-caspase-9 in the apoptosome (Xiong *et al.*, 2014). Mitochondria are the primary site of ROS generation in cells, and enhanced mitochondrial activity is usually associated with an increase in ROS production (Ray *et al.*, 2012). In addition to ROS production, mitochondrial activation can

manifest as an increase in the synthesis of localised mitochondrial proteins such as cytochrome c and cytochrome c oxidase (Sanchez-Alcazar *et al.*, 2000; Zorov *et al.*, 2014). The increased cytochrome c levels are an initial stress response which suggests that the copper complexes are somewhat toxic to mitochondria, and provide a signal to up-regulate mitochondrial protein synthesis (Sanchez-Alcazar *et al.*, 2000). Therefore in this case, the copper complexes up-regulates the synthesis of cytochrome c in the mitochondria which gradually releases into the cytosol to initiate apoptosome formation and eventually apoptotic cell death.

Alternatively, it is probable that mitochondria contain a constitutive protease that is involved in mitochondrial protein turnover, and whose inhibition results in higher protein levels due to impeded proteasomal degradation (Kowalski *et al.*, 2018). Thus it seems possible that the copper complexes may inhibit such a protease and consequently caused mitochondrial proteins such as cytochrome c to accumulate.

The baseline expression of Bcl-2 protein was high in HL-60 cells, poor in K562 cells and moderate in peripheral leukocytes. In comparison, anti-apoptotic Bcl-x was poorly expressed in HL-60, K562 cells and the leukocytes. The findings obtained from the proteome array of HL-60 control cells were in agreement with published literature, whereas that obtained from the proteome array of K562 cells were not (Campos and Pinto, 2019). Owing to the low expression levels of Bcl-2 in K562 and leukocytes, the changes induced by the copper-imidazo[1,2-*a*]pyridine complexes and camptothecin were considered not meaningful (Table 3.8).

The anti-apoptotic Bcl-2 protein is considered a negative prognostic factor (Sahu and Jena, 2011). The overexpression of Bcl-2 and Bcl-x are frequently observed in AML and CML (Tzifi *et al.*, 2012). Patients, who express high levels of Bcl-2 and Bcl-x are less responsive to chemotherapeutic agents, have resistance to apoptosis and a poor prognosis (Llambi and Green, 2011; Hata *et al.*, 2015; Campos and Pinto, 2019). Thus, increased Bcl-2 and Bcl-x expression may confer chemo-resistance in leukaemic cells. However, experimental studies have reported that the high levels of Bcl-2 in ALL and AML do not always correlate with chemo-resistance (Sahu and Das, 2002; Mérimo *et al.*, 2012).

Intrinsic apoptosis was favoured in HL-60 cells despite the massive amount of Bcl-2 in response to JD88, implying that it was not functional. This supports the argument that

increased Bcl-2 does not hinder or prevent apoptosis in these cells. These observations suggest that high levels of Bcl-2 alone do not regulate Bcl-2 effects but other protein interactions and mechanisms are involved.

Anti-apoptotic Bcl-2 and Bcl-x are localised in the outer mitochondrial membrane, the endoplasmic reticulum membrane and the nuclear envelope (Harris and Thompson, 2000). The increased levels of Bcl-2 may be as a result of Bcl-2 being trapped in either the endoplasmic reticulum or the nucleus. Follow up immunofluorescence experiments may help detect the sub-cellular localisation of Bax/Bcl-2 for a better understanding.

The interaction of Bcl-2 protein with pro-apoptotic Bcl-2 family members (e.g. Bax and Bad) and kinases play a key role in apoptosis (Tait and Green, 2010). They preserve the integrity of the mitochondrial membrane by forming a heterodimer with Bax thereby preventing mitochondrial pore formation (Peña-Blanco and García-Sáez, 2018). Bcl-2 binds and inactivates Bax, whereas when bound to Bad its activity is down-regulated (O'Neill *et al.*, 2016). In HL-60 cells, both Bcl-2 levels and Bax were increased by JD88 (Table 3.8). The increased expression of Bcl-2 by JD88 may be an attempt to overcome mitochondrial pore formation since higher levels of Bcl-2 will inactivate Bax and stabilise the mitochondrial membrane and preventing cytochrome c release (Shawgo *et al.*, 2008). The Bcl-2 levels were essentially higher in HL-60 cells than Bax and so the Bcl-2: Bax ratio was high. Even though clinical data suggests that Bcl-2: Bax ratio is important for the survival of cells in AML, there is no clear correlation between chemo-resistance and the ratio (Kulsoom *et al.*, 2018).

Despite the apparent increase of Bcl-2 by JD88 in HL-60 cells, the other copper complexes support the findings of previous studies which reported that copper compounds suppress the expression of Bcl-2. In 2014, a study reported the apoptotic activity of a phosphine containing copper (I) complex in B-cell acute lymphoblastic leukaemia (Bortolozzi *et al.*, 2014). This complex caused the down-regulation of Bcl-2 promoting the induction of the apoptotic cascade. Copper (II) complexes showed their ability to induce apoptosis in the MCF-7 breast cancer cell line by significantly increasing the expression of the Bax to Bcl-2 ratio (Salimi *et al.*, 2015). Similarly, Ma *et al.*, (2015) demonstrated the induction of apoptosis by three ternary copper (II) complexes in human lung cancer (A549), human oesophageal cancer (Eca109) and human gastric cancer (SGC7901) cell lines. Their apoptotic activity was related to their effect on the protein expression of Bax, Bcl-2 and p53

as detected by Western blotting where Bcl-2 was down-regulated and Bax and p53 were up-regulated (Ma *et al.*, 2015).

Members of the Bcl-2 family are important regulators of both apoptotic pathways and changes in the levels of anti- and pro-apoptotic Bcl-2 proteins determine whether cells will undergo apoptotic cell death (Hata *et al.*, 2015). The fact that the copper complexes did not stimulate Bcl-2 expression, with the exception of JD88 in HL-60 cells, suggest that the increased expression of pro-apoptotic Bax and Bad (Table 3.8) drove cells toward apoptosis since Bax homodimerisation was favoured rather than heterodimerisation.

3.4.4 Test compounds increase Smac/DIABLO and HTRA2/Omi protein expression

The proteome array data demonstrated that Smac/DIABLO was up-regulated by the copper-imidazo[1,2-*a*]pyridines in HL-60 and K562 cells (Table 3.9), especially by JD88.

Camptothecin caused no meaningful increase in Smac/DIABLO in either cell line but caused as 64% decrease in K562 cells. In peripheral leukocytes, Smac/DIABLO was also significantly increased, by the copper complexes but not camptothecin providing evidence for the initiation of normal apoptotic responses in the advent of cell stress (Table 3.9).

Table 3.9 Comparison of the changes in mitochondrial Smac/DIABLO and HTRA2/Omi expression in HL-60, K562 and leukocytes relative to untreated controls

Smac/DIABLO	HL-60	K562	Leukocytes	Comments
JD88	86%	31%	30%	Smac/DIABLO was up-regulated in all cells. Copper complexes increased Smac more than camptothecin
JD47R	19%	Nt	22%	
JD49R	Nt	4%	Nt	
Camptothecin	17%	-9%	Nt	
HTRA2/Omi	HL-60	K562	leukocytes	Comments
JD88	80%	-6%	-8%	Copper complexes increased HTRA2/Omi in only HL-60 cells. Camptothecin caused a decrease in HL-60 and K562 cells
JD47R	59%	Nt	-24%	
JD49R	Nt	-6%	Nt	
Camptothecin	-15%	-64%	Nt	

*Nt-compound not tested in these cells

The baseline expression of the serine protease HTRA2/Omi was moderate to high in HL-60 and K562 control cells and normal peripheral leukocytes. The copper-imidazo[1,2-*a*]pyridines significantly increased HTRA2/Omi expression in HL-60 cells, while no meaningful changes were observed in K562 and leukocytes. Camptothecin showed no

meaningful changes in HL-60 cells either but did significantly suppress HTRA2/Omi in K562 cells.

HTRA2/Omi is located in the intermembrane space of mitochondria, and is released to the cytosol in response to cell stress where it binds to and inhibits XIAP, promoting apoptosis (Fulda, 2014). Despite the increase in XIAP by JD88, the increase in HTRA2/Omi may together with Smac/DIABLO counteract its anti-apoptotic effect. The activation of caspase-9 in response to JD88 (Chapter 2; section 2.3.9) provides indirect evidence for this assumption. HTRA2/Omi directly cleaves cytoskeletal proteins and is essential in maintaining mitochondrial integrity (Yamauchi *et al.*, 2014). The data obtained provides additional evidence for the pro-apoptotic effects of HTRA2/Omi in cancer cells.

3.4.5 Copper-imidazo[1,2-*a*]pyridines suppress XIAP and cIAP-1

The baseline expression of XIAP was moderately high in HL-60 and K562 control cells and low-moderate in normal peripheral leukocytes. In HL-60 cells, JD88 significantly increased XIAP while JD47R showed a decrease which was considered as insignificant. The copper-imidazo[1,2-*a*]pyridines were able to suppress XIAP protein levels in K562 cells and leukocytes (Table 3.10). In comparison to the copper complexes, camptothecin suppressed XIAP protein levels in both cell lines. The decreased expression of XIAP prevents its normal function as inhibitor both the extrinsic and intrinsic apoptotic pathways (Rathore *et al.*, 2017).

The baseline expression of cIAP-1 and cIAP-2 in HL-60 cells and normal peripheral leukocytes were low. The cIAP-1 protein was better expressed than cIAP-2 in K562 cells. Expression and function of IAPs (XIAP as well as cIAP-1 and cIAP-2) are altered, due to genetic aberrations, increased mRNA or protein levels, or loss of their endogenous inhibitors in various cancers. These abnormalities are associated with poor prognosis and chemo-resistance in AML, CML, T-ALL and CLL (Helgason *et al.*, 2011; Baig *et al.*, 2016).

Table 3.10 Comparison of the changes in expression of select IAP proteins in HL-60, K562 and leukocytes relative to untreated controls

XIAP	HL-60	K562	leukocytes	Comments
JD88	29%	-26%	-8%	XIAP expression was suppressed by all compounds with the exception of JD88, which caused an increase in HL-60 cells
JD47R	-8%	Nt	-26%	
JD49R	Nt	-26%	Nt	
Camptothecin	-28%	-73%	Nt	
cIAP-1	HL-60	K562	leukocytes	Comments
JD88	1%	-41%	7%	cIAP-1 expression was significantly suppressed in K562 cells. No meaningful changes were observed in the other cells
JD47R	7%	Nt	-15%	
JD49R	Nt	-30%	Nt	
Camptothecin	13%	-90%	Nt	
cIAP-2	HL-60	K562	leukocytes	Comments
JD88	7%	-13%	-10%	cIAP-2 was poorly expressed and no significant changes in response to treatment were observed
JD47R	4%	Nt	-23%	
JD49R	Nt	-7%	Nt	
Camptothecin	12%	-15%	Nt	
Survivin	HL-60	K562	leukocytes	Comments
JD88	33%	28%	-21%	Survivin expression was suppressed by all treatments with the exception of JD88 which increased it in HL-60 and K562 cells.
JD47R	-26%	Nt	-29%	
JD49R	Nt	9%	Nt	
Camptothecin	-32%	-87%	Nt	
Livin	HL-60	K562	leukocytes	Comments
JD88	-5%	-1%	-15%	Livin expression was suppressed by all treatments in all cells however these changes were not meaningful
JD47R	-12%	Nt	-10%	
JD49R	Nt	-4%	Nt	
Camptothecin	-14%	-12%	Nt	

*Nt-compound not tested in these cells

XIAP is the most potent inhibitor among the IAP protein family and directly binds and inhibits caspase-3 and caspase-9 via its characteristic BIR domain (Zumbrägel *et al.*, 2017). Among the two copper complexes, JD88 increased the expression of XIAP in HL-60 cells which suggest that increased XIAP is a response mechanism to protect against apoptosis from JD88 treatment. However, Smac/DIABLO levels were increased following JD88 treatment (Table 3.13). Smac/DIABLO is XIAP's main antagonist, inhibiting XIAP's inhibitory function and allowing apoptosis to proceed (Fulda, 2014). In addition, Smac/DIABLO binds to cIAP-1 and cIAP-2 and decrease their function are other IAPs that binds to.

XIAP levels were decreased in response to JD47R and camptothecin while there is an anomaly with JD88 causing an increase in XIAP levels. However, the copper complexes increased Smac levels which restored the XIAP/Smac balance re-sensitised HL-60 cells to apoptosis. In K562 cells, as XIAP levels decreased in response to JD88, JD49R and camptothecin, Smac levels increased and together these changes pushed cells in a more pro-apoptotic state. In addition, cIAP-1 was suppressed by the copper-imidazo[1,2-*a*]pyridines and camptothecin contributing to the induction of apoptosis. The copper-imidazo[1,2-*a*]pyridines show their ability to overcome XIAP-mediated caspase inhibition by inducing intrinsic apoptosis (Chapter 2; section 2.3.6 and 2.3.9). Similarly to this study, Harmse *et al.*, (2019), reported on a significant decrease in XIAP and cIAP-1 protein expression in HT-29 cells after treatment with three copper-imidazo[1,2-*a*]pyridines JD88, JD46R and JD47R (Harmse *et al.*, 2019).

XIAP bind and directly suppress caspase catalytic activity. Like cIAP-1 and cIAP-2, XIAP also functions as E3 ubiquitin ligases. Extrinsic apoptosis mediated via death-receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, is thus inhibited by cIAPs (Fulda, 2014; Morotti *et al.*, 2017). When Smac/DIABLO binds to cIAP-1 and cIAP-2, they undergo auto-ubiquitination with subsequent proteosomal degradation resulting in the activation of caspase-8 found in this receptor complex. XIAP and cIAP-1 have been shown to undergo specific and functional cleavage by caspases to ensure the induction of apoptosis (Hörnle *et al.*, 2011; Vince *et al.*, 2018). cIAP-1 and cIAP-2 expression is transcriptionally regulated by the canonical and non-canonical NF- κ B pathways (Xia *et al.*, 2014).

The overexpression of survivin is considered a marker for poor patient prognosis in many cancers including AML (Chen *et al.*, 2016; Crivellaro *et al.*, 2016; Garg *et al.*, 2016). The expression levels of survivin was moderate in HL-60 and K562 control cell lines and the effect of the copper-imidazo[1,2-*a*]pyridines on its expression varied. In peripheral leukocytes, baseline expression of survivin was low and the copper-imidazo[1,2-*a*]pyridines suppressed its protein expression. Several *in vitro* studies have indicated that the down-regulation of survivin sensitizes cancer cells to chemotherapeutic agents (Li, 2013; Suzuki *et al.*, 2019). In the current study, survivin expression levels by itself did not appear to influence the response to the copper complexes.

Survivin is another member of the IAP protein family which is induced at a transcriptional level by NF- κ B, and can be activated indirectly by the PI3K/Akt pathway (Fulda, 2014).

Survivin is known to promote cell survival (Cheung *et al.*, 2013) and mitotic progression (Kitagawa and Lee, 2015). The anti-apoptotic effect of survivin is attributed to a direct interaction with XIAP where they synergistically inhibit caspase-9 and enhance the stability of XIAP by preventing auto-ubiquitination (Chandele *et al.*, 2004; Garg *et al.*, 2016). Survivin also indirectly inhibits caspase-3/7 through its interaction with Smac/DIABLO from blocking XIAP action (Zumbrägel *et al.*, 2017).

The increased expression of survivin in HL-60 and K562 cells by JD88 is not likely to compromise the inhibitory effect of Smac/DIABLO on XIAP. In contrast to JD88, JD47R decreased survivin expression in HL-60 cells. The transcription of survivin is not exclusively controlled by NF- κ B (Angileri *et al.*, 2008) but also by STAT and Kruppel like factor 5, (Gritsko *et al.*, 2006; Zhu *et al.*, 2006).

In HL-60 cells, K562 control cells and in peripheral leukocytes, the baseline expression of livin was very low. The copper complexes and camptothecin caused no significant changes to livin expression. The low levels of livin indicated that NF- κ B is not very active in the cells, This observation provided further evidence for the suppression of NF- κ B mediated transcription by the copper-imidazo[1,2-*a*]pyridines. Livin is another IAP member that binds and prevents Smac/DIABLO, caspase-3, caspase-7 and caspase-9, from carrying out their pro-apoptotic function (Fulda, 2014). The overexpression of livin is associated with an increase in NF- κ B activity (Rathore *et al.*, 2017).

The NF- κ B family of transcriptional activators consists of five separate proteins; NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65) and c-Rel, RelB. They are homologous proteins that contain a dimerization domain, a DNA binding domain and a nuclear localization signal (Barre *et al.*, 2010). NF- κ B is a homo- or heterodimeric complex that is formed by the Rel-like domain-containing proteins. When dimerized they bind at kappa-B sites in DNA and stimulate the transcription of pro-survival genes (Park and Hong, 2016; Morotti *et al.*, 2017) encoding for XIAP, cIAP-1, cIAP-2 as well as Bcl-2 and Bcl-x (Rathore *et al.*, 2017). NF- κ B thus ensures cancer cell survival (Fulda, 2014).

The overexpression of NF- κ B has been demonstrated in several cancer cell lines originating from leukaemia (Crivellaro *et al.*, 2015; Karabay *et al.*, 2018), lymphoma (Nagel *et al.*, 2014), multiple myeloma (Vrábel *et al.*, 2019), colorectal cancer (Slattery *et al.*, 2018), ovarian (Harrington and Annunziata, 2019) and breast cancers (Wang *et al.*, 2015a). Studies

investigated the molecular mechanisms which contribute to the increased activity of NF- κ B observed in AML cells which includes chromosomal mutations and gene translocations causing abnormal myeloid formation and NF- κ B activation (Kojima *et al.*, 2016). NF- κ B is a pleiotropic transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by various stimuli related to inflammation, immunity, differentiation, cell growth, tumourigenesis and apoptosis (Fulda, 2014). A proteomic study by Karabay *et al.*, (2018) demonstrated the constitutive activation of NF- κ B in CML, and its association with cell survival. In addition, the increased activity of NF- κ B prevented apoptosis in response to chemotherapy thus displaying its role in drug resistance (Karabay *et al.*, 2018).

The observed suppression of cIAP-1 protein levels indicate that the copper-imidazo[1,2-*a*]pyridines may inhibit IKK and subsequently decrease the transcriptional activity of the canonical NF- κ B pathway. Therefore, the associated meaningful decrease of XIAP, cIAP-1, Bcl-2 and Bcl-x by the copper complexes, with exception to the increase by JD88 in HL-60 cells, may be attributed to the decreased transcriptional activity of the canonical NF- κ B pathway.

CIAP proteins have both stimulatory and inhibitory functions in the regulation of NF- κ B signalling. They regulate the non-canonical NF- κ B pathway by acting as ubiquitin ligases. CIAP-1 and cIAP-2 cause ubiquitination and proteasomal degradation of NF- κ B inducing kinase (NIK) and thereby prevent NIK-mediated activation of RelB and p52 (Odqvist *et al.*, 2013). TNF-receptor signalling disrupts the NIK regulatory function and causes NIK to accumulate in the cytoplasm to levels where it promotes the phosphorylation and activation of IKK α . IKK α activity mediates the phosphorylation of p100, which targets the protein for processing by the proteasome, resulting in p52 production.

The low and absent expression of both cIAPs provides indirect evidence for the inhibition of the self-perpetuating activation loop of NF- κ B-mediated transcription, thereby favouring the entry of the cells into apoptosis. As a result, the copper-imidazo[1,2-*a*]pyridines are useful for their ability to suppress IAP expression with the exception of JD88 on XIAP expression in HL-60 cells.

3.4.6 Test compounds increase death receptor protein expression

The TNF death receptor superfamily proteins; Fas, TRAIL-R1/DR4, TRAIL-R2/DR5 and FADD were poorly expressed in HL-60 and K562 control cells whilst moderately expressed in peripheral leukocytes. Abnormal expression and down-regulation of death receptor proteins have been associated with several cancers highlighting their contribution to cancer cell survival (Fulda, 2014). In AML, expression levels of Fas, TRAIL-R1/DR4 and TRAIL-R2/DR5 death receptors as well as cytosolic FADD adaptor protein are low (Naimi *et al.*, 2018). Death receptors are mostly activated by specific ligands rather than small molecules. This contributes to the poor prognosis of AML, and resistance to death receptor-mediated apoptosis (Tzifi *et al.*, 2012; Saraei *et al.*, 2018).

Table 3.11 Comparison of changes in the expression of death receptors in HL-60, K562 and leukocytes relative to untreated controls

TRAIL-R1	HL-60	K562	leukocytes	Comments
JD88	16%	11%	-44%	Treatments caused poor increases in HL-60 and K562 cells while significantly decreasing expression in leukocytes. Camptothecin decreased TRAIL-R1 in K562 cells.
JD47R	19%	Nt	-50%	
JD49R	Nt	58%	Nt	
Camptothecin	18%	-86%	Nt	
TRAIL-R2	HL-60	K562	leukocytes	Comments
JD88	18%	295%	-43%	Treatments caused highly significant increases in K562 cells but not in HL-60 cells.
JD47R	2%	Nt	-31%	
JD49R	Nt	176%	Nt	
Camptothecin	14%	17%	Nt	
FADD	HL-60	K562	leukocytes	Comments
JD88	55%	15%	-28%	JD88 caused a significant increase in HL-60 cells and no meaningful change in K562 cells. Camptothecin suppressed expression in K562 cells. Copper complexes suppressed leukocyte expression.
JD47R	4%	Nt	-40%	
JD49R	Nt	8%	Nt	
Camptothecin	2%	-68%	Nt	
Fas/TNFRSF6/CD95	HL-60	K562	leukocytes	Comments
JD88	120%	9%	-17%	Copper complexes increase Fas expression in HL-60 cells but not in K562 cells.
JD47R	10%	Nt	2%	
JD49R	Nt	6%	Nt	
Camptothecin	55%	-10%	Nt	
TNF-R1/TNFRSF1A	HL-60	K562	leukocytes	Comments
JD88	14%	-3%	Nt	JD88 caused a significant increase (14%) in TNF-R1/TNFRSF1A in HL-60 cells but not in K562 cells or leukocytes.
JD47R	1%	Nt	-10%	
JD49R	Nt	8%	Nt	
Camptothecin	10%	-54%	Nt	

*Nt-compound not tested in these cells

In HL-60 cells, aside from the increase in FADD and Fas expression by JD88, no meaningful changes were induced in death receptor protein expression (Table 3.11). In K562 cells, JD88 increased the expression of TRAIL-R2 while JD49R increased the expression of TRAIL-R1 and TRAIL-R2. Expression of FADD, Fas and TNF were not significantly changed by the copper complexes or camptothecin. The expression of TRAIL-R1, TRAIL-R2 and FADD were significantly decreased by the copper complexes while the changes in the expression of Fas and TNF-R1 were not significant.

Death receptors transduce external apoptotic signals and an increase in their expression suggests activation of the extrinsic apoptotic pathway (Fulda and Debatin, 2006). This occurs via the formation of TNF-R1 and TRAIL associated cytoplasmic complex RIP1/FADD/Caspase-8 which drives caspase-8 activation and apoptosis (Chu, 2013). In K562 cells, the expression of poorly expressed TRAIL-R2 was significantly increased by JD49R which is in contrast to TRAIL-R1 (Table 3.11). TRAIL-R2 facilitates Smac/DIABLO mediated apoptosis (Eckhardt *et al.*, 2013). The increased expression of Smac/DIABLO activates death receptor/ligand systems like TNF- α /TNF-R1 or TRAIL-R2 via NF- κ B which in turn promote the assembly of the RIP1/FADD/Caspase-8 cytosolic cell death complex (Eckhardt *et al.*, 2013). The significant increase in TRAIL-R2 expression may be a survival response to cell stress. The significance of the increase in TRAIL-R2 requires further investigation.

JD88 caused no meaningful change to TNF-R1 expression in HL-60 cells, contributing to decreased NF- κ B transcriptional activity and despite increasing FADD adaptor protein levels, caspase-8 activity was poorly detected (Chapter 2; section 2.3.11). While, the copper complexes failed to induce caspase-8 activity in K562 cells (Chapter 2; section 2.3.11) since no meaningful changes in TNF-R1 or FADD expression were induced. These findings suggest that the copper complexes may have inhibited NIK and subsequently prevented the activation of the non-canonical NF- κ B pathway (Odqvist *et al.*, 2013). Future studies will include assays on NIK activity and transcription (CHIP assay and NIK kinase assay) to confirm whether NIK is being targeted by these copper complexes.

Despite failing to induce substantial caspase-8 activity, the increased expression of FADD and Fas as well as TRAIL by the copper-imidazo[1,2-*a*]pyridines implies that these increases may have occurred in response to cell stress and not necessarily be related to the activation of apoptosis.

3.4.7 Copper-imidazo[1,2-*a*]pyridines have minimal effect on phosphorylated p53

The baseline expression of the three forms of phosphorylated p53 was low in HL-60 and K562 cells and moderate in normal peripheral leukocytes. Of the three phosphorylated p53 species, phospho-p53 (S15) was significantly increased by JD88 in HL-60 cells. In response to the copper-imidazo[1,2-*a*]pyridines and camptothecin, no meaningful changes were observed in either of the cell lines. This may be due to the less frequent occurrence of p53 mutations in haematological cancers (Peller and Rotter, 2003). Approximately 50% of cancers express wtp53 which is inactivated by the deregulation of its regulatory proteins, whereas the other 50% have a p53 mutation or deletion (Yogosawa and Yoshida, 2018). As previously mentioned, the HL-60 and K562 cell lines encode for non-functional p53 proteins (Crivellaro *et al.*, 2015; Crivellaro *et al.*, 2016).

Table 3.12 Comparison of the changes phosphorylated p53 expression in HL-60, K562 and leukocytes relative to untreated controls

Phospho-p53 (S15)	HL-60	K562	leukocytes	Comments
JD88	52%	-7%	-48%	JD88 significantly increased phospho-p53 (S15) in HL-60 cells. In leukocytes, JD88 and JD47R suppressed its expression
JD47R	0%	Nt	-51%	
JD49R	Nt	-5%	Nt	
Camptothecin	23%	-14%	Nt	
Phospho-p53 (S46)	HL-60	K562	leukocytes	Comments
JD88	4%	14%	-48%	Phospho-p53 (S46) was suppressed in K562 cells by JD49R and camptothecin. In leukocytes, both JD88 and JD47R suppressed its expression
JD47R	4%	Nt	-27%	
JD49R	Nt	-25%	Nt	
Camptothecin	0%	-45%	Nt	
Phospho-p53 (S392)	HL-60	K562	leukocytes	Comments
JD88	12%	-5%	-10%	No meaningful changes of phospho-p53 (S392) were induced by the treatments
JD47R	1%	Nt	17%	
JD49R	Nt	18%	Nt	
Camptothecin	4%	0%	Nt	

*Nt-compound not tested in these cells

The tumour suppressor p53 is generally expressed at low levels in normal cells with a short half-life due to its negative regulation by ubiquitin ligases MDM2 and carboxy-terminus of Hsp70-interacting protein (CHIP) (Yue *et al.*, 2017). Nuclear localization of p53 causes the transcription of sequence-specific target genes such as p21 and Bax which induce apoptosis and cell cycle arrest (Crivellaro *et al.*, 2016). When activated, wtp53 undergoes post-translational modifications which result in p53 stabilization and evasion of MDM2

proteasome-mediated degradation (Hientz *et al.*, 2017). This p53/MDM2 degradation loop is not functional in cancers harbouring mutated p53 as MDM2 transcription is prevented (Zhang and Lozano, 2017).

The function of tumour suppressor p53 can be affected by changes in protein levels, changes in cellular compartmentalization and by post-translational modifications, such as phosphorylation, acetylation and ubiquitination (Crivellaro *et al.*, 2016). In response to stress, p53 is phosphorylated at serine sites by different kinases which have been specifically associated with the ability of p53 to induce an apoptotic response. The frameshift mutations cause an abnormal p53 protein that is not functional in HL-60 and K562 cells which contributes to the suppression of p53 transcription and cellular repair capacity.

Serine 15 is phosphorylated by the ataxia-telangiectasia mutated (ATM) kinases. Genotoxic stress, radiation or DNA damage signalling could account for the stabilisation of mutant p53 by phosphorylation at serine 15. Serine 46 is phosphorylated by multiple kinases including ATM, homeodomain interacting protein kinase 2 (HIPK2) and dual-specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) (Smeenk *et al.*, 2011). Serine 392 is phosphorylated by cyclin-dependent kinase 9 (CDK9) (Bagashev *et al.*, 2013).

The *BCR-ABL* fusion gene which characterises CML, stabilizes an I κ B α /p53 complex which is responsible for the sequestration of p53 in the cytoplasm of CML cells (Crivellaro *et al.*, 2015). I κ B α is a NF- κ B inhibitor which can bind either NF- κ B or p53 and this complex prevents p53-mediated apoptosis activity (Morotti *et al.*, 2017). Taken together, the overexpression of MDM2, the mutated p53 gene and the *BCR-ABL* fusion gene in K562 cells cause a loss in the transcriptional activity of wtp53 target genes and increases transcriptional activity of genes that drive cancer cell proliferation, cancer cell survival, and chemo-resistance (Yue *et al.*, 2017).

MDM2-mediated p53 inhibition is counteracted when the ATM kinase along with HIPK2 phosphorylates p53 at serine site 15 and 46. JD88 significantly increased the presence of phospho-p53 (S15) in HL-60 cells, causing an increase in stability of mutated p53, implying an increase of ATM kinase activity. Phospho-p53 (S46) particularly restores the pro-apoptotic tumour suppressor transcription function of p53 which ensues the induction of apoptogenic factors such as Bax, Noxa, Puma and TRAIL in response to chemotherapy (Zhang and Lozano, 2017). In response to DNA damage, several studies have indicated that

phospho-p53 (S46) is necessary to regulate apoptosis (Yogosawa and Yoshida, 2018). The copper-imidazo[1,2-*a*]pyridine JD88 caused a marginal increase in phosphorylated p53 (S46) levels in both cell lines, indicating some ATM and HIPK2 kinase activity (Smeenk *et al.*, 2011). The overexpression of p53 phosphorylated at serine site 392 has been linked with the poor prognosis of advanced tumours (Bagashev *et al.*, 2013). In contrast, phospho-p53 (S392) was not detected in either cell line. These data imply that the loss of p53 pro-apoptotic activity in HL-60 and K562 cells might be due to the lack of p53 phosphorylation particularly at serine 46. This lack of phosphorylation is possibly due to the inhibition of the kinases responsible for their modification/phosphorylation.

In comparison to our findings, Harmse *et al.*, (2019) demonstrated highly significant decreases in all three phosphorylated forms of p53 when treated with copper-imidazo[1,2-*a*]pyridines JD88 and JD47R in colorectal HT-29 cells. HT-29 cells harbour a gain of function (GOF) mutation, R273H which causes p53 to accumulate in the cells (Yue *et al.*, 2017). This explains the high expression of the phosphorylated forms of p53 in HT-29 colorectal cells in comparison to leukaemic cells.

3.4.8 Copper complexes cause differential changes on the expression of HSPs

The baseline expressions of all three heat shock proteins (HSP27, HSP60 and HSP70) were low in HL-60 and K562 control cells and moderate in normal peripheral leukocytes. The copper-imidazo[1,2-*a*]pyridines showed differential effects on HSP expression in all cells. In HL-60 cells, the increase of phospho-p53 (S15) by JD88 correlates to the increase in HSP expression which stabilizes p53.

Heat shock proteins are molecular chaperones whose expression is increased under stressful conditions such as hypoxic environments, oxidative stress and high temperatures (Chatterjee and Burns, 2017). HSPs are classified according to their molecular weight. For instance, the HSP27 molecule is 27 kDa in size. HSPs promote p53 function by binding and stabilizing p53. They also protect against p53 degradation by inhibiting constitutive MDM2 and CHIP E3 ligase activity (Li *et al.*, 2011). p53 stimulates heat shock factor-1 (HSF-1), a heat shock protein transcription factor.

Table 3.13 Comparison of changes in the expression of HSPs in HL-60, K562 and leukocytes relative to untreated controls

HSP27	HL-60	K562	leukocytes	Comments
JD88	6%	23%	-51%	Treatments caused no meaningful change in HL-60 cells, differential changes in K562 cells and significant decreases in leukocytes
JD47R	8%	Nt	-49%	
JD49R	Nt	-15%	Nt	
Camptothecin	11%	-26%	Nt	
HSP60	HL-60	K562	leukocytes	Comments
JD88	57%	-1%	18%	Treatments increased HSP60 in HL-60 cells and decreased it in K562 cells. No meaningful changes in leukocytes
JD47R	33%	Nt	-6%	
JD49R	Nt	-32%	Nt	
Camptothecin	153%	-41%	Nt	
HSP70	HL-60	K562	leukocytes	Comments
JD88	47%	-2%	-29%	Treatments increased HSP70 in HL-60 cells, decreases in K562 cells and leukocytes
JD47R	0%	Nt	-21%	
JD49R	Nt	-44%	Nt	
Camptothecin	37%	-52%	Nt	
Clusterin	HL-60	K562	leukocytes	Comments
JD88	93%	12%	-66%	Copper complexes increased clusterin in HL-60 cells, with no meaningful changes in K562 cells and decreases in leukocytes. Camptothecin caused a significant increase in HL-60 cells but not in K562 cells
JD47R	61%	Nt	-73%	
JD49R	Nt	18%	Nt	
Camptothecin	46%	-15%	Nt	

*Nt-compound not tested in these cells

Anti-apoptotic HSP27 and HSP70 (Garrido *et al.*, 2006) are known to be overexpressed in many cancer cells and this contributes to the development of resistance to chemotherapy and the evasion of apoptosis (Ma *et al.*, 2013; Kennedy *et al.*, 2014; Guo *et al.*, 2015; Calderwood and Gong, 2016).

HSP27 and HSP70 are anti-apoptotic since they promote cell survival (Li *et al.*, 2000; Schmitt *et al.*, 2003; Acunzo *et al.*, 2012). HSP27 inhibits both the intrinsic and extrinsic apoptotic pathways. It inhibits caspase-3 thus contributing to cell survival. It inhibits Bax by binding directly to the death domain associated protein which enhances Akt activity by inhibiting Bid or protein kinase C (PKC). HSP27 is also responsible for the proteasomal degradation of I κ B α thereby promoting NF- κ B transcription activity. The ability of the copper-imidazo[1,2-*a*]pyridines to suppress HSP expression in K562 cells suggests their

potential in driving cells to apoptotic cell death by overcoming drug resistance. This is consistent with a previous study by Zhu *et al.*, (2010) who demonstrated how silencing HSP27 in squamous cells resulted in apoptotic cell death by caspase-3 activation, thereby validating the anti-apoptotic function of HSP27 when expressed.

HSP70 particularly inhibits Apaf-1 from binding and forming the apoptosome complex with pro-caspase-9, thereby inhibiting intrinsic apoptosis (reviewed in (Wang *et al.*, 2014)). Another study demonstrates the potential of HSP70 inhibitors which were able to reduce the risk of drug resistance (Lazarev *et al.*, 2018). HSP70 inhibitors efficiently suppress drug resistance development in leukaemic cells (Pocaly *et al.*, 2007). The function of HSP60 is unclear. It is considered pro-apoptotic for its ability to cleave pro-caspase-3, and anti-apoptotic because its overexpression promotes cell survival rather than programmed cell death (Caruso Bavisotto *et al.*, 2017).

HSP27 binds to wtp53 causing its inactivation, whilst HSP70 stabilises mutant p53 promoting cancer cell growth (Wiech *et al.*, 2012). The observed increases of HSP expression in this study, particularly in HL-60 cells, showed no correlation with the poor expression of phosphorylated-p53 species, implying and confirming a non-functional p53 tumour suppressor protein in the cells investigated (Crivellaro *et al.*, 2016).

Clusterin is a cytoprotective protein which promotes cell survival in response to cell stress much like heat shock proteins and HO-1/HMOX1/HSP32 (Zoubeidi *et al.*, 2010). In this study, the baseline expression of clusterin was low in leukaemic HL-60 and K562 control cells and high in normal peripheral leukocytes. The overexpression of clusterin contributes to cell survival by evading apoptosis, cell proliferation, and invasion observed in cancer cells (Wang *et al.*, 2015b).

A study by Park *et al.*, (2008) demonstrated the up-regulation of clusterin by anti-cancer therapies conferring treatment resistance. Similarly to Park *et al.*, (2008), the copper-imidazo[1,2-*a*]pyridines in this study had increased the expression of clusterin in leukaemic cells while significantly decreasing it in leukocytes (Table 3.13). These varying observations imply that clusterin expression appears to be induced in response to drug treatment in specific malignant cells and not healthy cells. In a study by Wang *et al.*, (2015b) the silencing of clusterin was shown to induce apoptosis and inhibit HL-60 cell proliferation *in vitro* (Wang *et*

al., 2015b). Clusterin may be an effective approach to reverse resistance to treatment-induced apoptosis and the regulation of cancer.

3.4.9 Cell cycle regulatory proteins

The copper-imidazo[1,2-*a*]pyridines significantly increased the expression of the p21^{Cip1} in K562 cells, while causing no meaningful changes in HL-60 cells or peripheral leukocytes. The baseline expression of p21^{Cip1} was low in K562 control cells since the interaction of *BCR-ABL* with IκBα does not support the transcription of wtp53 target genes (Crivellaro *et al.*, 2016).

Table 3.14 Comparison of changes in the expression of cell regulatory proteins in HL-60, K562 and leukocytes relative to untreated controls

p21 ^{Cip1/CDKN1A}	HL-60	K562	leukocytes	Comments
JD88	6%	104%	1%	p21 was significantly increased in K562 cells by JD88, JD47R and camptothecin while no meaningful changes were observed in the other cells
JD47R	0%	Nt	17%	
JD49R	Nt	94%	Nt	
Camptothecin	17%	36%	Nt	
p27 ^{Kip1}	HL-60	K562	leukocytes	Comments
JD88	19%	10%	137%	p27 was significantly increased in leukocytes by the copper complexes
JD47R	1%	Nt	112%	
JD49R	Nt	12%	Nt	
Camptothecin	9%	-15%	Nt	
Claspin	HL-60	K562	leukocytes	Comments
JD88	81%	-9%	-12%	Claspin was significantly increased in HL-60 cells by JD88 while camptothecin decreased its expression. Camptothecin also decreased claspin levels in K562 cells.
JD47R	14%	Nt	-2%	
JD49R	Nt	-15%	Nt	
Camptothecin	-67%	-93%	Nt	

*Nt-compound not tested in these cells

Wild type p53 induces the transcription of p21^{Cip1} protein which is considered an important cell cycle regulator for promoting G₂/M phase cell cycle arrest by inhibiting CDK2 (Zhang and Lozano, 2017). In CML cells, the expression of p21^{Cip1} is decreased and following chemotherapy, p21 is restored (Quintas-Cardama *et al.*, 2017). Therefore, the increase in p21^{Cip1} protein expression in the K562 cells indicates the possible restoration of p53 transcriptional function (Table 3.14)

The baseline expression of p27 in HL-60 control cells was extremely low with no meaningful effect observed by the copper-imidazo[1,2-*a*]pyridines. The baseline expression levels of p27 in K562 control cells were low, and the copper-imidazo[1,2-*a*]pyridines caused further suppression. In contrast to the leukaemic cells, the expression level of p27 was significantly increased in leukocytes providing evidence for the stimulation of its tumour suppressor function. The high expression of p27 indicates cells with reduced growth rates and also enhanced protection against cytotoxic agents (Drexler and Pebler, 2003).

The tumour suppressor, p27, is defined as a CDK inhibitor important for regulating the cell cycle (James *et al.*, 2008). In normal cells, p27 is predominantly localised in the nucleus and in CML it is in the cytoplasm (Sharma and Pledger, 2016). The *BCR-ABL* fusion gene in CML has been reported to promote a GOF-mutation of cytoplasmic p27 (Agarwal *et al.*, 2014). Mutant p27 causes leukaemogenesis over wtp27 validating the tumour suppressor function of nuclear p27 in CML. BCR-ABL oncoprotein disrupts p27 transcription and expression and promotes the degradation of nuclear p27 (Agarwal *et al.*, 2008). In the pathogenesis of CML, nuclear p27 acts as a tumour suppressor supporting cancer cell growth arrest and apoptosis whilst cytoplasmic p27 acts as an oncogene (Crivellaro *et al.*, 2016).

The cell cycle regulatory protein, claspin, is expressed in response to DNA damage. During DNA replication and damage, claspin regulates ATR-dependent phosphorylation of checkpoint protein kinase-1 (Chk-1), activates it and subsequently causes S-phase checkpoint arrest preventing cell cycle progression (Scorah and McGowan, 2009). It also plays an independent role in cell proliferation by regulating DNA replication. The overexpression of claspin cause cancer cell proliferation as it stimulates cell survival mechanisms.

The baseline expression of claspin was high in leukaemic control cells compared to normal peripheral leukocytes. The copper-imidazo[1,2-*a*]pyridines suppressed claspin expression with the exception of JD88 which increased its expression. The decreased expression most likely inhibited its cell survival function to some extent. Claspin is frequently overexpressed in cancer cells when compared to normal cells which correlate to the proteome data obtained in this study.

3.4.10 In Summary:

Proteomic analysis revealed that copper-imidazo[1,2-*a*]pyridines increased the expression of cleaved caspase-3 in both leukaemic cell lines confirming the induction of apoptosis. The expression of inhibitor of apoptosis proteins, XIAP, cIAP-1, livin, and the anti-apoptotic proteins, Bcl-2 and Bcl-x, was decreased while simultaneously increasing pro-apoptotic Bad and Bax expression. HO-1/HMOX-1/HSP32 expression was notably increased in HL-60 cells. Increased expression of TRAIL-R2/DR5 death receptor indicated the possible dual activation of both the extrinsic and intrinsic apoptotic pathways; however, caspase-8 activation could not be demonstrated.

CHAPTER FOUR: Human apoptosis PCR Arrays

4.1 INTRODUCTION

Malignancy is caused as a result of the accumulation of DNA mutations, either acquired or inherited (Brown *et al.*, 2019). Mutations cause an alteration of gene expression profiles, with an increase in proto-oncogene expression and the suppression of tumour suppressor genes (Baylin and Jones, 2011). This allows them to accumulate thus maintaining their cancer-causing effects such as primary tumour development and tissue invasion, followed by angiogenesis and metastasis (Hanahan and Weinberg, 2011). Molecular diagnostic tools may be used to distinguish cancer cells on the basis of the presence or absence of receptor proteins, cancer driver mutations, or oncogenic fusion rearrangements (Brown *et al.*, 2019). Mutations and oncogenes cause changes in protein structure and function and, change expression levels by changing the copy number (Beroukhi *et al.*, 2010).

4.1.1 Chromosomal translocations

Chromosomal translocations are common in haematological malignancies and lymphoid tumours. The molecular mechanisms associated to them are complex and poorly understood (Mitelman *et al.*, 2007). Chromosomal translocations may alter the original locations of proto-oncogenes, causing changes to gene products by the generation of oncogenic fusion proteins and by the overexpression of proto-oncogenes (Samudio *et al.*, 2010). For example, the first specific chromosomal translocation identified in human cancer was the Philadelphia translocation [t(9;22)], of CML. It causes the fusion of the *BCR* (for breakpoint recombination) and *ABL* (encodes tyrosine kinase) genes (Deininger *et al.*, 2000). The fusion *BCR-ABL* gene encodes for the constitutively active p210 and p190 Bcr/Abl tyrosine kinases which are associated in the pathogenesis of CML (Hantschel, 2012).

4.1.2 Oncogenes and tumour suppressor genes

Oncogenes are transcriptional modifiers which act as activator or repressor proteins where they ultimately alter normal cellular behaviour (Wang *et al.*, 2018). They are derived from proto-oncogenes which have been mutated (Hanahan and Weinberg, 2011; Jacob *et al.*, 2018). Deregulation of one or two proto-oncogenes are sufficient to trigger the transformation of a cell (Shortt and Johnstone, 2012). Transformation occurs through the

simultaneous stimulation of cell proliferation and inhibition of apoptosis (Shortt and Johnstone, 2012).

Oncogenes and tumour suppressor genes associated with leukaemia are listed in Table 4.1 (O'Neill *et al.*, 2016; Trino *et al.*, 2016). Oncogenes like *MYC* induce proliferation; however, intrinsic apoptotic mechanisms such as those induced by the MDM2/p53 pathway counteract the mitotic stimulus mediated by *MYC* and suppress transformation (McMahon, 2014).

Oncogenes such as *BCL2* (encodes for Bcl-2) are potent inhibitors of apoptosis but poor inducers of cell proliferation and are insufficient to drive tumour formation as a single oncogenic event. However, the combination of *MYC* and *BCL2* together suppress apoptosis and drive proliferation resulting in cellular transformation (Shortt and Johnstone, 2012).

The entry of cells into the cell cycle is a precisely controlled event. Cyclins, CDKs (Johnson and Shapiro, 2010) and, the tumour suppressors p53 and retinoblastoma (Rb) protein (McCubrey *et al.*, 2017), are the central control checkpoint regulators of the cell cycle. Any alteration in the expression level of these gene products may result in uncontrolled proliferation and replication resulting in cancer (Zhu *et al.*, 2015a).

Table 4.1 Tumour suppressor genes and oncogenes associated with leukaemia

Genes	Function	Associated cancer
<i>TP53</i>	Tumour suppressor gene encodes for p53 protein which induces apoptosis and cell cycle arrest	Expressed in breast, lung, colorectal, brain, liver, blood and other cancers
<i>MYC1</i>	Critical role in regulating cell growth, differentiation and apoptosis	Increased expression in leukaemia and breast, stomach and lung cancers
<i>BCL2</i>	Prevent apoptotic cell death.	Increased expression in small cell lung cancer, prostate cancer, diffuse large B-cell lymphoma, multiple myeloma, AML and follicular B-cell lymphoma
<i>MDM2</i>	Binds and inhibits <i>TP53</i> gene product p53 causing changes in expression of target genes	Increased expression in leukaemia, sarcomas (connective tissue cancers) and other cancers

Abbreviations: *TP53*; tumour suppressor protein 53, *MYC1*, myc regulator proto-oncogene 1; *BCL2*, cancer oncogene; *MDM2*, negative regulator.

4.1.3 Transcription factors and regulators of apoptosis

Transcription factors are proteins which regulate gene expression by either promoting or suppressing the transcription of genes. Deregulation of anti-apoptotic signalling includes the up-regulation of anti-apoptotic Bcl-2, the IAPs, the PI3K/Akt pathway, and/or NF- κ B pathways. The PI3K/Akt pathway is a strong pro-survival pathway that is over-activated by loss-of-function mutation of the PTEN tumour suppressor gene (Fruman and Rommel, 2014) or a gain-of-function mutation of *RAS*, *BCR-ABL* or genes encoding for the epidermal growth factor receptor (*EGFR*) (Donev *et al.*, 2011; Chakrabarty *et al.*, 2013). The gene regulation of p53, NF- κ B, Bcl-2 and IAP are further discussed.

4.1.4 Tumour suppressor and transcription factor p53

Mutations and gene silencing of the *TP53* gene are common defects in several human cancers (Candi *et al.*, 2014a; Hientz *et al.*, 2017). In the event that *TP53* is mutated or silenced, cancer cells are able to evade apoptosis and cell cycle arrest, resulting in the accumulation of cells carrying mutant genes that proliferate and contribute to the development of cancer (Yue *et al.*, 2017). Since p53 is a tetramer, single mutant subunits will affect its activity significantly. The HL-60 cell line is described as p53 null where the *TP53* gene is deleted, whilst the K562 cell line contains a mutation where *TP53* is inactivated via a single base pair insertion at nucleotide 136 (Crivellaro *et al.*, 2015; Crivellaro *et al.*, 2016). Both cell lines have non-functional p53 protein. Following cell stress, p53 is phosphorylated at multiple residues thereby modifying its function as a transcription factor. These mutations suppress p53 transcription and cellular repair capacity since the frameshift causes an abnormal protein that is not functional.

Mutated *TP53* cause the failure to do cell-cycle checks, which leads to an increase of cells harbouring mutations, which in turn, enhances cancer progression (Yue *et al.*, 2017).

Approximately 50% of human tumours show mutations of the *TP53* gene (Zhang and Lozano, 2017) with rates of about 10% in haematological malignancies (Peller and Rotter, 2003). The frequency of *TP53* mutations is highly variable depending on the type of cancer (Leroy *et al.*, 2014). In leukaemia and lymphoma, *TP53* mutations are found more frequently at relapse (Leroy *et al.*, 2014). The negative regulator of p53, MDM2 coded for by *MDM2*, is either increased and/or mutated in AML and CML (Chatterjee *et al.*, 2010; Kojima *et al.*, 2016).

The p53 protein (coded for by the *TP53* gene) is a transcription factor central to the induction of apoptosis in stressed cells. It suppresses tumour formation and provides protection against DNA damage by inducing cell cycle arrest, DNA repair, or apoptosis (McCubrey *et al.*, 2017). Upon activation triggered by signals like hypoxia or DNA damage, p53 increases transcription of genes that are involved in DNA repair, cell cycle arrest or apoptosis (Riley *et al.*, 2008). These include *BAX* (*Bax*), *PUMA* (p53-up-regulated modulator of apoptosis; *Puma*), *FAS* (Fas receptor), (Hientz *et al.*, 2017), *BID* (*Bid*), *CASP1* (caspase-1), *CASP6* (caspase-6), *CASP10* (caspase-10), *CDKN1A* (cyclin-dependent kinase inhibitor 1A, p21^{Cip1}), *GADD45A* (growth arrest and DNA damage inducible alpha), *TNFRSF10B* (TRAIL-R2), *RB1* (Rb protein), *MDM2* (E3 ubiquitin-protein ligase MDM2), *EGFR* (epidermal growth factor receptor), *PCNA* (proliferating cell nuclear antigen), *PTEN* (phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase) and *TP63*, and *TP73* (TP53-related genes). In some instances, p53 represses the expression of certain genes like *CDC25* (cell division cycle 25 phosphatase), *HSP70* (heat shock protein 70 kDa), *HSP90* (heat shock protein 90kDa) and *BIRC5* (survivin) (Riley *et al.*, 2008).

Cell cycle progression is blocked by p53 by inducing the expression of p21^{Cip1} which blocks the activity of cyclin D/ CDK4 and subsequently the hyper-phosphorylation of the Rb protein (Zhu *et al.*, 2015a). When Rb is not phosphorylated, E2F-mediated gene transcription and cell cycle progression at the G₁ phase is inhibited (Cao *et al.*, 2004). E2 promoter binding factor (E2F) is a family of transcription factors that play a role in the regulation of the cell cycle and cell proliferation (Poppy Roworth *et al.*, 2015).

Death receptor pathways are inactivated in several cancers like leukaemias, hepatocellular carcinomas, neoplastic colon epithelium and melanomas by mutations and deletions. Mutations and deletions of the p53 target genes *FAS*, *APAF1*, *TNFRSF10A* and *TNFRSF10B* coding for Fas, Apaf-1, TRAIL-R1 and TRAIL-R2, respectively, have been reported to contribute to chemo-resistance (Jourdan *et al.*, 2009; Candi *et al.*, 2014b). The frequent deletion of the chromosomal region 8p21-22 in multiple myeloma prevents the transcription of TRAIL-R2 coded for by *TNFRSF10B* gene and increases anti-apoptotic Bcl-2, NF-κB and decoy receptor TRAIL-R4 mRNA levels (Duru *et al.*, 2015). The decreased expression of the *APAF1* gene has been proposed as a mechanism to escape intrinsic apoptosis (Tsujimoto *et al.*, 2005). The loss of expression of these p53 target genes contribute to the ability of cancer cells to evade apoptosis thereby facilitating metastasis.

4.1.5 NF- κ B transcription factors and gene expression

NF- κ B is an inducible transcription factor family that plays an important role in inflammation, autoimmune response, cell proliferation, and apoptosis by regulating the expression of genes in all of these processes (Liu *et al.*, 2017b). When activated, NF- κ B targets inflammation by directly increasing the transcription of inflammatory cytokines, chemokines and adhesion molecules (Feltham and Silke, 2017). Activated NF- κ B stimulates the transcription of anti-apoptotic genes both in the intrinsic and extrinsic pathways (Yang *et al.*, 2016; Witt and Vucic, 2017).

Under normal conditions, NF- κ B is bound to I κ B through a non-covalent interaction (Morotti *et al.*, 2017). The NF- κ B/I κ B complexes localize predominantly to the cytoplasm of cells, a small fraction can be detected within the nucleus where, by virtue of their inability to bind DNA, the NF- κ B/I κ B complexes are mostly inactive (Nagel *et al.*, 2014). NF- κ B/I κ B complexes exhibit DNA binding activity in response to a stimulus that post-translationally modifies the complex. The free active nuclear NF- κ B dimers become amplified and lead to gene transcription (Morotti *et al.*, 2017).

The NF- κ B transcription factor family consist of five homologous member genes and their protein subunits: *RELA* (p65), *RELB* (Rel b), *cREL* (Rel), *NF κ B1* (p50 and its precursor p105) and *NF κ B2* (p52 and its precursor p100). The protein subunits form homo- or heterodimers to form transcriptionally active or repressive subunits (Huguet *et al.*, 1997; Ferreira *et al.*, 1998). They share a highly conserved Rel homology domain (RHD), which is responsible for DNA binding and activation. Two NF- κ B pathways exist; the canonical and non-canonical (Stein and Baldwin, 2013).

In the nucleus, the canonical p65/p50 complex increases the transcription of anti-apoptotic genes that promote tumour cell survival, inflammation, invasion, and angiogenesis (Fulda, 2014; Morotti *et al.*, 2017). This includes the up-regulation of NF- κ B target genes *BCL2*, *BCL2LA1*, *XIAP*, *BIRC2* and *BIRC3*, *TRAF1* and *TRAF2* (Park and Hong, 2016) as well as proliferative genes such as *CCND1* (encoding for cyclin D1) and *IL6* (encoding for interleukin-6) (Oeckinghaus and Ghosh, 2009). RelA targets the genes encoding for interleukin-8 (*IL8*) and chemokine (C-X-C motif) ligand 2 (*CXCL2*); a cytokine involved in cancer metastasis and angiogenesis (Nowak *et al.*, 2008).

The p52/RelB heterodimer becomes active as a result of non-canonical NF- κ B pathway. The p52/RelB complex translocates to the nucleus to induce gene expression of the NF- κ B subunit p52 target genes which encode for TNF- α (*TNF*), proto-oncogenes cyclin D1 and c-myc (*MYC*), together with Bcl-xl (*BCL2L1*), Bcl-2 (*BCL2*) and XIAP (*XIAP*) (Barre *et al.*, 2010; Zhao *et al.*, 2012; Zhou *et al.*, 2015). The p52 homodimers are transcriptional repressors that play an important role to prevent aberrant expression of NF- κ B target genes, including those involved in inflammation (Liu *et al.*, 2017b).

Post-translational modifications such as phosphorylation, methylation, ubiquitination, acetylation, and isomerization of specific amino acid residues are described as methods of fine-tune regulating NF- κ B. This occurs by targeting the IKKs, the I κ Bs, the NF- κ B subunits or critical adaptor proteins that feed into NF- κ B pathways. Post-translational modifications are changes in a protein occurring after the translational process, either when a functional group is covalently added to the protein, or during the proteolytic and folding processes. These structural changes act as a mechanism for the specification of proteins and increase their variety. As an example, some reports describe how these post-translational modifications prevent RelA from binding to I κ B α , facilitating its binding to DNA and increasing its stability to activate the expression of downstream RelA target genes (Zhao *et al.*, 2012; Xia *et al.*, 2014).

4.1.6 Transcriptional regulation of IAP genes

Inhibitor of apoptosis proteins share the BIR domain located at the amino-terminus and a RING finger domain at the carboxy-terminus (Clark and Longo, 2018). IAP family members include XIAP (*BIRC4*), cIAP-1(*BIRC2*), cIAP-2 (*BIRC3*) and survivin (*BIRC5*) (Salvesen and Duckett, 2002). XIAP, the most potent IAP, contains three BIR domains with BIR3 directly binding to caspase-9, while the linker region close to BIR2 targets caspase-3 and -7 (Eckelman *et al.*, 2006). The IAP family members *BIRC2* and *BIRC3* are under transcriptional control of NF- κ B and also appear to regulate NF- κ B activity (Gyrd-Hansen and Meier, 2010). The overexpression of *BIRC3* activates NF- κ B directed transcription by promoting I κ B α degradation.

4.1.7 Regulation of Bcl-2 family of genes

The Bcl-2 family of genes are key regulators of apoptosis, which include anti-apoptotic (*BCL2*, *BCL2L1*, *BCL2A1*) and pro-apoptotic (*BAD*, *BAK*, *BAX*) Bcl-2 family members (Czabotar *et al.*, 2014). As mentioned above, transcription of several family members of the Bcl-2 genes are up-regulated by NF- κ B (Fulda, 2014). Bcl-2 protein expression has been shown to have an inhibitory effect on NF- κ B activity at important regulatory steps by stabilization of I κ B α , by inhibiting transactivation of RelA and by modulating nuclear levels of Rel family members (Xia *et al.*, 2014). The mechanisms by which Bcl-2 affects these regulatory steps are still unclear, although hyper-phosphorylation of I κ B α is associated with the stabilization of NF- κ B by Bcl-2 (Oeckinghaus and Ghosh, 2009) .

4.1.8 Aim and Objectives

Aim:

Determine the effect of the active copper-imidazo[1,2-*a*]pyridines on the apoptotic transcriptome of HL-60 and K562 leukaemic cells

Objectives:

Evaluate the effects of the most active imidazo[1,2-*a*]pyridines on gene expression using an apoptosis specific human gene array by performing real-time quantitative polymerase chain reactions (RT-qPCR). This objective will be fulfilled by:

- Isolating total RNA
- Transcribing mRNA to cDNA
- Performing qPCR array to quantify changes to individual mRNA species
- Analysing qPCR data using online GeneGlobe (Qiagen) analysis tool
- Identifying individual apoptotic regulatory genes affected by the copper- imidazo[1,2-*a*]pyridines

4.2 METHODS

4.2.1 Human apoptotic gene array

A gene (PCR) array panel was used to analyze the apoptotic response in treated and untreated leukaemic cells. This is a highly sensitive and useful method for gene expression profiling of specific panels of genes in cell signalling pathways using the real-time PCR method. In this study, the RT² Profiler Apoptosis PCR arrays (Qiagen) were used to analyze the effect of the copper-imidazo[1,2-*a*]pyridines on the expression of apoptotic genes. RT-qPCR is a sensitive gene analysis technique that ensures efficiency and accuracy thus it is common practice to perform once.

Real-time quantitative polymerase chain reactions were performed in a 96-well plate format (Appendix G, Figure G1) according to the manufacturer's protocol. Of the 84 genes, 16 were inclusive of the proteins evaluated in the proteome array and provide information regarding their gene expression status. The 84 apoptosis-related genes, their accession numbers corresponding to the gene names in full and abbreviations are provided in Appendix G; Table G1). The array also included five house-keeping reference genes and a panel of proprietary controls to monitor genomic DNA contamination (GDC), first strand synthesis (RTC) and real-time PCR efficiency (PPC). The panel requires the use and isolation of high quality cellular RNA and generous amounts of cDNA to provide for PCR amplification and analysis of gene expression as described below.

4.2.2 RNA extraction

The exposure period and treatment concentration required careful consideration since mRNA expression needed to be optimal following exposure to the copper-imidazo[1,2-*a*]pyridines. Since a treatment period of 18 hours caused an increase in the protein levels of pro-apoptotic markers, this was also selected as the treatment condition for gene expression profiling. HL-60 and K562 cells were treated with active copper-imidazo[1,2-*a*]pyridines at their respective IC₇₅ concentrations for 18 hours. Total RNA was first isolated from 4 x 10⁶ cells using microcentrifugation and the use of the Qiagen RNeasy Mini kit.

The RNeasy Mini Kit (Qiagen) was used to isolate total RNA from treated and untreated cells following the manufacturer's instructions. The Qiagen RNeasy method combines the selective binding properties of a silica-based membrane with the speed of microspin

technology. Cells are lysed in the presence of guanidine-thiocyanate which inactivates RNases to ensure the purification of undegraded RNA. The sample is then applied to an RNeasy mini spin column, where the total RNA binds to the membrane and contaminants are washed away. Total RNA is then eluted in 30 µl of RNase free water. With the RNeasy procedure, RNA molecules longer than 200 nucleotides are purified ensuring enrichment of mRNA.

For accurate and reproducible PCR Array results, it was important to avoid contamination of the assay with RNases. All procedures were conducted under RNase free conditions using RNase free laboratory consumables (plastics and pipette tips) and RNase free solutions. The use of sterile, disposable RNase free polypropylene tubes were used throughout the procedure. To set up and maintain a working environment that was RNase free, only fresh PCR-grade reagents (agarose) reserved for RNA only were used. Separate workspaces were used for PCR setup and post-PCR processing. The PCR workspace and laboratory ware (tube racks etc.) were decontaminated before each use with RNase away spray (Sigma-Aldrich) and hydrogen peroxide. Glassware was baked for 6 hour periods and solutions for gel electrophoresis were prepared using water that was DEPC treated and autoclaved.

4.2.3 Removal of genomic DNA

Total RNA was first isolated with the use of the Qiagen RNeasy Mini kit and this was followed by treatment with RNase-free DNase (Qiagen) to remove any residual genomic DNA. The RNase-Free DNase Set (cat. no. 79254) provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps with 350 µl of Buffer RWT followed by 80 µl of DNase I incubation mix for 15 minutes. The resultant RNA was subjected to a control PCR reaction to confirm the absence of genomic DNA contamination. Messenger RNA (mRNA) was then reverse transcribed to cDNA with the RT² First Strand Kit (Qiagen).

4.2.4 cDNA synthesis

cDNA was synthesized using the RT² First Strand cDNA synthesis kit (Qiagen) following the manufacturer's instructions. Two separate mixtures were prepared, firstly the genomic DNA elimination mixture (Table 4.2) for each RNA sample and secondly, the reverse transcription

mix (Table 4.3). The genomic DNA mixture was incubated at 42°C for 5 minutes and immediately placed on ice for 1 minute.

Table 4.2 Genomic DNA elimination mixture

Reagent	Volume
Total RNA	1 µg*
GE (5x gDNA Elimination Buffer)	2 µl
RNase-free water	variable
Final volume	10 µl

*The volume in microliters (µl) depends on concentration of the RNA in ng/µl

Table 4.3 Reverse-transcription cocktail

Reagent	Volume
5x Buffer BC3	4 µl
RE3 Reverse Transcriptase Mix	2 µl
Control P2	1 µl
RNase-Free Water	3 µl
Final volume	10 µl

The reverse transcriptase cocktail was centrifuged and added to the genomic DNA mixture to provide a final volume of 20 µl. This mixture was then incubated at 42°C for 15 minutes and thereafter at 95°C for 5 minutes to stop the reaction. cDNA was diluted with 91 µl of RNase-free water, according to the manufacturer's instructions. The cDNA was placed on ice and used immediately for the PCR Arrays.

4.2.5 Quantification and purity of total RNA

The concentration of total RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific™). The amount of UV light absorbed is used to determine the concentration of RNA in a solution by applying the Beer-Lambert law (Strong, 1952). The Beer-Lambert law is based on the direct correlation between absorbance and concentration, taking into account the extinction coefficient which is 40 µg/ml for RNA. The spectrophotometer was calibrated with RNase free water. Nanodrop technology uses one microliter of RNA was to determine the concentration of total RNA. The ratio of the absorbance readings at 260 nm and 280 nm

(A_{260}/A_{280}) of the total RNA provides an estimate of the purity since contaminating proteins absorbed in the UV spectrum decrease the $A_{260}:A_{280}$ ratio. Pure nucleic acids has an absorbance ($A_{260}:A_{280}$) ratio of 1.8 or greater. An absorption ratio, $A_{260}:A_{230}$ is used to indicate the presence of contaminants like organic compounds, or guanidium salts. This absorption ratio should be is in a range of 2.0-2.2.

4.2.6 Electrophoresis of RNA

The integrity and size distribution of total RNA was confirmed by performing a formaldehyde agarose gel electrophoresis gel with EtBr staining (Mansour and Pestov, 2013). Intact, undegraded RNA shows ribosomal RNA species as sharp bands on the gel. A degraded RNA sample appears as a smear with no distinction of bands.

A 1.5% formaldehyde agarose gel was used for resolving RNA. For this, 0.45 g of agarose powder (BIORAD) was dissolved in 21.6 mL of diethylpyrocarbonate (DEPC) treated Milli-Q water and microwaved to melt the agarose. Thereafter, 3 ml of $10 \times$ MOPS running buffer, 5.4 ml of formaldehyde (Sigma-Aldrich) and 3 μ l of a 10 mg/ml EtBr stock solution (Sigma-Aldrich) was added to the agarose. The gel was mixed thoroughly and poured onto the gel chamber and allowed to set for 20-30 minutes. Each RNA sample (4.5 μ l) was added to 14.5 μ l RNase free sample buffer and incubated at 55°C for 15 minutes. A 2 μ L volume of loading buffer (0.4% saturated aqueous bromophenol blue solution, 1 mM EDTA (pH 8.0), 50% glycerol and 0.4% xylene cyanol; DEPC treated) was mixed with the samples and the entire sample was loaded into the wells. The gel was run at 15 V in $1 \times$ formaldehyde running buffer for 30 minutes, and thereafter, taken up to 30 V for a further 1.5 hours until the tracker dyes (bromophenol blue) had moved to the end of the gel. The RNA was visualised by UV trans-illumination on the Bio-Rad ChemiDoc system (Bio-Rad, USA) using the ChemiDoc MP Imager and Image Lab™ software version 6.0 (BioTechniques, NY, USA).

4.2.7 Real-time PCR (gene expression profiling)

Real-time RT-PCR was carried out using the ABI 7500 Real-Time PCR System (Applied Biosystems® Incorporated) by using the RT² SYBR Green qPCR MasterMix kit (Qiagen). This method has high precision for the quantitative detection of mRNA (as cDNA). The kit consisted of 2 x RT² SYBR Green qPCR Mastermix (1350 μ l) contained HotStart DNA Taq Polymerase that was active after heat activation. The synthesized cDNA was mixed with the

Qiagen RT² SYBR Green/ qPCR master mix, to which nuclease-free water was also added. Reactions were performed in a volume of 25 μ l.

The same thermal cycle, optimized by Qiagen, was used for all primer sets: a pre-incubation for 10 minutes at 95°C, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 seconds, primer annealing at 60°C for 1 minute, and primer extension at 72°C for 10 seconds. Melting curves were derived after 40 cycles by a denaturation step at 95°C for 10 seconds, followed by annealing of fluorescent probe at 65°C for 15 seconds, and a temperature rise to 95°C and continuous fluorescence measurement. Final cooling was performed at 40°C for 30 seconds. Melting curve analyses of each sample was done using ABI 7500 Software v2.0.4 (Applied Biosystems® Incorporated). Data obtained as C_T values was exported as Excel files to the Qiagen web portal at GeneGlobe (www.qiagen.com/geneglobe) for analysis. Samples were assigned to controls and test groups.

4.2.8 RT-qPCR data analysis using GeneGlobe

The comparative cycle number cross point (C_T) method was used to compare the C_T values of test samples of interest with an untreated control sample. The C_T values were normalized to appropriate housekeeping genes (HKG's) (Livak and Schmittgen, 2001). Five housekeeping genes included; β 2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein, large, P0 (RPLP0), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β -actin (ACTB) were used to obtain the change in cycle threshold threshold (ΔC_T) value for each gene of interest. Hence, the ΔC_T is calculated as the C_T value of the gene of interest (GOI) less the average C_T value of the five reference genes (HKG's), followed by $\Delta\Delta C_T$ calculations which represents the difference between the ΔC_T of the test group less the ΔC_T of the control group (ΔC_T (Test Group) - ΔC_T (Control Group)). Fold changes were then calculated by the $2^{-\Delta\Delta C_T}$ method. This value represents the level of the expression of each gene in the drug-treated sample versus that in the untreated control sample. By convention, the experiments were performed once and the fold difference of 2 was used to indicate up- or down-regulation of gene target identification. Owing to the costs of performing PCR gene arrays, these experiments were performed once.

4.3 RESULTS

Following on from the results obtained in Chapter 2 and 3, an apoptotic gene panel was used to further identify specific changes in mRNA levels of 84 apoptosis-related genes in the two leukaemic cells lines treated with the most active copper-imidazo[1,2-*a*]pyridines.

4.3.1 RNA isolation

4.3.1.1 Total RNA purity

Total RNA was isolated from HL-60 and K562 treated and untreated cell lysates after an 18 hour exposure period at an IC₇₅ concentration. Since it is important to use high quality RNA samples when carrying out PCR array assays, RNA obtained from each treatment were subjected to spectrophotometric analysis to determine its concentration and purity using a Thermo Scientific™ NanoDrop spectrophotometer. Results are summarized in Table 4.4, and indicate that the A_{260}/A_{280} and A_{260}/A_{230} ratios were within the preferred range. An A_{260}/A_{280} ratio of 1.8-2.0 indicates the absence of protein and is regarded as pure quality RNA. An A_{260}/A_{230} ratio greater than two indicates the absence of organic contaminants.

Table 4.4 Concentration and absorbance ratios of total RNA in HL-60 and K562 diluted cell lysates

Cell line	Sample	RNA concentration (ng/μl)	A260	A280	260/280	260/230
HL-60	Control	2575.5	64.39	32.45	1.98	2.30
	JD47R	2803.8	70.09	36.05	1.94	2.26
K562	Control	2773.0	69.33	35.60	1.95	2.29
	JD88	2422.8	60.57	30.29	2.00	2.30

4.3.1.2 RNA gel electrophoresis

High quality undegraded RNA samples are essential for PCR array analysis. The quality of the RNA samples were analysed and further confirmed by gel electrophoresis as indicated in Figure 4.1. RNA was stained with EtBr to visualize the integrity of 18S and 28S RNA bands using UV trans-illumination. These two bands are indicative of intact RNA. There is no distinct band for mRNA species are observed due to the heterogeneity of the sizes of mRNA.

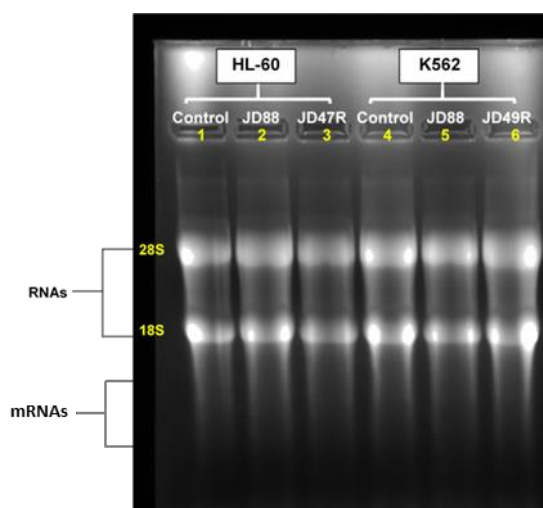


Figure 4.1 RNA quality and integrity by formaldehyde agarose gel analysis. Samples of total RNA were fractionated on a 1.5% agarose gel in MOPS buffer and stained with EtBr. HL-60 samples were loaded in Lanes 1 (untreated control), 2 (JD88) and 3 (JD47R), and K562 samples were loaded in lanes 4 (untreated control), 5 (JD88) and 6 (JD49R). RNA was visualised by UV Transillumination on the Bio-Rad ChemiDoc system (Bio-Rad, USA) using the ChemiDoc MP Imager and Image Lab™ software 6.0 (BioTechniques, NY, USA).

4.3.2 Human apoptosis PCR array

The PCR arrays included 84 apoptosis related genes. Four samples, i.e. untreated controls of the HL-60 and K562 cell lines, and the most active copper complex per cell line (JD47R treated HL-60 cells and JD88 treated K562 cells), were subjected to qPCR. The cDNA was subjected to data quality control checks using the GeneGlobe software program for monitoring genomic DNA contamination (GDC), the first strand synthesis (RTC) along with real-time PCR efficiency (PPC). All six arrays passed these checks (Appendix E; Table E2).

The data representing changes in gene expression are presented as heat maps, scatter plots and tables indicating the fold increase or decrease. Heat maps provide a visualization of the fold changes in gene expression between the treated and control samples. The heat maps mirrors the PCR array layout (Appendix G; Figure G1); blue blocks represent decreased gene expression and red blocks represent increased gene expression. The intensity of the colours correlates with the extent of the change in expression.

Changes in gene expression were analysed by the GeneGlobe software (Qiagen, Germany), by comparing the normalised fold changes (increase or decrease) of the treated cells against the control cells. Group 1/ Group 2 on the Y-axis of scatter plots indicate the treatment groups (JD88 and JD47R). Those genes that were differentially expressed, showing a more

than two fold change in expression is shown in the section 4.3.3 and 4.3.4. A summary of all the apoptosis-related genes present in the array, together with their corresponding protein is represented in Appendix G; Table G3. Microscopic evaluation of the cells, prior to harvesting, showed that the cells had intact membranes and no obvious changes in cell morphology indicating that the qPCR data are not representative of dead cells (Appendix G; Figure G2 and G3).

4.3.3 Gene expression analysis of HL-60 cells

4.3.3.1 The effect of JD47R on apoptotic gene expression of HL-60 cells

After treatment of HL-60 cells with JD47R relative to control cells, a total of 47 genes (56.0%) that were evaluated in the gene array were differentially expressed (Figure 4.2), with the expression of five genes being increased and 42 genes being suppressed (Table 4.5).

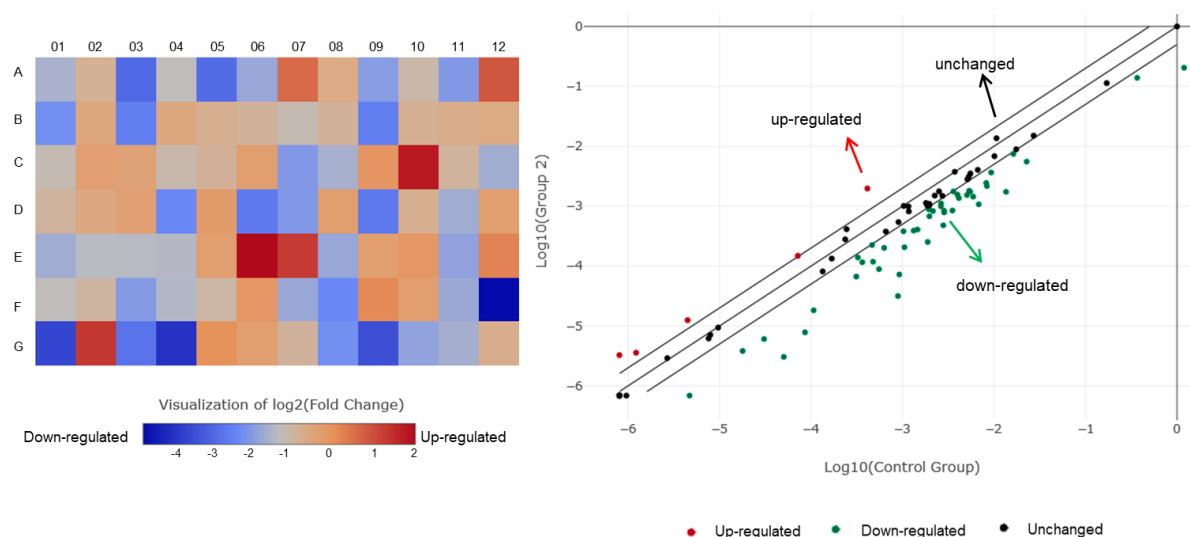


Figure 4.2 Changes in gene expression of HL-60 cells treated with JD47R. Cells were treated with JD47R at an IC_{75} concentration of $4.0 \mu M$ for 18 hrs. **A.** The heat map representing fold changes in gene expression. The colour intensity correlates with the extent of the changes in expression. **B.** The scatter plot compares the normalized expression of individual genes on the array between JD47R treated HL-60 and control cells, by plotting them against each other to visualise changes in expression. Each data point represents an individual gene with normalised expression. The central solid line indicates unchanged gene expression, while the other two lines indicate the selected fold regulation threshold (+/-2). An increase in gene expression greater than 2 fold is indicated by the red points, while a decrease in gene expression greater than 2 fold is indicated by the green points. Black points represent genes with changes in expression level less than 2 fold.

Table 4.5 Genes with changes in expression levels of HL-60 cells treated with JD47R

Gene Symbol	Gene product	GenBank Accession no.	Fold increase/ decrease
ABL	c-Abl tyrosine kinase	NM_005157	-2.9
AKT1	Akt kinase/ PKB	NM_005163	-7.8
APAF1	Apaf-1	NM_001160	-2.4
BAD	Bad	NM_004322	-7.5
BAG1	Bag-1	NM_004323	-3.4
BAX	Bax	NM_004324	-3.9
BCL2	Bcl-2	NM_000633	-4.1
BCL10	Bcl-10	NM_003921	-2.0
BCL2A1	Bfl-1	NM_004049	+2.1
BCL2L1	Bcl-x	NM_138578	-4.7
BCL2L11	Bim	NM_006538	-5.8
BIK	Bik	NM_001197	-2.2
BIRC3	cIAP-2	NM_001165	-5.8
BNIP3	Bnip-3	NM_004052	-2.2
CASP1	Caspase-1	NM_033292	-2.1
CASP2	Caspase-2	NM_032982	-4.1
CASP3	Caspase-3	NM_004346	-3.0
CASP5	Caspase-5	NM_004347	+4.1
CASP7	Caspase-7	NM_001227	-3.1
CD40	CD40/ TNFRSF5	NM_001250	-5.1
CD70	CD70/ TNFSF7	NM_001252	-6.2
CFLAR	cFLIP	NM_003879	-4.2
CIDEB	CIDE-B	NM_014430	-6.3
CYCS	Cytochrome c	NM_018947	-3.1
DFFA	ICAD	NM_004401	-3.1
DIABLO	Smac/DIABLO	NM_019887	-2.5
FADD	FADD	NM_003824	-2.6
FAS	Fas	NM_000043	-2.6
GADD45A	Gadd45 α	NM_001924	+4.8
HRK	Harakiri	NM_003806	+2.8
IGF1R	IGFR	NM_000875	-3.4
LTBR	TNFRSF3	NM_002342	-3.6
NAIP/BIRC1	NAIP	NM_004536	-2.3
NOD1	NOD-1	NM_006092	-4.0
NOL3	NOL-3	NM_003946	-2.7
TNF	TNF- α	NM_000594	-3.4
TNFRSF10A	TRAIL-R1/DR4	NM_003844	-5.1
TNFRSF1A	TNF-R1	NM_001065	-3.3
TNFRSF1B	TNF-R2	NM_001066	-28.2
TNFRSF9	CD137	NM_001561	-6.8
TNFRSF21	DR6	NM_014452	-12.7
TNFRSF25	DR3	NM_003790	+2.9
TNFSF10	TRAIL ligand	NM_003810	-16.5
TP73	Tumour suppressor p73	NM_005427	-4.7
TRADD	TRADD	NM_003789	-11.0
TRAF2	TRAF-2	NM_021138	-3.6
TRAF3	TRAF-3	NM_003300	-3.0

4.3.4 Gene expression analysis of K562 cells

4.3.4.1 The effect of JD88 on apoptotic gene expression of K562 cells

When K562 cells were treated with JD88, 39 genes (46.4%) were differentially expressed, with 16 genes showing an increase in expression and 23 genes showing a decrease in expression (Table 4.6) among the 84 genes in the apoptotic-specific array (Figure 4.3).

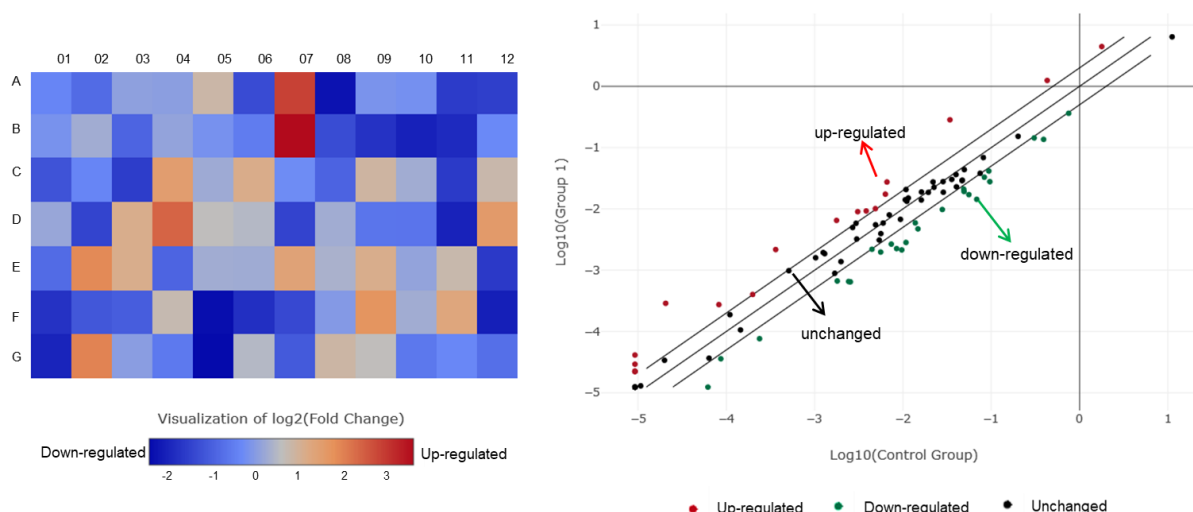


Figure 4.3 Changes in gene expression of K562 cells treated with JD88. Cells were treated with JD88 at an IC_{75} concentration of $7.1 \mu M$ for 18 hrs. **A.** The heat map representing fold changes in gene expression. The colour intensity correlates with the extent of the changes in expression. **B.** The scatter plot compares the normalized expression of individual genes on the array between JD88 treated K562 and control cells, by plotting them against each other to visualise changes in expression. Each data point represents an individual gene with normalised expression. The central solid line indicates unchanged gene expression, while the other two lines indicate the selected fold regulation threshold (+/-2). An increase in gene expression greater than 2 fold is indicated by the red points, while a decrease in gene expression greater than 2 fold is indicated by the green points. Black points represent genes with changes in expression level less than 2 fold.

Table 4.6 Genes with changes in expression levels of K562 cells treated with JD88

Gene symbol	Gene product	GenBank Accession no.	Fold increase/ decrease
BAG1	Bag-1	NM_004323	-2.3
BAG3	Bag-3	NM_004281	+8.4
BAK1	Bak	NM_001188	-4.5
BCL2	Bcl-2	NM_000633	-2.8
BCL2A1	Bfl-1	NM_004049	-2.7
BIK	Bik	NM_001197	+14.0
BIRC2	cIAP-1	NM_001166	-2.3
BIRC3	cIAP-2	NM_001165	-3.1
BIRC5	Survivin	NM_001168	-3.8
BIRC6	Apollon/Bruce	NM_016252	-3.5
BNIP3	Snip-3	NM_004052	-2.1
BRAF	Braf-1	NM_004333	-2.8
CASP1	Caspase-1	NM_033292	+3.2
CASP4	Caspase-4	NM_001225	+2.1
CASP6	Caspase-6	NM_032992	-2.9
CASP9	Caspase-9	NM_001229	-2.5
CASP14	Caspase-14	NM_012114	+2.5
CD27	TNFRSF7	NM_001242	+2.4
CD40	CD40/ TNFRSF5	NM_001250	+6.0
CFLAR	cFLIP	NM_003879	-2.6
CYCS	Cytochrome c	NM_018947	-3.8
DAPK1	DAPK	NM_004938	+3.3
DIABLO	Smac/DIABLO	NM_019887	+4.2
FADD	FADD	NM_003824	+2.4
HRK	Harakiri	NM_003806	+3.0
IL10	IL-10	NM_000572	+2.4
MCL1	Mcl-1	NM_021960	-2.9
NAIP/BIRC1	NAIP	NM_004536	-3.1
NFKB1	NF- κ B (p50)	NM_003998	-2.0
PYCARD	PYCARD	NM_013258	-4.8
RIPK2	RIPK2	NM_003821	-3.3
TNF	TNF- α	NM_000594	-2.4
TNFRSF1A	TNF-R1	NM_001065	+2.8
TNFRSF1B	TNF-R2	NM_001066	-4.0
TNFRSF10B	TRAIL-R2	NM_003842	+3.7
TNFSF8	CD30 ligand	NM_001244	-5.0
TNFRSF21	DR6	NM_014452	-3.7
TNFRSF25	DR3	NM_003790	+4.5
TP73	Tumour suppressor p73	NM_005427	+2.0

4.4 DISCUSSION

The human apoptotic PCR arrays showed meaningful changes in expression levels of genes encoding apoptosis associated proteins when cells were exposed to copper-imidazo[1,2-*a*]pyridines. Among these were genes coding for pro- and anti-apoptotic proteins in the Bcl-2 family, the TNF-receptor family, IAPs and the caspase family of cysteine proteases. To resolve the cell death process the genes were grouped according to their functions. In the context of this study, a discussion of the expression level of each and every gene in the array for the two cells lines is beyond the scope and aims of this study.

It is possible that the changes in expression of genes are time and concentration dependent. Therefore, the data obtained provides information at a specific time point and longer incubation times may have caused a different set of changes. The most pronounced noticeable effect of the copper-imidazo[1,2-*a*]pyridines, was a decrease in the expression of anti-apoptotic genes.

4.4.1 Copper-imidazo[1,2-*a*]pyridines changes the expression levels of Bcl-2 genes in HL-60 and K562 cells

The Bcl-2 family of genes encode structurally related proteins which regulate apoptosis (Pfeffer and Singh, 2018). Deregulation of their gene products is associated with malignancies and resistance to therapeutic interventions (Tzifi *et al.*, 2012). The qPCR data showed that the expression of four Bcl-2 related genes *BAG1*, *BCL2*, *BNIP3* and *HRK* were affected by the copper complexes in both leukaemic cell lines (Table 4.7). The expression of *BAG1*, *BCL2* and *BNIP3* were suppressed while *HRK* was elevated.

Table 4.7 Changes in mRNA expression of Bcl-2 genes in HL-60 and K562 cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	<i>Gene product</i>	<i>JD47R</i>	<i>JD88</i>
<u>Anti-apoptotic BCL2 genes</u>			
<i>BAG1</i>	Bag1	-3.4	-2.3
<i>BAG3</i>	Bag3	NE	+8.4
<i>BCL10</i>	Bcl-10	-2.0	NE
<i>BCL2</i>	Bcl-2	-4.1	-2.8
<i>BCL2L1</i>	Bcl-x	-4.7	NE
<i>BCL2A1</i>	Bfl-1/A1	+2.1	-2.7
<i>BRAF</i>	Braf-1	NE	-2.8
<i>MCL1</i>	Mcl-1	NE	-2.9
<u>Pro-apoptotic BCL2 genes</u>			
<i>BAD</i>	Bad	-7.5	NE
<i>BAK1</i>	Bak	NE	-4.5
<i>BAX</i>	Bax	-3.9	NE
<i>BCL2L11</i>	Bim	-5.8	NE
<i>BIK</i>	Bik	-2.2	+14.0
<i>BNIP3</i>	Bnip-3	-2.2	-2.1
<i>HRK</i>	Harakiri	+2.8	+2.9

*NE= no effect

4.4.1.1 Changes in *BCL2* family of genes common to both HL-60 and K562 cells

a) *BCL2* gene expression

The decreased expression of the *BCL2* gene translated to a reduction in protein levels of its gene product Bcl-2 in the proteome array analysis (Chapter 3; section 3.4.3). Bcl-2 is an anti-apoptotic protein that prevents mitochondrial outer membrane permeability to occur by binding and inhibiting both Bak and Bax and, the BH3-only proteins by interacting with their BH3 domains (Green and Llambi, 2015). Anti-apoptotic Bcl-2 protein is regulated at both the transcriptional and post-translational levels which include phosphorylation, ubiquitination and proteasomal degradation events (Kutuk and Letai, 2008; Geng *et al.*, 2011; Shamas-Din *et al.*, 2011; Vucic *et al.*, 2011).

b) *HRK* gene expression

The product of the *HRK* gene is the BH3-only Harakiri (Hrk) protein, a regulator of cell death that physically interacts and inactivates anti-apoptotic proteins Bcl-2 and Bcl-x by forming inactive complexes of Bcl-2-Hrk and/or Bcl-x-Hrk (Inohara *et al.*, 1997). The increase in *HRK* mRNA levels in both cell lines is beneficial since it inactivates anti-apoptotic Bcl-2 and will promote apoptosis.

c) *BAG1* suppression by copper complexes

The anti-apoptotic gene *BAG1*; was suppressed by the copper complexes in both cell lines. When overexpressed, Bag-1 (Bcl-2-associated athanogene) protein binds and enhances the activity of Bcl-2, thereby promoting cell survival and metastasis (Karpel-Massler *et al.*, 2015). The suppression of Bag-1 gene will further decrease Bcl-2 activity. Bag-1 provides a link between growth factor receptors and anti-apoptotic pro-survival mechanisms. Bag-1 is shown to activate Akt kinase (Kizilboga *et al.*, 2019). The overexpression of Bag-1 increases Bad phosphorylation via Akt kinase activation, implicating Akt in its anti-apoptotic function in leukaemic cells. The decreased expression of Bag-1 disrupts its interaction with kinases like Akt. The decreased expression of Akt further impedes this survival strategy exploited by cancer cells to inhibit the pro-apoptotic function of Bad.

d) Suppression of *BNIP3* expression

BNIP3 was suppressed in both cell lines treated with the copper complexes. *BNIP3* encodes for the pro-apoptotic protein Bnip-3 (Bcl-2/E1B-19K-interacting protein 3; initially called Nip3) which is localised to the outer membrane of mitochondria and is elevated in response to hypoxia (Singh *et al.*, 2018). At the mitochondria, the BH3 domains of Bnip-3 suppress the function of anti-apoptotic Bcl-2 proteins by heterodimerising with Bcl-2 and Bcl-x through its transmembrane domain (Ray *et al.*, 2000; Chinnadurai *et al.*, 2008). In addition to its pro-apoptotic functions, Bnip-3 was reported to trigger autophagy and mitophagy (mitochondrial autophagy) (Thomas *et al.*, 2011).

The transcription of *BNIP3* is controlled by a variety of transcription factors like HIF-1 α , E2F-1 and FoxO3. Therefore, the mRNA levels may be suppressed as a result of transcription factor activation which is most likely a cytoplasmic event. Most transcription factors are sequestered in the cytoplasm and so this would mean that transcription factors needed for

BNIP3 expression was somehow inhibited by the copper complexes. It is not possible to distinguish which transcription factor. The suppression of *BNIP3* excludes Bnip-mediated cell death by the copper-imidazo[1,2-*a*]pyridines in both HL-60 and K562 cells.

4.4.1.2 Changes in *BCL2* genes that show opposite effects in HL-60 and K562 cells

a) Anti-apoptotic *BCL2A1* (Bfl-1)

Another notable effect in HL-60 cells was the increased expression of the anti-apoptotic gene, *BCL2A1* (B-cell lymphoma 2-related protein A1). *BCL2A1* encodes for Bfl-1 protein which is the Bcl-2-related anti-apoptotic gene expressed in fetal liver. Bfl-1 is overexpressed in a variety of cancers including leukaemia, myeloma and lymphoma and contribute to cancer progression (Vogler, 2012).

Bfl-1 exerts its anti-apoptotic function by sequestering pro-apoptotic Bcl-2 proteins (multi-domain and BH3-only) and inhibiting their pro-apoptotic function (Llambi and Green, 2011). Bfl-1 binding prevents exposed BH3 alpha helices of Bax and Bak heterodimers from forming the mitochondrial outer membrane pore that triggers apoptosis. In addition, it sequesters direct activator BH3-only proteins (e.g. Bid, Bim and Puma), which serve as upstream sensors of cell stress and prevent them from activating Bax/Bak. Bfl-1 binding can also inhibit the indirect activation of Bax/Bak by sensitizer BH3-only proteins (e.g. Bad and Noxa), which dissociate the inhibitory complexes between anti-apoptotic proteins and activated Bax and Bak monomers.

The transcription of *BCL2A1* is primarily regulated by NF- κ B (Vogler, 2012). The Bfl-1 protein is post-translationally regulated by rapid ubiquitin-mediated proteasomal degradation resulting in a short half-life (Rooswinkel *et al.*, 2014). Mutations of *BCL2A1* prevents ubiquitination and prolongs the half-life of the protein (Fan *et al.*, 2010). These results further suggest that either increased transcription rate or increased transcript stability were the cause of the increased *BCL2A1* levels in HL-60 cells.

An increase of *BCL2A1* expression is associated with chemo-resistance and reduced sensitivity to the induction of apoptosis (Xia *et al.*, 2006). While leukaemic cells are susceptible to Bcl-2 targeted therapy with venetoclax, they do up-regulate Bfl-1 cellular levels which contribute to chemo-resistance (Olsson *et al.*, 2007). Therefore, Bfl-1 increase may be a response to the inhibition or decrease of Bcl-2 activity (Brocco *et al.*, 2017;

Valentin *et al.*, 2018). This demonstrates that cancer cells hardly rely on one survival factor to survive, as evidenced by their capacity to rewire their anti-apoptotic dependence on Bcl-2 proteins (Guerra *et al.*, 2018). The detection of increased *BCL2A1* levels in HL-60 cells indicated resistance to the pro-apoptotic effects of the copper complexes. This is indirect evidence that Bcl-2 is an indirect target of the copper complexes. Although this requires further investigation, the inhibition of Bfl-1 may improve the anti-cancer effects of Bcl-2 inhibitors.

In contrast to HL-60 cells, the transcription of *BCL2A1* was suppressed in K562 cells. This could likely be a characteristic response of the specific cell line to the copper complex. The copper complexes suppressed *NFKB1* (Table 4.8), the transcription factor responsible for activation of the canonical signalling pathway and the subsequent transcription of target genes like *BCL2A1*.

b) Copper complexes suppress *BIK* expression in HL-60 cells and extensively increase it in K562 cells

BIK (Bcl-2-interacting killer) which encodes for the BH3-only pro-apoptotic protein, Bik, was suppressed by JD47R. The protein levels of Bik were not determined as it was not contained within the proteome array conducted. Bik binds to Bcl-2 or Mcl-1 complex and displaces pro-apoptotic Bak (Shamas-Din *et al.*, 2011). Bak is then able to homodimerise with Bax and enable the release of cytochrome c from the mitochondria into the cytosol. This promotes both Mcl-1 and Bcl-2 degradation and subsequently causes intrinsic apoptosis (O'Neill *et al.*, 2016). *BIK* is usually undetectable or expressed at low levels in cancers (Valdez *et al.*, 2008; Ferreira *et al.*, 2015), however in AML it is up-regulated (Stirewalt *et al.*, 2008). Consistent with Stirewalt *et al.*, (2008), the HL-60 cells in this study expressed high levels of *BIK* which was suppressed following JD47R treatment.

BIK mRNA levels were greatly increased (> 14 fold) in K562 cells which was in contrast to its effect in HL-60 cells. This disparity is likely caused by the differences in molecular profiles of the two cell lines. Bik mRNA and protein is thought to be increased in response to genotoxic stimuli by transcription factors E2F and p53-dependent and p53-independent mechanisms (Hur *et al.*, 2006; Real *et al.*, 2006). This 14-fold increase of *BIK* expression is an important effect. Since *BIK* is pro-apoptotic, its increased expression may help to

overcome the anti-apoptotic state of the K562 cells, mitigate the effects of increased *BAG3* (8-fold) and promote cell death.

4.4.1.3 Changes of *BCL2*-related genes in HL-60 cells

In HL-60 cells, the copper-imidazo[1,2-*a*]pyridine JD47R suppressed the expression of 10 out of 15 pro- and anti-apoptotic Bcl-2 genes, suggesting that JD47R targets the transcription of Bcl-2 related genes. Four anti-apoptotic genes were suppressed as well as five pro-apoptotic genes *BAD*, *BAX*, *BCL2L11*, *BIK* and *BNIP3* with *BAD* being highly suppressed. *HRK* was the only pro-apoptotic gene to be increased. The suppression of mRNA levels indicates that the activity of the transcription factors may have been inhibited by the copper complexes. Given the fact that JD47R was a potent inducer of intrinsic apoptosis (Chapter 2; section 2.3.10) these gene array results should be interpreted with caution.

a) Suppression of anti-apoptotic *BCL2L1* (Bcl-x)

The expression of the gene encoding for the anti-apoptotic protein Bcl-x is *BCL2L1*, which much like Bcl-2, inhibits the pro-apoptotic function of Bax/Bak. The gene expression of *BCL2L1* was suppressed by JD47R thereby contributing to the induction of apoptosis by the copper complexes. Bcl-x expression is controlled by STAT3 (Maeda *et al.*, 2018). It is likely that STAT3 activity may have been hindered by the copper complex in this cell line upstream of Bcl-x signalling.

b) Suppression of the *BCL10* gene

The *BCL10* gene encoding for Bcl-10 (B-cell lymphoma/leukaemia 10) protein, was suppressed by JD47R. Bcl-10 commonly promotes cancer cell growth (Ruland *et al.*, 2001; Mahanivong *et al.*, 2008; Jiang *et al.*, 2011; Kuo *et al.*, 2012). Bcl-10 promotes cancer growth by forming a CARMA-BCL10-MALT1 (CBM) complex that activates canonical NF- κ B signalling induced by DNA damage and, prevents apoptosis (Luo *et al.*, 2019). The CBM complex initiates cell survival by activating the IKK signalling complex (includes IKK α , IKK β and IKK γ), which integrates upstream signals and causes phosphorylation of I κ B, enabling NF- κ B translocation to the nucleus thereby promoting gene transcription (Park and Hong, 2016). In this context, the copper complexes suppressed and prevented *BCL10* from triggering aberrant NF- κ B activation and promoting HL-60 cell survival via the canonical pathway.

c) Decreased expression of pro-apoptotic BAD and BAX with BAD being highly suppressed

BAD encodes for the pro-apoptotic BH3-only protein, Bad, which indirectly activates pro-apoptotic effectors (Bak and Bax) by binding and deactivating the anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-x) (Green and Llambi, 2015). Bad is usually activated by post-transcriptional modification which is dephosphorylation. Bad activity is negatively regulated by phosphorylation through the serine/threonine kinase Akt which sequesters it and, prevents its pro-apoptotic function (Pennington *et al.*, 2018). Akt prevents Bad from increasing the permeability of the mitochondrial outer membrane and the subsequent release of pro-apoptotic proteins cytochrome c and Smac/DIABLO into the cytosol. *AKT* suppression by JD47R predicted a decrease of Akt kinase which will affect the activity of Bad protein (Table 4.7). The observed decrease in Bad mRNA may have been as a result of mRNA degradation. Nevertheless, JD47R was able to increase Bad protein expression (Chapter 3; Table 3.3) and decrease mitochondrial membrane permeability (Chapter 2; section 2.3.9), suggesting that the function of Bad protein was not affected in HL-60 cells.

BAX encodes for the pro-apoptotic effector protein, Bax, which is responsible for the loss of mitochondrial outer membrane integrity and necessary for MOMP (Green and Llambi, 2015). The suppression of *BAX* by JD47R predicted a decreased apoptotic response however, its effect in increasing Bax protein expression suggested otherwise (Chapter 3; Table 3.3). The half-life of Bax is fairly long (~12 hours) (Song *et al.*, 2019). Therefore, the pro-apoptotic effect of Bax will outlive the decrease in the formation of new protein as cells will be dead before that becomes significant. There is some indication that mRNAs are degraded during apoptosis thus providing evidence for the reduced mRNA expression of stress-responsive genes like *BAD* and *BAX* (Thomas *et al.*, 2015).

d) Suppression of pro-apoptotic *BCL2L11* gene

The expression of *BCL2L11* was highly suppressed in HL-60 cells. *BCL2L11* encodes for Bim, a Bcl-2-interacting mediator of cell death. Bim is a BH3-only protein that directly activates effectors Bak and Bax through transient binding and promotes MOMP (Green and Llambi, 2015). Bim can also form a complex and inhibit the anti-apoptotic Bcl-2 (Aranovich *et al.*, 2012). When Bim is displaced from Bcl-2, increased levels of Bfl-1 and Mcl-1 sequester and prevent it from activating Bax and Bak, and committing the cell to apoptosis

(Niu *et al.*, 2016). It has been suggested that when any BH3-only protein binds an anti-apoptotic protein, the effect is to promote apoptosis by promoting Bax/Bak rather than to inhibit it (Leber *et al.*, 2018).

The transcription factor, FoxO1, promotes apoptosis by up-regulating pro-apoptotic members like Bim and Fas ligand, which in this study may have been defective (Gilley *et al.*, 2003). The half-life of Bim is ~ 3 hours, and the inhibition of Bim would take longer than 1 hour to be observed, indicating an increase in degradation (Frank *et al.*, 2015). Despite the decreased mRNA levels, apoptosis proceeded which indicates that the mRNA levels are not directly dictating the activity of the protein.

4.4.1.4 Effect of copper complexes on BCL2 genes in K562 cells

In K562 cells, JD88 suppressed 5 out of 8 anti-apoptotic and 2 out of 6 pro-apoptotic Bcl-2-related genes. Of interest was the substantial increase of Bcl-2 associated athanogene 3 (*BAG3*) and *BIK* expression levels in the K562 cells. *BAG3* and *BIK* were increased by 8.4 and 14-fold, respectively, which indicates that these genes are important responders to copper complex induced cell stress.

a) Gene expression of *BAG3*

The *BAG3* gene which encodes for the anti-apoptotic protein; Bag-3, was substantially increased in the K562 cell line. In normal cells, Bag-3 expression is very low, while its expression is increased in leukaemias (Yang *et al.*, 2007; Cesaro *et al.*, 2010; Rosati *et al.*, 2011; Rosati *et al.*, 2012). In lymphoid and myeloid leukaemic cell lines (Jurkat, U937, HL-60, K562) *BAG3* is commonly overexpressed (Seo *et al.*, 2005; Valdez *et al.*, 2008; Liu *et al.*, 2009; Chen *et al.*, 2010).

Bag-3 promotes the extracellular signal-related kinase (ERK)/MAPK or PI3K/Akt signalling pathways via heat shock proteins (Rosati *et al.*, 2015; Carrizzo *et al.*, 2016). It acts as a co-chaperone that positively interacts with the ATPase domain of HSP70 (Rauch *et al.*, 2017) and participates in promoting cell survival and autophagy (Colvin *et al.*, 2014). The large increase in *BAG3* mRNA may facilitate the formation of stable HSP complexes which promote protein folding and so overcome the unfolded protein effects in cancer cells (Calderwood and Gong, 2016), thus reducing stress and promoting cell survival. The increased gene expression of *BAG3* together with the increase in heat shock protein

expression in Chapter 3 (Table 3.4), may act in concert as a response to cell stress induced by the copper complexes.

The increased expression of Bag-3 contributes to resistance to therapy. *BAG3* expression is increased in cancers treated with proteasome inhibitors (bortezomib) and several cytotoxic drugs such as fludarabine and etoposide (Chiappetta *et al.*, 2007; Du *et al.*, 2008; Wang *et al.*, 2008a; Liu *et al.*, 2009). The increased mRNA levels of *BAG3* in this study are consistent with these reports, suggesting that the copper complexes may possibly interfere with the function of the proteasome, similar to bortezomib. Bag-3 protein levels and its subcellular location need to be determined by western blot and immunofluorescence assays to validate this notion. These findings indicate that Bag-3 may be a feasible drug target in leukaemic cells (Chiappetta *et al.*, 2007; Rosati *et al.*, 2015; Carrizzo *et al.*, 2016).

b) Gene expression of *BRAF*

Suppression of *BRAF* gene (coding for B-raf protein kinase) encoding for Braf-1 protein was observed in K562 cells (Table 4.7). *BRAF* is a downstream signal transduction oncogene of the EGFR and RAS signalling pathways (Morkel *et al.*, 2015; Dankner *et al.*, 2018). Its suppression by the copper complexes implies a loss of anti-apoptotic function of this gene which contributes to lowering the apoptotic threshold in K562 cells.

c) Suppression of *MCL1*

In K562 cells, JD88 suppressed the transcription of *MCL1*. *MCL1* encodes for the pro-survival protein Mcl-1 which suppresses apoptosis by trapping the BH3 pro-apoptotic proteins. Mcl-1 is highly oncogenic and is amplified and overexpressed in haematological malignancies (Opferman *et al.*, 2005; Campbell *et al.*, 2010; Glaser *et al.*, 2012; Carrington *et al.*, 2017; Luedtke *et al.*, 2017). An increased expression of Mcl-1 is often associated with chemo-resistance and relapse in cancer patients (Carrington *et al.*, 2017).

MCL1 together with *BCL2* and *BCL2L1* are the most documented anti-apoptotic genes in leukaemias and are considered as essential survival factors of leukaemic cells (Tzifi *et al.*, 2012). Their overexpression confers increased resistance to cytotoxic agents which results in poor survival rates due to a poor response to chemotherapy (Beroukhim *et al.*, 2010; Mehta *et al.*, 2013). Cell death is preceded by the decreases in the expression of these Bcl-2 anti-

apoptotic genes, which was consistent with the observations of this study (Chapter 2; section 2.3.7 and 2.3.10).

Several growth factors and cytokines that act on the MAPK, PI3K/Akt and JAK/STAT signal transduction pathways and downstream transcription factors have been shown to induce Mcl-1 transcription (Booy *et al.*, 2011; Steelman *et al.*, 2011; Chou *et al.*, 2013; Shenoy *et al.*, 2014; Young *et al.*, 2018). In contrast, the E2F-1 transcription factor directly represses the Mcl-1 promoter. Therefore, the decreased expression of *MCL1* may have been mediated by the inactivation of the transcription factors stimulating Mcl-1 transcription. Future studies dissecting the relative contributions of these different players in regulating Mcl-1 gene and protein levels in specific conditions may help develop more effective therapeutic strategies for the treatment of specific leukaemias.

d) *BAK1* suppression

The pro-apoptotic gene *BAK* which encodes for Bak was suppressed by JD88. Studies have shown that the decreased expression of Bak contribute to the development of cancers as well as resistance (Kondo *et al.*, 2000; Sorbello *et al.*, 2003; Tong *et al.*, 2004; Zohny and El-Shinawi, 2011; Busacca *et al.*, 2013). Thus, the suppression of *BAK* mRNA may be a resistance response to copper complexes. Bak is bound to anti-apoptotic protein Bcl-2 or Mcl-1 and counteracts their anti-apoptotic function. Since the decreased expression of multiple BH3-only proteins are considered to be weak promoters of tumour formation (Labi *et al.*, 2014), the down-regulation of apoptotic effector *BAK* did not prevent mitochondrial-mediated apoptosis in cell culture, as evidenced by the loss of MMP and the induction of caspase-9 activity (Chapter 2; section 2.3.9 and 2.3.10).

The expression of Bak is up-regulated upon cell stress by the tumour suppressor p53 (McCubrey *et al.*, 2017). However, there is insufficient data about how the *BAK* gene is regulated. Some studies suggest that the expression of *BAK* can be induced by both tumour suppressor p53 and p73 (Graupner *et al.*, 2011). The decrease in the phosphorylated p53 at serine site 15 and 46 (Chapter 3; section 3.4.7), may be associated with the reduced transcription of p53 target genes i.e. *BAK*. It is also probable that other transcription factors such as WT1 (Wilm's tumour suppressor protein 1) decreased *BAK* transcription (Morrison *et al.*, 2005).

4.4.1.5 Summary of copper complexes and Bcl-2 related proteins

Leukaemic cells have been reported to express high levels of pro-apoptotic Bcl-2 family members. Sarosiek and Letai., (2016) have demonstrated how the overall survival of AML cells are based on the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins. The survival of the tumour cells are therefore highly dependent on the presence of anti-apoptotic Bcl-2 proteins resulting in oncogenic addiction as reviewed by Pagliarini *et al.*, (2015). Targeting *MCL1* and *BCL2* in AML cells have proven to be successful in inducing cell death *in vitro* and *in vivo*. Several Bcl-2 inhibitors have been evaluated in pre-clinical studies with varying success rates. Sabutoclax (Wei *et al.*, 2010), and maritoclax (Doi *et al.*, 2014) showed no specificity towards their targets in clinical trials (Beere and Green, 2017), while venetoclax was recently approved for clinical use in chronic lymphocytic leukaemia by the FDA (Luedtke *et al.*, 2017; Jain *et al.*, 2019).

The decrease in the expression of Bcl-2-related genes implies that there is an overall decrease in the functioning of the transcription factors responsible for regulating their transcription such as NF- κ B. The exceptions of *BAG3* and *BIK* suggest transcription of these genes is regulated by other means. *BAG3*, *BIK* and *BCL2A1* need further investigation.

4.4.2 Copper-imidazo[1,2-*a*]pyridines suppresses TNF-family related genes in leukaemic cells

Following treatment with the copper complexes, TNF- α and TNF- receptor complexes were decreased in both cell lines. The copper complexes suppressed the mRNA levels of TNF- α , the receptors for TNF- α , *TNFRSF1B* and *TNFRSF21* while increasing *TNFRSF25* in both cell lines. The substantial decrease of *TNFRSF1B*, *TNFRSF21* and *TRADD* in HL-60 cells are of particular interest.

Table 4.8 Changes in mRNA expression of TNF-family related genes in leukaemic cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	<i>Gene product</i>	JD47R	JD88
<i>TNF</i>	TNF- α	-3.4	-2.4
<i>TNFRSF1A</i>	TNF-R1	-3.3	+2.8
<i>TNFRSF1B</i>	TNF-R2	-28.3	-3.9
<i>TNFRSF3/LTBR</i>	LTBR	-3.6	NE
<i>TNFRSF5/ CD40</i>	CD40	-5.1	+6.0
<i>TNFRSF9</i>	CD137	-6.8	NE
<i>TNFRSF21</i>	TNFRSF21/DR6	-12.7	-3.7
<i>TNFRSF25</i>	TNFRSF25/DR3	+3.0	+4.5
<i>TNFRSF7/ CD27</i>	CD27	NE	+2.4
<i>TNFSF7/ CD70</i>	CD70	-6.2	NE
<i>TNFSF8</i>	CD30	NE	-5.0
<i>TRADD</i>	TRADD	-11.0	NE
<i>TRAF2</i>	TRAF-2	-3.6	NE
<i>TRAF3</i>	TRAF-3	-3.0	NE
<i>RIPK2</i>	RIPK-2	NE	-3.3
<i>NFKB1</i>	NF- κ B (p50)	NE	-2.0

*NE= no effect

4.4.2.1 Common TNF-associated genes

The copper-imidazo[1,2-*a*]pyridines affect the transcription of several genes associated with the tumour necrosis factor pathways in both cell lines.

a) TNF- α gene

The *TNF* gene encodes the multifunctional pro-inflammatory cytokine TNF- α (Balkwill, 2009) and its transcription is suppressed by the copper-imidazo[1,2-*a*]pyridines in both cell lines. TNF- α binds to its trimeric receptors; TNF-R1 or TNF-R2 in mediating a variety of immune responses, including cell survival (Martínez-Reza *et al.*, 2017). TNF- α stimulates the innate immune response and induces the transcriptional up-regulation of itself and the pro-inflammatory cytokine; IL-6, causing a pro-inflammatory feedback loop (Ham *et al.*, 2016).

b) TNF-R1 and TNF-R2 genes

There are two TNF receptors, TNF-R1 (coded by *TNFRSF1A*) and TNF-R2 (coded by *TNFRSF1B*). TNF-R2 gene transcription was suppressed in both cell lines, but in HL-60 cells

in a most remarkable fashion (28-fold suppression) indicating that TNF-R2 is vastly overexpressed in HL-60 cells.

Although TNF-R1 mRNA levels were only suppressed in HL-60 cells, the proteomic data indicates a decrease in TNF-R1 levels. Despite the positive effect of JD88 on TNF-R1 transcription, the proteomic data has indicated that there is a decrease in the protein expression of TNF-R1.

c) *TNFRSF21* (DR6)

The death receptor 6 (DR6) encoded by *TNFRSF21* is a member of the TNF-receptor superfamily. The transcription of DR6 was highly suppressed in both cell lines, but more so in HL-60 cells (12-fold) than in K562 cells (3.7-fold).

Apoptotic signals that stimulate DR6 is mediated by TRADD, FADD and RIPK (Klíma *et al.*, 2011). The decreased expression of these genes further contributes to the suppression of NF- κ B signalling in the leukaemic cells. Consequently, if one of the proteins within the complex is suppressed it will decrease the formation of the complex and thus decrease its activity. These observations provide an explanation for the lack of activation of the extrinsic pathway as seen in Chapter 2; section 2.3.11. Importantly, the suppression of *TRADD* and *TRAF2/3* in HL-60 and the suppression of RIPK2 in K562 cells implies that the copper complexes suppressed cell signalling pathways which regulate gene expression, proliferation, apoptosis and cell survival (Ruspi *et al.*, 2014; Wu *et al.*, 2015).

d) *TNFRSF25* (DR3)

Of interest was the increased expression of the death receptor 3 (DR3) encoded by *TNFRSF25* in both cell lines. This receptor is associated with TNF signalling and the activation of NF- κ B (Slebioda *et al.*, 2011). The ligand for TNFRSF25 is a TNF-like protein (TL1A) that exists either as a membrane-anchored protein or a soluble cytokine (Migone *et al.*, 2002). While no meaningful increases in components of active TNF-receptor complexes were evident, the increase in DR3 mRNA may be a cellular response to overcome death signals.

e) *CD40*

The copper complexes showed notable differences in the transcription of *CD40* in the two cell lines. In HL-60 cells, the transcription of *CD40* was greatly suppressed by 5.1-fold. In contrast, *CD40* mRNA transcription in K562 cells was elevated by JD88.

The decrease of *CD40* as a member of the TNF-R superfamily prevents *CD40* mediated cell survival. The ligation of *CD40* with its ligand (coded by *CD40LG*) leads to the activation of the NF- κ B transcription factor and the transcription of pro-survival genes *Bcl-x*, *Bfl-1*, *cFLIP* and *survivin* (Travert *et al.*, 2008; Song *et al.*, 2011; So and Croft, 2013). This suggests that continuous signalling by this cell-surface molecule is required to maintain the viability of leukaemic cells (Troeger *et al.*, 2007; Travert *et al.*, 2008; Elgueta *et al.*, 2009).

Despite the up-regulation of *CD40* in K562 cells, JD88 failed to increase the transcription of *CD40LG* thus preventing *CD40* mediated cell survival. The decreased transcription of *Bcl-x* and *Bfl-1* are assumed to be related to the inhibition of the NF- κ B pathway as *BCL2L1* and *BCL2A1*, respectively, are target genes of canonical NF- κ B (Lee *et al.*, 1999; Travert *et al.*, 2008).

f) *CD27/ CD70* complex

Some TNF/TNF-R complexes inhibit anti-apoptotic protein members of the *Bcl-2* family. The *CD70* cytokine known as a TNF-superfamily, member 7 (TNFSF7) is the ligand for the receptor *CD27/ TNFRSF7*. Since *CD70* mRNA levels were suppressed in HL-60 cells, and its ligand was increased in K562 cells, it implies that the protein responsible for this signal transduction may have been dysregulated.

This signal transduction pathway is mediated by a *CD27*-binding protein known as *Siva-1* (Prasad *et al.*, 1997). *Siva-1* induces apoptosis via inhibition of anti-apoptotic proteins *Bcl-2* and *Bcl-x* (Barkinge *et al.*, 2009). *Siva-1* was found to be a transcriptional target of two transcription factors, *p53* and *E2F-1* (Fortin *et al.*, 2004). *MDM2* may modulate the expression of *Siva-1* through regulation of the stability and activation of *p73* and *E2F1* transcription factors, which represent a *p53*-independent mechanism of *Siva-1* regulation (Ray *et al.*, 2011). Upon the overexpression of *MDM2* in leukaemia, *MDM2* also binds to *p73* which results in the suppression of *p73* transcriptional activity (Ray *et al.*, 2011; Trino *et al.*, 2016; Khurana and Shafer, 2019). Given that there is a reduced expression of *Siva-1* in

leukaemic cells, further studies are necessary to verify whether Siva-1 down-regulation may be involved in aberrant p53 signalling pathway due to defective p53 transcriptional machinery in leukaemia.

4.4.2.2 TNF-associated genes in HL-60 cells

In HL-60 cells, further suppression of the NF- κ B pathway is supported by a down-regulation of other proteins associated with the TNF signalling pathway like TRADD, TRAF-2 and TRAF-3. This in turn further suppresses NF- κ B activation. Another two less common TNF receptors that were suppressed are *CD137* and *LTBR*.

a) *CD137*

TNFRSF9 encodes for the cell surface protein CD137 known to be overexpressed in leukaemia, lung cancer and lymphoma cells promoting cell growth and survival (Palma *et al.*, 2004; Zhang *et al.*, 2007; Ho *et al.*, 2013). In HL-60 cells, the mRNA level of this gene was suppressed by JD47R. Its expression is regulated by transcription factors AP-1 and NF- κ B (Kim *et al.*, 2003; Glorieux and Huang, 2019). CD137 (*TNFRSF9*) mediates its action via association with TRAF-2 leading to the activation of NIK which activates NF- κ B via the IKK α complex (McPherson *et al.*, 2012). NF- κ B induces transcription of Bcl-x, Bcl-2 and Bfl-1 and thus promotes survival. The observed decrease was probably due to AP-1 and NF- κ B inactivation hence preventing cell survival. No effect on this gene was seen in K562 cells.

b) *LTBR*

Lymphotoxin-beta receptor (*LTBR*) is a member of the TNF-R superfamily whose transcription was suppressed in HL-60 cells. This receptor binds to TRAF2/3 and mediates stimulation of both canonical and non-canonical NF- κ B signalling pathways, leading to the activation of NF- κ B complexes i.e. NF- κ B p65/p50 and non-canonical NF- κ B p52/RelB (Bista *et al.*, 2010). As the qPCR data indicated (Table 4.8), the copper complexes inhibited NF- κ B transcription mediated by LTBR.

4.4.2.3 TNF-associated genes in K562 cells

In K562 cells, TRADD and TRAF-2/3 are not affected by the copper complexes, indicating that this pathway may not be as efficiently affected as in HL-60 cells. However, JD88 did decrease the transcription of RIPK2, CD30, NF- κ B and PYCARD in K562 cells.

a) *RIPK2*

The *RIPK2* gene encodes for serine/threonine protein RIPK2 is an important component of the NF- κ B signalling pathway. JD88 was able to suppress RIPK2 by 3.3-fold in K562 cells. While RIPK2 is an important component of TNF receptor complexes, this decrease protects cells from favouring cell survival.

b) *TNFSF8/CD30*

The gene *TNFSF8* encodes for the TNF ligand superfamily member, CD30L. This gene was suppressed in K562 cells only. *TNFSF8* expression is likely linked to the activation of the PI3K/Akt pathway given the interaction of its receptor (*TNFRSF8*) with TRAFs (So and Croft, 2013). This suppression suggests the reduced activation of Akt and subsequent NF- κ B signalling in K562 cells.

c) *PYCARD*

The expression of the PYCARD (pyrin-like domain (PYD) and CARD domain containing) gene is decreased in K562 cells. PYCARD is an adaptor molecule that primarily facilitates caspase-1 activation within the inflammasome. The down-regulation of PYCARD dampens caspase-1 activation but also impinges on the NF- κ B pathway. stimulates the transcription of NF- κ B, AP-1, MAPK and caspase-8 in their respective signalling pathways (Hasegawa *et al.*, 2009; Taxman *et al.*, 2011). In particular, PYCARD mediated NF- κ B activation is thought to occur by interacting with RIPK2 (Franchi *et al.*, 2009; Man and Kanneganti, 2016). Further study is required to clarify the exact mechanism.

d) *NFKB1* (NF- κ B p50)

In K562 cells, *NFKB1* encoding for the canonical NF- κ B subunit (p50) was suppressed by JD88. The mRNA transcript levels of canonical NF- κ B as well as NF- κ B regulated target genes; *BIRC2*, *BIRC3*, *BIRC5*, *BCL2*, *BCL2A1*, *MCL1*, *TNF* and *CFLAR* were decreased in K562 cells (Table 4.7 and 4.9). NF- κ B target genes were also suppressed in HL-60 cells.

The NF- κ B signal transduction pathway is dysregulated in a variety of cancers including haematological malignancies (Xiao and Fu, 2011), and is an important signalling component for the survival of leukaemic cells (Rushworth *et al.*, 2010; Crivellaro *et al.*, 2015). The expression of several anti-apoptotic genes; *BCL2*, *BCL2L1*, *XIAP*, *BIRC2*, *BIRC3*, *BIRC5*,

CFLAR and *TNF* are increased by either the canonical or non-canonical NF- κ B transcription factors (Verstrepen *et al.*, 2008).

While the actual NF- κ B mRNA levels are not directly associated with the protein activity of NF- κ B, the transcription of the *NFkB1* gene is of more importance. In addition to NF- κ B target genes mentioned, NF- κ B activity leads to the transcriptional induction of members of the NF- κ B/ I κ B pathway such as p50, RelA and I κ B. Therefore, to control and limit NF- κ B activation, it transcribes I κ B and re-inhibits NF- κ B by translocating to the cytoplasm, forming an auto-regulatory feedback loop, oscillating NF- κ B activity. The suppression of these genes suggests the copper complexes disrupted this auto-regulatory feedback loop.

In leukaemic cells, NF- κ B is constitutively active (Park and Hong, 2016) as a result of chromosomal translocations or mutations leading to myeloid transformation (Bosman *et al.*, 2016), autocrine/ paracrine cytokine signalling, as well as increased expression and activation of NF- κ B regulators (Gallipoli *et al.*, 2013; Kagoya *et al.*, 2014).

4.4.3 Copper-imidazo[1,2-*a*]pyridines induce differential effects on death receptor gene expression

Table 4.9 Changes in the mRNA levels of death receptor genes in leukaemic cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	Gene product	JD47R	JD88
<i>CFLAR</i>	c-Flip	-4.2	-2.6
<i>FADD</i>	FADD	-2.6	+2.4
<i>FAS</i>	Fas/CD95	-2.6	NE
<i>FASLG</i>	Fas ligand	NE	NE
<i>TNFSF10</i>	TRAIL	-16.5	NE
<i>TNFRSF10A</i>	TRAIL-R1	-5.1	NE
<i>TNFRSF10B</i>	TRAIL-R2	NE	+3.7

*NE= no effect

4.4.3.1 Suppression of *CFLAR* in both leukaemic cell lines

The *CFLAR* gene coding for c-FLIP, was suppressed by the copper complexes in both cell lines. Under normal conditions, caspase-8 is bound to c-FLIP, forming a heterodimer to prevent its apoptotic function (Feltham and Silke, 2017). The up-regulation of c-FLIP maintains cell survival despite TNF-receptor stimulation (Chiewchengchol *et al.*, 2016). The

decrease in c-FLIP is thought to enable caspase-8 homodimers to undergo auto-activation thereby promoting its apoptotic function (Kong *et al.*, 2017). The implications of decreased c-FLIP mRNA needs to be investigated since the apoptotic assays failed to show any meaningful activation of caspase-8.

Caspase-8 gene expression was not altered by the copper complexes and neither was it explicitly active in both cell lines (Chapter 2, section 2.3.11). Caspase-8 plays an important role in the extrinsic apoptotic pathway as well as activating pro-inflammatory caspase-1 (Man *et al.*, 2013; Gurung *et al.*, 2014).

4.4.3.2 Death receptor gene expression in HL-60 cells

In HL-60 cells, as reported in Table 4.9, the genes related to the death receptor pathway *CFLAR*, *FADD*, *FAS*, *TNFSF10* and *TNFRSF10A* were suppressed by JD47R. Two genes, death receptor ligand *TNFSF10* (encoding for TRAIL) and *TNFRSF10A* (encoding for TRAIL-R1) were highly suppressed by 16.5 and 5.1 fold, respectively. TRAIL is a member of the TNF ligand superfamily which induces apoptosis in cancer cells upon binding to death receptors TRAIL-R1 and TRAIL-R2 (*TNFRSF10B*). The adaptor molecule FADD allows for the recruitment and activation of caspase-8 cascade upon stimulation of death receptors (Fas receptor, TNF receptors, TRAIL receptors and *TNFRSF25*) (Fulda, 2014). The suppression of these genes correlates with their poor protein expression (Chapter 3; Table 3.3) and the inability of the copper complexes to activate the extrinsic apoptotic pathway in treated HL-60 cells (Chapter 2; section 2.3.11).

4.4.3.3 Death receptor gene expression in K562 cells

Conversely in K562 cells, JD88 increased the transcription of *TNFRSF10B* and *FADD* which corresponded to the increase in their protein levels (Chapter 3; Table 3.4). Despite these increases of *FADD* and *TNFRSF10B* protein and gene expression, JD88 failed to induce caspase-8 activity. These increases may be part of an adaptive response to cell stress in order to avoid cell death. Other studies have shown that K562 cells are resistant to TRAIL-induced apoptosis (Shiiki *et al.*, 2000; Wuchter *et al.*, 2001; Riccioni *et al.*, 2005).

4.4.4 Copper-imidazo[1,2-*a*]pyridines alter the gene expression of IAPs and the IAP antagonist, Smac/DIABLO, in leukaemic cells

Table 4.10 Changes in mRNA levels of anti- and pro-apoptotic genes

		HL-60	K562
		Fold change	Fold change
Genes	Gene product	JD47R	JD88
<u>Anti-apoptotic genes</u>			
<i>BIRC1</i>	NAIP	-2.3	-3.1
<i>BIRC2</i>	cIAP-1	NE	-2.3
<i>BIRC3</i>	cIAP-2	-5.8	-3.1
<i>BIRC4/XIAP</i>	XIAP	NE	NE
<i>BIRC5</i>	Survivin	NE	-3.8
<i>BIRC6</i>	APOLLON/ Bruce	NE	-3.5
<u>Pro-apoptotic genes</u>			
<i>DIABLO</i>	Smac/DIABLO	-2.5	+4.2

*NE= no effect

4.4.4.1 Gene expression of common anti-apoptotic IAPs in HL-60 and K562 cells

The transcription of some IAPs was found to be suppressed by the copper complexes as shown in Table 4.10. In both leukaemic cell lines, the expression of *BIRC1* (encoding for NAIP) and *BIRC3* (encoding for cIAP-2) was suppressed by the copper complexes. While in K562 cells, the mRNA expression of *BIRC2* (cIAP-1), *BIRC5* (survivin) and *BIRC6* (APOLLON) were decreased by JD88. The down-regulation of the NF- κ B dependent anti-apoptotic gene products IAPs appear to sensitize cells to apoptosis.

a) *BIRC1* (NAIP)

BIRC1 was suppressed in both leukaemic cell lines. The gene product NAIP (neuronal apoptosis inhibitory protein) is a member of the IAP family which regulates innate immunity (Abadía-Molina *et al.*, 2017). NAIP is commonly overexpressed in inflammatory diseases and haematological malignancies (Grzybowska-Izydorczyk *et al.*, 2010; Finlay *et al.*, 2017). NAIP possesses a BIR domain and a NOD and a leucine rich repeat domain making its functions distinct from the other IAP members. NAIP is a member of the NOD-like receptor family and modulates the assembly of the inflammasome playing an important role in the inflammatory process (Davoodi *et al.*, 2010; Mazrouei *et al.*, 2012).

b) *BIRC3* (cIAP-2)

The decrease in *BIRC3* mRNA supports the observation that the copper-imidazo[1,2-*a*]pyridines disrupt the auto-activation loop of NF- κ B-mediated transcription, thereby inhibiting cell survival. The suppression of cIAP-2 attenuates poly-ubiquitination of RIPK, an upstream event necessary for NF- κ B signalling (Rathore *et al.*, 2017). As a result, phosphorylation of the IKK complex is reduced, and signal transduction is diminished. Furthermore, the low protein expression of cIAP-2 in HL-60 cells, and the decrease in cIAP-2 protein levels in K562 cells (Chapter 3; section 3.4.5) may prove to be an important effect of the copper complexes on leukaemic cells. These findings were consistent with Harmse *et al.*, (2019) who reported a meaningful decrease in cIAP-1/2 levels by copper-imidazo[1,2-*a*]pyridines in HT-29 cells, implying decreased transcriptional activity of NF- κ B.

4.4.4.2 Changes of IAP genes in K562 cells

a) *BIRC2* (cIAP-1)

Cellular IAP-1 and -2 play an important role in the regulation of both canonical and non-canonical NF- κ B pathways (Sun, 2017). Engagement of TNF-R1 by its ligand recruits cIAP-1, TRADD, TRAF2 and RIPK. RIPK is ubiquitinated by the cIAP-1/2 which activates the canonical NF- κ B signalling complex (IKK) (Hu and Sun, 2016). The suppression of *BIRC2* mRNA levels implies decreased transcriptional activity of canonical NF- κ B suggesting that the copper-imidazo[1,2-*a*]pyridines may inhibit IKK. In contrast, IAPs have negative effects on the non-canonical NF- κ B pathway (Blankenship *et al.*, 2009; Sun, 2017). IAP-mediated ubiquitination of TRAF2/3 stabilizes the protein, which itself has E3 ligase activity (Xie, 2013). TRAF2 associated to cIAP-1/2, in turn, ubiquitinates NIK leading to its degradation (Shi and Sun, 2018). The inhibition of NIK prevents activation of the IKK complex.

b) *BIRC5* (survivin)

The decreased transcription of the IAPs by the copper complexes corroborated with their proteomic data, providing evidence for the general cytotoxic effect of the copper complexes on the leukaemic cells. An exception was observed with *BIRC5* in K562 cells (Chapter 3; section 3.4.5). The decreased *BIRC5* mRNA levels were not consistent with the increased expression of its gene product, survivin, as reported in Chapter 3 (section 3.4.5). The lack of correlation between the mRNA levels of *BIRC5* and its protein levels may indicate that the

protein is degraded at different rates. Survivin is an IAP that functions as a caspase inhibitor and as a component of mitosis whereby G₂/M phase of the cell cycle is positively regulated (Garg *et al.*, 2016). The inhibition of survivin would therefore sensitize cells to apoptosis rather than promote cell proliferation and survival.

c) ***BIRC6* (APOLLON)**

The expression of *BIRC6* which encodes for the IAP protein APOLLON was decreased by JD88 in K562 cells. Elevated *BIRC6* expression is associated with cell survival, an unfavourable response to chemotherapy and relapse (Ismail *et al.*, 2012; Hu *et al.*, 2015; Li *et al.*, 2016; Luk *et al.*, 2017). Through its BIR domain, APOLLON inhibits the caspase-3, -6, -7 and -9 and ultimately apoptosis. APOLLON contains an ubiquitin-conjugating domain which exhibits E2 and E3 ligase activity (Bartke *et al.*, 2004), facilitates proteasomal degradation of pro-apoptotic proteins Smac/DIABLO and HTRA2/Omi (Qiu and Goldberg, 2005; Salehi *et al.*, 2017). This demonstrates the importance of *BIRC6* expression in regulating cell proliferation.

4.4.4.3 **Overview of copper complexes on *BIRC* genes**

In leukaemia, the constitutively active transcription factor NF- κ B contributes to the up-regulation of IAP target genes such as *BIRC2*, *BIRC3* and *BIRC4*, *BIRC5* and *BIRC6* thereby favouring cell survival (Baumgartner *et al.*, 2002; Park and Hong, 2016). The members, XIAP, cIAP-1, cIAP-2 and survivin of the IAP family are known to inhibit caspase activity, particularly caspase-3 (Zeligs *et al.*, 2016). The qPCR data indicated that some of these pro-survival genes were suppressed in HL-60 and K562 cells following treatment. The suppression of these NF- κ B target genes indicates NF- κ B nuclear translocation via IKK was prevented by the copper complexes in both cell lines.

4.4.4.4 **Pro-apoptotic IAP antagonist; Smac/DIABLO**

The expression of *DIABLO*, encoding for the mitochondrial pro-apoptotic protein Smac/DIABLO was suppressed by JD47R in HL-60 cells (Table 4.10). E2F-1 increases the transcription of Smac/DIABLO, which also targets IAPs for proteasomal degradation (Fulda, 2014; Poppy Roworth *et al.*, 2015). The suppression of *DIABLO* predicted a decrease in protein expression, however, after the same 18 hour treatment period; Smac/DIABLO protein levels were increased (Chapter 3; section 3.4.4). The search for effective Smac-mimetic

agents is warranted to overcome these genetic alterations in various types of leukaemia (Saleem *et al.*, 2013; Saraei *et al.*, 2018).

Conversely, in K562 cells, *DIABLO* expression was increased by JD88 which correlated with the increased protein expression (Chapter 3; section 3.4.4). The opposing effects on *DIABLO* expression by the copper complexes on the two cell lines were probably related to each cell's unique response to the treatment. The increase in Smac/*DIABLO* protein expression serves as evidence that the copper complexes were able to sensitize K562 cells (gene and protein level) and HL-60 cells (at a protein level) to apoptosis.

Smac/*DIABLO* is an important facilitator of apoptosis and decreased levels contribute to chemo-resistance in haematological cancers (Rathore *et al.*, 2017). Smac/*DIABLO* interacts with and inhibits XIAP, cIAP-1, cIAP-2, survivin and Apollon to inhibit their anti-apoptotic effects (Fulda, 2014). Thus, the increase of Smac/*DIABLO* together with the decreased expression of *BIRC* genes would allow cells to overcome the anti-apoptotic state and facilitate apoptosis.

4.4.5 Copper-imidazo[1,2-*a*]pyridine suppresses *AKT1* mRNA levels in HL-60 cells

Table 4.11 Changes in mRNA levels of Akt kinase in HL-60 and K562 cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	Gene product	JD47R	JD88
<i>AKT1</i>	Akt/ Protein kinase B	-7.8	NE

*NE= no effect

In HL-60 cells the transcription of *AKT1* was substantially suppressed (Table 4.11). *AKT1* encodes for the serine/ threonine kinase, Akt, which is the downstream target of the PI3K signal transduction pathway (Weinberg, 2013). The PI3K/ Akt pathway is one of the most frequently altered pathways in cancer (Fruman and Rommel, 2014; Danielsen *et al.*, 2015). Akt is recruited to the membrane by direct binding of its Pleckstrin Homology domain to PI3K (Manning and Toker, 2017).

The transcriptional regulation of *AKT1* remains unclear. Studies have suggested that *AKT1* is transcriptionally up-regulated by the JAK/STAT3 pathway (Zhang *et al.*, 2013). It is likely that the decrease in Akt mRNA will translate to a decrease in protein expression. Akt protein

levels will need to be confirmed by western blot and immunofluorescence to establish its function in leukaemic cell death.

Akt is a kinase and its ability to phosphorylate determines the activity of a series of downstream proteins that have multiple effects on the cell (Weinberg, 2013). Due to Akt regulating diverse cellular functions, it is an important therapeutic target for the treatment cancers.

a) Anti-apoptotic effects

Akt mediates cell survival through phosphorylation of pro-apoptotic Bad, decreasing its ability to bind anti-apoptotic Bcl-2 and Bcl-x. Akt phosphorylates caspase-9 and prevents the initiation of the caspase cascade (Weinberg, 2013). Akt inhibits pro-apoptotic s target genes Bim and Fas generated by FoxO-mediated transcription (FoxO1, FoxO3a and FoxO4) (Gilley *et al.*, 2003; Zhang *et al.*, 2011). Akt phosphorylates and activates MDM2, promoting its translocation to the nucleus, where it triggers p53 degradation (Manning and Toker, 2017). (Trino *et al.*, 2016).

b) Cell proliferation and cell cycle progression

Activated Akt stimulates cell proliferation and cell cycle progression through the phosphorylation of the CDK inhibitors, p21^{Cip1} and p27^{Kip1} (Xu *et al.*, 2012). CDK inhibitors are crucial for the regulation of the DNA damage response and functions by inhibiting the completion of the cell cycle (Karimian *et al.*, 2016; Tak *et al.*, 2019). Akt can indirectly inhibit p21^{Cip1} expression through MDM2, which down-regulates p53-mediated transcription of p21^{Cip1}.

Akt directly phosphorylates the DNA damage effector kinase, Chk1 and promotes cell cycle progression through mitosis (Tonic *et al.*, 2010; Xu *et al.*, 2012). Phosphorylation blocks the checkpoint function by stimulating Chk1 translocation to the cytosol, where it is sequestered. ATM and ATR (ataxia telangiectasia and Rad3-related protein) kinases thus have less nuclear Chk1 available for sensing DNA damage (Wang *et al.*, 2011a).

4.4.6 Copper-imidazo[1,2-a]pyridines suppress *ABL* in HL-60 cells

Table 4.12 Changes in mRNA expression of *ABL*

		HL-60	K562
		Fold change	Fold change
Genes	Gene product	JD47R	JD88
<i>ABL1</i>	c-Abl tyrosine kinase	-2.9	NE

*NE= no effect

In HL-60 cells, the copper complex JD47R suppressed *ABL1* mRNA expression (Table 4.12). The *ABL* gene encodes for c-Abl tyrosine kinase which is ubiquitously expressed in most tissue and cell types (Lin and Arlinghaus, 2008). In CML, the recombined Philadelphia chromosome codes for gene product BCR -ABL, the constitutively active driver onco-protein which stimulate proliferation. *BCR-ABL* which is overexpressed in CML. BCR-ABL is the target gene for TKI inhibitors such as imatinib (Soverini *et al.*, 2018). ABL tyrosine kinase activity induces resistance to extrinsic and intrinsic apoptosis (Colicelli, 2010).

Despite the HL-60 cell line is considered BCR-ABL negative, active c-Abl kinase in haematopoietic malignancies is similar to the *BCR-ABL* oncogene in CML. The decreased transcription of c-Abl tyrosine kinase suggests that the copper complexes may have disrupted tyrosine kinase signalling and possibly inhibited downstream. signalling cascades like MAPK, STAT, and PI3K /Akt pathways that regulate cell survival, cell proliferation, and inhibition of apoptosis of leukemic cells (Hantschel, 2012; Patel *et al.*, 2017).

4.4.7 Copper-imidazo[1,2-a]pyridines show varying effects on caspases

Table 4.13 shows that the effect of the copper complexes on caspase expression is mostly random and inconsistent. Since the two compounds tested have similar structures, similar IC₅₀ values and share the ability to induce apoptotic cell death, one can argue that they should have a similar effect on caspase gene transcription. However, in the HL-60 cell line, *CASP1*, *CASP2*, *CASP3* and *CASP7* encoding for caspases-1, -2, -3 and -7 were suppressed by JD47R while *CASP5* encoding for caspase-5 was increased. In contrast, in K562 cells, *CASP1*, *CASP4* and *CASP14* expression was increased while that of *CASP6* and *CASP9* was decreased.

Table 4.13 Changes in mRNA levels of caspases in leukaemic cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	<i>Gene product</i>	<i>JD47R</i>	<i>JD88</i>
CASP1	Caspase-1	-2.1	+3.2
CASP2	Caspase-2	-4.1	NE
CASP3	Caspase-3	-3.0	NE
CASP4	Caspase-4	NE	+2.1
CASP5	Caspase-5	+4.1	NE
CASP6	Caspase-6	NE	-2.9
CASP7	Caspase-7	-3.1	NE
CASP8	Caspase-8	NE	NE
CASP9	Caspase-9	NE	-2.5
CASP14	Caspase-14	NE	+2.5

*NE= no effect

The *CASP* genes encode for eleven members of the caspase family. Initiator caspase gene products (CASP2, CASP8, CASP9 and CASP10) are activated before the executioner caspase gene products (CASP3, CASP6 and CASP7). These caspases play a pivotal role in the activation and execution phases of apoptosis (Shalini *et al.*, 2015).

4.4.7.1 Gene expression of caspases in HL-60 cells

a) Gene expression of CASP3

In HL-60 cells, despite the decreases in gene expression, an increase in caspase-3 activity was observed. This was observed in both cell lines as evidenced by the apoptotic assays conducted in Chapter 2 (section 2.3.7). In addition, proteome analysis revealed an increase in cleaved caspase-3 providing further confirmation for caspase-3 activity (Chapter 3; Table 3.3 and 3.4). However, intact, non-cleaved caspase-3 was not increased which corresponds to the decrease in mRNA of the caspase-3 levels. This indicates that the mRNA levels are not necessarily a direct measure of the amount of protein present in a cell at a specific time, nor does it indicate if that protein is active or inactive. Proteins are degraded via the proteasome after ubiquitination and interference with this process will cause expressed protein to persist in the cell (Alberts *et al.*, 2015).

b) Gene expression of CASP2

Caspase-2 was down-regulated at the mRNA level following treatment with JD47R. Caspase-2 is an initiator caspase (Miles *et al.*, 2017) and is implicated in the regulation of cell death

that is induced by DNA damage, endoplasmic reticulum stress and mitotic catastrophe (Lopez-Cruzan *et al.*, 2016). Studies on caspase-2 are confounded by the fact that it may have tumour promoting tendencies (Shalini *et al.*, 2015).

c) Gene expression of *CASP1*, *CASP4* and *CASP5*

Caspase-1 as well as caspase-4 and caspase-5 are associated with the inflammatory programmed cell death pathway called pyroptosis (Vanaja *et al.*, 2015). The transcription of these inflammatory caspases is regulated by canonical NF- κ B, the mediator of the inflammatory response (Sagulenko *et al.*, 2018). Given the decrease in caspase-1 expression in HL-60 cells, the copper complexes did not contribute to canonical NF- κ B activity. Activation of canonical NF- κ B with IL-1 α elevates the expression of caspase-1, and this effect can be blocked by inhibiting NF- κ B activation, which blocks inflammasome function (Vanaja *et al.*, 2015).

Given the considerable fold increase in caspase-5 mRNA (4-fold) caused by JD47R, it is likely to be important in the cell death pathway of HL-60 cells. Caspase-5 together with the pro-inflammatory caspase-1 in the inflammasome indicates that it forms part of the inflammatory caspase subfamily and is regulated by pro-inflammatory factors such as lipopolysaccharide and interferon gamma (Bian *et al.*, 2011).

There is evidence that inflammatory caspases can cause the cleavage of caspase-3 and initiate apoptosis (Miao *et al.*, 2018; Sagulenko *et al.*, 2018). Caspase-5 was shown to reduce hypoxia-induced apoptosis in hepatocytes which indicates that it may be an important regulator of apoptosis (Zhu *et al.*, 2012). However, interrelationship of apoptotic caspases, inflammatory caspases and pyroptosis remains unclear (Man and Kanneganti, 2016; Man *et al.*, 2017). Based on these results of the gene array, the role of caspase-5 in cell death pathways in this cell line requires further investigation. In addition, protein levels of caspase-5 need to be investigated by western blotting and immunofluorescence determine its activation status.

4.4.7.2 Gene expression of caspases in K562 cells

a) Suppression of effector *CASP6*

The mRNA expression of *CASP6* (encoding caspase-6) was decreased in K562 cells. Caspase-6 is an effector caspase that is activated by caspase-3 in the proteolytic cascade (McIlwain *et al.*, 2013). Activated caspase-6 is able to cleave and activate caspase-8 (Kominami *et al.*, 2012). This cleavage might be required for amplification of apoptotic signals through the cascade but is not essential for the execution of cell death associated with caspase-3 activation. Although caspase-6 activity was not measured in this study the lack of caspase 8 activity indicates that it was not activated in K562 cells (Chapter 2; section 2.3.11).

b) Gene expression of initiator *CASP8* and *CASP9*

Despite the decrease in caspase-9 gene expression in K562 cells, caspase-9 activity was potently induced (Table 4.13). This was observed in both cell lines as evidenced by the apoptotic assays conducted in Chapter 2 (section 2.3.10). The transcription of caspase-9 genes are regulated by transcription factor activator protein-4 (AP-4) (Tsujimoto *et al.*, 2005; Jung and Hermeking, 2009). This suggests that AP-4 may have been dysregulated in K562 cells contributing to the decreased transcription of caspase-9. E2F-1 is activated as cells pass through the cell cycle checkpoint to S phase, to induce apoptosis.

The copper complexes failed to induce meaningful caspase-8 activity in both cell lines (Chapter 2; section 2.3.11), therefore supporting the lack of effect on caspase-8 gene expression shown in Table 4.13. The transcription of caspase-8 is stimulated by E2F-1 (Cao *et al.*, 2004; Huang *et al.*, 2016). E2F-1 regulates S phase entry of the cell cycle. It is likely that the activity of E2F-1 was defective in the leukaemic cells investigated.

c) Gene expression of inflammatory *CASP1* and *CASP4*

The increased mRNA expression of caspase-1 and 4 are likely to be an important event in K562 cell death (Table 4.7). In line with these observations, we reported that *NFκB1* is suppressed (2-fold) in K562 cells while, IL-10 is elevated by 2.4-fold in Table 4.6. NF-κB is a key transcription factor that is required for the induction of a several inflammatory genes, including those encoding for TNF-α, IL-1β, IL-6, IL-12 and cyclooxygenase-2 (Liu *et al.*, 2017b). While the production of anti-inflammatory cytokines, IL-10 and IL-13, are important

for resolving inflammation (Burmeister and Marriott, 2018). These observations indicate that canonical NF- κ B was not activated and no inflammatory cytokines (e.g. *TNF*) were actively transcribed in K562 cells. In cases such as with caspase-1, where the copper complexes caused opposite effects between the two cell lines, it is most likely a reflection of the unique sequence of events in that cell line.

d) Gene expression of non-apoptotic *CASP14*

In contrast to the other caspases, caspase-14 expression and activation is associated with cell differentiation rather than the direct induction of apoptosis (Denecker *et al.*, 2008). Caspase-14 mRNA levels were increased in K562 cells. *CASP4* is a transcriptional target of GATA-3, a transcription factor of the GATA family (Asselin-Labat *et al.*, 2011). GATA proteins recognize G-A-T-A nucleotide sequences in target gene promoters and activate or repress those genes (Chou *et al.*, 2010; Zheng and Blobel, 2010). The overexpression of caspase-14 is reported to significantly decrease cell proliferation and delay tumour cell growth in several cancers including ovarian, breast, epithelial carcinomas and gastric cancer (Koenig *et al.*, 2005; Krajewska *et al.*, 2005; Asselin-Labat *et al.*, 2011; Li *et al.*, 2015b). It is thus possible that the copper complexes increased the transcriptional activity of GATA-3 in K562 cells to promote caspase-14 mRNA expression.

4.4.7.3 Suppression of cytochrome c (*CYCS*) in HL-60 and K562 cells

Cytochrome c is an important factor required once released from the mitochondria for the activation of caspase-9 (Xiong *et al.*, 2014). Cytochrome c mRNA levels were suppressed by the copper complexes by 3.1-fold in HL-60 cells and 3.8-fold in K562 cells (Table 4.5 and Table 4.6, respectively); however HL-60 cells showed an increase in cytochrome c protein and in K562 cells there was no significant change (Chapter 3; Table 3.3 and Table 3.4). Since the copper complexes caused mitochondrial damage as evidenced by the loss of MMP, the damaged mitochondria are possibly degraded by a specialised form of autophagy called mitophagy (Mammucari and Rizzuto, 2010), ensuring the maintenance of functional mitochondria that can produce ATP. This in turn decreases the transcription of mitochondrial proteins mRNA such as cytochrome c (*CYCS*) which occurs in mitochondrial DNA.

4.4.8 Copper-imidazo[1,2-*a*]pyridines alters the expression of tumour suppressor and pro-apoptotic genes

Table 4.14 Changes in mRNA expression of pro-apoptotic genes in leukaemic cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	Gene product	JD47R	JD88
<i>TP53</i>	Tumour suppressor p53	NE	NE
<i>TP73</i>	Tumour suppressor p73	-4.7	+2.0
<i>GADD45A</i>	Gadd45A	+4.8	NE
<i>DAPK</i>	DAPK	NE	+3.3
<i>DFFA</i>	ICAD	-3.1	NE
<i>CIDEB</i>	CIDE-B	-6.3	NE

*NE= no effect

4.4.8.1 Expression of tumour suppressor genes *TP53* and *TP73*

The *TP53* gene encodes for tumour suppressor p53 which regulates the transcription of apoptotic genes including caspases, death receptors and Bcl-2-family members. In this study, no changes in *TP53* mRNA levels were detected in either cell line (Table 4.14), which is consistent with HL-60 cells being a p53 null cell line and K562 cells harbouring a p53 single base pair mutation (Crivellaro *et al.*, 2016).

The *TP73* gene encodes for tumour suppressor protein p73, which is analogous to p53. Tumour suppressor p73 induces the expression of pro-apoptotic proteins PUMA, NOXA, and Siva-1, and activates transcription of p21^{Cip1}, Bax and GADD45 and induces apoptosis irrespective of p53 status. The variances in p73 gene expression in the two cell lines may well be an indication of their genetic differences. Future investigations on p73 protein levels using western blot and immunofluorescence are necessary to verify the effects of the copper complexes on its expression in these leukaemic cells.

4.4.8.2 Gene expression of *GADD45A*

The protein GADD45 α (Growth Arrest and DNA Damage-induced gene 45 alpha) is encoded by *GADD45A* which is induced by DNA damage and is regulated by p53 in normal cells (Liebermann and Hoffman, 2008). The copper complexes were still able to induce *GADD45A* transcription in HL-60 cells with negative p53 status (Table 4.14). This has been previously

reported in colon and breast cancer cell lines (Tamura *et al.*, 2012). GADD45 α was induced following treatment with JD47R and DNA damage. *GADD45A* actively participates in apoptosis or cell cycle arrest and DNA repair by inducing G₂/M phase arrest through the inhibition of Cdc2 kinase activity via the MAPK pathway (Zhan, 2005).

4.4.8.3 Gene expression of *DAPK*

The death-associated protein kinase, DAPK is a tumour suppressor and regulator of apoptosis (Singh *et al.*, 2016). An increased expression of *DAPK* mRNA levels is likely to promote apoptosis (Table 4.14). This suggests that DAPK may contribute to the apoptotic effect of the copper complexes in K562 cells by sensitizing them to apoptosis. The death domain of DAPK is crucial in mediating its apoptotic function and kinase activity (Shiloh *et al.*, 2014). The exact mechanism by which this occurs remains elusive. Further investigations inhibiting DAPK would indicate whether it is crucial in the induction of apoptosis in K562 cells. Validation approaches like western blot or immunohistochemistry to examine the protein expression of DAPK are necessary.

4.4.8.4 Gene expression of *DFFA* and *CIDEB*

The gene *DFFA* (DNA fragmentation factor subunit alpha) encodes for ICAD. Apoptosis is characterised by DNA fragmentation and chromosomal degradation (Elmore, 2007). DFF40 and its inhibitor DFF45/ICAD, are two subunits of the DFF complex (Inohara *et al.*, 1998). *CIDEB* (cell death-inducing DFF45 (DNA fragmentation factor 45)-like effector B) encodes for CIDE-B, a member of apoptosis-inducing factors, which was decreased with borderline significance in HL-60 cells (Table 4.14). In turn, *DFFA* was suppressed. The N-terminal region of CIDE is homologous to the CIDE N-domains in DFF40 and DFF45/ICAD. CIDE-B regulates the cleavage of DFF45/ICAD by caspase-3 releasing DFF40/CAD from the complex, leading to DNA fragmentation and nuclear condensation (Liang *et al.*, 2003; Yoshida *et al.*, 2006).

4.4.9 Copper-imidazo[1,2-*a*]pyridines suppresses anti-apoptotic genes in HL-60 cells

Table 4.15 Changes in mRNA expression of anti-apoptotic genes in leukaemic cells

		HL-60	K562
		Fold change	Fold change
<i>Genes</i>	Gene product	JD47R	JD88
<i>IGF1R</i>	IGF1 receptor	-3.4	NE
<i>NOD1</i>	NOD-1	-4.0	NE
<i>NOL3</i>	NOL-3	-2.7	NE

*NE= no effect

4.4.9.1 Suppression of *IGF1R* gene

The overexpression of *IGF1R*, encoding for insulin-like growth factor I receptor (IGF1R), and the activation of its downstream signalling molecules have been linked to the inhibition of apoptosis, increased resistance to cytotoxic chemotherapeutic drugs and to poor prognosis in several cancers (Chapuis *et al.*, 2010; Shi *et al.*, 2010). In response to the copper complexes, *IGF1R* was suppressed in HL-60 cells, preventing cell survival while not affecting K562 cells (Table 4.15). This receptor binds insulin-like growth factor with a high affinity and has tyrosine kinase activity leading to auto-phosphorylation. Phosphorylation of IGF1R leads to PI3K/Akt and the Ras/MAPK pathway activation ultimately leading to increased cellular proliferation (Chapuis *et al.*, 2010; Shi *et al.*, 2010). Activated PI3K/Akt triggers the anti-apoptotic effects of IGF1R through phosphorylation and inactivation of Bad, increased protein synthesis and increased glucose metabolism (Chapuis *et al.*, 2010; Manning and Toker, 2017).

4.4.9.2 Suppression of *NOD1*

The copper complex JD47R decreased the mRNA levels of *NOD1*, encoding for NOD-1 protein (nucleotide-binding oligomerization domain containing 1). The encoded protein plays a role in innate immunity by initiating inflammation (Monie, 2017). NOD-1 harbours a CARD domain that facilitates RIPK2 interaction, leading to the activation of IKK and NF- κ B and subsequent induction of pro-inflammatory cytokines (Kim *et al.*, 2010; Witt and Vucic, 2017). The suppression of *NOD1* (Table 4.15) will decrease cytokine expression and protect cells from cell survival caused by NF- κ B activation.

4.4.9.3 Gene expression of NOL3

NOL3 encodes for an apoptotic repressor which has been shown to inhibit multiple modes of cell death. *NOL3* was suppressed by JD47R in HL-60 cells (Table 4.15). The overexpression of *NOL3* is implicated in AML (Stanley *et al.*, 2017). The gene product, NOL-3 (nucleolar protein 3 (apoptosis repressor with CARD domain)) has a unique ability to inhibit both the intrinsic and extrinsic apoptosis pathways (Toth *et al.*, 2017). It suppresses caspase-2/-8 enzymatic activity via the CARD domain and interacts with FADD via its death effector domain, to prevent DISC assembly. NOL-3 prevents intrinsic apoptosis by inactivating Bax and preventing mitochondrial release of pro-apoptotic factors. Lastly, it inhibits the TNF-signalling pathway by abrogating the recruitment of RIPK to the TNF-R1 complex (Kung *et al.*, 2014).

4.4.10 In Summary:

The copper-imidazo[1,2-*a*]pyridines were effective inducers of intrinsic apoptotic cell death and changed the expression levels of key genes, important for cell survival and cell death. The reduced expression of Bcl-2 related genes, *BIRC* genes and *CFLAR* indicates that the copper complexes disrupted NF- κ B transcriptional activity to promote apoptosis. This is further supported by a suppression of various components of the TNF signalling pathways on both an mRNA and a protein level in both HL-60 and K562 cells. These copper complexes may be useful tools to better understand the complexity of cell death signalling networks in leukaemic cell death in response to the copper complexes.

CHAPTER FIVE: Conclusion

The purpose of this study was to identify new compounds that may be beneficial in the treatment of leukaemias such as acute promyelocytic leukaemia and chronic myelocytic leukaemia. In this context, a library of imidazo[1,2-*a*]pyridines and metal coordinated imidazo[1,2-*a*]pyridines were evaluated. These compounds were synthesized and their structures verified by collaborators in the School of Chemistry, University of the Witwatersrand. Once active compounds were identified, the mode of cell death was determined and compounds that caused cell death by apoptosis were identified. Apoptotic proteome arrays and qPCR apoptotic panels were used to better understand the mechanism/s of action and effects of the active compounds on cell death signalling pathways. Two cell lines were selected for this study HL-60 cells representing acute promyelocytic leukaemia and K562 cells representing chronic myelocytic leukaemia.

5.1 Copper-imidazo[1,2-*a*]pyridines were selectively active toward HL-60 and K562 cells

Copper-imidazo[1,2-*a*]pyridines JD88, JD47R and JD49R were identified as the most active against HL-60 and K562 cells with IC₅₀ values below 6.0 μM. JD47R was the most active copper complex in HL-60 cells (IC₅₀ = 2.1 μM), while JD88 was the most active in K562 cells (IC₅₀ = 1.7 μM). The positive control camptothecin was cytotoxic to HL-60 cells with an IC₅₀ value of 7.0 nM compared to an IC₅₀ value of 2.8 μM in K562 cells. Unfortunately none of the complexes tested showed activity comparable to camptothecin in HL-60 cells. Compared to the leukaemic cell lines, the copper complexes were less toxic to normal unstimulated peripheral leukocytes suggesting that the leukaemic cells were more sensitive. No single copper complex was equally active in both cell lines which made it necessary to select specific copper complexes for further evaluation in each cell line.

The anti-leukaemic action of copper complexes depends on their molecular structure and even minor structural modifications affected their cytotoxic properties. Several structural features were important for activity: these included a cyclohexyl linked to the amidogen group of the imidazo[1,2-*a*]pyridine base structure and the addition of a bromine atom at 6-position of the imidazo[1,2-*a*]pyridine core. This study showed that for the HL-60 and K562 cell lines, copper complexes with a halogen atom (Br or Cl) in 6-position are more potent

than those with a halogen at the 5-position. Since JD88 was the most potent in both cell lines, it indicated that the substitution of a bromine atom at C6 was the most beneficial modification, increasing the cytotoxic activity.

5.2 Copper-imidazo[1,2-*a*]pyridines induce intrinsic apoptosis

Morphological analysis of cells treated with the most active copper complexes indicated that apoptotic cell death is a likely end point. Thus membrane blebbing, apoptotic nuclei and nuclear fragmentation were prominent features in treated cells after 24 hours of exposure. This was confirmed by increased Annexin-V binding and caspase-3/7 activity. JD88 and JD47R caused the most Annexin-V binding in HL-60 cells (66%) and JD49R caused the most Annexin-V binding in K562 cells (57%). Differences between the extents of the PS exposure on the outer cell membrane between the compounds can most likely be attributed to the variations in the molecular signatures of the two cell lines.

The induction of caspase-3/7 activity was the highest in HL-60 cells. K562 cells appeared to be more resistant to the induction of apoptosis than the HL-60 cells. This is consistent with previous studies which have shown K562 cells to be less sensitive to genotoxic stress than other leukaemic cell lines such as HL-60 and Jurkat cells (Bourogaa *et al.*, 2011; Watanabe *et al.*, 2012; Hui *et al.*, 2014; Liu *et al.*, 2015). Taken together, the translocation of PS and the increase of caspase-3/7 activity provided evidence that the active copper-imidazo[1,2-*a*]pyridines caused apoptotic cell death in both cell lines, albeit less in K562 cells.

The loss of MMP and the activation of caspase-9 are both indicators for activation of the intrinsic mitochondrial apoptotic pathway. JD88, JD47R and JD49R showed a complete loss of MMP. In both cell lines, the activity of caspase-9 was increased by the active copper complexes. Despite exposing cells to various incubation periods, no significant caspase-8 activity was observed at any time point, further providing evidence for the activation of the intrinsic apoptotic pathway.

These results are consistent with another study on these complexes which confirmed the induction of intrinsic apoptosis by the copper-imidazo[1,2-*a*]pyridine complexes JD88, JD46 and JD47R *in vitro* in colorectal cancer cells (Harmse *et al.*, 2019). Hence, copper-imidazo[1,2-*a*]pyridines were effective inducers of intrinsic apoptosis in both leukaemic and colorectal cancer cell lines.

The HL-60 and K562 cell lines have different doubling times and through exploratory experiments it appeared that the K562 cells were more resistant to cell death than the HL-60 cells, hence treatment periods for K562 cells were longer than HL-60 cells. Furthermore, different treatment periods were established based on the effects the copper complexes had on the various apoptotic markers such as Annexin-V/PI and MMP depolarisation as they were performed at different time periods.

The depolarisation of MMP is an early event of apoptosis. In HL-60 cells, after 14 hours, treated cells showed a loss in MMP whereas in K562 cells it occurred after 18 hours of exposure. Similarly, the externalisation of PS is an early event of apoptosis which does not depend on caspase activation and was measured in HL-60 and K562 cells at 14 and 24 hours, respectively. Whether membrane flipping occurs prior to the disruption of MMP is unclear and yet to be determined.

The loss in MMP suggests that the copper complexes have a rapid effect on mitochondrial activity confirming their role in mitochondrial mediated apoptosis. The change in MMP is linked to the change in mitochondrial membrane permeability triggering the release of mitochondrial cytochrome c into the cytosol. Once in the cytoplasm, cytochrome c binds to Apaf-1 and activates procaspase-9. Similarly, caspase-9 activity was detected at 14 and 24 hours in HL-60 and K562 cells, respectively, which in turn activated downstream effector caspase-3/7.

Caspase-3/7 activity was detected in HL-60 and K562 cells at 18 and 24 hours, respectively. Caspase-3 and caspase-7 are proteolytic enzymes which cause the cleavage of cytoskeletal proteins, disruption in the nuclear membrane and the release of CAD from ICAD to allow DNA fragmentation (McIlwain *et al.*, 2013). These proteolytic events are responsible for the morphological changes characteristic of apoptotic cells.

5.3 Apoptosis proteome and genome array profiling

In order to obtain a more complete understanding of the mechanism involved in the apoptotic process, an apoptotic proteome array was employed to determine the effects of the copper-imidazo[1,2-*a*]pyridines on the expression of apoptotic-regulatory proteins. The proteomic analysis provided insight in the effect of the compounds on regulatory proteins of the apoptotic process. An apoptosis specific human gene array was similarly used to evaluate the

effects of the most active imidazo[1,2-*a*]pyridines on gene expression. The human apoptotic PCR arrays showed meaningful changes in expression levels of genes encoding for apoptosis related proteins when cells were exposed to the copper-imidazo[1,2-*a*]pyridines for 18 hours.

5.3.1 Copper-imidazo[1,2-*a*]pyridines elevate cleaved caspase-3 protein levels confirming the induction of apoptosis

Proteomic analysis showed a dramatic increase in cleaved caspase-3 protein in both cell lines. The increased expression of cleaved caspase-3 confirms the validity of the caspase-3 functional assays and supports the conclusion that the copper complexes cause cell death by apoptosis in HL-60 and K562 cells.

5.3.2 Copper-imidazo[1,2-*a*]pyridines increased expression of pro-apoptotic Bax and Bad whilst decreasing anti-apoptotic Bcl-2 proteins and genes

The copper complexes increased the expression of pro-apoptotic Bad and Bax whilst decreasing anti-apoptotic Bcl-2 proteins. The increased expression of Bad and Bax by the copper complexes appears to have out-competed the effects of anti-apoptotic Bcl-2 and Bcl-x proteins (Chapter 3; section 3.4.3, Table 3.8) and indicated that these compounds were able to drive cells toward a pro-apoptotic state and activate the intrinsic mitochondrial pathway. The increase in Bad expression will promote its binding to Bcl-2, inactivate it, and favour apoptosis. These findings are supported by the data obtained from apoptotic marker assays which show the disruption of MMP and activation of caspase-9 in HL-60 and K562 cells.

Gene array data indicated a decrease in a number of Bcl-2 transcripts in both cell lines as well as a decrease in Bad and Bax in the two cell lines. This indicates that mRNA transcripts cannot always be used as a direct predictor of the amount of protein present after a specific treatment. Gene array data indicated a massive increase in pro-apoptotic Bik in K562 cells and this requires further investigation on the protein level. It may well be that an increase in Bik overcomes the anti-apoptotic effect caused by the decreased mRNA levels of pro-apoptotic Bcl-2 proteins. The Hrk gene expression which was increased in both cell lines could cause an increase of this protein. The Hrk protein forms inactive complexes with Bcl-2 and Bcl-x, mitigating their anti-apoptotic effects.

5.3.3 Copper-imidazo[1,2-*a*]pyridines increase HO-1/HMOX/HSP32 expression in response to cell stress

In HL-60 cells, the proteome profile identified HO-1/HMOX-1/HSP32 as an interesting potential anti-cancer target. Increased HO-1/HMOX-1/HSP32 expression, in response to cancer chemotherapy, is emerging as a mechanism of resistance to cancer chemotherapy (Lin *et al.*, 2015; Cao *et al.*, 2016; Salerno *et al.*, 2017). The increased expression of HO-1/HMOX-1/HSP32 may have reduced the cytotoxic potential of the copper-imidazo[1,2-*a*]pyridines in HL-60 cells. However, the mechanism by which this occurred will require further investigation. Combination studies with HO-1/HMOX1/HSP32 inhibitors may provide useful insight as to whether increased HO-1/HMOX1/HSP32 protects cells against the copper-imidazo[1,2-*a*]pyridines or aids the apoptotic effect of them (Harmse *et al.*, 2019). The use of HO-1/HMOX-1/HSP32 inhibitors in combination may increase the sensitivity of leukaemic cells to the cytotoxic effects of the copper complexes and other cytostatic agents.

5.3.4 Copper complexes suppress IAP expression while simultaneously increasing IAP antagonist, Smac/DIABLO expression, in HL-60 and K562 cells

In both cell lines, the increased expression of pro-apoptotic proteins and the suppression of IAP proteins favoured apoptosis. In HL-60 cells, the protein levels of XIAP was decreased by JD47R but increased by JD88. Despite the increase in XIAP by JD88, the concomitant increases in pro-apoptotic HTRA2/Omi together with Smac/DIABLO mitigated the anti-apoptotic effect of XIAP and re-sensitized HL-60 cells to enter apoptosis. In K562 cells, both copper complexes caused substantial decreases in XIAP as well as elevated Smac/DIABLO expression, together promoting apoptosis. The XIAP mRNA levels were not affected in either cell line suggesting the possible inhibition of its degradation. Upon the loss of MMP, Smac/DIABLO is released from mitochondria and directly binds to IAPs, inhibiting their function, and thereby allowing apoptosis to proceed (Lopez and Tait, 2015).

The XIAP protein is a potent inhibitor of caspase-3, -7 and -9 followed by cIAP-1 and cIAP-2, which are considerably weaker. Copper-imidazo[1,2-*a*]pyridines; specifically JD88, was able to induce caspase-3/7 and caspase-9 activity as previously described (Chapter 2; section 2.3.7 and 2.3.10). Collectively, these results indicate that JD88, JD47R and JD49R have decreased the IAPs in HL-60 and K562, thereby overcoming their anti-apoptotic effects.

The protein levels of cIAP-1 and cIAP-2 were suppressed by the copper-imidazo[1,2-*a*]pyridines which indicated that cIAP-1 and 2 synthesis was inhibited in HL-60 and K562 cells. Anti-apoptotic cIAP-1 and cIAP-2 protein and mRNA levels were suppressed in K562 cells. In HL-60 cells, copper complexes showed no meaningful changes on IAPs but did suppress *BIRC1* and *BIRC3* mRNA levels coding for cIAP-2 and NAIP, respectively. The decrease in IAPs indicates suppression in NF- κ B signalling, since the transcription of these proteins is under NF- κ B control.

5.3.5 Copper complexes down-regulated key genes encoding for anti-apoptotic proteins

The down-regulation on select genes which encode for anti-apoptotic proteins Bcl-2, Bcl-x, cFLIP and IAPs indicated modulation of apoptotic and cell survival pathways in both HL-60 and K562 cells. Together the decreased mRNA expression of Bcl-2 related genes, *BIRC* genes and *CFLAR* indicates a decrease of NF- κ B transcriptional activity. It is evident that the copper complexes disrupt NF- κ B transcriptional activity to promote apoptosis. This is further supported by a suppression of various components of the TNF signalling pathways on both an mRNA and a protein level (Figure 5.1). A detailed description of the TNF signalling process linked to NF- κ B and the effects of JD47R and JD88 on these pathways are discussed below.

5.3.6 The anti-apoptotic role of TNF α in HL-60 and K562 leukaemic cells

Proteomic results from Chapter 3, section 3.4.6, show that TNF-R1 was decreased in HL-60 and K562 cells treated with the active copper-imidazo[1,2-*a*]pyridines. The increase in TNF-receptor expression suggests the activation of the extrinsic apoptotic pathway and NF- κ B pathway (Ham *et al.*, 2016; Savva *et al.*, 2016). The NF- κ B family of transcription factors are involved in immune activation, inflammation, cell survival and proliferation (Park and Hong, 2016; Liu *et al.*, 2017b). TNF-R1 recruits the TRADD adaptor protein which in turn leads to NF- κ B activation. NF- κ B promotes the transcription of anti-apoptotic IAP proteins which prevent TNF-R1-mediated apoptosis (Vince *et al.*, 2009).

The anti-apoptotic cIAP-1 and cIAP-2 proteins facilitate TNF-R1-induced activation of NF- κ B and regulate their own transcription (Fulda, 2014). Since these IAP proteins were decreased by the copper complexes (Chapter 3; Table 3.10), it follows that TNF-R1 induced activation of NF- κ B may have been inhibited, thereby preventing cell proliferation (Ham *et*

al., 2016). It is likely that this decrease further enhanced the pro-apoptotic status of both HL-60 and K562 cells. Inhibited NF- κ B is thought to divert cells to TNF-induced apoptosis (Savva *et al.*, 2016). The lack of caspase-8 activity in HL-60 cells and the poor activity of caspase-8 in K562 cells indicate that apoptosis was not induced via TNF-signalling, despite the increased protein expression of TRAIL-R, FADD and Fas (Chapter 3; section 3.4.6). These results are consistent with the apoptotic effects observed using direct apoptotic activity assays in Chapter 2; section 2.3.10 and 2.3.11.

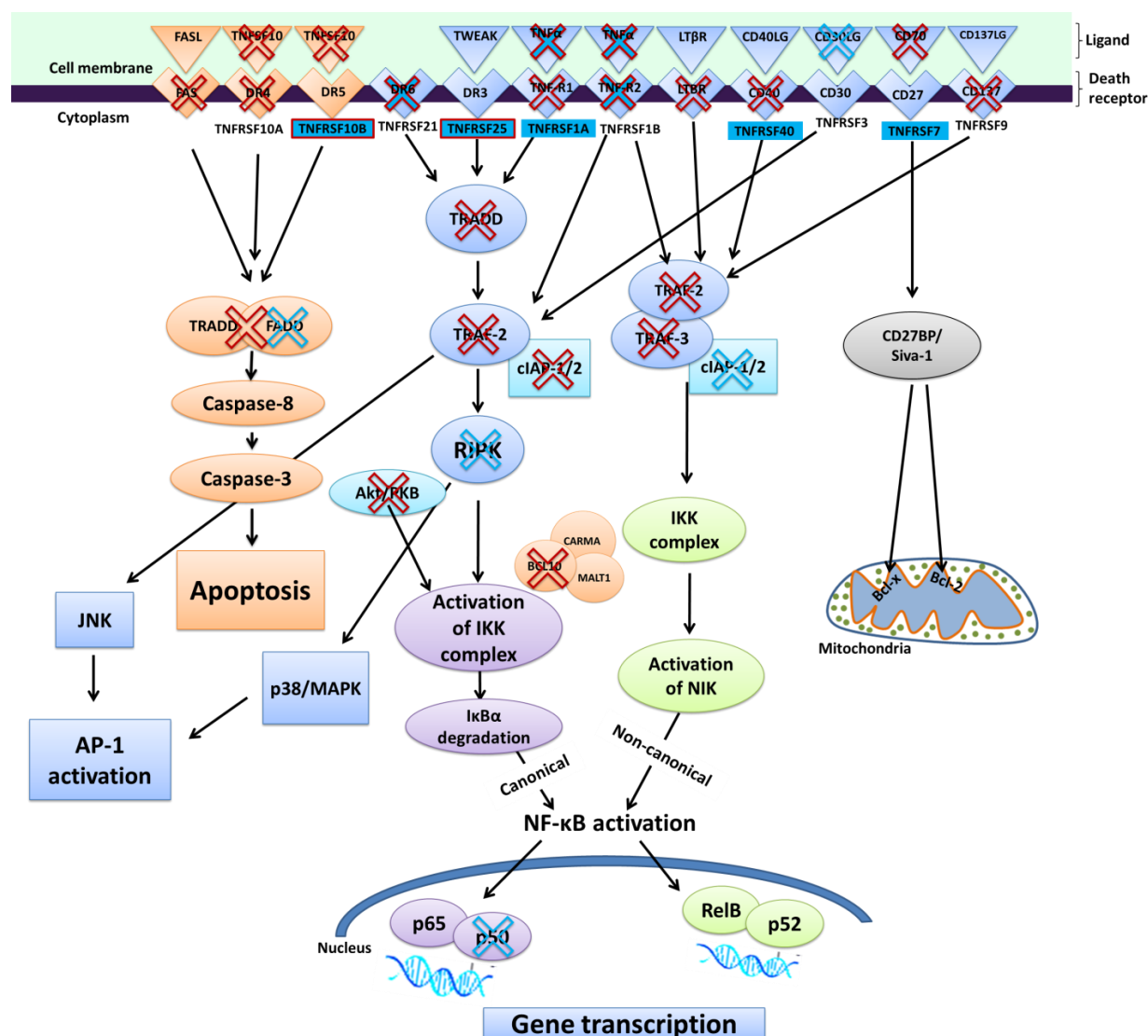


Figure 5.1 TNF-receptor signalling pathways. The schematic illustrates death receptors and their respective ligands involved in TNF signalling. The signal transduction of TNF-receptors mediates the activation of the transcription factors NF- κ B and AP-1 through IKK and JNK/p38MAPK, respectively. NF- κ B is regulated by Akt by the phosphorylation and activation of IKK. TRADD and FADD have a central regulatory role in apoptosis mediated by caspase-8. The effects of the copper complexes on TNF-related genes in both HL-60 (red) and K562 (blue) cells are illustrated. Crosses represent the suppression of genes while highlights represent increases in gene expression. Figure adapted from: www.cellsignal.com/contents/science/cst-pathways/science-pathways).

Analysis of the gene array data showed suppression of TNF-R1 mRNA levels in HL-60 cells and TNF-R2 mRNA levels in both HL-60 and K562 cells. The most remarkable decrease of TNF-R2 (28-fold) in HL-60 cells indicates that this receptor may play an important role in stimulating and ensuring the survival of these leukaemic cells. Further analysis of the TNF-R1 related transcripts indicate that numerous components of this pathway were inhibited by the copper complexes.

The engagement of TNF- α to TNF-R1 facilitates the interaction with adaptor protein TRADD through its death domain to recruit TRAF2, cIAP-1/2 and RIPK (Ham *et al.*, 2016). The expression of TNF- α mRNA was suppressed in both cell lines. TNF- α activates both TNF-R1 and TNF-R2. TNF-R1 causes activation of TRADD which in turn cause the association of TRAF2 with cIAP-1/2. TNF-R2 activation bypasses TRADD and recruits of existing TRAF2/cIAP-1/cIAP-2 complexes (Vince *et al.*, 2009). The TRAF2-cIAP-2 complex causes RIPK ubiquitination by the E3 ubiquitin ligases, cIAP-1 and 2, which in turn phosphorylates and causes the proteasomal degradation and dissociation of I κ B α from NF- κ B. The degradation of I κ B α , allows for the translocation of NF- κ B to the nucleus where it functions as a transcription factor. This constitutes the canonical NF- κ B pathway (Ruspi *et al.*, 2014). The TRAF2-cIAP-2 pathway is also activated by TNFRSF21, which was massively decreased in both cell lines (Figure 5.1).

Alternatively, the TNF-R2 signalling can activate the non-canonical NF- κ B signalling pathway via the formation of the TRAF2-TRAF3-cIAP-1/2 complex. This complex activates IKK which in turn activates the protein kinase NIK activating the non-canonical NF- κ B pathway. The TRAF2-TRAF3-cIAP complex can also be activated by LTBR, CD40 CD30 and CD137 of which CD40, CD30 and CD137 mRNA transcripts were decreased by the copper complexes, suggesting the suppression of the non-canonical NF- κ B pathway.

TNF-R1 and TNF-R2 signalling also activates the stress-activated p38 MAPK and JNK pathways, which activate AP-1, a transcription factor promoting inflammation and cell proliferation (Xie, 2013). The suppression of TNF-R1 signalling in HL-60 cells is associated with the decreased expression of TRADD and TRAF2/3 mRNA (Table 4.8). Despite the mRNA levels of TNF-R1 being elevated in K562 cells (Table 4.8), the protein expression of TNF-R1 was suppressed (Chapter 3; section 3.4.6), implying that TNF-R1 signalling did not occur in both the leukaemic cell lines when exposed to the copper complexes.

In addition, TRADD can recruit FADD, which subsequently recruits caspase-8 to initiate the caspase cascade that activates extrinsic apoptosis (Wang *et al.*, 2008b). In HL-60 cells, TRADD mRNA was impressively suppressed, indicating further inhibition of TNF-R1 signalling in HL-60 cells. The function of cIAP-1 and 2, causing RIPK ubiquitination, reduces TNF α -induced apoptosis by inhibiting caspases (Xie, 2013). The inhibition of cIAP-1 and 2 prevents the ubiquitination of RIPK, favouring the formation of the RIPK-FADD-caspase-8 complex, sensitising cells to caspase-8 activation (Feltham and Silke, 2017).

In addition, TRADD can recruit FADD, which subsequently recruits caspase-8 to initiate the caspase cascade that activates extrinsic apoptosis (Wang *et al.*, 2008b). In HL-60 cells, TRADD mRNA was impressively suppressed, indicating further inhibition of TNF-R1 signalling in HL-60 cells. The function of cIAP-1 and 2, causing RIPK ubiquitination, reduces TNF α -induced apoptosis by inhibiting caspases (Xie, 2013). The inhibition of cIAP-1 and 2 prevents the ubiquitination of RIPK, favouring the formation of the RIPK-FADD-caspase-8 complex, sensitising cells to caspase-8 activation (Feltham and Silke, 2017).

The poor caspase-8 activation HL-60 cells and the lack of caspase-8 activity in K562 cells support the evidence that cIAP-1/2 functioning is defective when cells are treated with copper complexes. The same is true when NF- κ B signalling is defective or inhibited (Brenner *et al.*, 2015; Ting and Bertrand, 2016). Ubiquitination of RIPK is thus important for NF- κ B activation, as for TNF-signalling. In turn, the adaptor TRADD and the ubiquitin ligase TRAF2 are important for efficient RIPK ubiquitination (Vince *et al.*, 2009). In addition to the massive TNF-R2 decrease, TRAF2 as well as TRAF3 mRNA levels were decreased in HL-60 cells which further reduce NF- κ B signalling. This may provide an explanation for why apoptosis occurred more readily in HL-60 cells as compared to K562 cells.

Evidence indicates that the biological effects of TRAF-signalling appear to be mediated through the activation of both the canonical and non-canonical NF- κ B pathway (Song *et al.*, 2011; Ruspi *et al.*, 2014). Activation of the canonical (classical) NF- κ B pathway requires the activation of IKK α complex by Akt, which subsequently phosphorylates I κ B α and targets it for ubiquitin mediated proteolysis (Zhou *et al.*, 2015). Akt enables phosphorylation and promotes nuclear translocation of the NF- κ B p65/p50 heterodimer (Morotti *et al.*, 2017). Since Akt was markedly suppressed in HL-60 cells (Table 4.10), this further supports a decrease in NF- κ B transcriptional activity. The transcriptional products of the canonical NF-

κ B pathway in turn positively regulates its own activity via the formation of the cIAP-1/2 /TRAF-2/3 complex that cause ubiquitination of RIPK (Michel *et al.*, 2014).

When IAPs or TRAF-2 are inhibited, the pathway reverts to the non-canonical activation of NF- κ B (McPherson *et al.*, 2012). The non-canonical pathway depends on the activation of NIK by TNF-R2 signalling. This activates the IKK α complex to achieve phosphorylation and ubiquitination of p100 NF- κ B processing, leading to the formation of NF- κ B p52/RelB dimers that mediate gene transcription (Odqvist *et al.*, 2013).

The suppression of upstream members of the NF- κ B pathway; *TNFRSF1A*, *TNFRSF1B*, *TNF*, *TRAF2* and *TRAF3* contributes to the decreased transcriptional activity of both NF- κ B pathways. TRADD is a component of TNFR1-receptor signalling (Brenner *et al.*, 2015) and the suppression caused by the copper complexes will further impair TNF signal transduction in cells.

Decreased TNF-signalling prevents NF- κ B from entering the nucleus to transcribe pro-survival genes encoding anti-apoptotic factors such as cIAP-1/2 (Ruspi *et al.*, 2014). Instead of being cleaved, NF- κ B remains trapped in the cytoplasm bound to its inhibitory protein I κ B. In this study, the suppression of *BIRC* genes and their gene products serve as further evidence of suppressed NF- κ B signalling by the copper complexes (Chapter 3; section 3.4.5 and Chapter 4; section 4.4.4).

TNF-R2 is associated with cell survival signalling and hence found to be over-expressed in leukaemia (Hui *et al.*, 2006; Govindaraj *et al.*, 2014; Vanamee and Faustman, 2017). TNF-R2 signalling results in the formation of complexes that generate cytokines and pro-survival proteins, necessary for an inflammatory response. JNK kinases play an important role in stress signalling pathways activated by such chemokines and cytokines (Lin *et al.*, 2015). Noteworthy, the TNF-R mediated NF- κ B and JNK-signalling pathways may offer novel therapeutic targets to treat various types of inflammatory diseases including cancers that rely on these pathways to escape apoptosis (Vanamee and Faustman, 2017).

Copper-imidazo[1,2-*a*]pyridines differentially affected the Akt-JNK/p38 MAPK pathway and the NF- κ B pathways in both cell lines. A comparison of the effects of the active copper complexes on both the proteome and the gene array indicates that the two cell lines respond differently and that there may be important differences in apoptotic signalling. One important difference is that of Akt. Akt transcription was suppressed in HL-60 cells but not in K562

cells. Akt exerts its cell survival and anti-apoptotic effects through crosstalk with pathways like NF- κ B and JNK/p38 MAPK (Rane *et al.*, 2010; Song *et al.*, 2016).

The stress-activated JNK/p38 pathway is inhibited by Akt (Zhang *et al.*, 2014). Akt directly phosphorylates the apoptosis signal-regulated kinase 1 (ASK-1), which is upstream of the JNK/p38 pathway (Shiizaki *et al.*, 2013). Activated ASK-1 phosphorylates and inhibits Bcl-2, thereby promoting apoptosis (Zhang *et al.*, 2014). Akt blocks the activation of ASK-1. Suppression of Akt in HL-60 cells will thus facilitate the inhibition of Bcl-2 and promote apoptosis.

Akt regulates NF- κ B signalling by phosphorylating and activating IKK α to free NF- κ B (p65) and allow it to translocate to the nucleus and subsequently activate transcription of its target genes (Ghoneum and Said, 2019). The suppression of *AKT1* and consequently its protein decreases NF- κ B activation and therefore indirectly prevents IAP transcription making cells more susceptible to apoptosis (Lin *et al.*, 2010; Kanwar *et al.*, 2011). This combined with the suppression of TNF-R2 signalling further suppress the activity of NF- κ B and decreases NF- κ B mediated cell survival. The suppression of canonical NF- κ B regulated target genes *BCL2*, *BIRC2*, *BIRC3*, *CFLAR* and itself (*NFKB1*) further contributes to the decreased transcriptional activity of NF- κ B.

The mRNA transcript levels of non-canonical NF- κ B regulated target genes such as TNF- α was decreased signifying an inhibitory effect on NIK and preventing non-canonical NF- κ B transcriptional activity. Evidence from the qPCR data on TRAF2 and TRAF3 indicates that inhibition of non-canonical NF- κ B may be central to the apoptotic effect of the copper-imidazo[1,2-*a*]pyridines which needs to be further explored.

5.3.7 Overall effect of the copper complexes on apoptosis related proteins and genes

The copper-imidazo[1,2-*a*]pyridine complexes were able to induce apoptotic cell death by stimulating the intrinsic apoptotic pathway. Considering the evidence, the effect of the copper-imidazo[1,2-*a*]pyridine complexes on anti-apoptotic proteins (Bcl-2, Bcl-x), pro-apoptotic proteins (Smac/DIABLO, Bad and Bax) and IAPs (XIAP, cIAP-1 and cIAP-2 and livin) indicate that they affected cell death signalling pathways in HL-60 and K562 cells. Changes in the expression levels of these apoptotic proteins provided clues to the sequence of

intracellular events leading to apoptotic cell death. Figure 5.2 and 5.3 summarizes the mechanism of action and potential targets of the copper-imidazo[1,2-*a*]pyridines thus far.

It is likely that the overall balance of pro-apoptotic and anti-apoptotic proteins and not any particular protein target determines if a cell will survive or undergo apoptosis. The variations in responses of the two cell lines to the copper complexes are most likely a reflection of the unique molecular signature of each cell line. Importantly, it indicates that none of these proteins can be singled out as the direct molecular target of the copper complexes.

This study further indicated that mRNA levels do not always correlate with the actual amount of protein present. The transcription level is thought to suggest whether or not protein is present and estimate the level of protein expression which may serve to be useful for identifying potential targets for follow-up studies at a protein level (Vogel and Marcotte, 2012). The discrepancy between mRNA expression and protein levels is often due to post-transcriptional modifications which can influence the half-lives of specific proteins in a cell (Schrimpf *et al.*, 2009). There is evidence for an association of copper complexes and the decreased degradation of ubiquitinated proteins in breast, leukaemic and colorectal cancer cells (Hindo *et al.*, 2009; Zuo *et al.*, 2013; Ng *et al.*, 2014; Zhang *et al.*, 2015). Assays to determine the effects of the complexes on ubiquitin ligases and proteasome activity should be included in future studies with these copper complexes.

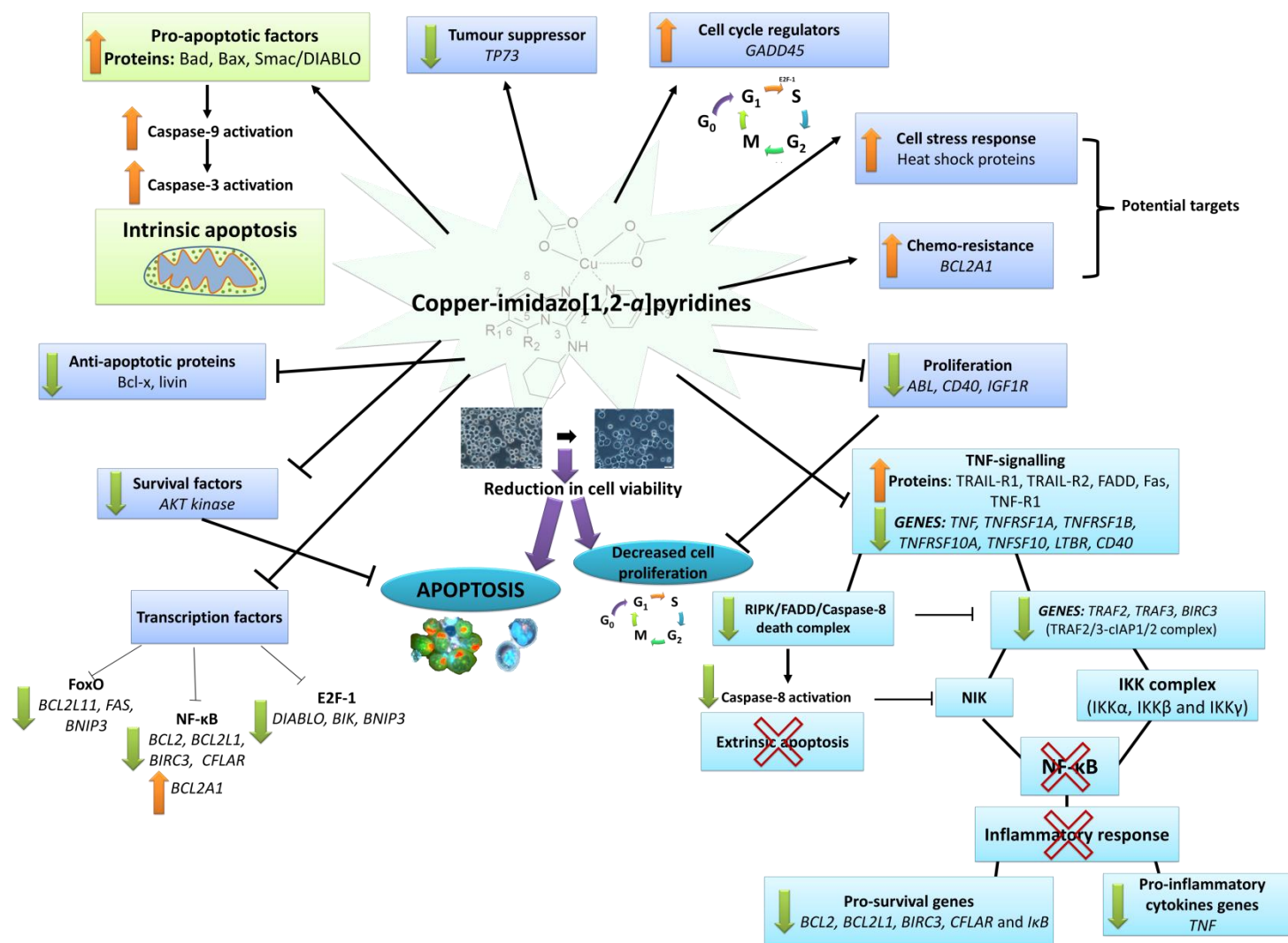


Figure 5.2 Summary of copper-imidazo[1,2-*a*]pyridine complexes effects on HL-60 cells. Several proteins and genes which are affected by the copper complexes, contributing to the apoptotic effects of treated HL-60 cells. Green arrows represent increased expression and orange arrows represent decreased expression. *Italicised* text represents apoptotic genes and normal text represents apoptotic proteins. Red crosses indicated no activation.

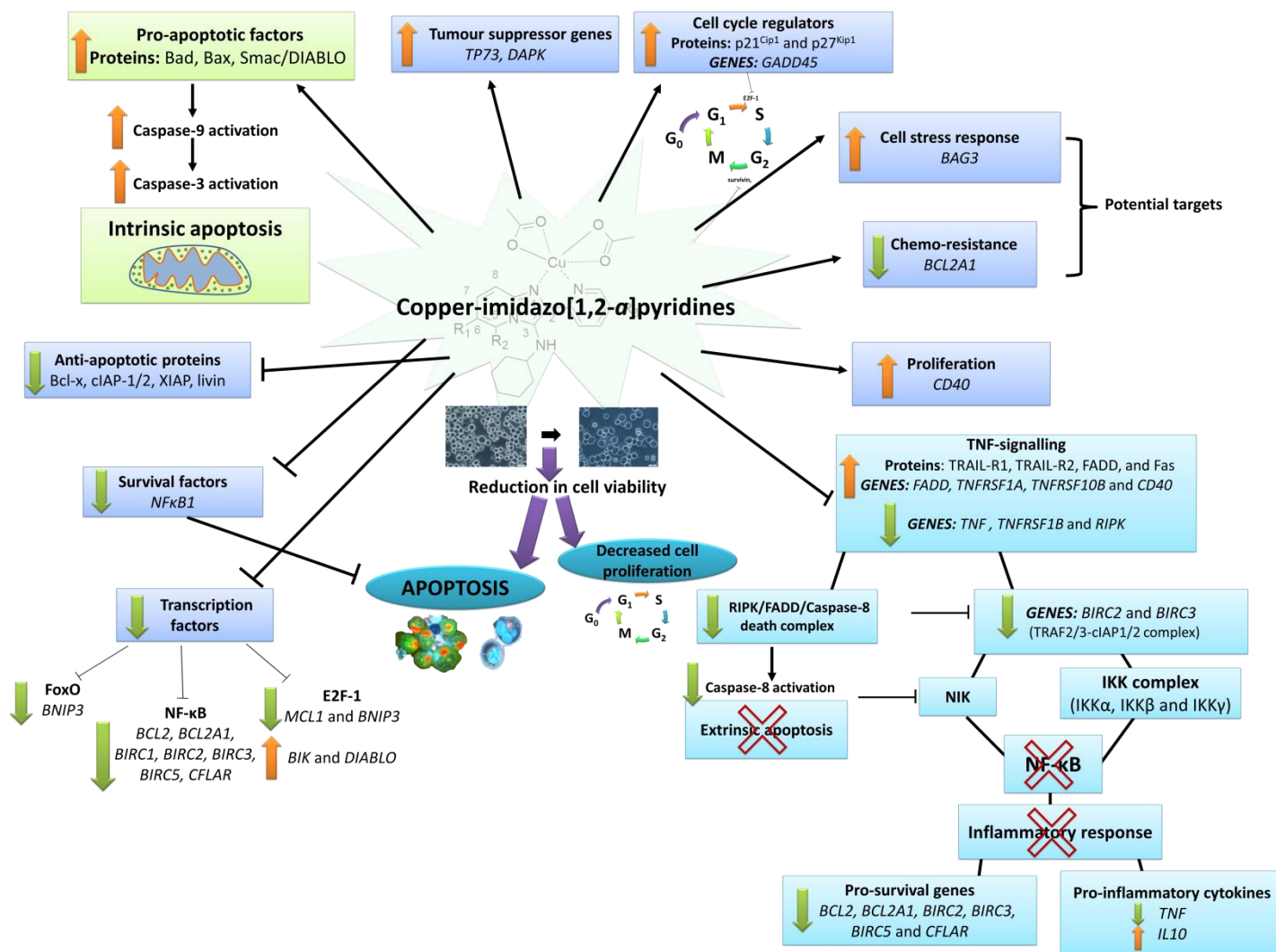


Figure 5.3 Summary of copper-imidazo[1,2-a]pyridine complexes effects on K562 cells. Several proteins and genes which are affected by the copper complexes, contributing to the apoptotic effects of treated K562 cells. Green arrows represent increased expression and orange arrows represent decreased expression. *Italicised* text represents apoptotic genes and normal text represents apoptotic proteins. Red crosses indicated no activation.

5.4 Limitations

A major limitation of the study that the assays were performed in cell lines and events in cell culture cannot directly be translated to clinical efficacy. The clinical efficacy has to be further investigated in pre-clinical animal models. This will also enable an evaluation of pharmacokinetic and pharmacodynamics.

One of the challenges of assays used to measure apoptosis is the variation between cell lines and cancer types that it would take for the initiation of apoptosis. Each cell line responds to apoptotic stresses at different time points. It is therefore important to perform time studies to ensure appropriate sampling time-points for specific assays. Due to time and cost constraints, there is difficulty in establishing a complete profile of each test compound and apoptotic assay. In order to enable direct comparisons of the effectiveness of the compounds, a single time point was used in each of the apoptotic assays. To obtain more meaningful results, more than a single time-point (12, 18, 24 hours) was included for evaluation where feasible.

As this study evaluated proteins and genes specific to apoptosis, expanding it to evaluate other modes of cell death (such as autophagy) may provide a more complete understanding of the cell death processes affected by the copper complexes. Another limitation in this study is the prohibitive costs attached to the gene arrays. Despite, qPCR being a well-accepted assay which is typically accurate and sensitive, the associated costs prevented the gene array experiments from being repeated.

The inconsistencies between the cell lines and discrepancies observed with the protein and gene array results indicate that protein and qPCR array data by themselves do not supply sufficient evidence to make final conclusions regarding the potential molecular targets of the copper complexes. These observations will need to be followed up with further experiments.

5.5 Future work

The study identified three copper-imidazo[1,2-*a*]pyridine compounds showing promising activity as lead compounds against leukaemic cells. Pre-clinical studies will determine pharmacodynamics and pharmacokinetic properties *in vivo*. Further pre-clinical development should include acute and chronic toxicity evaluation.

These studies have provided a foundation for further investigation of how these complexes mediate cell death. In response to cell stress, other copper complexes are known to generate ROS and stimulate apoptosis. Therefore, it may be useful to determine whether these copper complexes cause an increase in ROS. Following on from this, *in vitro* combination studies can be performed with HO-1/HMOX1/HSP32 inhibitors and the copper complexes to further evaluate the role of HO-1/HMOX1/HSP32 in leukaemic cells and other cancer cells lines to determine if the apoptotic threshold is reduced by this combination.

Importantly, intrinsic apoptosis was favoured in both leukaemic cell lines despite the over-expression of Bcl-2, implying that it was not functional and may be trapped in the ER or the nucleus. The localisation of Bcl-2 proteins determines whether it will be active. Bcl-2 can be verified by follow up experiments with immunofluorescence to determine its intracellular location. This can be achieved by use of ER tracker as well as a mitochondrial marker.

Knock-out studies or inhibitory studies can be performed to determine if high levels of XIAP and survivin are essential for leukaemic cell survival. The study also revealed the increased expression of Smac/DIABLO by the copper complexes which may translate into novel therapeutic approaches such as Smac mimetics which mimic the binding site of Smac/DIABLO and stimulate apoptotic cell death.

Effects of the complexes on the expression levels of specific proteins can be further verified by western blots and immunofluorescence studies. Immunofluorescence studies will provide additional information regarding the sub-cellular location of affected proteins. Finally, the mRNA expression levels of identified target genes from the apoptotic regulatory array need to be validated by RT-qPCR.

5.6 In conclusion

This study has identified the copper-imidazo[1,2-*a*]pyridines, JD88, JD47R and JD49R as new lead compounds in the search for novel anti-leukaemic agents. In both cell lines, these copper complexes induced cell death that was mediated by DNA fragmentation and activation of the intrinsic apoptotic pathway. The low IC₅₀ values make it possible to achieve a total cell kill within the range of clinically achievable and acceptable concentrations, supporting the therapeutic potential of copper-imidazo[1,2-*a*]pyridines in the treatment of leukaemias (Smith and Houghton, 2013; Liston and Davis, 2017).

Intrinsic apoptosis in both leukaemic cell lines was facilitated by the decreased expression of key cell survival proteins (IAPs, and Bcl-2) and simultaneous increase in pro-apoptotic protein expression (Bad, Bax and Smac/DIABLO). This appears to be mediated by the transcriptional activity of both NF- κ B pathways, which may be central to the apoptotic effect of the copper complexes. The complexes may have had an inhibitory effect on cytoplasmic signalling complex IKK and/or NIK in these cells. In HL-60 cells, the decreased transcription of Akt kinase suggests an inhibitory effect on its downstream link with transcription factor NF- κ B, enhancing the pro-apoptotic effects in these cells.

These copper complexes serve as valuable tools to better understand the intricacy of cell death signalling pathways in leukaemic cells. Future studies will further investigate these mechanisms on the leukaemic cells. These studies may reveal alternative drug targets and lead to efforts to enhance their efficacy. They may also lead to the development of newer imidazo[1,2-*a*]pyridine derivatives that may target specific pathways more potently and provide the rationale to combine therapies with specific targets. Finally, the findings presented in this study provide the necessary incentive for further verification of the copper complexes as anti-leukaemic agents both *in vitro* and *in vivo*.

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APPENDIX

Appendix A: Human ethics waiver certificate

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg



Ref: W-CJ-130311-3

11/03/2013

TO WHOM IT MAY CONCERN:

- Waiver:** This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).
- Investigator:** Dr L Harmse, Ms Z Ismail (student no 348581).
- Project title:** The anti-cancer effects of azaindoles and imidazopyridines on leukaemic cell lines.
- Reason:** This is a laboratory study using cell lines including K562, Jurkats, HL60, HEK93, MCF7, MDM, HT29 and Caco-2. No humans are involved.



Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

Appendix B: MTT calibration curves

HL-60:

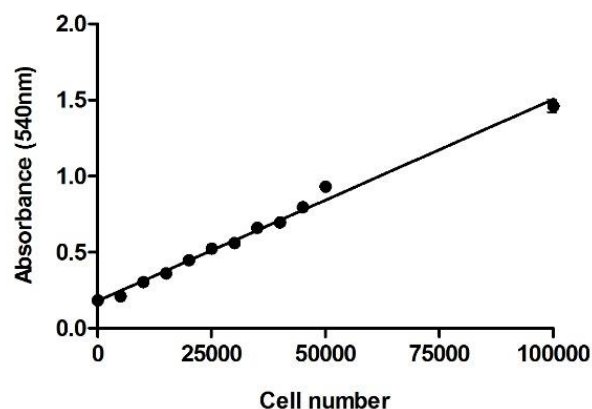


Figure B1 Calibration curves of HL-60 cells after 48 hour incubation period using the MTT cytotoxicity assay

K562:

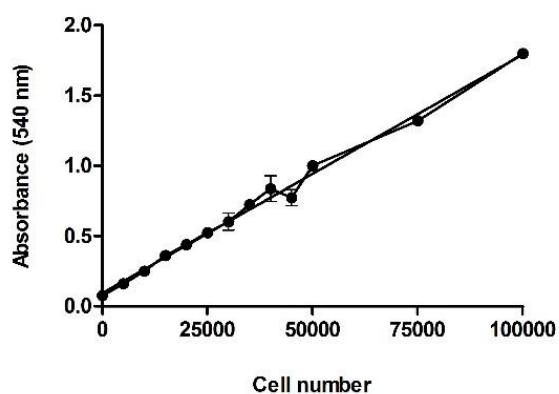


Figure B2 Calibration curves of K562 cells after 48 hour incubation period using the MTT cytotoxicity assay

Appendix C: Human ethics clearance certificate for isolating human leukocytes



R14/49 Miss Zeenat Ismail

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170509

NAME: Miss Zeenat Ismail
(Principal Investigator)
DEPARTMENT: Pharmacy and Pharmacology
University of the Witwatersrand

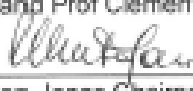
PROJECT TITLE: The Apoptotic of Copper Imidazo [1,2-a] Pyridine
Complexes on Leukaemic Cells

DATE CONSIDERED: 26/05/2017

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr Leonie Harmse and Prof Clement Penny

APPROVED BY: 
Professor P. Cleaton-Jones Chairperson, HREC (Medical)

DATE OF APPROVAL: 12/07/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/3rd floor, Philip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially review May and will therefore be due in the month of May each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

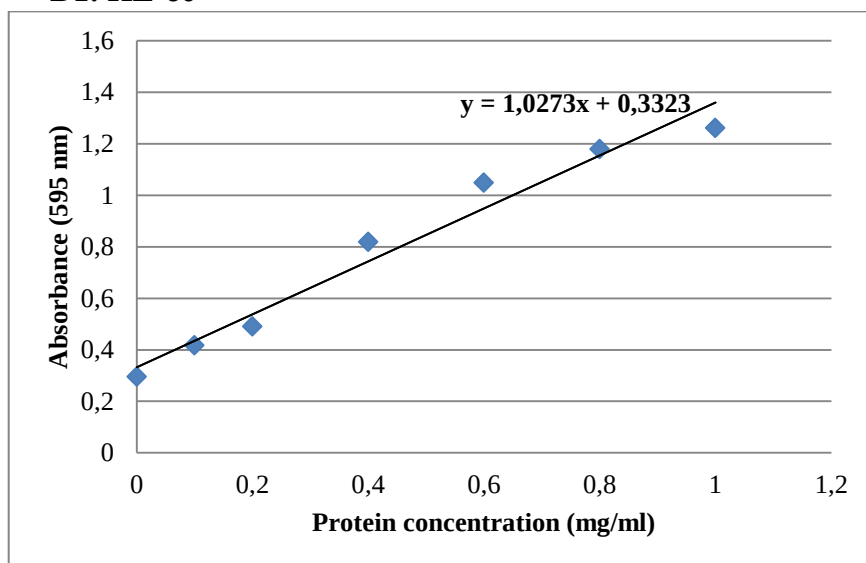
Principal Investigator Signature _____

Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D: Protein standard curves

D1: HL-60



D2: K562

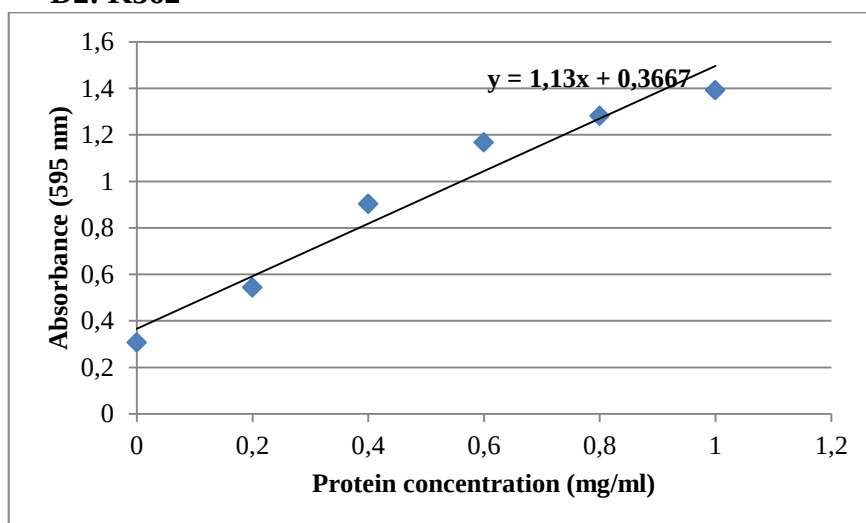
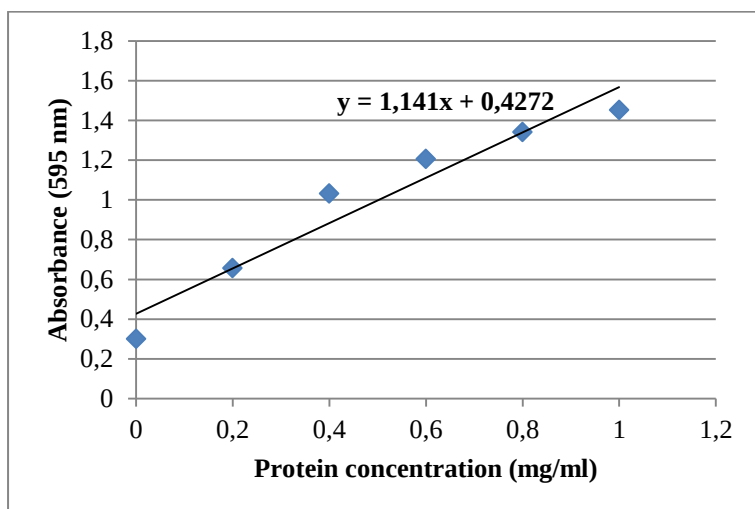


Figure D1-2 Representative standard curves for protein determination of the caspase-3/7 assay in HL-60 and K562 cells

D3: HL-60



D4: K562

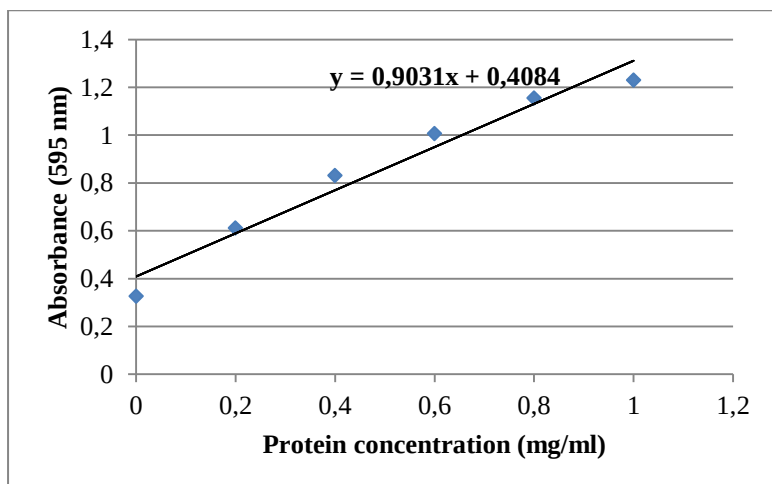


Figure D3-4 Standard curve for protein determination of the caspase-9 assay in HL-60 and K562 cells

HL-60:

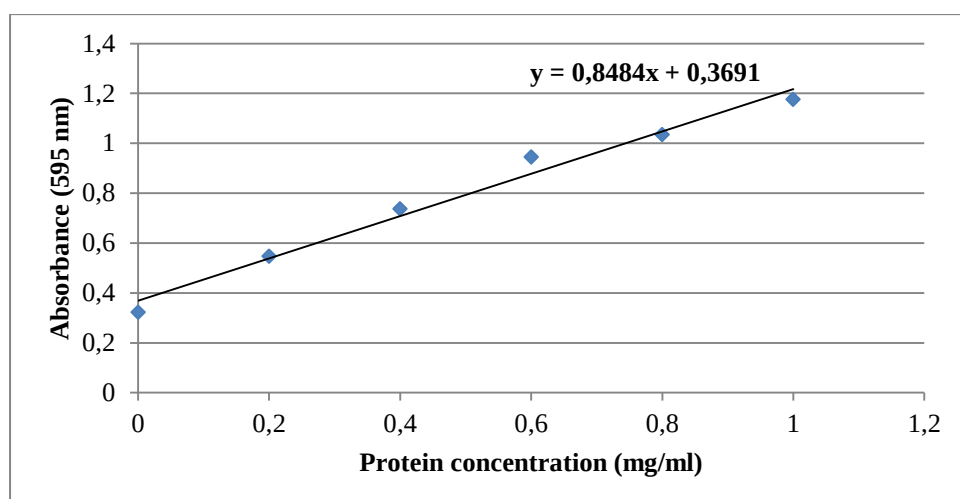


Figure D5 Standard curve for protein determination in the human apoptosis array for HL-60 cells

K562:

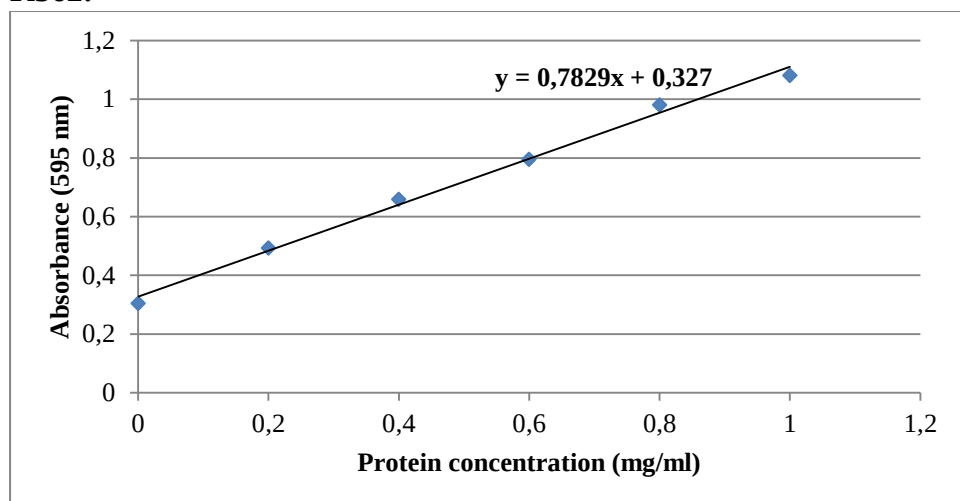


Figure D6 Standard curve for protein determination in the human apoptosis array for K562 cells

Leukocytes

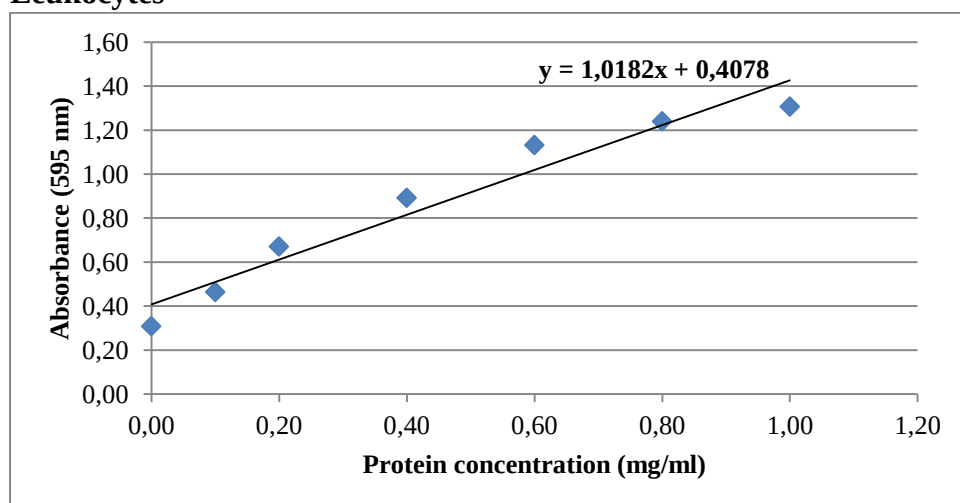


Figure D7 Standard curve for protein determination in the human apoptosis array for Leukocytes

Appendix E: List of test compounds

Table E1 Test compounds and varying substituent groups on imidazo[1,2-*a*]pyridine core

Imidazo[1,2- <i>a</i>]pyridine test compounds	Transition metal coordination complex	Substituent additions to imidazo[1,2- <i>a</i>]pyridine core	Halogen (Br/Cl) substituents on 2-pyridyl ring of copper complexes
JD52	No coordination complex	Varying the substituents at the R ₂ and R ₃ positions of the imidazo[1,2- <i>a</i>]pyridine core	Br substituent at 6-position of the imidazo[1,2- <i>a</i>]pyridine core
JD60			
JD64			
JD74			
JD75			
JD77			
JD80			
JD81			
JD119			
JD122			
JD124			
JD137			
JD142			
JD196			
JD224			
JD88	Copper coordination complex	Cyclohexyl moiety to amine arm	
JD88M	Copper coordination complex		
JD96	Zinc coordination complex		
JD96M	Zinc coordination complex		
JD100	Platinum coordination complex	Amidogen moiety to amine arm	
JD218	No coordination complex	Benzyl moiety to amine arm	
JD236	Platinum coordination complex		
JD252	Zinc coordination complex		
JD253	Copper coordination complex		
JD249	No coordination complex	Ethyl glycinate moiety to amine arm	
JD254	Zinc coordination complex		
JD255	Platinum coordination complex		
JD256	Copper coordination complex		
JD248	No coordination complex	Adamantyl moiety to amine arm	
JD257	Zinc coordination complex		
JD258	Copper coordination complex		
JD259	Platinum coordination complex		
JDPrCl ₂	Platinum coordination complex	Cyclohexyl moiety to amine arm	
JD34R	Copper coordination complex	Cyclohexyl moiety to amine arm	Halogen substituent
JD35R			Inactive, not analysed
JD36R			Inactive, not analysed
JD37R			Br on the R ₂ substituent group
JD38R			Inactive, not analysed
JD39R			Inactive, not analysed
JD40R			Inactive, not analysed
JD42R			Inactive, not analysed
JD43R			Inactive, not analysed
JD47R			Br at 5-position
JD49R			Cl at 6-position
JD46R			Un-substituted analogue

Appendix F: Caspase-9 supplementary images

HL-60:

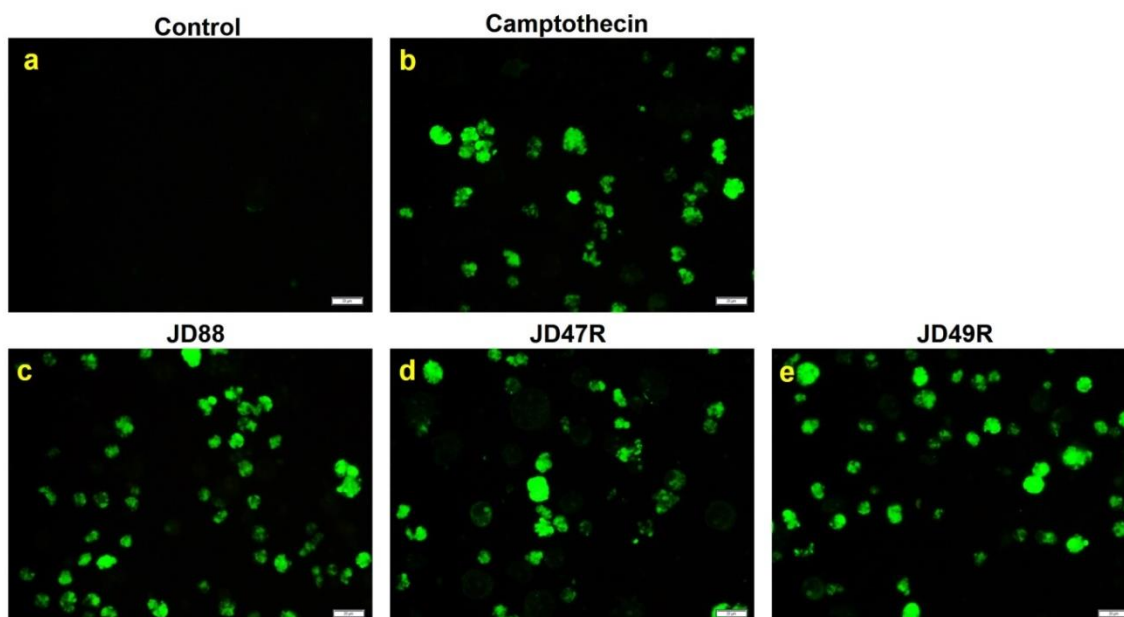


Figure F1. Copper-imidazo[1,2-*a*]pyridines stimulate caspase-9 activity in HL-60 cells. Cells were treated with IC₇₅ concentration of JD88, JD47R, JD49R and camptothecin and exposed for 14 hrs. Images were captured with the Olympus DP72 digital camera. Magnification: 400X, Exposure: 340 ms; ISO: 400; Scale bar: 20 μ m.

K562:

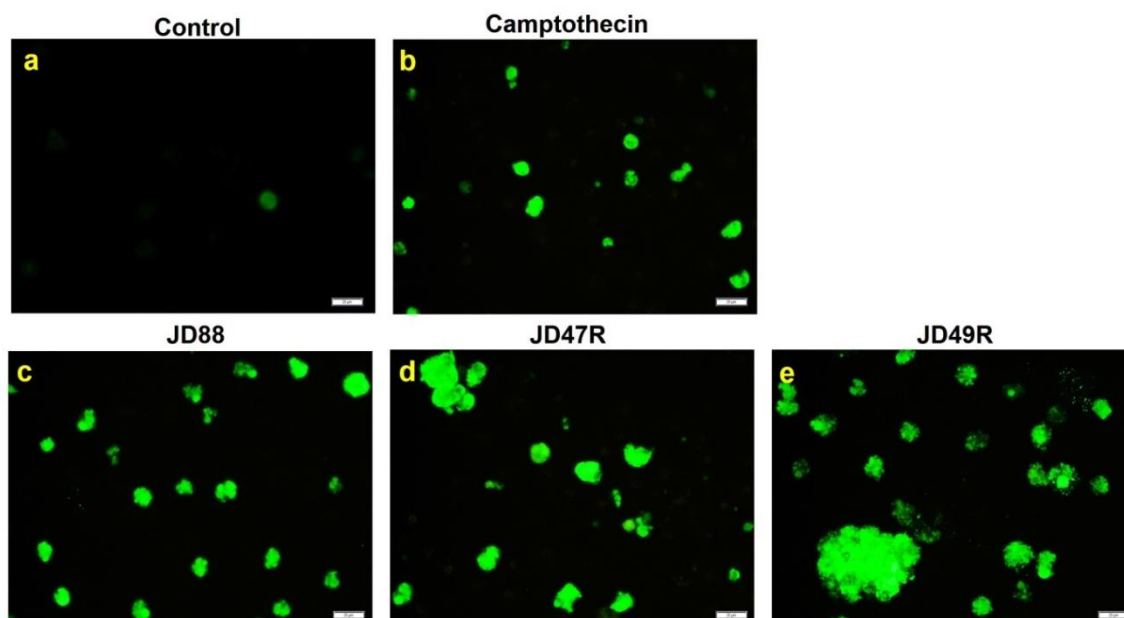


Figure F2. Copper-imidazo[1,2-*a*]pyridines stimulate caspase-9 activity in K562 cells. Cells were treated with IC₇₅ concentration of JD88, JD47R, JD49R and camptothecin and exposed for 24 hrs. Images were captured with the Olympus DP72 digital camera. Magnification: 400X, Exposure: 340 ms; ISO: 400; Scale bar: 20 μ m.

Appendix G PCR gene arrays

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABL1	AIFM1	AKT1	APAF1	BAD	BAG1	BAG3	BAK1	BAX	BCL10	BCL2	BCL2A1
B	BCL2L1	BCL2L10	BCL2L11	BCL2L2	BFAR	BID	BIK	BIRC2	BIRC3	BIRC5	BIRC6	BNIP2
C	BNIP3	BNIP3L	BRAF	CASP1	CASP10	CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7
D	CASP8	CASP9	CD27	CD40	CD40LG	CD70	CFLAR	CIDEA	CIDEB	CRADD	CYC5	DAPK1
E	DFFA	DIABLO	FADD	FAS	FASLG	GADD45A	HRK	IGF1R	IL10	LTA	LTBR	MCL1
F	NAIP	NFKB1	NOD1	NOL3	PYCARD	RIPK2	TNF	TNFRSF10A	TNFRSF10B	TNFRSF11B	TNFRSF1A	TNFRSF1B
G	TNFRSF21	TNFRSF25	TNFRSF9	TNFSF10	TNFSF8	TP53	TP53BP2	TP73	TRADD	TRAF2	TRAF3	XIAP
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure G1 The 96-well layout of the human apoptosis PCR Array, each well representing an individual gene. There are 84 genes across the array (A01 to G12), with the H row comprising of the quality controls which include HKG, HGDC, as well as RTC and PPC.

Table G1 The accession numbers corresponding to the gene names (full) and abbreviations are provided here.

Position	Accession ID	Symbol	Description	Gname
A01	NM_005157	ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	ABL/ITK7/bcr/abl/c-ABL/c-ABL1/p150/v-abl
A02	NM_004208	AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	AIF/CMT2D/CMTX4/COWCK/COPX6/NADMR/NAMSD/PDCD8
A03	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	AKT/CWS6/PKB/PKB-ALPHA/PRKBA/RAC/RAC-ALPHA
A04	NM_001160	APAF1	Apoptotic peptidase activating factor 1	APAF-1/CED4
A05	NM_004322	BAD	BCL2-associated agonist of cell death	BBC2/BCL2L8
A06	NM_004323	BAG1	BCL2-associated athanogene	BAG-1/HAP/RAP46
A07	NM_004281	BAG3	BCL2-associated athanogene 3	BAG-3/BIS/CAIR-1/MFM6
A08	NM_001188	BAK1	BCL2-antagonist/killer 1	BAK/BAK-LIKE/BCL2L7/CDN1
A09	NM_004324	BAX	BCL2-associated X protein	BCL2L4
A10	NM_003921	BCL10	B-cell CLL/lymphoma 10	CARMEN/CIPER/CLAP/IMD37/c-E10/mE10
A11	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2/PPP1R50
A12	NM_004049	BCL2A1	BCL2-related protein A1	ACC-1/ACC-2/ACC1/ACC2/BCL2L5/BFL1/GRS/HBPA1
B01	NM_138578	BCL2L1	BCL2-like 1	BCL-XL/S/BCL2L/BCLX/BCLXL/BCLXS/bcl-X/PPP1R52/bcl-xl/bcl-xs
B02	NM_020396	BCL2L10	BCL2-like 10 (apoptosis facilitator)	BCL-B/Boo/Divva/bcl2-L-10
B03	NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)	BAM/BIM/BOD
B04	NM_004050	BCL2L2	BCL2-like 2	BCL-W/BCL2-L-2/BCLW/PPP1R51
B05	NM_016561	BFAR	Bifunctional apoptosis regulator	BAR/RNF47
B06	NM_001196	BID	BH3 interacting domain death agonist	FP497
B07	NM_001197	BIK	BCL2-interacting killer (apoptosis-inducing)	BIP1/BP4/NBK
B08	NM_001166	BIRC2	Baculoviral IAP repeat containing 2	API1/HIAP2/Hiap-2/MIHB/RNF48/c-IAP1/cIAP1
B09	NM_001165	BIRC3	Baculoviral IAP repeat containing 3	API1/API2/CIAP2/HAIPI1/HIAP1/MALT2/MIHC/RNF49/c-IAP2
B10	NM_001168	BIRC5	Baculoviral IAP repeat containing 5	API4/EPR-1
B11	NM_016252	BIRC6	Baculoviral IAP repeat containing 6	APOLLON/BRUCE
B12	NM_004330	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	BNIP-2/NIIP2
C01	NM_004052	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NIIP3
C02	NM_004331	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	BNIP3a/NIX
C03	NM_004333	BRAF	V-raf murine sarcoma viral oncogene homolog B1	B-RAF1/BRAF1/NS7/RAFBI
C04	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	ICE/IL1BC/P45
C05	NM_001230	CASP10	Caspase 10, apoptosis-related cysteine peptidase	ALPS2/FLUCE2/MCH4
C06	NM_012114	CASP14	Caspase 14, apoptosis-related cysteine peptidase	-
C07	NM_032982	CASP2	Caspase 2, apoptosis-related cysteine peptidase	CASP-2/ICH1/NEDD-2/NEDD2/PPP1R57
C08	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase	CPP32/CPBP32B/SCA-1
C09	NM_001225	CASP4	Caspase 4, apoptosis-related cysteine peptidase	ICE(rel)II/ICEREL-II/ICH-2/MIH1/TX/TX
C10	NM_004347	CASP5	Caspase 5, apoptosis-related cysteine peptidase	ICE(rel)III/ICEREL-III/ICH-3
C11	NM_032992	CASP6	Caspase 6, apoptosis-related cysteine peptidase	MCH2
C12	NM_001227	CASP7	Caspase 7, apoptosis-related cysteine peptidase	CASP-7/CMH-1/ICE-LAP3/ICE2/MCH3
D01	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase	ALPS2B/CAP4/Casp-8/FLICE/MACH/MCH5
D02	NM_001229	CASP9	Caspase 9, apoptosis-related cysteine peptidase	APAF-3/APAF3/ICE-LAP6/MCH6/PPP1R56
D03	NM_001242	CD27	CD27 molecule	S152/S152-LPFS2/T14/TNFRSF7/Tp55
D04	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	Bp50/CDW40/TNFRSF5/p50
D05	NM_000074	CD40LG	CD40 ligand	CD154/CD40L/HIGM1/GIM/IMD3/T-BAM/TNFSF5/TRAP/gp39/hCD40L
D06	NM_001252	CD70	CD70 molecule	CD27L/CD27LG/TNFSF7
D07	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	CASH/CASP8AP1/CLARP/Casper/FLAME/FLAME-1/FLAME1/FLIP-I-FLICE/MRIT/c-FLIP/c-FUPLI/c-FUPLR/c-FUPLS
D08	NM_001279	CIDEA	Cell death-inducing DFFA-like effector a	CIDE-A
D09	NM_014430	CIDEB	Cell death-inducing DFFA-like effector b	-
D10	NM_003805	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	MRT34/RAIDD
D11	NM_018947	CYC5	Cytochrome c, somatic	CYC/HCS/THC4
D12	NM_004938	DAPK1	Death-associated protein kinase 1	DAPK
E01	NM_004401	DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	DFF-45/DFF1/CAD
E02	NM_019887	DIABLO	Diablo, IAP-binding mitochondrial protein	DFNAG4/SMAC
E03	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain	GIG3/MORT1
E04	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	ALPS1A/APO-1/APT1/CD95/FAS1/FASTM/TNFRSF6
E05	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	ALPS1B/APT1LG1/APTL/CD178/CD95-L/CD95L/FASL/TNFSF6
E06	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha	DDIT1/GADD45
E07	NM_003806	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	DPS/HARAKIRI
E08	NM_000875	IGF1R	Insulin-like growth factor 1 receptor	CD221/IGF1R/IGF1R/ITK13
E09	NM_000572	IL10	Interleukin 10	CSIF/GVHDS/IL-10/IL10A/TGIF
E10	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	LT/TNFB/TNFSF1
E11	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	CD18/D125370/LT-BETA-R/TNF-R-III/TNFR/TNFR-RP/TNFR2-RP/TNFR3/TNFRSF3
E12	NM_021960	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	BCL2L3/EAT/MCL1-ES/MCL1/MCL15/Mcl-1/TM/bcl2-L-3/mcl1/EAT
F01	NM_004536	NAIP	NLR family, apoptosis inhibitory protein	BIRC1/NLRB1/ps1NAIP
F02	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1/KBF1/NF-kB1/NF-kappa-B/NF-kappaB/NFKB-p105/NFKB-p50/NFKappaB/p105/p50
F03	NM_006092	NOD1	Nucleotide-binding oligomerization domain containing 1	CARD4/CLR7.1/NLRCL1
F04	NM_003946	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	ARC/FCM/MYP/NOP/NOP30
F05	NM_013258	PYCARD	PYD and CARD domain containing	ASC/CARD5/TMS/TMS-1/TMS1
F06	NM_003821	RIPK2	Receptor-interacting serine-threonine kinase 2	CARD3/CARDIAK/CKK/GiG30/RICK/RIP2
F07	NM_000594	TNF	Tumor necrosis factor	DIF/TNF-alpha/TNFA/TNFSF2
F08	NM_003844	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	APO2/CD261/DRA/TRAILR-1/TRAILR1
F09	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	CD262/DRS/KILLER/KILLER/DRS/TRAIL-R2/TRAILR2/TRICK2/TRICK2A/TRICK2B/TRICKX/ZTNFR9
F10	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	OCIF/OPG/PDBS/TR1
F11	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	CD120a/EPF/MSS/TBP1/TNF-R/TNF-R-I/TNF-R55/TNFA/TNFR1/TNFR1-d2/TNFR55/TNFR60/p55/p55-R/p60
F12	NM_001066	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	CD120b/TBPII/TNF-R-II/TNF-R75/TNFB/TNFR1B/TNFR2/TNFR80/p75/p75TNFR
G01	NM_014452	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	BM-018/CD358/DR6
G02	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	APO-3/DDR3/DR3/LARD/TNFRSF12/TR3/TRAMP/WSL-1/WSL-LR
G03	NM_001561	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	4-1BB/CD137/CDw137/ILA
G04	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	APO2/Apo-2L/CD253/TL2/TRAIL
G05	NM_001244	TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	CD153/CD30L/CD30LG
G06	NM_000546	TP53	Tumor protein p53	BCC7/LFS1/P53/TRP53
G07	NM_005426	TP53BP2	Tumor protein p53 binding protein, 2	53BP2/ASPP2/BBP/P53BP2/PPP1R13A
G08	NM_005427	TP73	Tumor protein p73	P73
G09	NM_003789	TRADD	TNFRSF1A-associated via death domain	HS_89862
G10	NM_021138	TRAF2	TNF receptor-associated factor 2	MGC45012/TRAP/TRAP3
G11	NM_003300	TRAF3	TNF receptor-associated factor 3	CAP-1/CAP1/CD40bp/CRAF1/IAES/LAP1
G12	NM_001167	XIAP	X-linked inhibitor of apoptosis	API3/BIRC4/IAP-3/ILP1/MIHA/XLP2/hiAP-3/hiAP3
H01	NM_001101	ACTB	Actin, beta	BRWS1/P51TP5BP1
H02	NM_004048	B2M	Beta-2-microglobulin	-
H03	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD/HEL-5-162eP
H04	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
H05	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E/LP0/PO/PLP0/RPPO
H06	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	SA_00104	RTC	Reverse Transcription Control	RTC
H08	SA_00104	RTC	Reverse Transcription Control	RTC
H09	SA_00104	RTC	Reverse Transcription Control	RTC
H10	SA_00103	PPC	Positive PCR Control	PPC
H11	SA_00103	PPC	Positive PCR Control	PPC
H12	SA_00103	PPC	Positive PCR Control	PPC

Table G2 Assessment of quality data control (QDC) of RT² Profiler PCR array data

Test performed	Test Result
1. PCR Array Reproducibility	All Samples Passed
2. RT Efficiency	All Samples Passed
3. Genomic DNA Contamination	None-All Samples Passed

Table G3 Description of apoptosis-related genes and their corresponding protein

GENE	Description	Protein
<i>ABL1</i>	Bcr/abl oncogene	Bcr/abl
<i>AIFM1</i>	Apoptosis inducing factor	AIF
<i>AKT1</i>	Protein kinase B	Akt/ PKB
<i>APAF1</i>	Apoptotic peptidase activating factor 1	Apaf-1
<i>BAD</i>	Bcl-2-associated agonist of cell death	Bad
<i>BAG1</i>	Bcl-2-associated athanogene	Bag1
<i>BAG3</i>	Bcl-2-associated athanogene 3	Bag3
<i>BAK1</i>	Bcl-2-antagonist/killer 1	Bak
<i>BAX</i>	Bcl-2-associated X protein	Bax
<i>BCL10</i>	B-cell CLL/lymphoma 10	Bcl-10
<i>BCL2</i>	B-cell CLL/lymphoma 2	Bcl-2
<i>BCL2A1</i>	Bcl-2-related protein A1	Bfl-1
<i>BCL2L1</i>	Bcl-2-like 1	Bcl-xl
<i>BCL2L10</i>	Bcl-2-like 10 (apoptosis facilitator) //cell survival/blocks bax	Bcl-2-l-10
<i>BCL2L11</i>	Bcl-2-like 11 (apoptosis facilitator)	Bim
<i>BCL2L2</i>	Bcl-2-like 2	Bcl-w
<i>BFAR</i>	Bifunctional apoptosis regulator	Bfar
<i>BID</i>	BH3 interacting domain death agonist	Bid
<i>BIK</i>	Bcl-2-interacting killer (apoptosis-inducing)	Bik
<i>BIRC2</i>	Baculoviral IAP repeat containing 2	cIAP-1
<i>BIRC3</i>	Baculoviral IAP repeat containing 3	cIAP-2
<i>BIRC5</i>	Baculoviral IAP repeat containing 5	Survivin
<i>BIRC6</i>	Baculoviral IAP repeat containing 6	Bruce
<i>BNIP2</i>	Bcl-2/adenovirus E1B 19kDa interacting protein 2	Bnip-2
<i>BNIP3</i>	Bcl-2/adenovirus E1B 19kDa interacting protein 3	Bnip3
<i>BNIP3L</i>	Bcl-2/adenovirus E1B 19kDa interacting protein 3-like	Bnip3L
<i>BRAF</i>	V-raf murine sarcoma viral oncogene homolog B1	Braf-1
<i>CASP1</i>	Caspase-1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	Caspase-1
<i>CASP2</i>	Caspase-2, apoptosis-related cysteine peptidase	Caspase-2
<i>CASP3</i>	Caspase-3, apoptosis-related cysteine peptidase	Caspase-3
<i>CASP4</i>	Caspase-4, apoptosis-related cysteine peptidase	Caspase-4
<i>CASP5</i>	Caspase-5, apoptosis-related cysteine peptidase	Caspase-5
<i>CASP6</i>	Caspase-6, apoptosis-related cysteine peptidase	Caspase-6
<i>CASP7</i>	Caspase-7, apoptosis-related cysteine peptidase	Caspase-7
<i>CASP8</i>	Caspase-8, apoptosis-related cysteine peptidase	Caspase-8
<i>CASP9</i>	Caspase-9, apoptosis-related cysteine peptidase	Caspase-9
<i>CASP10</i>	Caspase-10, apoptosis-related cysteine peptidase	Caspase-10

CASP14	Caspase-14, apoptosis-related cysteine peptidase	Caspase-14
CD27	CD27 molecule / Tumour necrosis factor receptor superfamily, member 7	CD27/ TNFRSF7
CD40	CD40 receptor/ Tumour necrosis factor receptor superfamily, member 5	CD40/ TNFRSF5
CD40LG	CD40 ligand/ Tumour necrosis factor (ligand) superfamily, member 5	CD40L/ TNFSF5
CD70	CD70 molecule/ CD27 ligand/ TNF (ligand) superfamily, member 7	CD27L/ TNFSF7
CFLAR	CASP8 and FADD-like apoptosis regulator	c-FLIP
CIDEA	Cell death-inducing DFFA-like effector a	CIDE-A
CIDEB	Cell death-inducing DFFA-like effector b	CIDE-B
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	CRADD
CYCS	Cytochrome c	Cytochrome c
DAPK1	Death-associated protein kinase 1	DAPK
DFFA	ICAD	ICAD
DIABLO	Diablo, IAP-binding mitochondrial protein	Smac/DIABLO
FADD	Fas (TNFRSF6)-associated via death domain	FADD
FAS	Fas/CD95 receptor	Fas/CD95
FASLG	Fas Ligand	Fas L
GADD45A	Growth arrest and DNA-damage-inducible, alpha	Gadd45A
HRK	Harakiri	Harakiri
IGF1R	Insulin-like growth factor 1 receptor	IGF1R
IL10	Interleukin-10	IL-10
LTA	Lymphotoxin alpha (TNF (ligand) superfamily, member 1)	TNFSF1
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	TNFRSF3
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	MCL-1
NAIP	NLR family apoptotic inhibitory protein	NAIP
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NF-kB
NOD1	Nucleotide-binding oligomerization domain containing 1	NOD-1
NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	NOL-3
PYCARD	PYD and CARD domain containing	PYCARD
RIPK2	Receptor-interacting serine-threonine kinase 2	RIPK-2
TNF	Tumour necrosis factor alpha	TNF
TNFRSF10A	Tumour necrosis factor receptor superfamily, member 10a	TRAIL-R1
TNFRSF10B	Tumour necrosis factor receptor superfamily, member 10b	TRAIL-R2
TNFRSF11B	Tumour necrosis factor receptor superfamily, member 11b	TNFRSF11B
TNFRSF1A	Tumour necrosis factor receptor superfamily, member 1A	TNFR1
TNFRSF1B	Tumour necrosis factor receptor superfamily, member 1B	TNFR2
TNFRSF21	Tumour necrosis factor receptor superfamily, member 21	TNFRSF21
TNFRSF25	Tumour necrosis factor receptor superfamily, member 25	TNFRSF25
TNFRSF9	Tumour necrosis factor receptor superfamily, member 9	CD137
TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10/ TRAIL ligand	TRAIL

<i>TNFSF8</i>	Tumour necrosis factor (ligand) superfamily, member 8/ CD30 ligand	CD30
<i>TP53</i>	Tumour protein p53	p53
<i>TP53BP2</i>	Tumour protein p53 binding protein, 2	p53bp2
<i>TP73</i>	Tumour protein p73	p73
<i>TRADD</i>	TNFRSF1A-associated via death domain	TRADD
<i>TRAF2</i>	TNF receptor-associated factor 2	TRAF-2
<i>TRAF3</i>	TNF receptor-associated factor 3	TRAF-3
<i>XIAP</i>	X-linked inhibitor of apoptosis	XIAP

HL-60:

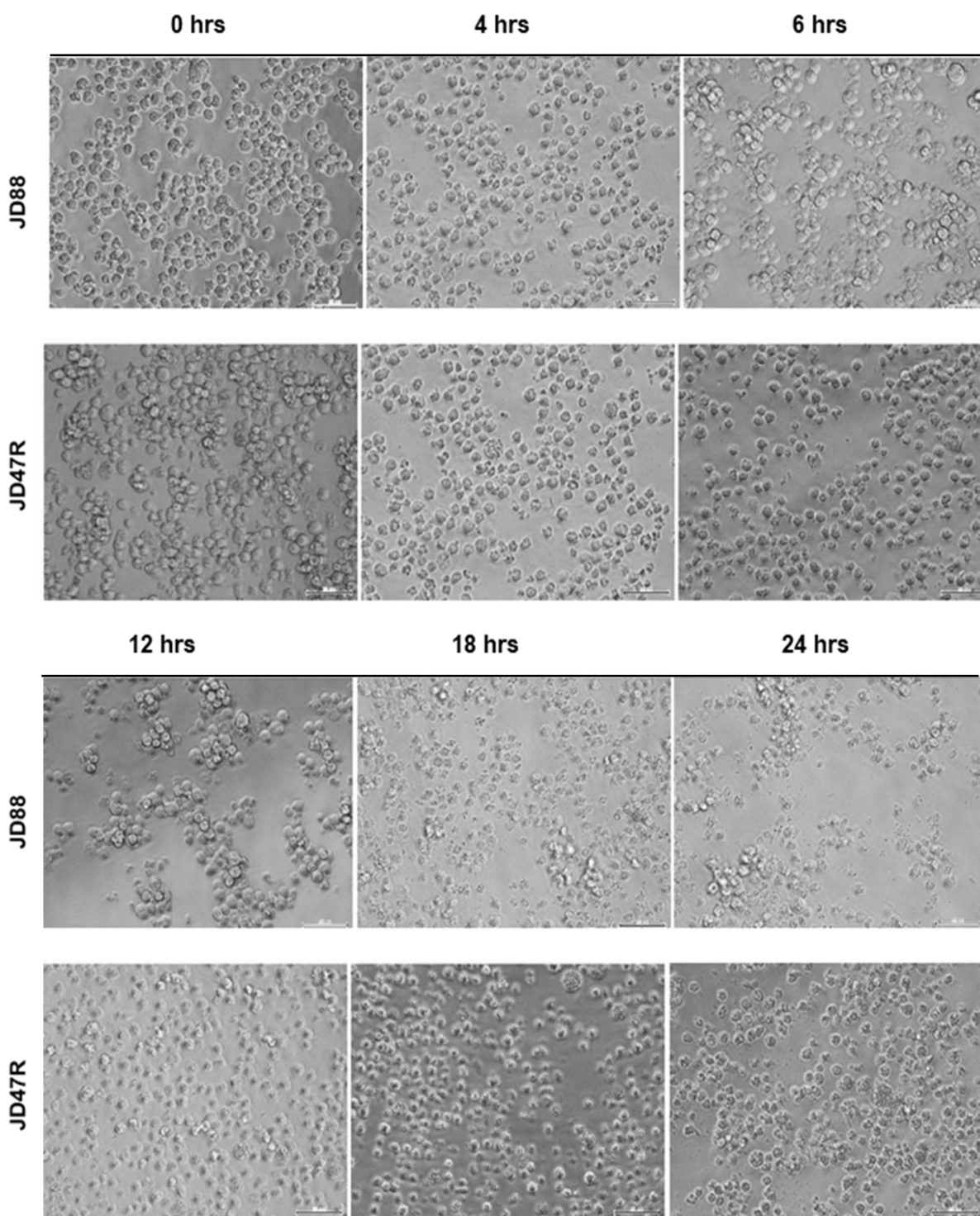


Figure G2 The effect of the copper-imidazo[1,2-a]pyridine complexes on HL-60 cell morphology over a 24 hour period at IC_{75} concentrations. Images were captured at 200 $\times$ magnification with the Nikon Optiphot light microscope. Scale bars: 50 μ m.

K562:

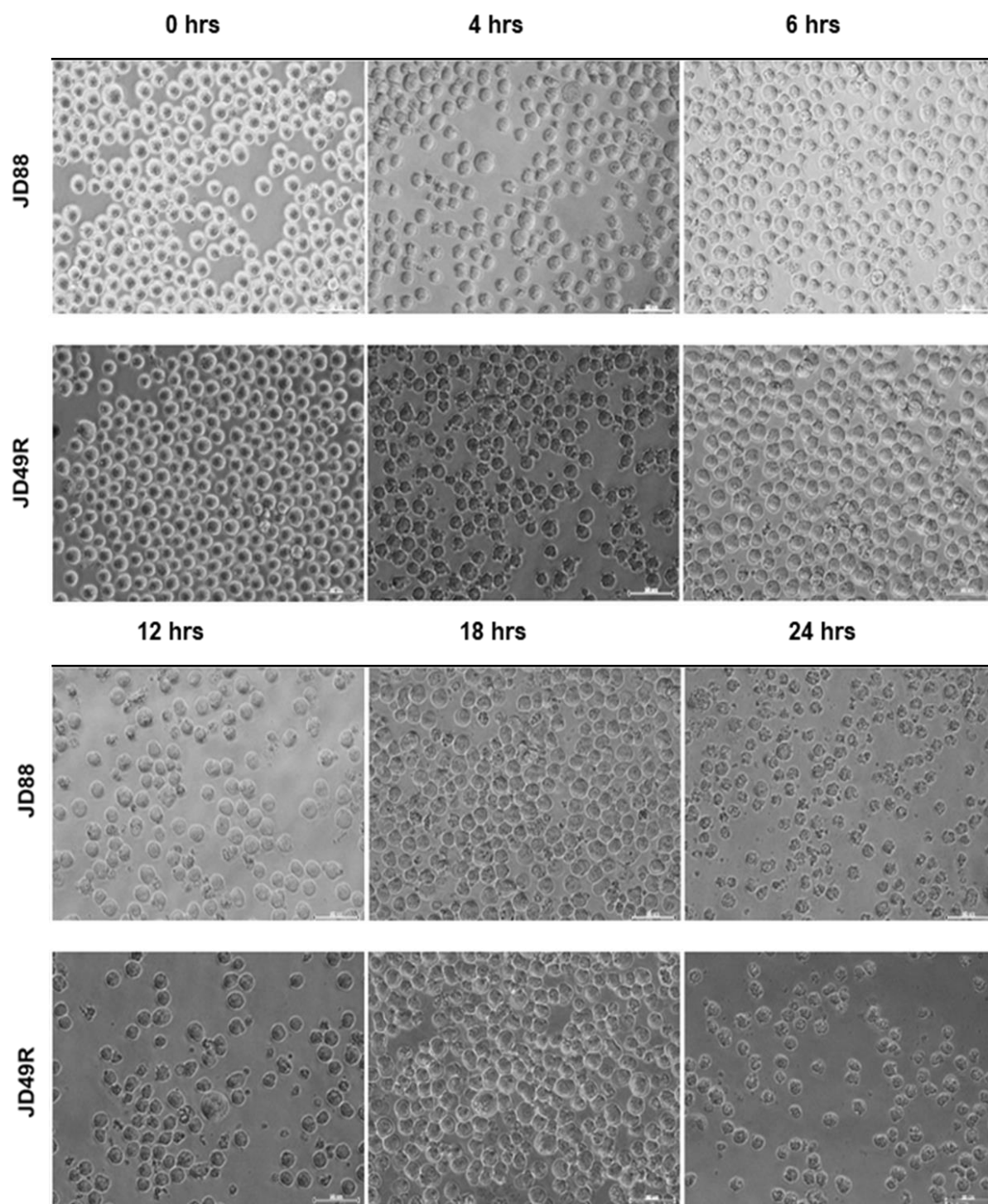


Figure G3 The effect of the copper-imidazo[1,2-*a*]pyridine complexes on K562 cell morphology over a 24 hour period at IC₇₅ concentrations. Images were captured at 200× magnification with the Nikon Optiphot light microscope. Scale bars: 50 μm.