An investigation into the effects of metformin in combination with Clofarabine for the treatment of leukaemia using T-lymphoblastic leukaemia cell lines

by

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Dissertation

Submitted in fulfilment of the requirements for the degree

Masters of science

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

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September 2020

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Abstract

T-cell acute lymphoblastic leukaemia (T-ALL) is an aggressive cancer that develops from the expression of a high number of immature progenitors that encode T-cell development. T-ALL is a significant contributor to mortality in children and adults, and accounts for 15% of paediatric acute lymphoblastic leukaemia (ALL) and 25% of adult ALL cases. Clofarabine is a chemotherapeutic drug used to treat multiple leukaemia including T-ALL. Cellular metabolic alterations can result in resistance to clofarabine treatment. Advanced chemotherapeutic strategies are required to improve clofarabine activity. Metformin, an antidiabetic drug, is of interest for novel drug combination therapy with clofarabine as it can enhance metabolic processes required in enhancing clofarabine effectiveness. Our results by XTT assay indicated that metformin alone significantly inhibited cell proliferation by 17-82% in CEM and by 20-51% in THP1 cells. The presence of metformin seems to enhance the sensitivity of CEM cells to clofarabine but induced slight resistance to clofarabine in THP1. Using Western blotting we looked at the expression of nucleoside metabolism enzymes. In CEM cells, in comparison to untreated control, metformin-treated cells showed decreased expression of thymidine kinase (TK-1) to 85±3% and 40±5% in 5mM treatment at 48 and 72 hours exposure, respectively. Thymidylate synthase (TS) levels were also decreased to 73±5% and 56±45 in 5mM treatment at 48 and 72 hours exposure, respectively, in comparison to untreated controls. There was no statistically significant change in TK-1 expression in THP1 cells. As clofarabine depends on deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) for its effectiveness, we investigated the effect of metformin on

expression levels of dCK and dGK. We found a change in expression levels of dCK and dGK with 5mM metformin at 72 hours which caused a 69% (31±1%) and 56% (44±10%) reduction in CEM cells. In THP1 cells we found an increase of 22% (78±5%) in dCK with 2.5mM metformin at 24 hours treatment. Apoptosis with both metformin and metformin/clofarabine treatment was not evident, either through caspase-3 cleavage or DNA fragmentation in CEM and THP1 cell lines. In conclusion, metformin enhances clofarabine effectiveness in ALL CEM but slightly induces clofarabine resistance in AML THP1 cells.

Dedication

I dedicate this to my mother Miss Thifhelimbilu Florence Mulaudzi and my brother Mmbengeni Nemuvumoni and my sister Khathutshelo Nemuvumoni and my late great grandmother Miss Mmbengeni Elissa Lukheli.

Acknowledgement

I wish to thank God for giving me strength and keeping me in healthy state throughout the year to complete this study. I am extremely grateful to my supervisor Prof D. Mavri-Damelin for her support, guidance, and her mentorship throughout the project and her kindness and also for being patient, which make it easy for me to pursue this degree.

I would also like to express my gratitude to my co-supervisor Dr L.H Damelin for his assistance and input throughout this project.

I would also like to thanks Dr Vanessa Meyer for giving us THP1 cell lines and I would like to thank Dr Kaur for granting us the access to her lab to use Floid microscope. I would also like to extend my gratitude to Dr K Mothlatlego and my advisor Dr R. Jivan for their essential inputs. Their assistance was essential for me to pursue this study.

Over the two years' period in functional genetics lab I was fortunate to work with generous colleague's/lab mates. I would like to give thanks Tebogo, Eunice, Kerry and all 2019 honours I shared lab with for being friendly and welcoming, generous, stimulating discussions, motivation and for great memories in the last 2 years. It was very easy to work with these brilliant individuals.

Lastly, I have to thank my uncle Mr Mmbangiseni Robert Tshikovhi for his love and support and also for providing me with accommodation when I was in need. I could not have completed this dissertation without your support.

I wish to thank National Research Fund (NRF) for financial support.

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Abbreviations of units	**Symbol interpretation**
%	Percentage
µl	Microliter
^{0}C	Degree celcius
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myelogenous Leukaemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APAF-1	Cytosolic apoptotic protease-activating factor-1
APRT	Adenine phosphoribosyl transferase
APS	Ammonium persulfate
Ara-A	9-β-D arabinofuranosyl-adenine
ATP	Adenosine triphosphate
bp	base pair

CDKN2A	Cyclin-dependent kinase inhibitor 2A
CH2THF	5,10-Methylene tetrahydrofolate
CLL	Chronic Lymphoblastic Leukaemia
Clof	Clofarabine
CML	Chronic Myelogenous Leukaemia
CR	complete remission
dATP	Deoxyadenosine triphosphate
dCDP	Deoxycytidine diphosphate
dCK	Deoxycytidine kinase
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
dGK	Deoxyguanosine kinase
dGTP	Deoxyguanosine triphosphate
DHFR	Dihydrofolate reductase
DLL	Delta like ligand
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dTMP	Deoxythymidine monophosphate

dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
EBV	Epstein Barr virus
ECD	Extracellular domain
ENTs	Equilibrative nucleoside transporter
EPT-ALL	T-lineage Progenitor-T-ALL
FDA	Food and drug administration
g	gram
hCNT	Human concentrative nucleoside transporter
hENT	Human equilibrative nucleoside transporter
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HTLV-1	Human T-lymphocytes virus type 1
ICD	Intracellular domain
IL-7R	Interleukin-7 receptor
IMP	Inosine monophosphate
INK4A	Inhibitor of CDK4

JAK	Janus kinase
kDa	Kilodalton
Met	Metformin
ml	Millilitre
MTOR	Mammalian target of rapamycin
NECD	NOTCH extracellular domain
NICED	NOTCH intracellular domain
OR	Overall response
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol-4,5 biphosphate 3 kinase
PIP2	Phosphatidylinositol 4,4-biphosphate
PIP3	Phosphatidylinositol 3,4,5-biphosphate
PKB	Protein Kinase B
pre-T ALL	Precursor-T ALL
PRPP	Phosphoribosyl pyrophosphate
PTEN	Phosphate and Tensin homolog
RB	Retinoblastoma

RBCs	Red blood cells
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
RTK	Receptor tyrosine kinase
SCT	Stem cell transplant
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
TEMED	Tetramethylenediamine
TK-1	Thymidine kinase
TS	Thymidylate synthase
v/v	Volume per volume
w/v	Weight per volume
WBCs	White blood cells
XG	Revolution per minute
XTT	The.2,3-bis-(2-methoxy-4-nitro-5sulfophenyl)-2H-tetrazolium-5-carboxanilide
β	Beta

1. Introduction

Cancer is the uncontrolled growth of abnormal cells that results in tumor formation in different parts of the body (WHO, 2018). Cancer can be caused by genetic alteration such as mutations, that can be caused by various internal factors such as errors in DNA replication and/or external factors such as chemical (such as alcohol), biological (viral infections such as HPV) and physical carcinogens (such as ultraviolet rays) (WHO, 2018). Cancers are named according to the organs or the cells they affect or where they begin, these include carcinoma that arises from epithelial cells, sarcoma which arise from connective tissues, and lymphomas and leukaemia, which are of relevance to this project and are discussed in detail below (Varricchio, 2004).

1.1 Blood cell formation

Blood cells are produced from the soft sponge-like tissue found inside the bone called bone marrow (Soni and Yadav, 2014). Stem cells are specialized cells in human with the ability to differentiate into different cell types, this includes matured blood cells. Stem cells first differentiate into blast cells (immature blood cells) then form mature blood cell types (red blood cells, platelets and white blood cells) that leave the bone marrow and enter the bloodstream (Figure 1.1). Mature blood cell types have different functions: Red blood cells are responsible for oxygen transportation throughout the body and also act as a carrier of carbon dioxide to the lungs to be removed out, platelets are responsible for blood clotting

and can therefore facilitate wound repair and white blood cells are required for the acquired immune system to protect the body against invading antigens (Hartenstein, 2006; Luckheeram*et al.,* 2011). These act by recognizing and eliminating non-native antigens together with the development of immunologic memory and acquiring tolerance to self-antigens (Luckheeram*et al.,* 2011). Thus, this category includes all types of white blood cells, namely lymphocytes, granulocytes, and monocytes (Hartenstein, 2006).

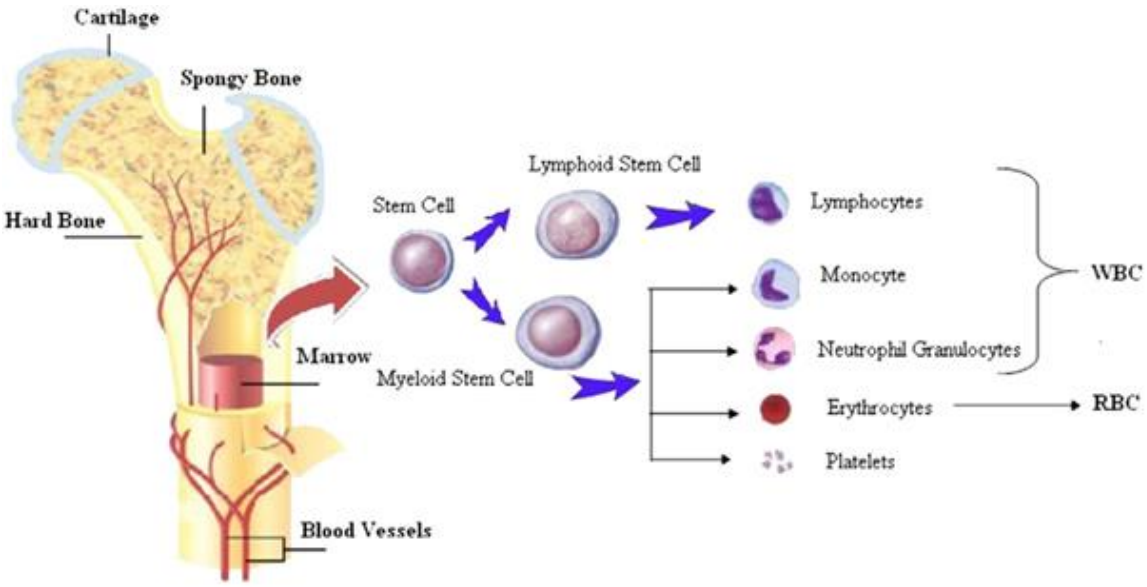

Figure 1.1: Formation of blood cells. Development of blood cells from bone marrow. Blast stem cells undergo a series of transformations to become mature blood cells. They elongate into different types of blood cells (red blood cells (RBC), platelets, and white blood cells (WBC)), adopted from Sami and Yadav, 2014.

Lymphocytes constitute the lymphoid tissue whereby they mature from lymphoblasts (immature white blood cells) (Camara *et al.,* 2012). Lymphocytes are comprised of T-lymphocytes and B-lymphocytes derived from the thymus and bone-marrow respectively, and natural killer cells (NK cells) (Luckheeram*et al.,* 2011). Both T and B lymphocytes form part of the adaptive immune-system that works hand-in-hand with the innate immune system (Camara *et al.,* 2012). The B-lymphocytes form antibodies that can attach to the invading molecule to be targeted and destroyed by the immune system providing adaptive immunity (Camara *et al.,* 2012). There are different T-lymphocytes and they all present different specialized functions (Luckheeram*et al.,* 2011). Helper T cells ($CD4^+$ T) and Cytotoxic (killer) T cells ($CD8^+$ T) are the main constituent of the T-lymphocytes subtypes (Luckheeram*et al.,* 2011). Naive $CD4^+$ T cells differentiate and develop into distinct effector subtypes after interacting with the antigen presented by major histocompatibility complex II (MHC II) molecules on the surface of the antigen-presenting cells (APCs), to help with the activation of immune response (Tao *et al.,* 1997). After the activation of $CD4^+$ T cells, B-cells can produce antibodies that induce macrophage synthesis and microbicidal activity for active immune responses (Zhu and Paul, 2019).

The $CD4^+$ T cells also play a crucial role in the development and maintenance of the cytotoxic T lymphocytes (also known as $CD8^+$ T-cells, or CTLs) (Kamamoto *et al.,* 2011). The immunologic response CTLs is to destroy external environmental body invading molecules (such as viruses, bacteria etc.) and cancerous cells (Arens

and Schoenberger, 2010). Aberrant production of immature stem cells can result in malignant blood cancer called leukaemia (Grigoropoulos *et al*., 2013).

1.2 Leukaemia

Leukaemia is considered as one of the most lethal types of cancer with an estimated 437 035 (2.4%) cases in 2018, ranking it in sixth place among various human malignancies (Bray *et al.,* 2018). Leukaemia has been associated with at least 309 006 (3.2%) cancer-related deaths worldwide (Bray *et al.,* 2018). It has been also considered the most common neoplasm in children, and about 30% of cancer-related death cases are caused by leukaemia in children and adolescents under the age of 14 years worldwide (Liu *et al.,* 2015). Leukaemia can affect any age group but is more likely in men than women and develop more often in Caucasians compared to other races (Mahbub *et al.,* 2013). In addition, a recent cancer report presented by GLOBACAN (2018) indicated that leukaemia is one of the top 10 leading types of cancer in South Africa. Leukaemia has a recorded age-standardized rate of 5.5 and 3.4 per 100 000 in male and female in South Africa, respectively (GLOBACAN, 2018).

Leukaemia is a malignancy of the haemopoietic system that initiates when the bone marrow synthesizes abnormal immature stem cells also called leukemic blast cells (Grigoropoulos *et al.,* 2013). When cells become old or damaged, leukaemia cells do not die but continue to grow and overcrowd the blood system (Isoda *et al.,* 2014). Overcrowding of blood system with immature blast cells limits blood flow and that makes it difficult for red blood cells to transport oxygen throughout

the body (Zhang *et al.,* 2009). Leukaemia hematological malignancies affect the cells of all haematopoietic lineages (Qin *et al.,* 2012).

Leukaemogenesis can occur by endogenous and exogenous exposure to various environmental factors, and genetic susceptibility (Liu *et al.,* 2015). However, there are no exact known causes of leukaemia (Arber *et al.,* 2016). One well studied environmental risk factor is the exposure to ionizing radiation, which most evidence of risk association based on documented incidence of leukaemia cases following the atomic bombing of Nagasaki and Hiroshima in 1945 (Pfresten *et al.,* 1987). In addition, individuals who are working with radiation on daily bases show increased risk, and patients undergoing radiotherapy for cancer therapy are at risk of acquiring leukaemia as a secondary cancer (Yoshinga *et al*., 2004; Shuryak *et al*., 2006). Continuous exposure to benzene also increases chances for leukaemia development, in a dose dependent manner (Brandt *et al*., 1978; Schnatter *et al*., 2012). Lifestyle also plays a great role in leukaemia development, this include smoking and being overweight (Pogoda *et al*., 2002; Calle *et al*., 2004). In addition, parental alcohol drinking can increase the risk of leukaemia development in the fetus (Menegau *et al*., 2007). Viral infection is also associated with leukaemogenesis this includes Human T-lymphotropic virus type 1 (HTLV-1) and Epstein Barr Virus (EBV) infection (Brady *et al*., 2007; Tsukasaki and Tobinai 2014). Genetic alterations are also associated with leukaemia development, this includes genetic translocation, deletion or inversion, which are discussed later (Greaves, 1997).

There are four different major groups of leukaemia that are grouped according to the cell type they affect and progression of the particular type of tumor, namely; Chronic Myelogenous Leukaemia (CML), Acute Myelogenous Leukaemia (AML), Chronic Lymphoblastic Leukaemia (CLL) and Acute Lymphoblastic Leukaemia (ALL) (Davis *et al.,* 2014). The CML and AML are types of myeloid leukaemia of granulocytes and monocytes that synthesizes macrophages (Gore *et al.,*2014; Short *et al.,* 2018). AML is characterized by extensive overexpression of immature granulocytes and monocytes from blood stem cells accumulating blood-stream system (Short *et al.,* 2018). CML is characterized by overexpression of mature, but abnormal, myeloid white blood cells (Gore *et al.,* 2014). CLL is regarded as clonal malignancy of B cell lymphocytes that accumulate in the blood system (Saidano *et al.,* 2012). Lastly, ALL which is the focus of this study, is a haematopoietic malignancy resulting from immature B and T- lymphoid cells (Banelli *et al.,* 2012). Seeing how ALL mostly affects young children (2 -5 years) and adults (50+) having less chance of survival (50%) (Banelli *et al.,* 2012), this motivated consideration of investigating alternative chemotherapy approaches.

1.2.1 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) develops from premature forms of lymphocytes, and initiates in B-cells and T-cells during different stages of maturation (Chonghaile *et al.,* 2014). ALL affects both adults and children, where it is common in children between the ages of 2 to 5 years, representing about one-third of all childhood cancers (Winter *et al.,* 2014). Recent treatment approaches have improved outcome but 20% of pediatric patients still die of this disease, and adults have a 50% mortality rate (Marks and Rowntree, 2017).

The most severe form of the disease occurs with relapse after remission, and it has been a challenge to evade this therapy obstruction (Pui and Evans., 2006). Assessment of the chances for relapse is critical in selecting a therapy that will avoid neurotoxicity and support a high rate of tumor remission (Pui and Evans., 2006). The state of the disease influences the selection of the treatment to be used (Evans and Relling., 2004).

1.2.1.1 T cell-acute lymphoblastic leukaemia

This study will be focusing on T-ALL, which is an aggressive cancer that develops from the expression of a high number of immature progenitors that encode T-cell development (Girardi *et al.,* 2017). This can occur in different development stages of T-cells (Girardi *et al.,* 2017). These blast T-cells tend to infiltrate and over-crowd the bone marrow, lymph nodes and also diffuse into the central nervous system which leads to a more severe form of the disease (Bongiovanni *et al.,* 2017).

T- ALL is a significant contributor to mortality in children and adults that contribute about 25% of adult ALL and 15% of pediatric ALL cases (Eduardo *et al.,* 2017; Van Vlierberghe *et al.,* 2012). It has also been considered to have a high risk of relapse which leads to a significant clinical problem (Yadav *et al.,* 2016).

T-ALL is classified into different sub-groups namely: early cortical, late or mature T-ALL (Ferrando *et al.,* 2002). Precursor -T ALL (pre-T-ALL) comprises about 20% of cases and mature T-cell ALL comprise about 15% to 20% cases (Ferrando *et al.,* 2002). A new subtype of T-ALL has more recently been discovered, named early T-lineage progenitor T-ALL (ETP-ALL) which does not express some T-cell surface marker, but has been associated with the expression of myeloid and stem cell markers (Inuki *et al.,* 2012).

1.2.1.2 Contributors to the initiation and progression of T-ALL

T-cell development requires several steps to reach matured, functional cells (Vlierberghe and Ferrando, 2012). Genetic alterations that modifies normal pathways regulating cell growth, proliferation, survival, and differentiation during thymocyte development are discussed and this includes; the NOTCH signaling pathway, cyclin-dependent kinase inhibitor 2A (CDKN2A locus) deletion, and phosphatidylinositol-4,5 biphosphate 3-kinase (PI3K)/ AKT also known as protein kinase B (PKB)/mammalian target of rapamycin (mTOR) (P13K/AKT/mTOR) pathways (Vlierberghe and Ferrando, 2012).

Neurogenic locus homolog protein 1 (NOTCH1) signaling has been associated with enhancement of leukaemia malignancies through transcriptional upregulation of anabolic pathways, ribosome biosynthesis, protein translation, and nucleotide and amino acid metabolism (Weng *et al.* 2006).

The NOTCH signaling pathway is a complex pathway involving regulation of various molecules (Pui *et al.,* 2008). The NOTCH signaling pathway involves intercellular interaction between two cells (Pui *et al.,* 2008). Signal sending cells has more delta like ligand (DLL) for signal transmission and receiving cell has more NOTCH receptor than DLL (Pui *et al.,* 2008). The NOTCH receptor has 3 components NOTCH extracellular domain (NECD), NOTCH intracellular domain (NICD) and transmembrane component which links the ECD and ICD.

The NOTCH1 becomes activated when the DLL binds to the ECD of the NOTCH receptor and this triggers the cleavage of the ECD by the ADAM10 metalloprotease (S2 cleavage) (Martin and Ferrando, 2019). Furthermore, the γ-secretase cleaves the ICD off the transmembrane portion of the NOTCH receptor (S3 cleavage) (Palomero *et al.,* 2006). The ICD becomes free in the cytosol, where it binds with other proteins forming a complex, this includes RBPJ DNA-binding, MAML transcriptional coactivators and the complex further binds to p300 (Pui *et al.,* 2008). The entire complex translocate to the nucleus, regulates the expression of the NOTCH target genes including MYC oncogene that code for transcription factors, p21 and cyclin D3 and the NICD can be down-regulated and proteasome deregulated to effectively shut-down the pathway as the NOTCH target genes, has been expressed (Weng *et al.,* 2006).

Although abnormal triggering of the NOTCH1 pathway is the most significant cause of T-ALL formation, some other mechanisms leading to T-ALL are caused by genetic alteration such as; cyclin-dependent kinase inhibitor 2A (CDKN2A locus) deletion in chromosome band 9p21 (Weng *et al.,* 2004). *CDKN2A* is a gene that encodes p16 (a tumor suppressor protein), INK4A (an inhibitor of CDK4) and p14/ARF (a tumor suppressor). These multiple proteins arise through alternate reading frames within the CDKN2A locus (Weng *et al.,* 2004). In some cases of T-ALL, the t (12;14) (p13; q11) and t (7;12) (q34; p13) translocation can enhance Cyclin D2 (CCND2) cycle regulator expression, which then inhibits members of the retinoblastoma (RB) protein family including RB1 and CDKN1B, and this leads to uncontrolled cell cycle progression (Remke *et al.,* 2009).

Other vital pathways that can be upregulated and lead to ALL include; phosphatidylinositol-4,5 biphosphate 3-kinase (PI3K)/ AKT also known as protein kinase B (PKB)/mammalian target of rapamycin (mTOR) (P13K/AKT/mTOR). The PI3K/Akt and mTOR signaling pathways are essential for cell growth and survival during cellular stress (Datta *et al.,* 1999). These pathways are interconnected in such a way that they can be regarded as a single pathway (Portal et al., 2014). Extracellular signaling molecule (e.g. growth factor), activates the receptor tyrosine kinase (RTK) embedded in the cell membrane to initiates the pathway (Portal et al., 2014). The RTK undergoes auto-phosphorylation that attracts the PI3K into the membrane to bind directly to the phosphotyrosine (Portal et al., 2014). The PI3K becomes activated and detach from the phosphotyrosine to phosphorylate phosphatidylinositol 4,4-biphosphate (PI-4,5-P_2 or PIP2) which is another protein embedded into the cell membrane, this protein is phosphorylated into phosphatidylinositol 3,4,5 triphosphate (PI3,4,5-P_2 or PIP3) (Portal et al., 2014).

Once PIP3 is activated it synthesizes Akt via enzyme phosphoinositide-dependent kinase1 (PDK1) (Fruman *et al.,* 1998; Fesno-Varaja *et al.,* 2003). Akt is essential for cell survival as it promotes cell proliferation and cellular growth, it also inhibits apoptosis molecules such as procaspase-9 thus it enhance cancer progression (Manning and Cantley, 2007). Furthermore, Akt activates mTOR that is also crucial for protein synthesis and cell growth, cell cycle survival and apoptosis inhibition (Hay and Sonenberg, 2004). Thus, upregulation and activation of mTOR may support tumor growth by enhancing protein synthesis.

A normal PI3K/Akt and mTOR signaling pathway is regulated by positive (e.g growth factors) and negative upstream regulators (Shaw *et al.,* 2004). The main negative regulators are phosphate and tensin homolog (PTEN), which inhibits the pathway via blocking of PI3K-Akt pathway progression (Shaw *et al.,* 2004). This subsequently enhances apoptosis (Shaw *et al.,* 2004). A PTEN deficiency has been linked with the tumor progression including in T-cell malignancy (Sansal and Sellers, 2004). The activity of PTEN against P13K/Akt signaling pathway has been associated with the maintenance of the haematopoietic stem cells and preventing leukemiogenesis (Yilmaz *et al.,* 2006; Zhang *et al.,* 2006). The PTEN deficiency has been associated with 5% to 10% of T-ALL cases (Palomero *et al.,* 2007). Enforced PTEN expression (expression of PTEN from unusual site) in T-ALL cell lines has been demonstrated to induce apoptosis through inhibition of PI3K/Akt signaling pathway (Shade *et al.,* 2006).

Another vital pathway that is linked with supporting T-ALL tumor progression is interleukin-7 receptor (IL-7R)/Janus kinase (JAK); signal transducer and activator of transcription (STAT) (IL-7R/JAK/STAT) signaling pathway (Ribeiro *et al.,* 2018). Interleukin-7 is a cytokine synthesized in the thymus and bone marrow that is associated with lymphoid development (Freedom-Jeffery *et al.,* 1995; Peschon *et al.,* 1994; Jiang *et al.,*2005). The STAT family members are regarded as transcriptional factors, that are activated upstream by JAK kinase proteins (Becerra-Diaz *et al.,* 2011). The pathway is initiated by binding of IL-7 into the cell surface receptor (IL-7R), and this enhances the JAKs kinases activity through the tyrosine residue phosphorylation in the cytokine receptor (Becerra-Diaz *et al.,*

2011; Montano *et al.,* 2018). This then leads to the attachment of the cytoplasmic STAT onto the cytokine receptors and STAT is phosphorylated by the JAK proteins at tyrosine residues (Becerra-Diaz *et al.,* 2011). The phosphorylated STAT proteins detach from the receptors, forming heterodimers or homodimers among themselves (Becerra-Diaz *et al.,* 2011). These proteins then translocate to the nucleus where they bind DNA sequences and gene expression that supports increased cell proliferation rate, growth and cell cycle progression (Montano *et al.,* 2018; Ribeiro *et al.,* 2018).

1.2.1.3 Treatment of T-ALL

Pediatric ALL treatment has achieved considerable success in more recent years with at least 50% survival rate (Pui *et al.,* 2013; David *et al.,* 2018). Adult ALL treatment has improved over the years, with 40% of adults responding well to the treatment (Stock *et al.,* 2008). However, adolescents and young adults are still showing a greater response to intensive chemotherapy regimens than adults (Stock *et al.,* 2008). As such there is considerable room for improvement on current therapeutic strategies.

Treatments for ALL includes; induction of remission, consolidation, continuation and allogeneic hemopoietin stem cell transplant, radiotherapy followed by supportive care (Greaves, 1997; Inaba *et al.,* 2013).

1.2.1.3.1. Induction of remission

An initial treatment applied after diagnosis is given to the patients for 4-6 weeks. The aim of the treatment is to eradicate most of the leukaemic cells and restore normal haematopoetic process (Bassan and Hoelzer, 2011). The drugs applied for chemotherapy are vincristidine and asparaginase which can be given with anthracycline (Inaba *et al.,* 2013). The therapy is considered successful when remission is achieved (Indaba *et al al*., 2013).

1.2.1.3.2. Consolidation

The stage after induction of remission treatment to destroy remaining leukaemia cells (Stanulla and Schruppe, 2009). This is applied for 20-30 weeks, and the drugs usually given are methotrexate in high dose with mercaptopurine (Indaba *et al.,* 2013). After consolidation therapy the continuation therapy is applied to prevent relapse and this is referred as the maintenance therapy (Indaba *et al.,* 2013). This therapy is given for 2 years, the drugs used are mercaptopurine daily and methotrexate weekly, which can be applied with or without vincristine (Indaba *et al.,* 2013).

1.2.1.3.3. Stem cell transplant (SCT)

In this treatment damaged blood stem cells are destroyed and replaced with the new healthy blood-forming cells from a donor (Eapen *et al.,* 2010). The main objective is to replace unhealthy haematopoietic blood-forming cells with functional cell that will attack tumor cells. Chemotherapy is used as a pre-

treatment to suppress bone marrow formation, followed by transplant with effective blood-forming cells (Indaba *et al.,* 2013). Thus, the new healthy stem cells grow to form a new bone marrow and destroy the remaining cancer cells. In ALL the SCT applied is allogenic SCT in which chemotherapy is also used to destroy normal blood cells in bone marrow to weaken the immune system to avoid transplant failure (Baldazzi *et al.,* 2005). This suppresses the immune system and it may result in a high risk of infection and bleeding (Eapen *et al.,* 2010). The patients must stay in the high hygienic environment for some time for immune system recovery. Age and general health conditions may also be a limiting factor to undergo this treatment (Indaba *et al.,* 2013).

1.2.1.3.4. Radiation therapy

This type of therapy operates by applying high-energy rays to the ALL tumors to destroy or kill them (Greaves., 1997). Radiation chemotherapy can be applied differently according to the type of tumor treated. For ALL treatment the machine used emit rays from outside the body to target tumors, known as external beam radiation (Tisseverasinghe *et al.,* 2016). This is normally applied to cancer cells that have spread to the fluid around the brain and spinal cord. It is also applied prior to an SCT. This type of treatment has some considerable side effects such as skin changes, hair loss, fatigue, upset stomach, and diarrhea.

1.3 Nucleotide metabolism

Nucleotides are essential for many different cellular processes (Zhang *et al.,* 2015). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are polymers of nucleotides and have other crucial roles in a cellular mechanism such as being energy carriers, enzyme cofactors or signaling molecules that are involved in and regulate cellular metabolism, proliferation and repair (Voorde *et al.,* 2014). Nucleotides can be synthesized through *de novo* or *salvage pathway* for cellular requirements (Hatse *et al.,* 1999). Nucleotide metabolism is also a target for a specific class of cancer chemotherapy agents, as follows.

1.3.1 Pyrimidine nucleotide metabolism

De novo synthetic pathways exist for the pyrimidines (uracil (RNA), thymine (DNA) and cytosine (RNA and DNA). For Uracil synthesis of uridine-5'-monophosphate (UMP) first arises from orotate with 5'-phosphoribosyl-1-pyrophosphate (PRPP) as a cofactor for the reaction. UMP is converted into uridine-5'-diphosphate (UDP) and then uridine-5'-triphosphate (UTP) via nucleotide kinase activity. For cytosine, cytidine-5' (CTP) synthase converts UTP into CTP. Ribonucleotide reductase (RNR) catalyzes UDP and cytidine-5'-diphosphate (CDP), which results in 2'-deoxyuridine-5'-diphosphate (dUDP) synthesis forming dUTP, and 2'-deoxycytidine-5'-diphosphate (dCDP) synthesis forming dCTP, which are incorporated into RNA and DNA respectively (Voorde *et al.,* 2014). For thymine, thymidylate synthase (TS) methylate 2'-deoxyuridine-5'-monophosphate (dUMP) to thymidine-5'-monophosphate (dTMP). In this reaction 5,10-methylene tetrahydrofolate

(CH_2THF) acts as a single carbon donor and as reductant (Voorde *et al.,* 2014). The nucleotide kinase phosphorylation results in the formation of thymidine-5'-triphosphate (dTTP) and 2'-deoxycytidine-5'-triphosphate (dCTP) required for DNA polymerization (Voorde *et al.,* 2014).

Alternatively, pyrimidines can be synthesized via the pyrimidine salvage pathway, whereby thymidine acts as a substrate for thymidine kinase 1 (TK-1) and is required for thymidine-5'-monophosphate (dTMP) synthesis. Likewise, uridine can be a substrate for UMP, and cytidine yielding CMP that can also act a precursor for UMP production (Voorde *et al.,* 2014).

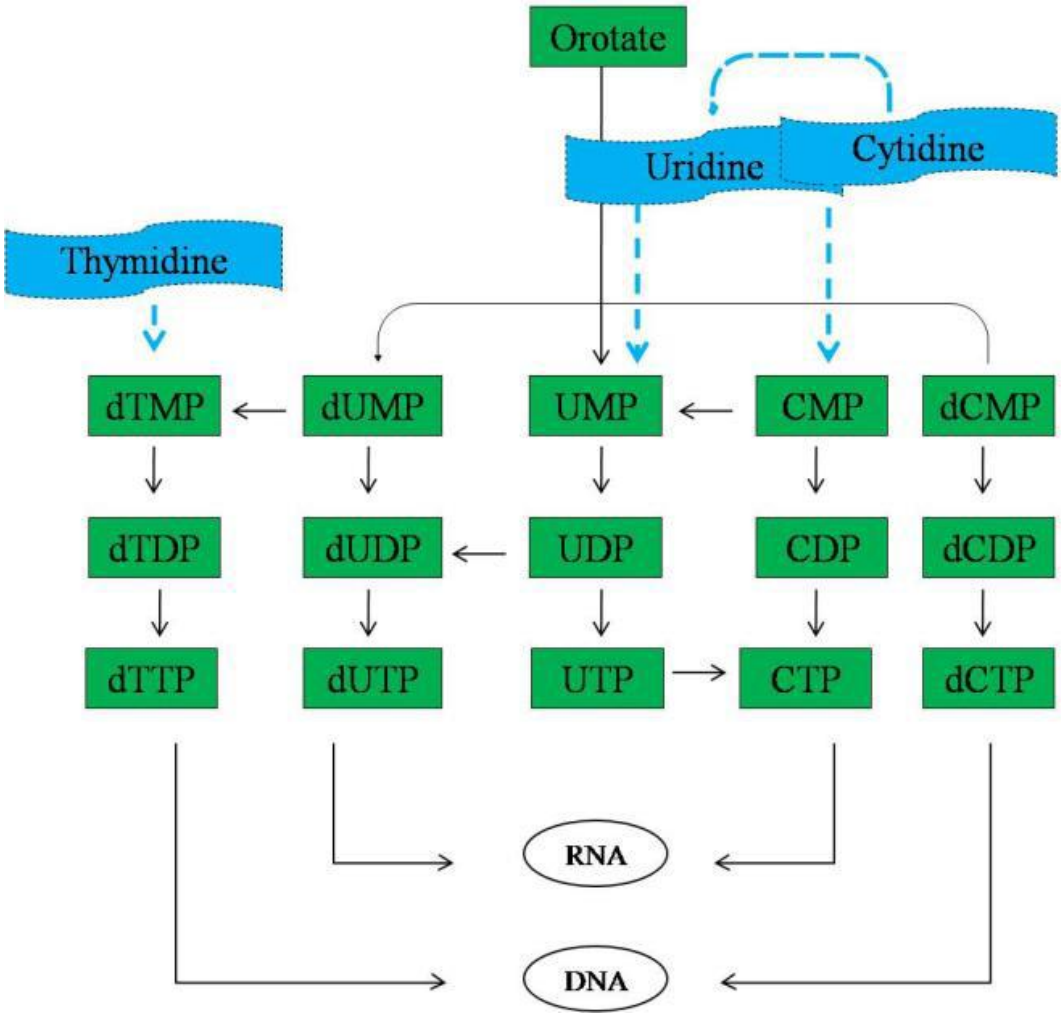

Figure 1.2: Schematic diagram representing pyrimidine de novo and salvage biosynthesis in mammalian cells. De novo pathway nucleotide precursors are denoted by green boxes joined by solid arrows. Salvage pathway nucleotide precursors are indicated by blue boxes joined by dashed blue arrows, adapted from Mynhardt *et al*., 2018.

As mentioned above, TS is a ubiquitous enzyme required for de novo synthesis of deoxythymidine monophosphate (dTMP/thymidylate) (Berger and Berger, 2006). TS catalyses reductive methylation of dUMP to dTMP, with CH_2THF a folic acid acting as one-carbon methyl donor (Rose *et al.,* 2002). Subsequently the dTMP is further phosphorylated into dTTP that is incorporated into DNA for DNA synthesis and repair (Kholodar *et al.,* 2018). In addition, dihydrofolate (DHF) is formed as a byproduct of dTMP in a 1:1 ratio with dTMP concentration, that is recycled to CH_2THF and acts as methyl donor for dTMP formation (Figure 1.3) (Di Cresce and Koroptnick., 2014). The TS cycle is the sole denovo pathway responsible for dTTP synthesis (Berger and Berger, 2006). The dTTP is the only dNTP molecule that does not require RNR for its biosynthesis and that can cause complications against clofarabine agent that aim for dNTP pool reduction via RNR inhibition (Berger and Berger, 2006). TS is highly accumulated in cancer cells and it plays a critical role in chemotherapy resistance in T-ALL for patients treated with drugs targeting dNTP pools reduction.

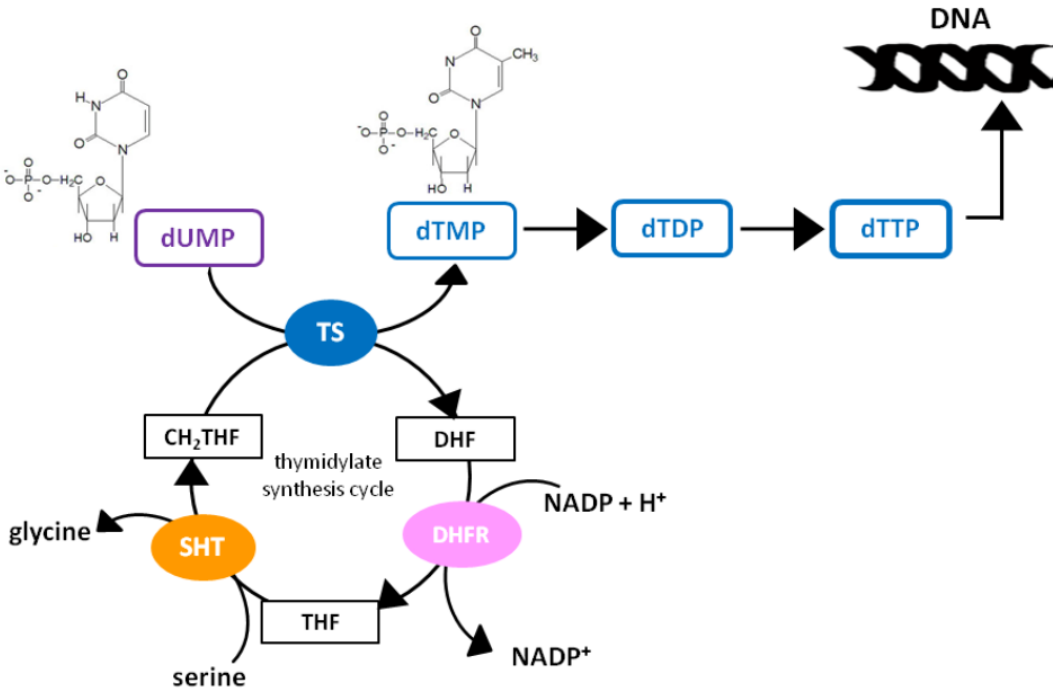

Figure 1.3: Role of TS cycle in providing dTTP for DNA synthesis and repair. The TS reductively methylate dUMP to dTMP. The DHF is synthesized as a byproduct of dTMP synthesis with equal ratio. The DHF is then recycled back via TS cycle into CH_2THF to be used again by TS to produce dTMP, adapted from Di Cresce, 2014.

TK-1 is a cell cycle regulated enzyme localized in the cytosol, it helps cells to recycle deoxyribonucleotides from degraded DNA fragments via salvage pathway (Welin *et al*., 2004). TK-1 couples, phosphate group from ATP molecule into thymidine, forming dTMP. The dTMP is further phosphorylated into di and triphosphate forms (dTDP, dTTP), respectively (Petersen B, 2010). Then dTTP is incorporated into DNA for DNA synthesis and repair (Begegni *et al*., 2012). Just like TS, TK-1 is also highly upregulated in proliferating cells and hits the peak during the S-phase with very low accumulation in quiescent cells (Ke and Chang, 2004). Thus, both TS and TK-1 are considered cancer biomarkers and they can modulate drug resistance in RNR drug targets as mentioned above (Ke and Chang, 2004).

1.3.2 Purine nucleotide metabolism

The *de novo* syntheses of purine nucleotides require inosine-5'-monophosphate (IMP) as a precursor for adenosine-5'-monophosphate (AMP) or guanosine-5'-monophosphate (GMP). Succinyl AMP converts IMP to AMP and GMP synthase convert IMP to GMP. Nucleotide kinase activity synthesizes adenosine-5'-diphosphate (ADP) and ATP from AMP and guanosine-5'-diphosphate (GDP) and GTP from GMP. RNR catalyzes synthesis of 2-deoxyguanosine-5'-diphosphate (dGDP) and 2'-deoxyadenosine which are further metabolized into 2'-deoxyguanosine-5'-triphosphate (dGTP) and 2'-deoxyadenosine-5'-triphosphate (dATP), which are incorporated into DNA for further DNA syntheses. Purines can also be synthesized via the purine salvage pathway, whereby inosine (Ino) and guanosine (guo) are catabolized into their respective nucleobases; hypoxanthine (Hx) and guanine by purine nucleoside phosphorylase (PNP). Adenine phosphoribosyl transferase (APRT) catalyzes adenine to AMP and hypoxanthine-guanine phosphoribosyl transferase (HGPRT) catabolizes Hx and guo to IMP and GMP respectively (Hatse *et al.,* 2014).

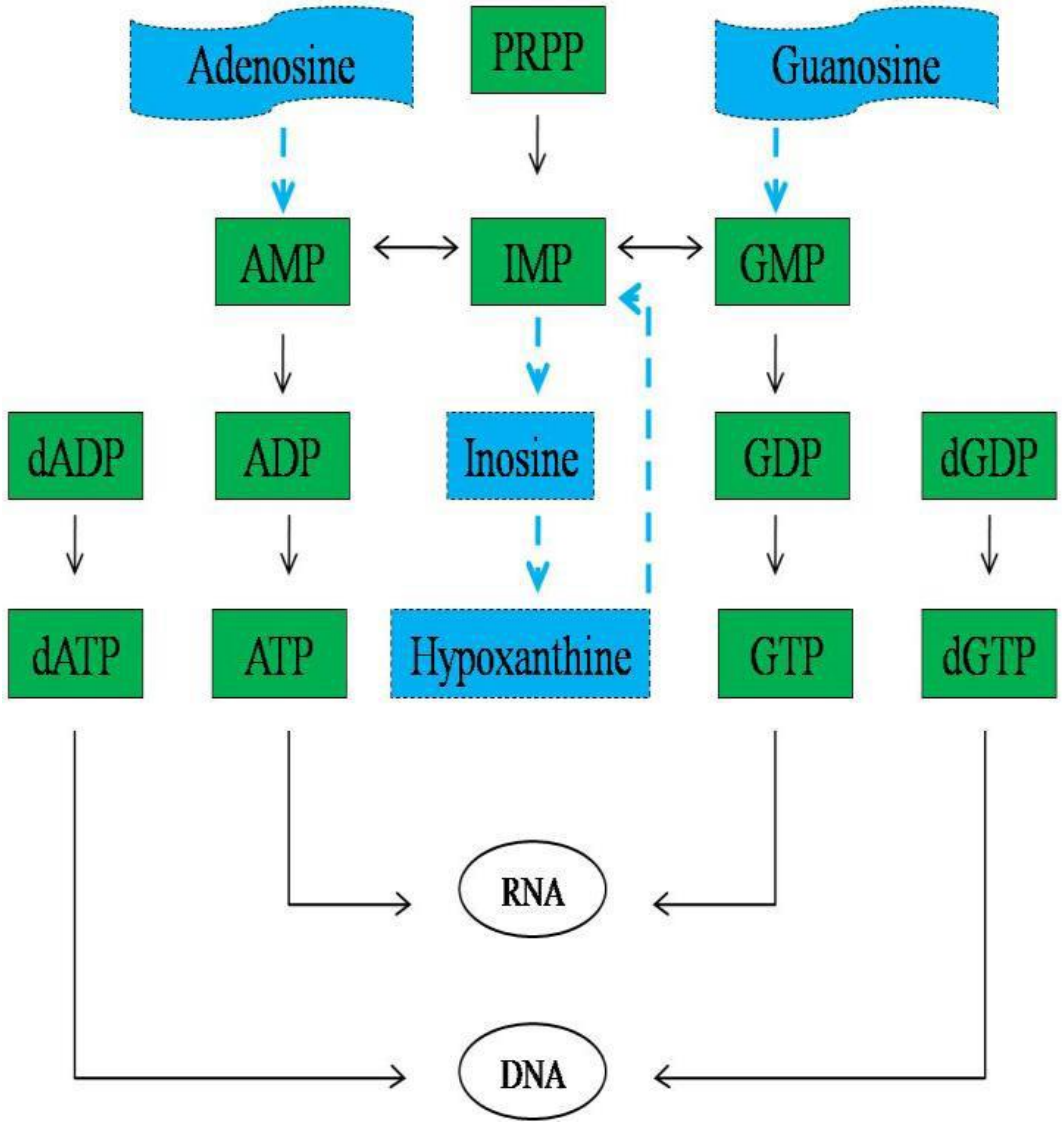

Figure 1.4: Schematic diagram representing purine de novo and salvage biosynthesis in mammalian cells. De novo pathway nucleotide precursors are denoted by green boxes joined by solid arrows. Salvage pathway nucleotide precursors are indicated by blue boxes joined by dashed blue arrows, adapted from Mynhardt *et al*., 2018.

1.3.3 Nucleoside and nucleotide analogues in cancer therapy

Nucleoside and nucleotide analogues are artificially modified chemical compounds that mimic their physiological counterparts (Jordhein *et al.,* 2013). One major mechanism through which these analogues exert their cytotoxic activity is through incorporation into the nucleic acid macromolecules resulting in inhibition of cellular division (Jordhein *et al.,* 2013).

Nucleosides analogues are transported into the cell via specialised nucleoside transporters, the SLC29 a solute carrier family also named equilibrative nucleoside transporters (ENTs) (Young *et al.,* 2008). The ENTs are divided into 4 subtypes; ENT1, ENT2, ENT3, and ENT4 which are responsible for nucleoside and nucleobase uptake for salvage pathways of nucleotide synthesis (Young *et al.,* 2008). Analogues are also transported through the same route as endogenous nucleosides (Young *et al.,* 2008). In addition, ENT1 and ENT2, which are primarily situated on the plasma membrane, transport various types of purines and pyrimidines, and ENT2 also transports nucleobases such as uracil (Baldwin *et al.,* 2004; Yao *et al.,* 2011). ENT3 predominantly functions intracellularly, it is located in the lysosomal membrane and it also transports several purines and pyrimidine nucleosides (Baldwin *et al.,* 2005). ENT4 is situated in the plasma membrane and transports adenosine (Zhou *et al.,* 2010).

Post-absorption, the nucleoside analogue drug metabolites are phosphorylated into their active forms to form mono-, di and triphosphorylated nucleosides via nucleoside monophosphate kinase, nucleoside diphosphate kinase and nucleoside

triphosphate kinase, respectively (Jordheim *et al.,* 2013). The analogues become cytotoxic when they inhibit target enzymes activities such as TS, DNA polymerase, and RNR (Jordheim *et al.,* 2013). In addition, cytotoxicity occurs when they are incorporated into newly synthesized DNA and RNA chains inducing direct cell death or apoptosis (Jordheim *et al.,* 2013). There are several approved nucleoside and nucleotide analogues for cancer chemotherapy (Parker., 2007).

Over the years, different nucleoside analogues has been developed for different cancer treatments. In the late 1950s cytosine arabinoside (cytarabine, Ara-C) was synthesized to treat AML (Tellman, 2006). Tumor cytotoxicity of Ara-C applied alone was not quite as aggressive as expected, thus it has to be combined with other agents such as anthracyclines to achieve satisfactory results (Gale and Cline, 1977). However, this gave rise to other promising arabinosyl-purine homologues such as 9-β-D arabinofuranosyl-adenine (Ara-A) (Zhenchuk *et al.,* 2009). Ara-A was found to be less active due to its rapid deamination by adenosine deaminase (ADA) (LePage *et al.,* 1973). This then led to the synthesis of ADA-resistant halogenated arabinosyl derivatives such as 2-fluoro-9-β-arabino-furanosyladenine (fludarabine) (Zhenchuk *et al.,* 2009). The insolubility characteristic of fludarabine was a setback for its efficiency (Zhenchuk *et al.,* 2009). Thus, the 5'-monophosphate derivative (fludarabine monophosphate, Fara-A, Fludara®) was developed, which is converted into fludarabine by endogenous phosphatase (Figure 1.5) (Zhenchuk *et al.,* 2009). The derivative was later associated with the phosphorylase cleavage of 2-fluoroaadenine, which is a toxic compound with inadequate tumor cytotoxicity (Struck *et al.,* 1982). The halogenated analogues

were found to pose cytotoxic effects that then led to the production of 2-chlordeoxyadenosine (cladrine, CdA), (Figure 1.5) (Christensen *et al*., 1972). However, CdA was found to pose reduced oral bioavailability due to its instability in the acidic gastric environment and susceptible to enzymatic cleavage (Lindemalm *et al.,* 2004). In the early 1990s, the next-generation deoxyadenosine analogue, clofarabine (2-chloro-2'-arabinofluoro-2'-deoxyadenosine, CAFdA, Cl-F-ara-A) was developed to overcome limitations posed by earlier nucleoside analogues (Figure 1.5) (Montgomery *et al.,* 1992). The aim was to combine the structural features of cladrabine and fludarabine. Clofarabine exerted cytotoxic effect in both non-proliferating human lymphocytes and rapidly proliferating cells, with the resistance to phosphorylitic cleavage and was deamination-stable in an acidic environment (Zhenchuk *et al.,* 2009).

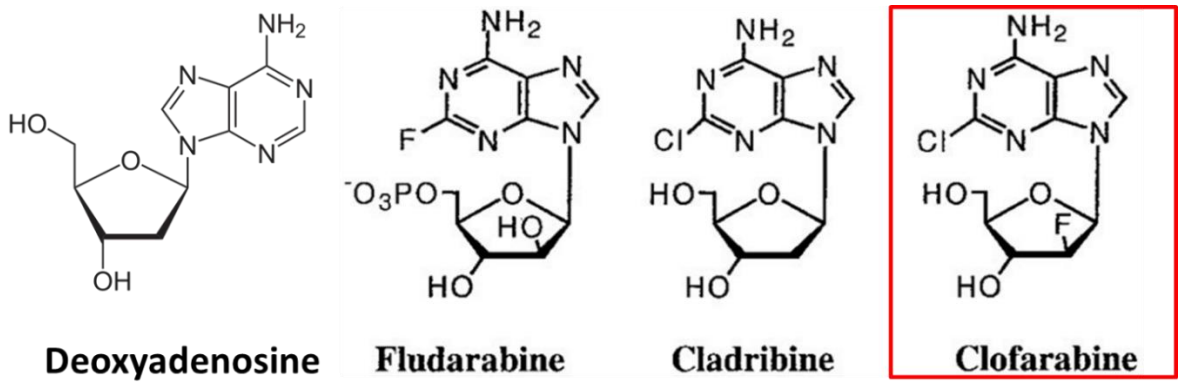

Figure 1.5: Chemical structure of clofarabine and its congeners/counterparts, adapted from, Zhenchuck *et al.,* 2009.

1.3.4 Clofarabine

Clofarabine is described as a new-generation deoxyadenosine analogue modified to enhance activity and significantly reducing toxicity posed by its congeners (Galmarini *et al.,* 2008). Clofarabine was approved in 2004 by the United States food and drug administration (FDA) for the treatment of leukaemia in pediatric patients, under commercial names Clolar™, Genzyme (Zhenchuk *et al.,* 2009). The drug was later approved by European commission approval in 2006, with commercial names, Evoltra® or Bioenvision (Zhenchuk et al., 2009). Clofarabine has a broad-spectrum anti-cancer activity in leukaemia and in some few solid tumors (e.g breast cancer) (Jena *el al.,* 2006). There are still ongoing studies of clofarabine against adult malignancy and other types of cancer.

Preclinical trials showed significant in vivo anticancer activities against various human tumors including prostate xenograft tumors (Waud *et al.,* 2000; Carson *et al.,* 1992). Following on, results from preclinical trials performed in mice and dogs gave passage to phase I and phase II clinical studies (Pession *et al*., 2010).

Phase I clinical trial for drug dosage and safety was conducted at The University of Texas M.D Anderson Cancer Center, in patients with relapse and refractory tumors (Kantarijan *et al*., 2003). Twenty-five patients with ALL and AML were treated with clofarabine (17 ALL and 8 AML, respectively) (Jeha *et al*., 2003). The treatment was applied with different doses ranging from 11.25 to 70 mg/m^2/d via intravenous infusion (Jeha *et al*., 2006). Hyperbilirubinemia and skin rash were the dose limiting factors at 70 mg/m2/d with the maximum tolerate-dosage at 52 mg/kg/d.

At least, 32% of patients shown to respond well to the drug (Jeha *et al*., 2006). Only 20% of patients posed complete remission (CR) (one AML patient and 4 ALL patients) (Jeha *et al*., 2006).

Phase II clinical trial establishing drug efficacy and safety analysis was investigated in patients with refractory ALL and AML (Kearns *et* al., 2006; Jeha *et al*., 2006). In the trial 61 patients with refractory ALL participated in the study (Kearns et al., 2006). Patients with refractory ALL administered 52 mg/m^2/day of clofarabine intravenously for five consecutive days (Kearns et al., 2006). From the study an overall response (OR) rate was 26% achieved. However, febrile neutropenia, seizures, sepsis and hepatoxicity were the most frequent adverse events (Kearns *et al*., 2006). The study conducted by Jeha and colleagues in 42 patients with refractory AML applying the same dosage as in ALL (52 mg/m^2/day) also retrieved 26% OR (Jeha *et al*., 2006). Unfortunately, five patients died as a result of adverse events (Jeha *et al*., 2006).

1.3.4.1 Mechanism of action

Administered clofarabine enters leukaemia cell membranes via different routes, including both two types of active nucleoside transporter, human equilibrative nucleoside transporter (hENT) and human concentrative nucleoside transporter (hCNT), and also enters the cells via passive diffusion across lipid membranes (Zhenchuk *et al.,* 2009). These, membrane nucleoside transporters are of importance in drug efficacy as demonstrated that clofarabine uptake is 10 fold higher by transport competent than transport-deficient human leukaemia cells

(King *et al.,* 2006). In addition, human cells expressing hCNT3 manifest higher rate of clofarabine uptake in contrast to cells expressing hCNT2, hENT1 and hENT2, furthermore transport-deficient and hCNT1 expressing cells exhibit no uptake as hCNT1 is specific for pyrimidines (Zhenchuk *et al.,* 200).

Inactive unphosphorylated clofarabine upon cell entry is phosphorylated by intracellular kinases into its monophosphate derivatives, primarily by deoxycytidine kinase (dCK) a cytosolic enzyme involved in the salvage pathway of DNA synthesis and also, by deoxyguanosine kinase (dGK) a mitochondrial kinase (Jeha *et al.,* 2006; Sjoberg *et al.,* 1998). Monophosphate clofarabine is further phosphorylated intracellularly into diphosphate form and finally, to active triphosphate clofarabine (clofarabine-3P) (Pui and Jeha, 2005). In addition, unprocessed forms remain intracellularly as clofarabine reservoir (Pui and Jeha, 2005).

Clofarabine executes anti-cancer activities via three main mechanisms namely; inhibition of DNA synthesis, RNR and direct induction of apoptosis (Yamchi *et al.,* 2015). Clofarabine triphosphate basically competes with the dATP for DNA incorporation, and Clofarabine-3P incorporation into the DNA results in termination of DNA repair and DNA elongation processes (Figure 3) (Galmarini *et al.,* 2008). Furthermore, this active form also binds to the allosteric site of regulatory subunits of RNR, thus reducing the in tracellular dNTP pool (Zhenchuk *et al.,* 2009; Faderl *et al.,* 2005). Specifically, with the most reduction in dCTP, dATP and dGTP pools in that order and with minor effect on dTTP pool formation

(Zhenchuk *et al.,* 2009; Faderl *et al.,* 2005). The inhibition of dNTP pool by active clofarabine, also self-increase its effectiveness in DNA synthesis inhibition (Chang and Cheng, 1980). Clofarabine-3P can also replace dATP and directly bind to cytosolic apoptotic protease-activating factor-1 (APAF-1) that leads to activation of caspase-9, which subsequently activates the caspase cascade (Genini *et al.,* 2000). Moreover, clofarabine-3P can disrupt the mitochondrial transmembrane potential, resulting in the release of cytochrome *c* and apoptosis-inducing factor (AIF) (Genin *et al.,* 2000). The combined actions of APAF-1, caspase-9, cytochrome c and AIF form the apoptosome complex, which is responsible for induction of programmed cell death (Figure 1.6) (Zhenchuk *et al.,* 2009). Clofarabine-3P has been demonstrated to induce dose and time dependent inhibition of the death suppressor proteins B-cell lymphoma-extra-large (Bcl-xL) and myeloid cell leukaemia-1 (Mcl-1) and dephosphorylation of anti-apoptotic kinase Akt (Tallman, 2006) (Figure 1.6).

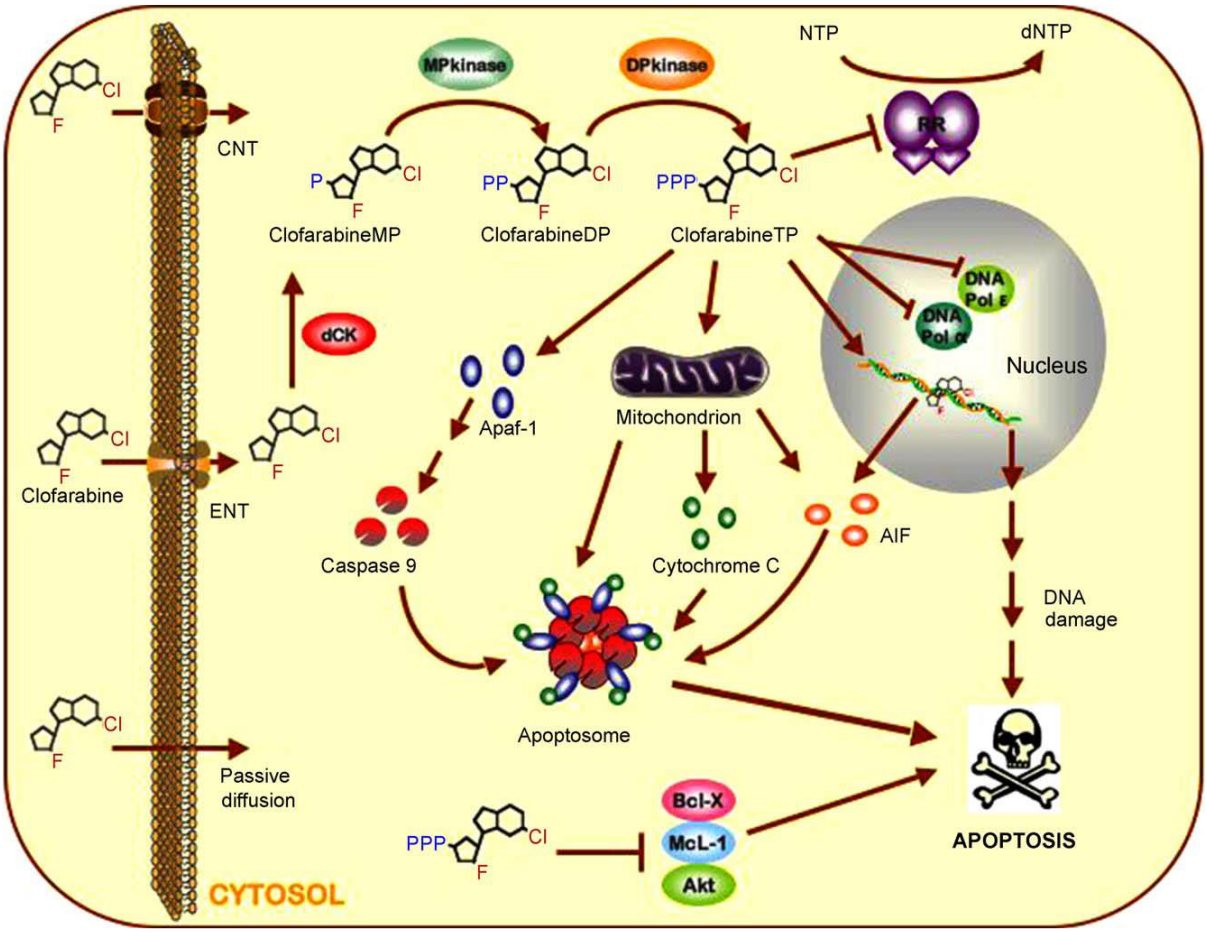

Figure 1.6: Mechanism of action for clofarabine. Clofarabine is transported into cells via concentrative and equilibrative transport (ENTs and CNTs). The intracellular clofarabine is phosphorylated into its monophosphate form by dCK and/or dGK and further, serially phosphorylated by other kinases to its active clofarabine triphosphate (Clofarabine-3P), adapted from Zhenchuck *et al.,* 2009.

1.4. Improving chemotherapy

T-ALL has shown a tendency for relapse and chemo-resistant to some therapeutic drugs (Vlierberghe and Fernando, 2012). Aggressive chemotherapy is required to treat T-ALL and this comes with considerable long-term side effects, such as neurotoxicity (Vlierberghe and Fernando, 2012).

dCK an intracellular kinase is crucial in the deoxyribonucleoside salvage pathway and which important in maintaining normal DNA metabolism, as mentioned earlier (Zhenchuk *et al*., 2009; Zhong *et al*., 2016). dGK kinase operates in the same manner as dCK, catalyzing phosphate transfer between ATP and 2'-deoxynucleoside (Jeha *et al.,* 2006; Sjoberg *et al.,* 1998). The dCK and dGK both have the ability to phosphorylate different types of nucleoside analogues to monophosphate forms subsequently their active forms (Ewal *et al*., 2008). This includes therapeutic nucleoside analogues such as fludarabine, nelarabine, cladribine, gemcitabine, deoxyadenosine and our drug of interest clofarabine and many others (Sjoberg *et al*., 1998; Ewald *et al*., 2008; Lonnetti *et al*., 2016). Accumulation of dCK and dGK can increase the amount of activated nucleoside analogous (Zhenchuck *et al.,* 2009). In addition, they can be used as biomarker for drug efficacy and therapeutic drug resistance (Zhenchuck *et al.,* 2009).

Clofarabine activation requires phosphorylation as previously described. Thus, adequate accumulation of dCK and dGK in leukaemia cells treated with clofarabine is required for drug activation. The accumulation of dCK and dGK increases their activity with increase in clofarabine activation and anticancer effectiveness. In

addition, downregulation of dCK/dGK expression can cause therapeutic resistance (Zhenchuck *et al.,* 2009).

Our laboratory has demonstrated that metformin treatment can enhance the expression of various enzymes involved in nucleotide metabolism including dCK in oesophageal squamous cell carcinoma (OSCC) cell lines (Mynhardt *et al.,* 2018). This could potentially increase accumulation of dCKand dGK in cancer cells and improve therapy.

1.5 Metformin

Metformin (N', N'-dimethylbiguanide) is a drug commonly used to treat type-2 diabetes, as well as other disorders where insulin resistance is observed (Gong *et al.,* 2012). Metformin was approved by the FDA in 1995 and is a relatively inexpensive drug in South Africa with a well-established track record (Whitburn *et al.,* 2017; Fortez *et al.,* 2014). In addition, metformin has been described to have low side effects with low-incidence of lactic acidosis (with 1 in 10 000 cases), which usually affects people with poor renal function (Lalau and Race, 1999). Metformin has several additional health benefits such as weight reduction, reducing plasma lipids levels, and prevention of some vascular complications (Defrenzo and Goodman, 2012). It is also used in other disorders such as in metabolic syndrome and in the treatment of polycystic syndrome (PCOS) and clinically as antiviral agent (Scarpello and Howlett, 2008; Rotella *et al.,* 2006).

1.5.1 Metformin in cancer

Metformin has been associated with some anti-cancer activities according to epidemiological studies conducted by Libby and colleagues (Libby *et al.,* 2009). In addition, Evans and colleagues, were the first to demonstrate that metformin can lower the risk of cancer (Evans *et* al., 2005). Metformin has been also demonstrated to significantly reduce the incidence of breast, liver, colon, pancreas and prostate cancer by 30%-50%, in diabetic patients taking metformin (Decensi *et al.,* 2010). Moreover, one study comparing diabetic patients treated with metformin with those treated with insulin and sulfonylureas for period of 5 years, demonstrated that those who were treated with metformin had a significantly lower rate of cancer-related death in comparison with those treated with insulin or sulfonylureas (Bowkwres *et al.,* 2006).

Metformin plays a role in the regulation of the 5' adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways, which are involved in the regulation of protein synthesis and cell proliferation (Kozka *et al.,* 1995). Mitogenic-responsive pathways (Ras/ERK, P13K/Akt) activates mTOR and it is also captivated by pathways that enhance intracellular energy and nutrients such as amino acids (Ben Sahra *et al.,* 2010). AMPK can disrupt the regulation of mTOR by inhibiting ATP synthesis and inducing inhibition of cell growth. This developed the interest in many laboratories to investigate the role of mTOR on cell proliferation and cancer incidence in patients treated with metformin (Ben Sahra *et al.,* 2010).

Metformin has been shown to reduce cell proliferation in Oesophageal squamous cell carcinoma in vitro and cells were shown to accumulate in Go/G1 phase of the cell cycle (Damelin *et al*., 2014). In addition, metformin has also been demonstrated to reduce chemotherapy-resistance leukaemia cell viability (Rodriguez-Lirio *et al.,* 2015). Moreover, an epidemiological study was conducted in 2529 women with breast cancer treated with neoadjuvant chemotherapy (Jiralerspong *et al*., 2014). From the study, 68 diabetic patients taking metformin showed 24% pathological complete response (pCR) rate compared to 87 diabetic patients not taking metformin with 8% pCR rate and 2374 non-diabetic patients with 16% pCR (Jiralerspong *et al*., 2014).

1.6 Problem statement

In leukaemia, T-ALL is regarded as a highly lethal and aggressive hematological malignancy (Marks and Rowntree, 2017). T-ALL contributes to morbidity in children and adults, with 25% of adult ALL and 15% of pediatric ALL cases (Eduardo *et al.,* 2017). Resistance to chemotherapy agents causes the disease to become more severe and there is a high risk of relapse, which is a significant clinical problem, thereby leading to poor prognosis (Bongiovanni *et al.,* 2017; Yadavetal *et al.,* 2016). The high doses of clofarabine can also lead to severe acute and long-term side effects (Gollard and Selco, 2013). Therefore, a novel combination therapy may allow the usage of smaller doses of clofarabine, and may aid in overcoming drug resistance and side effects.

2. Hypothesis

The chemotherapeutic agent, clofarabine depends on dCK and dGK for efficient activity. This study hypothesizes that metformin may enhance the sensitivity of T-ALL to clofarabine through upregulating dCK and dGK expression.

2.1 Aim

Investigate the effect of metformin in combination with therapeutic clofarabine on T-lymphoblastic leukaemia cell lines.

2.2 Objectives

(i) Investigate the cytotoxicity of metformin with clofarabine on cancer cells using XTT assay and microscopy.

(ii) Assess the expression level of enzymes involved in nucleotide metabolism using western blot.

(iii) Conduct cell death assessment using DNA laddering, and by western blotting for caspase 3.

3. Materials and methods

3.1 Reagents

General laboratory chemicals and reagents as well as the cell culture reagents were purchased from Sigma Aldrich (St Louis, Missouri, USA), unless otherwise specified. All plastic ware used for tissue culture and other experimentation were purchased from Sigma Aldrich (St Louis, Missouri, USA) and NEST (Wuxi, Jiansu, China).

3.2 Cell Culture

The human T-ALL CEM and AML THP1 cell lines were cultured in a RPM1 medium 1640 supplemented with 10% foetal bovine serum (FBS) (ThermoFisher Scientific, Illinois; USA) and 100 U/ml penicillin/0.1 mg/ml streptomycin. The cells were maintained in a humidified atmosphere at 37 °C with 5% carbon dioxide. These cell lines were a kind gift from Dr L.H Damelin (National Institute for Communicable Diseases) and Dr Vanessa Meyer (School of Molecular and Cell Biology, University of Witwatersrand, Johannesburg). Cells were routinely sub-cultured in a 30 ml flask once the cells had reached 70-90% confluence.

3.3 Cell Count

To ensure the number of cells was constant for each treatment during an experiment, a cell count was performed. Trypan-blue staining (0.2% in 1x PBS, filter sterilized) was used to count viable cells, which appeared clear, in comparison to dead cells that stain blue. This is based on the principle that viable

cells have intact cell membranes that are able to prevent the penetration of certain dyes such as trypan-blue, whereas dead cells have damaged permeable cell membranes allowing the dye to pass through (Sittampalam et al., 2016). The cell-trypan blue (1:1) mixture was injected onto a Neubaur haemocytometer and viewed under a light microscope at 10x magnification, whereby the average number of cells was counted. The number of cells required to be seeded onto the wells of the 96-well plates was calculated using the following formula: [Number of cells] / [(Average of cells counted) (2) (104 cells/ml)] x [volume of media required] = number of cells/ml

3.4 Cytotoxicity assay

The.2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was used to evaluate cell proliferation rate. Cells were seeded in 96 well plates at density of 500 00 cells/ml in complete cell culture medium, with 50 µl per well. After 24 hours 50 µl of different final metformin concentrations (0, 1.25, 2.5, 5 or 10 mM, respectively) was added in complete cell culture medium. The plates were incubated in a CO_2 incubator for 24, 48 and 72 hours respectively. After various times in culture medium, 50 µl XTT reagent mixture (10mM PMS solution in 4 ml XTT solution (4g dissolved in 4 ml medium) was added. The absorbance at 450 nm was determined after 2 hours of incubation using Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific, Illinois; USA). Four technical replicates were conducted and the cell proliferation rate was expressed as percentage in reference to control untreated cells (n=4±SD).

3.5 Cell imaging by microscope

For cell imaging, 500 000 cells/well were seeded into 12 well plates. For metformin alone treatment, cells were treated with different concentrations ranging from 0.625 mM to 10 Mm. CEM cells were treated for 72 hours and THP1 cells for 24 hours. The difference in incubation periods between two cell lines was influenced by results obtained from the XTT assays. For drug combination cells were co-treated with metformin and clofarabine and incubated for 24 hours. After treatment cell images were captured using the Floid® microscope (ThermoFisher Scientific, Illinois; USA) at 10 x magnification.

3.6 Western blot analysis of enzymes involved in cellular metabolic pathway

3.6.1 Drug treatment and cells collection

To determine if metformin affects the expression level of enzymes involved in nucleotide metabolism, we assessed the expression level of TS, TK-1, dCK and dGK in metformin treated T-ALL CEM cell lines using immunoblotting technique. Cells were seeded into tissues culture flasks and when they reach 70-80% confluency cells were treated with 1.25 mM and 5 mM metformin for 48 and 72 hours, respectively. After the drug treatment cells were harvested by centrifugation at 2000 xg for 5 minutes at 4^0C. The cells were resuspended in 1 ml 1x PBS and collected by centrifugation at 11000 xg for 10 minutes at 4 ^{0}C. The pellets were stored at -70 ^{0}C, until required.

3.6.2 Protein extraction and quantification

The frozen pellets were thawed on ice and resuspended in 50-100 µl of 1x radioimmunoprecipitation (1xRIPA) buffer with protease inhibitors (10 µg/ml phenylmethylsulfonyl fluoride (PMSF) and l µg/ml lupeptin) added just before use. Samples were placed on a rotator for 1 hour at 4 ^{0}C to ensure mixing and full cell lysis. After cell lysis, samples were vortexed followed by centrifugation at 12000 xg for 5 minutes at 4 ^{0}C to pellet cell debris. The supernatant was split into 0.2 ml tubes and stored at -70 ^{0}C.

Bramhall assay was used to quantify protein concentration (Bramhall, 1969). Whatman no. 1 filter paper was incubated in dH_2O for 20 minutes then incubated in 45 ml of 95% ethanol, 100% ethanol and 100% acetone for 5 minute intervals respectively, with gentle shaking. The filter paper was then dried under an extraction hood. The standard curve was determined using 1 µg/ml BSA stock in 1x RIPA buffer (1, 3, 6, 12, 16, and 20 µl, also 2 µl of each protein samples). The paper was dried under a fume hood for ~10 minutes then incubated for 45 minutes in 45 ml of 7.5% trichloroacetic acid (TCA) to precipitate and fix the proteins to the filter paper. The filter paper was rinsed in 5 ml of coomassie blue (3 mM coomassie brilliant blue G-250 prepared in 50% (v/v) methanol and 10% (v/v) acetic acid) dye with gently shaking for 5 minutes to remove excess TCA and 1x RIPA from the filter paper. Filter paper was stained in coomassie blue dye for 1 hour with moderate shaking and then destained for 1 hour with a destain solution (10% (v/v) acetic acid and 10% (v/v) methanol). The filter paper was dried under the extraction hood

and the protein circles were cut and placed into tubes with 5ml of elution buffer (12% (v/v) glacial acetic acid, 10% (v/v) methanol) and incubated in the dark for 2 hours with moderate shaking. The BSA standards and cell samples (160 µl of each) including the elution buffer as a blank was added, in triplicates, into wells of 96-well plate. The solution was mixed by shaking the plate for 30 seconds before reading at 595 nm using the Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific, Illinois; USA). The absorbance readings were exported into Microsoft Office Excel 2016 to plot the BSA standards against their respective concentrations. Thereafter, a linear trend line was fitted and unknown concentrations calculated using the equation of the standard curve.

3.6.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After protein quantification, equal amounts of protein (45 µg) was boiled (100 ^{0}C) in SDS 4x protein loading buffer (40% (v/v) glycerol; 240 mM Tris-Hcl ;8% (w/v) SDS; 0,04% (w/v) bromophenol blue; 5% (v/v) β-mercaptoethanol, Ph 6.8;) in 4:1 ratio for 5 minutes. Samples were electrophoresed at a constant voltage of 120 volts for an hour, on a 15% running and 5% stacking gel (Table 2.1), in running buffer (192 mM glycine; 25 mM Tris-Hcl; 3.5 mM SDS; Ph 8.8). The PageRuler Prestained protein ladder (ThermoFisher Scientific, Illinois; USA) was also loaded as the molecular weight marker. The region of the gel containing the protein of interest was cut and prepared for immunoblotting. The gel was stained in coomassie blue dye (3 mM coomassie blue; 50% (v/v) methanol; 10% (v/v) acetic

acid) for an hour and thereafter destained (10% (v/v) acetic acid and 10% (v/v) methanol). overnight, in order to visualise the quality of protein bands.

Table 2.1: Polyacrylamide gel recipe for SDS-PAGE

Reagents	**Resolving gel (10 ml)**	**Stacking gel (5 ml)**
	15%	**5%**
ddH2O	2.3 ml	3.4 ml
29:1 Acry/Bisacrylmide	5.0 ml	830 µl
Tris (1.5 M pH 8.8)	2.5 ml	-
Tris (1.0 M pH 6.8)	-	630 µl
10% SDS	100 µl	50 µl
10% APS	100 µl	50 µl
TEMED	4 µl	4 µl

3.6.4 Western blot analysis

After SDS-PAGE, protein was transferred onto nitrocellulose membrane in ice cold western blotting transfer buffer (25 mM glycine; 192 mM Tris; 20% methanol, pH 8.3) at a constant current of 300 mA for 2 hours in a cold room of 4 ^{0}C. After transfer, the gel was stained with coomassie and destained to check the quality of

protein transferred. The membrane was incubated in blocking buffer (5% BSA in 1x TBS/T) to prevent non-specific binding for an hour at room temperature, with gentle agitation at 15 rpm. Afterwards, the membrane was washed three times with cold 1x TBS/T at 5 minute intervals and then incubated in primary antibody in 1:1000 ratio unless specified. All primary antibodies used were purchased from Santa Cruz Biotechnology (Dallas; USA) unless otherwise specified. Mouse monoclonal anti-TS (sc-33679), rabbit polyclonal anti-TK-1: Cell Signalling Technology, #8960 (Danver; USA), mouse monoclonal anti-dCK (sc-393099), rabbit polyclonal anti-dGK Sigma Aldrich,#R34177 (St Louis, Missouri, USA), rabbit polyclonal anti-β-actin (sc-130656) in 1:2000 ratio and for caspase 3 activation analysis it was incubated with the rabbit polyclonal anti-caspase 3 (sc-56053) then diluted in 5% BSA overnight at 4 ^{0}C. Afterwards the blots were washed with 1X TBS/T five times at 5 minute intervals. The blots were incubated for an hour at room temperature in secondary antibodies (Santa Cruz Biotechnology, Dallas, USA) mouse IgG*K* BP – HRP: sc-516102) (for TS and dCk, 1: 2000) and mouse anti-rabbit IgG-HRP: sc-2357 (for TK-1, dGK, caspase 3 and β-actin in 1:2000 ratio) horse-radish peroxidase secondary labelled antibodies. Detection of antibody binding was detected using Pierce ECL Plus Western blotting detection reagent (ThermoFisher Scientific, Illinois; USA). Blue X-ray film was used to detect the bands, followed by rinsing in the developer solution (6.4 M metol; 0.6 M sodium sulphite; 80 mM hydroquinone; 45 mM sodium carbonate; 34 mM potassium bromide) and fixer solution (0.8 mM sodium thiosulfate; 0.2 mM sodium metasulfite). The images were captured with a scanning/copy machine system for

densitometry analysis using the Image Studio Lite version 5.2 software. The statistical significance was calculated using a paired students t-test, whereby the $P<0.05$ was considered significant, n=3±SD.

3.7. DNA fragmentation

3.7.1 Drug treatment and cell harvesting

Cell were seeded, as described above in 30 ml cell culture flasks for 48 hours then cells were treated with 1.25 mM and 5 mM metformin (met) for 48 and 72 hours, respectively. One plate was treated with 10 µM gemcitabine as a positive control for 24 hours. Cells were also treated with 1.25 µM and 5 µM clofarabine (clof) with or without metformin. After drug treatment, the cell culture media was centrifuged to harvest the cells at 4000 xg for 5 minutes at 4 ^{0}C and the supernatant discarded. The cells were washed by resuspending in 1 ml cold 1x PBS and collected by centrifugation at 11000 xg for 10 minutes at 4 ^{0}C. The pellets were stored at -70 ^{0}C.

3.7.2 DNA extraction with phenol extraction

Cell pellets were re-suspended in 50 µL 1x PBS buffer then 450 µL lysis buffer (100mM Tris-HCl, pH 8.0; 100 mM EDTA, 2% SDS, and 20µg/mL RNase A) was added and samples incubated at 37 ^{0}C for 1 hour. Proteinase K (100 µg/mL) was added and cells were incubated at 50 ^{0}C for a further 2 hours and vortexed every 20 minutes. Then 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added into each tube, and mixed well for 5 minutes. The mixture was centrifuged

at 13 000 xg for 10 minutes and then aqueous phase pipetted into new tubes. A tenth of the volume of 3 M sodium acetate was added into the aqueous phase. Thereafter, two volumes of 100% ice cold ethanol was added and mixed thoroughly, then stored in -20 ^{0}C overnight to precipitate the DNA. The following day the mixture was centrifuged at 13 000 xg for 30 minutes and the supernatant discarded. The DNA was washed with 70% ethanol, centrifuged at 13 000 xg for 10 minutes and supernatant discarded. The excess ethanol was removed by pipetting and air dried for 10 minutes. The pellet was dissolved in 100 µl Tris-HCl (pH 8.0) buffer and rotated for 1 hour to dissolve DNA. The DNA was quantified using a Nanodrop ND-100 V3.8.1 spectrophotometer (ThermoFisher Scientific, Illinois; USA).

3.7.3 Agarose gel electrophoresis

Equal amounts of DNA samples were electrophoresed on 2% (w/v) agarose gel in 1xTAE buffer with ethidium bromide (0,2 µg/ml) at 100 volts for 45 minutes against the molecular weight GeneRuler 1kb DNA ladder (ThermoFisher Scientific, Illinois; USA). Images were captured under ultra-violet (UV) light using the Bio-Rad Gel Doc System and Labworks 3.0 software.

3.8 Statistics analysis

Each experiment value was expressed as mean ± standard deviation. The statistical significance of the difference between the values of control and treated groups was determined using simple one-way ANOVA analysis of variance using GraphPad Prism 8.0.2 software (Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significance difference.

4. Results

4.1 Metformin reduces CEM and THP-1 human leukaemia cell line cell proliferation rate

Metformin has been shown to decrease cell proliferation of leukaemia cell lines (CEM, $10E_1$ –CEM cells which are CEM cells transfected with a vector containing a Bcl-2 cDNA and R5-CEM derived from parental CEM) that are resistance to most therapeutic drugs (Rodrigues *et al.*, 2015). In our study we employed the XTT assay to assess the effect of metformin on the cell proliferation rate of CEM and THP1 leukaemia cell lines. We exposed cells to different concentrations of metformin 0,625-10 mM range. Overall, metformin reduced cell proliferation as compared to untreated cells but to a different extent in the two cell lines in a dose-and- time dependent manner (Figure 4.1).

For the CEM cell line, at all concentrations of metformin at 24 hours there was no statistical significant difference except at 10 mM treatment which shows 34 ± 0.5% significant increase in cell proliferation (Figure 4.1). At 48 hours, there was no change at 0.625 mM and 1.25 mM metformin treatment, however, with 2.5 mM metformin there was a 54 ± 6% decrease, at 5 mM metformin a 75 ± 4% decrease and with 10 mM metformin exposure there was 82 ± 4% decrease in CEM cell proliferation rate. Furthermore, at 72 hours metformin treatment there was 27 ± 1% decrease in cell proliferation at 0.625 M, and 1.25 and 2.5 mM metformin treatment displayed 41% ± 1% and 51 ± 0.63% decrease in cell proliferation rate, respectively. 10 mM showed 82% ± 0.60% decrease in cell proliferation rate.

The THP1 cells were less sensitive to metformin treatment. The most effective metformin concentrations were 0.625 mM at 24 hours exposure and 10 mM at 72 hours exposure, displaying 45 ± 1% and 51 ± 4% decrease in cell proliferation, respectively (Figure 4.1). However, the THP1 cells showed no significant change in proliferation rate at 48 hours treatment in all concentrations (Figure 4.1).

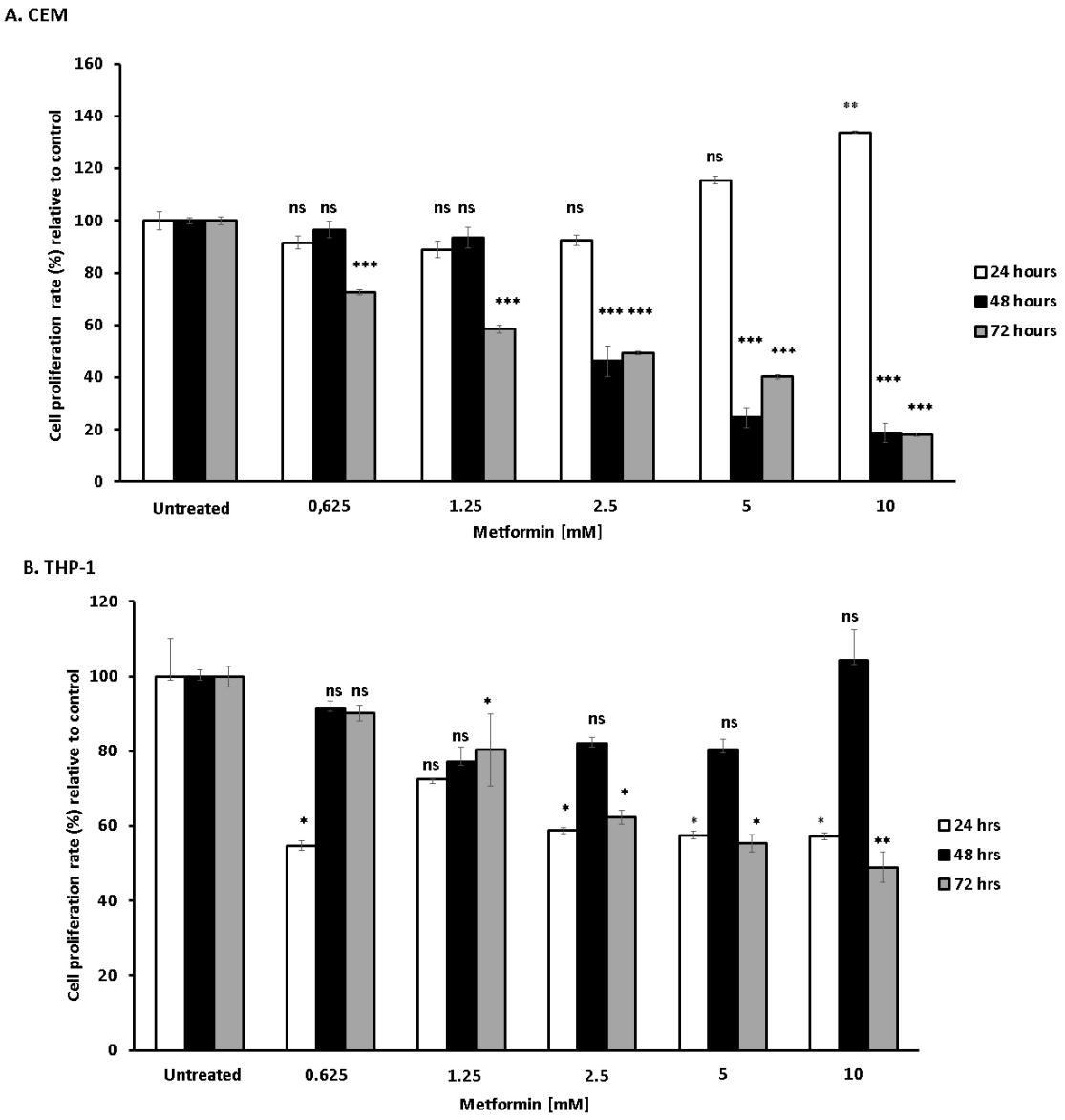

Figure 4.1: Metformin reduces human leukaemia cell proliferation rate in time and dosage dependent manner. The leukaemia cell lines were exposed to metformin for the times and at the concentrations indicated, and cell proliferation rate was assessed with the XTT assay. The values are shown relative to the untreated cells (n = 4 ± SD); ns (not significant), ***P<0.001, **P<0.01, * P<0.05.

To determine whether metformin impairs leukaemia cell proliferation rate or cell viability, 500 000 cells/well were seeded and Floid® microscope imaging was used for image capturing (Figure 4.2 and 4.3). Images of CEM cells treated with metformin alone for 72 hours showed an inhibition of cell proliferation at 0.625 mM and 1.25 mM treatment compared to untreated control. More interestingly, images of cells treated with 2.5mM, 5 mM and 10mM metformin concentrations clearly depict the reduction in cell number compared to untreated (Figure 4.2). It is possible that higher exposure of metformin may exert cytotoxic effects that could be exploited for cancer therapy.

In THP1 cell line, the microscope imaging showed that metformin inhibits cell proliferation after 24 hours of metformin treatment in all concentrations compared to untreated except for 1.25 mM and 5mM which are lot less than untreated (Figure 4.3). In addition, time point zero (T_0) and untreated cells showed that cells cluster but after treatment with metformin, cells detach from each other (Figure 4.2 and 4.3).

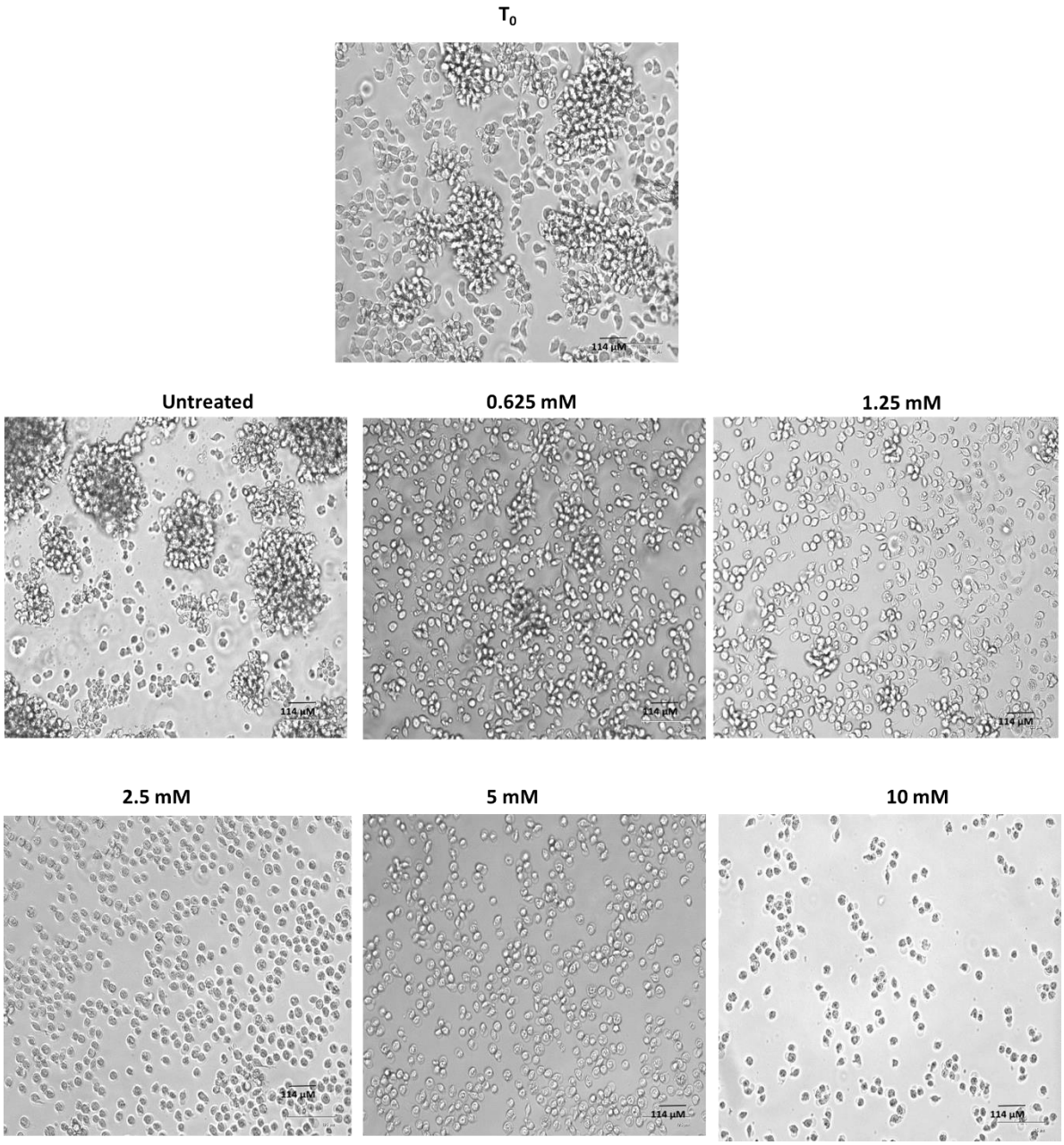

Figure 4.2: Metformin inhibits cell proliferation of CEM cell line. Images of CEM cells treated with metformin. Images were captured using Floid® microscope at 10 X magnification (scale bar = 114 µM).

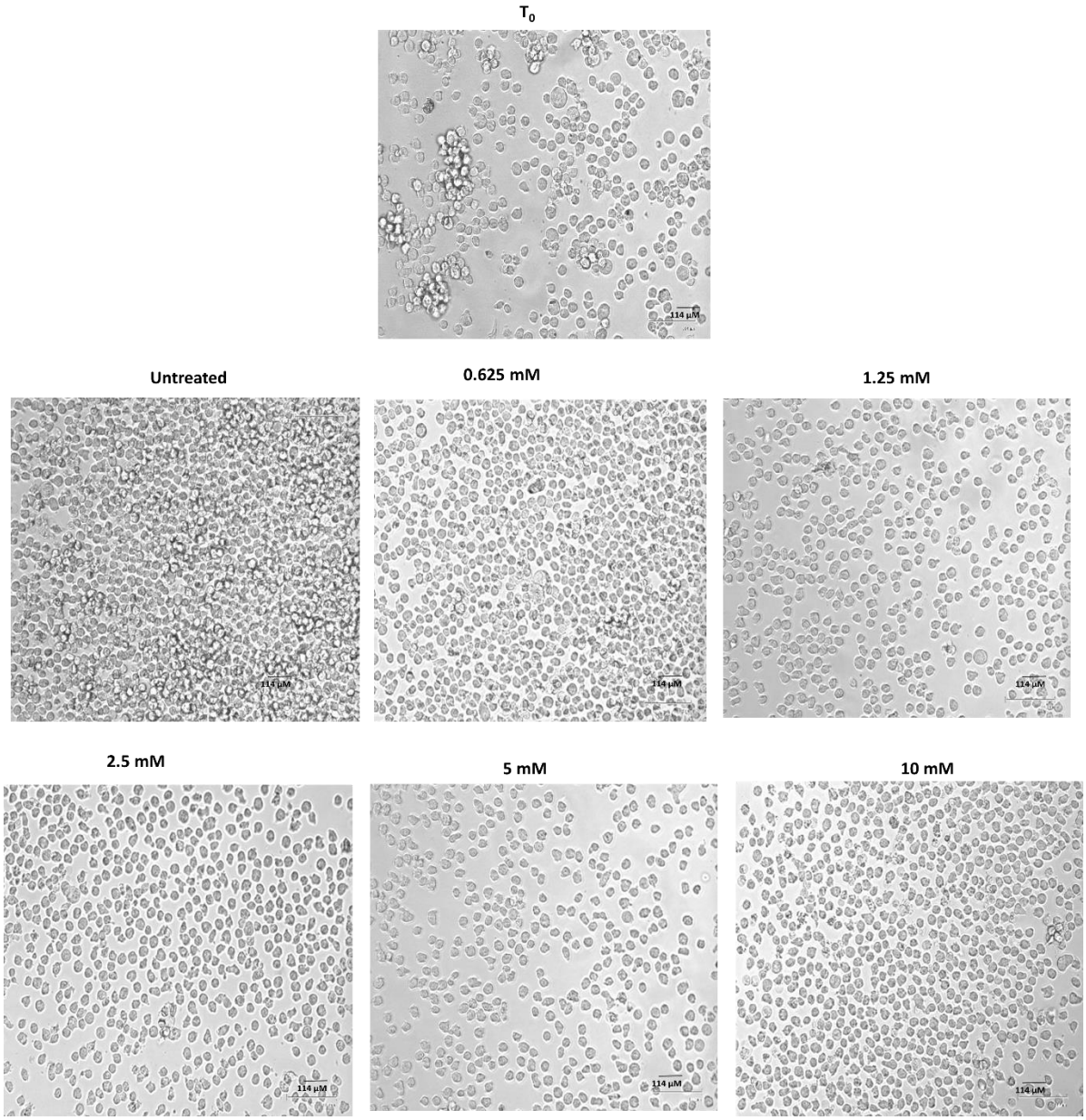

Figure 4.3: Metformin inhibits cell proliferation of THP-1 cell lines. THP1 cells treated with metformin. Images were captured using Floid® microscope at 10 X magnification (scale bar = 114 µM).

4.2 Clofarabine alone exerts cytotoxic effects on leukaemia cell lines

Clofarabine induces DNA damage in cancerous cells and therefore exerts cytotoxicity effects on CEM and THP1 leukaemia cells (Zhenchuck *et al.,* 2009). The cytotoxic effect of clofarabine on leukaemia cells was analysed using XTT assay. CEM and THP1 were treated with a range (0.078125-20 µM) of clofarabine concentrations for 24, 48 and 72 hours (Figure 4.4). From both cell lines, clofarabine was shown to induce cytotoxicity in a dosage and time dependent manner. In CEM cells, 20µM clofarabine treatment showed the most significant effect with 76% ± 1% reduction in cell viability at 72 hours exposure (Figure 4.4). In THP1 cells, the most significant clofarabine concentration was 5µM exhibiting 92% ± 2% cytotoxicity at 72 hours exposure (Figure 4.4).

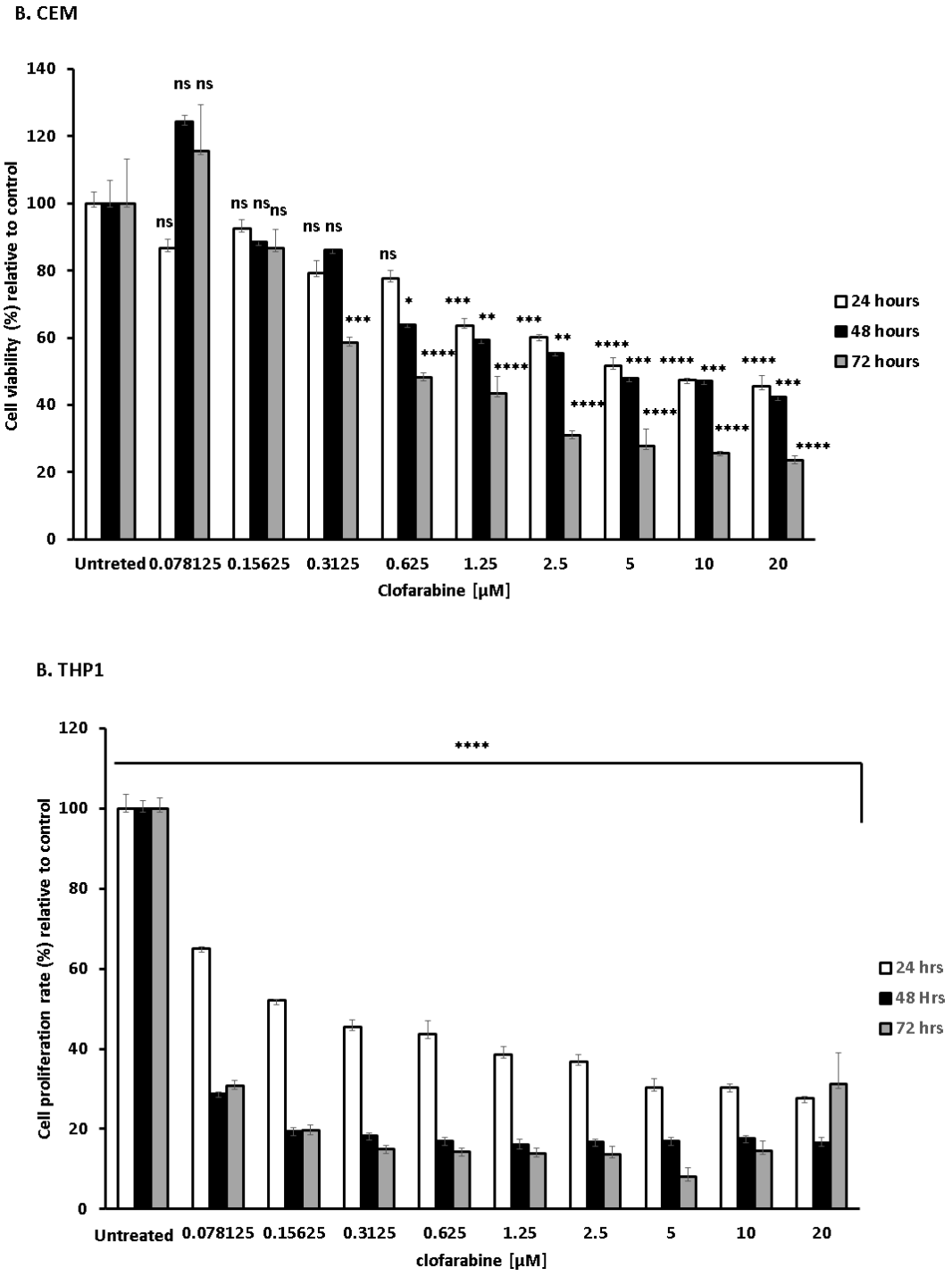

Figure 4.4: Assessing the effect of clofarabine on cell viability. CEM and THP1 cell lines were exposed to different clofarabine concentrations, at different time point as indicated. Clofarabine cytotoxicity was assessed with the XTT assay (n=4 ± SD): ns (not significant), ****P<0.0001, ***P<0.001, **P<0.01, * P<0.05.

4.3 Metformin enhances sensitivity in ALL CEM cells but not AML THP-1 cells

In the present study, leukaemia cell lines were co-treated with clofarabine and metformin to assess whether metformin modifies the effect of clofarabine on cell viability in comparison to clofarabine alone. A lower clofarabine concentrations which did not show maximal effect were chosen for drug combination with metformin, as determined by the assays in 4.1 and 4.2 above. Moreover, these concentrations had some effect on leukaemia cells and could show enhanced effect combined with metformin. The CEM cell line was co-treated with 0.325 or 1.25 μM clofarabine combined with 1.25 mM or 5 mM for 72 hours (Figure 4.5). Our results showed that 0.325 μM clofarabine and 1.25 mM 0r 5 mM metformin co-treated CEM cells decrease in cell viability by 66 ± 1% (Figure 4.5). There was 57 ± 1% and 59 ± 1% reduction in cell viability at 1.25 μM clofarabine and 1.25 mM or 5 mM metformin co-treated CEM cells, respectively (Figure 4.5).

The THP1 cell lines co-treated with metformin and 1.25 μM clofarabine for 24 hours had slightly increased cell proliferation rate compared to cells treated with clofarabine alone (Figure 4.5). The THP1 cells co-treated with 0.078 μM clofarabine and 1.25 or 5 mM metformin exhibited no significant difference in cell proliferation rate compared to cells treated with 0.078 μM clofarabine alone (Figure 4.5). However, THP1 cells co-treated with clofarabine and metformin showed resistance in comparison to clofarabine alone, with 1.25 μM clofarabine and 1.25 mM showing a 14 ± 3% increase in cell viability and 1.25 μM and 5 mM

metformin exhibiting a 20 ± 4% increase in cell viability, in comparison to clofarabine alone (Figure 4.5).

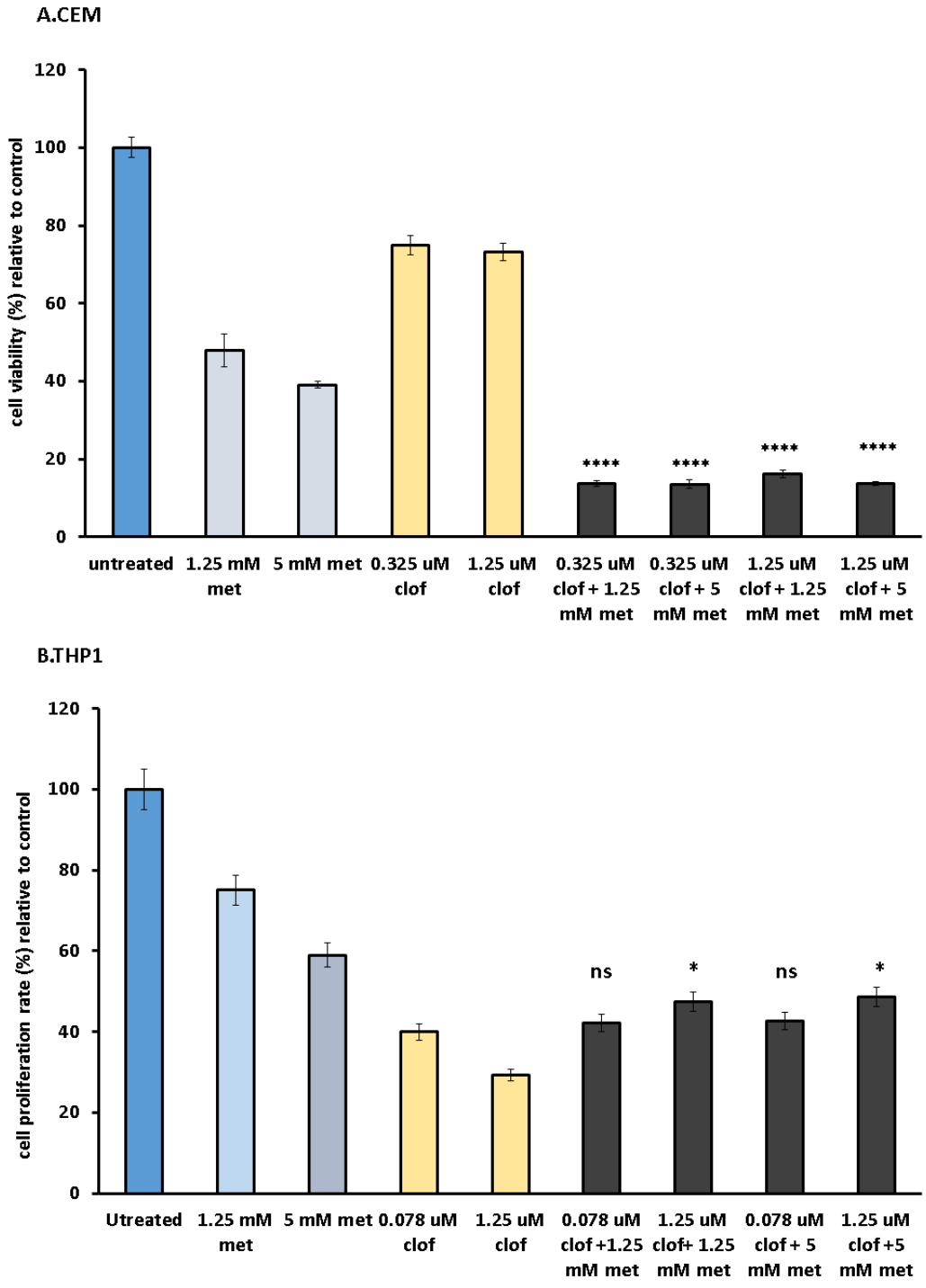

Figure 4.5: Metformin enhances sensitivity of human ALL CEM cells to clofarabine but slightly induces AML THP1 cells resistance to clofarabine. Cells were treated with metformin alone or in-combination with clofarabine. The CEM cells were treated for 72 hours and THP1 for 24 hours with drugs concentrations

indicated. Cell proliferation rate was then evaluated with XTT assay. The values are shown relative to cells treated with clofarabine alone (n = 4 ± SD): ns (not significant), ****P<0.0001, ***P<0.001, * P<0.05.

The decrease in metformin-induced clofarabine cell proliferation rate was also evident in the microscopy images of leukaemia cells co-treated with metformin and clofarabine (Figure 4.6 and 4.7). Metformin was shown to enhance sensitivity of CEM cells to clofarabine in dosage dependent manner, with the greater response at 1.25 μM clofarabine and 5mM metformin treatment in comparison with clofarabine alone. In THP1 cells, our results showed that cells co-treated with clofarabine and metformin for 24 hours exhibited no significant difference compared to cells treated with clofarabine alone (Figure 4.7). In addition, THP1 cells co-treated with 1.25 μM clofarabine and 1.25 or 5 mM metformin seem to be less than cells treated with clofarabine alone, and this contradicts the XTT assay results.

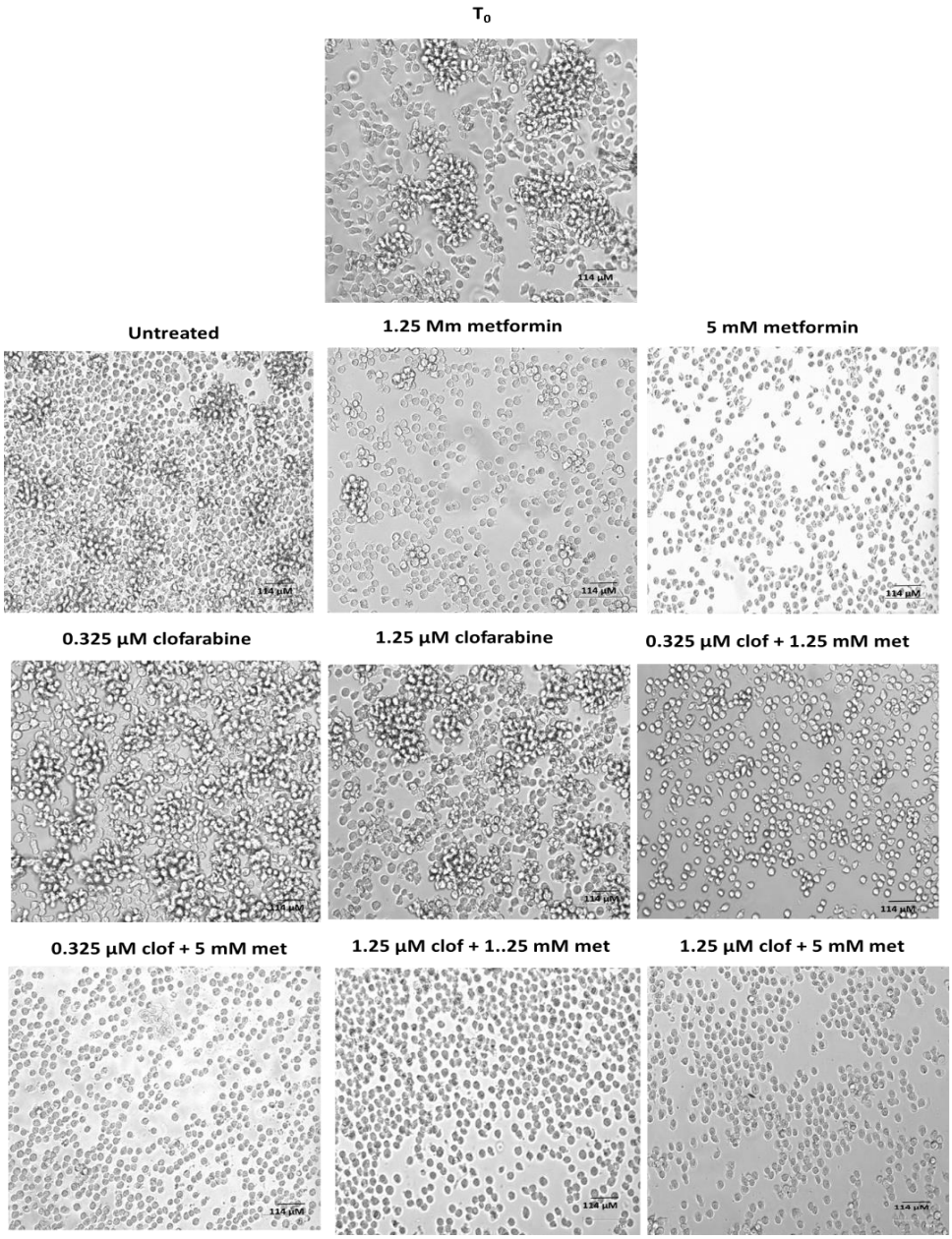

Figure 4.6: Metformin enhances sensitivity of CEM leukaemia cells to clofarabine. The CEM cells were treated for 72 hours with clofarabine alone or in combination with metformin. Images were captured using Floid® microscope at 10 X magnification (scale bar = 114 µM).

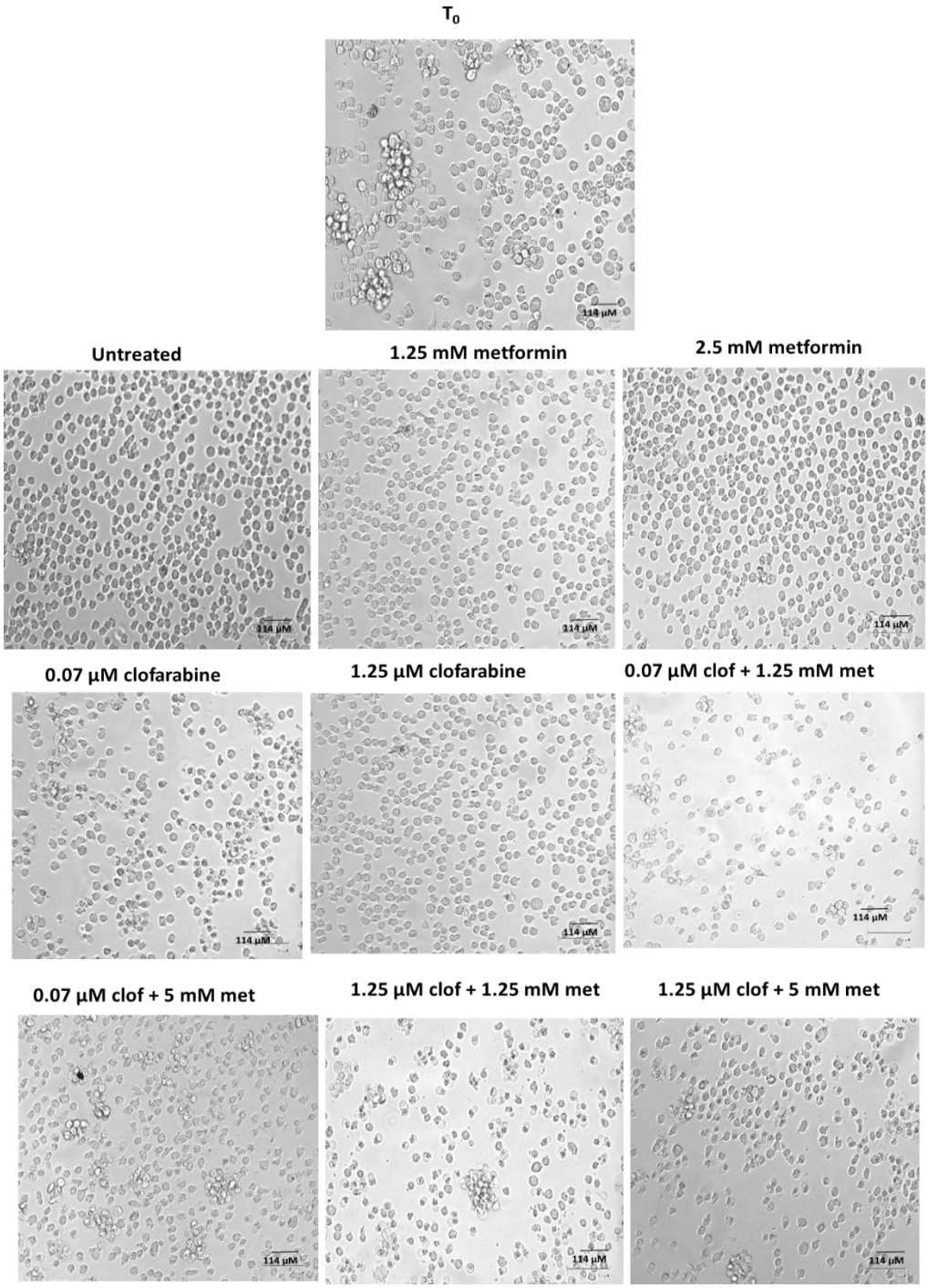

Figure 4.7: Metformin enhances sensitivity of THP1 leukaemia cells to clofarabine. THP1 cells were treated for 24 hours with clofarabine alone or in combination with metformin. Images were captured using Floid® microscope at 10 X magnification (scale bar = 114 µM).

4.4 Analysis of TS and TK-1 enzyme expression levels in metformin treated cell lines

TS is a critical enzyme for dTMP synthesis and essential in DNA replication/synthesis (Berger and Berger, 2006). The cytosolic TK-1 is a salvage enzyme that produces dTMP for DNA synthesis and repair (Petersen B, 2010). As for TS and TK1 Western blots, specific concentrations and time points which showed optimal effectiveness in both cell lines were selected from XTT assay results for Western blot analysis (Figure 4.1). In our study looking at TS expression following metformin treatment at 48 and 72 hours, Western blot results show that CEM cells treated with 1.25 mM metformin had no statistically significant difference in TS expression level compared to untreated CEM cells (Figure 4.8). For 5mM metformin there was a 27% (73 ± 5%) and a 44% (56 ± 45%) decrease after 48 and 72 hours, respectively. For TK-1 expression, in CEM cells treated with 1.25 mM metformin there was a 37% (137 ± 10%) increase at 48 hours with no statistically significant change at 72 hours metformin exposure. At 5mM metformin there was a 15% (85 ± 3%) and a 60% (40 ± 5%) decrease in TK-1 expression in CEM cells treated for 48 and 72 hours, respectively (Figure 4.8). In the THP1 cell lines treated with 2.5 mM metformin for 24 hours, there was no statistically significance in expression levels of TK-1 compared to untreated THP1 cells (Figure 4.8).

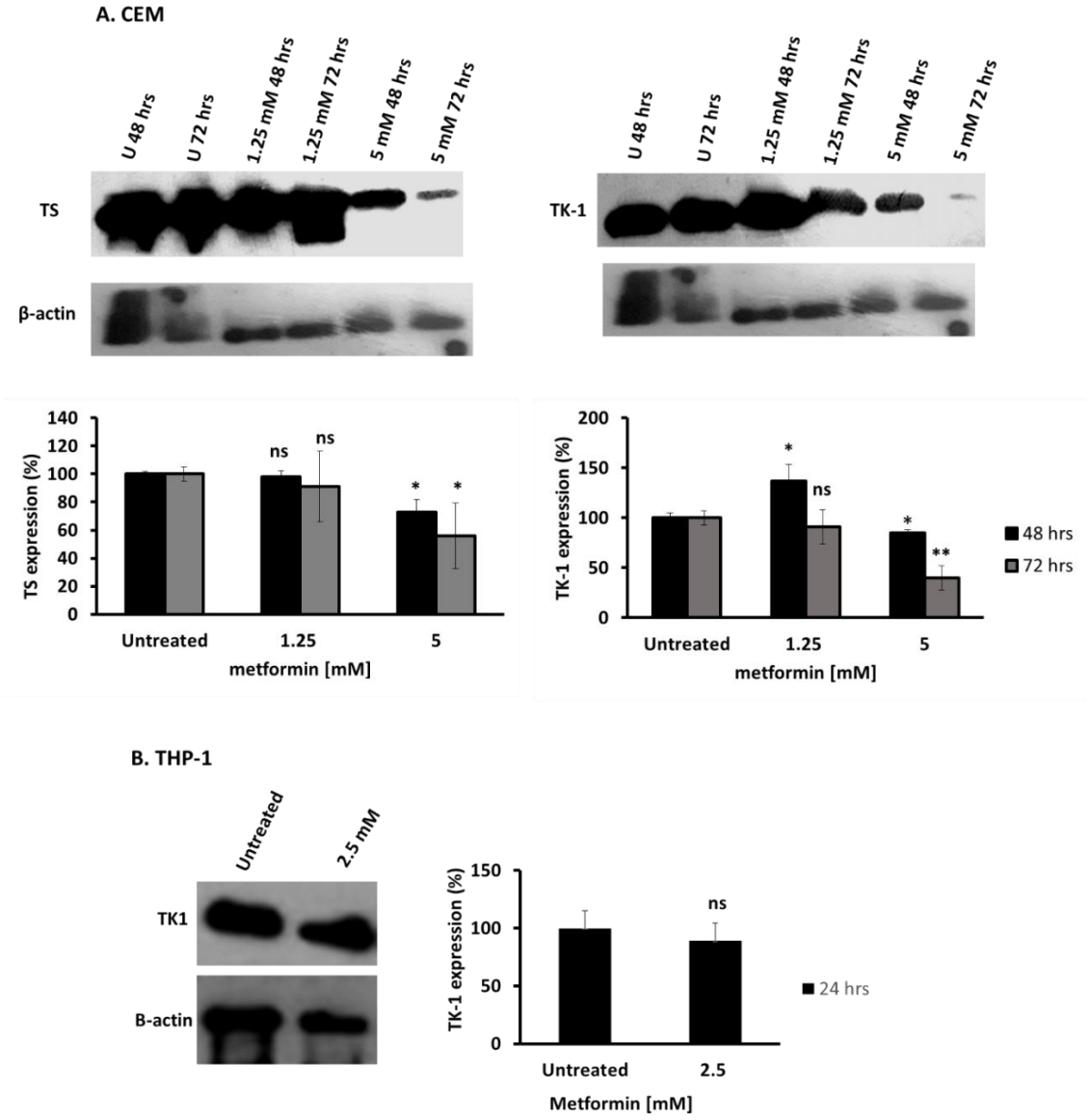

Figure 4.8: The effect of metformin on cell growth regulatory proteins. CEM and THP1cell lines were exposed to different metformin concentrations in different time points as indicated. Immunoblot shows a decrease in expression level of TK-1 and TS (n=3 ± SD): ns (not significance), * P<0.05.

4.5 Metformin alters expression level of dCK and dGK intracellular kinases

Upon cell entry inactive clofarabine gets phosphorylated by intracellular kinases into its monophosphate derivatives, primarily by dCK a cytosolic enzyme involved in the salvage pathway of DNA synthesis and also by dGK a mitochondrial enzyme (Jeha *et al.,* 2006; Sjoberg *et al.,* 1998). Specific dosage and time points which shows optimal effectives in both cell lines were selected from XTT assay results for Western blot analysis (Figure 4.1). We investigated the effect of metformin on expression levels of dCK and dGK in CEM and THP1 cell line using western blotting (Figure 4.9). In CEM cells, western blot results showed that with 1.25 mM metformin treatment there was no statistically significant changes in dGK expression level after 48 hours, however there was a 49% (59 ± 3%) decrease after 72 hours, when compared to untreated CEM cells. There was also no statistically significant difference in dGK expression level after 5 mM metformin treatment at 48 hours compared to untreated CEM cells. However, there was a 56% (44 ± 10%) decrease in dGK expression levels after 5 mM treatment at 72 hours exposure. For dCK expression level, in CEM cells treated with 1.25 mM metformin, there was no statistical significance in dCK expression levels at 48 and 72 hours exposure. There was a 31% (69 ± 5%) and a 69% (31 ± 1%) decrease in dCK expression after 5 mM metformin treatment at 48 and 72 hours, respectively (Figure 4.9). Western blot results in THP1 cells treated with 2.5 mM metformin showed a 22% (78 ± 5%) decrease at 24 hours exposure (Figure 4.9).

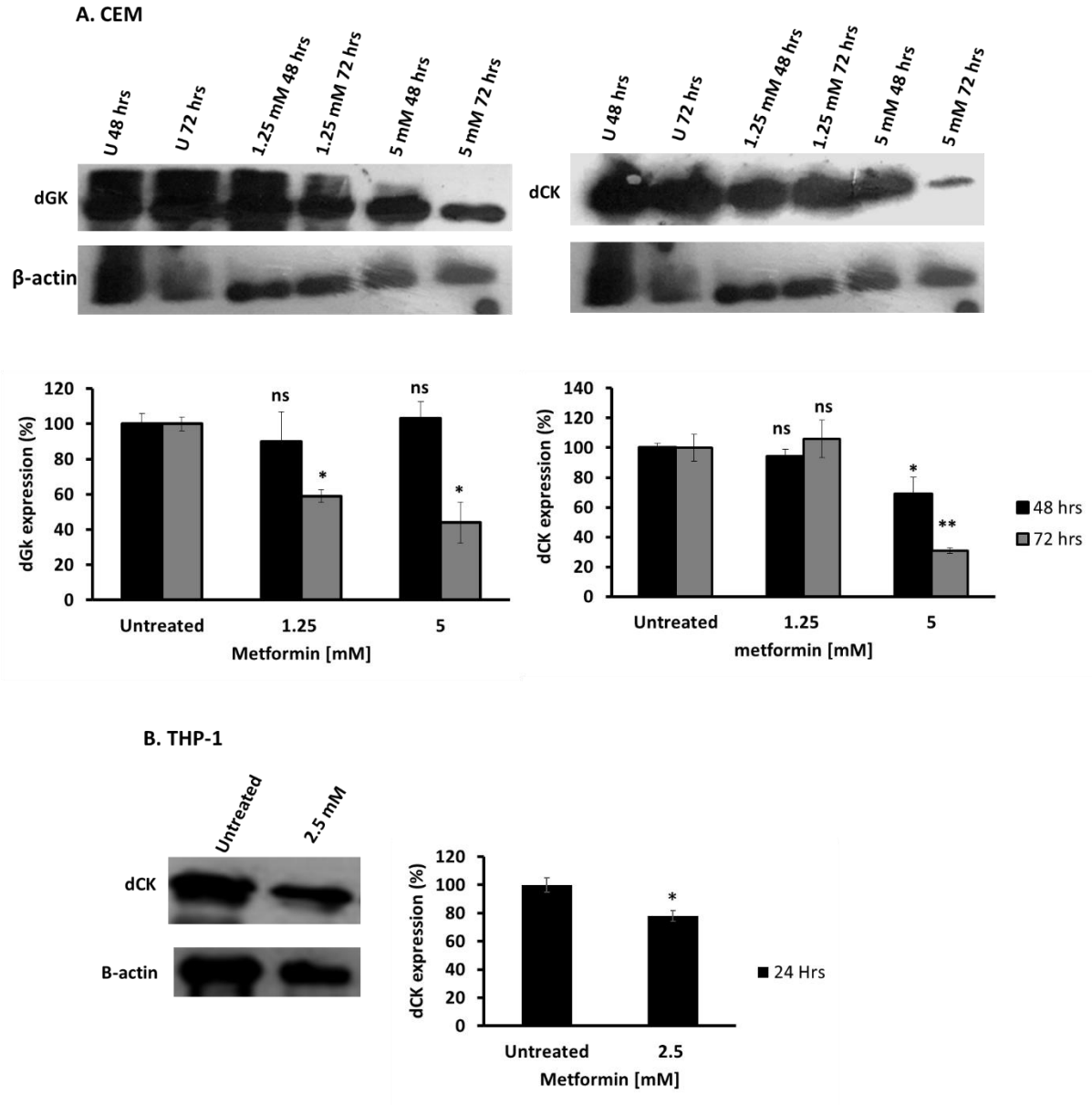

Figure 4.9: The effect of metformin on expression level of cell growth regulatory proteins. CEM and THP1 cell line were exposed to different metformin concentrations in different time points as indicated. Immunoblot shows changes in dCK and dGK expression level (n=3 ± SD). ns (not significant), $^{**}P<0.01$.

4.6 Metformin does not induce leukaemia cell death via caspase-3 activation

Apoptotic cell death was investigated in leukaemia cells treated with metformin, by assessing inactive and active caspase-3 using western blot analysis. To detect caspase-3 activation, a polyclonal antibody that recognizes both active (17-20 kDa) and inactive (35 kDa) caspase-3 was used. The results indicated that active caspase-3 was not detected in metformin treated CEM and THP1 cells (Figure 4.10). Moreover, we have previously shown that metformin does not induce cell death but rather inhibits proliferation. In addition, CEM cell line shows little inactive caspase-3 (35 kDa) with 5mM metformin at 72 hours treatment.

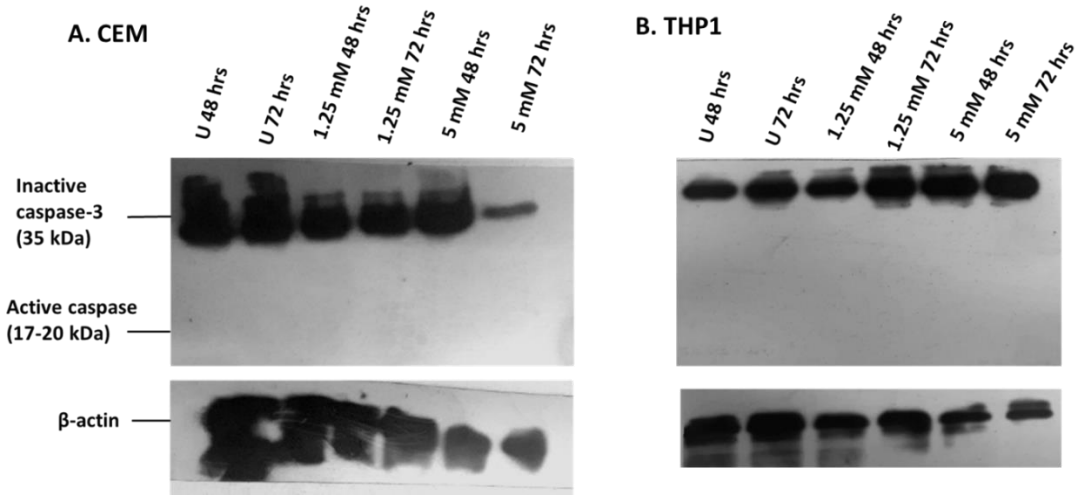

Figure 4.10: Analysis of caspase-3 activation by western blot. Apoptosis through caspase-3 cleavage was not evident in metformin treated (a) CEM and (b)THP1 cell lines. A single band at 35 kDa representing uncleaved (inactive) caspase-3 was prevalent in both cell lines. There was no cleaved/active (17-20 kDa) caspase-3 retrieved.

4.7 Metformin does not induce DNA fragmentation in leukaemia cell lines

A pivotal feature of apoptosis is the DNA fragmentation induced by caspase activated DNase (CAD), which occurs via, caspase cascade including caspase-3 (Wong R, 2011). The activation of CAD results in cleavage of the DNA forming DNA fragments of approximately 200 base pairs known as a DNA ladder. We investigated if metformin induces apoptosis using DNA laddering in CEM and THP1 treated with different concentrations of metformin in different time points as indicated (Figure 4.11). Our findings show that DNA laddering was not evident in both cell lines (Figure 4.11). In addition, gemcitabine did not produce DNA laddering .

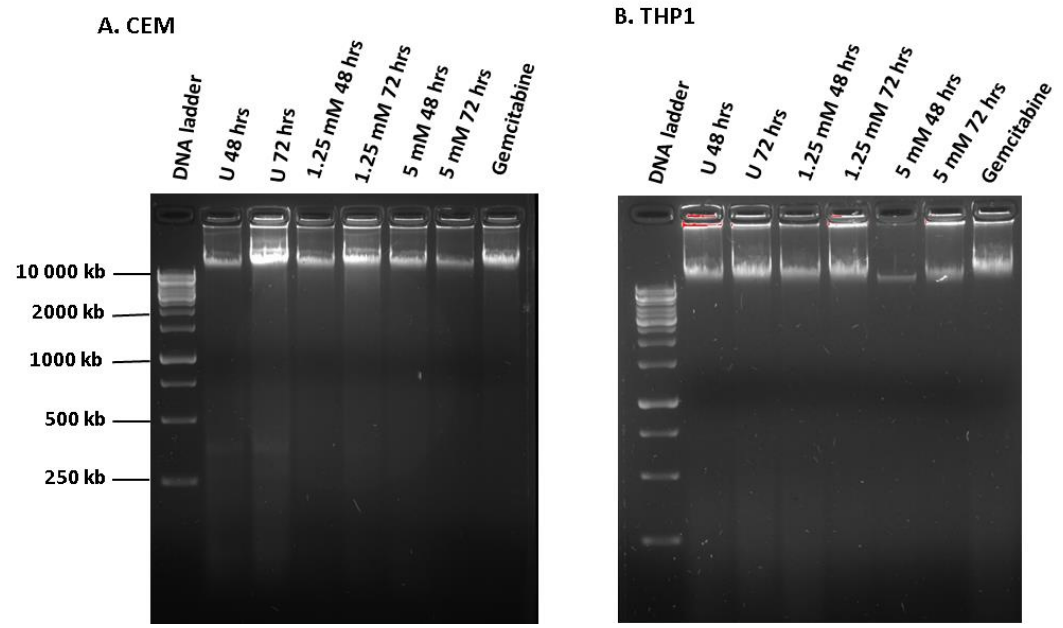

Figure 4.11: Metformin does not induce DNA fragmentation. The CEM and THP1 cell lines were treated with range of metformin concentrations, as indicated. The CEM cells were treated for 72 hours and THP1 for 24 hours. There was no pronounced DNA laddering induced by metformin in both cell lines.

We assessed if metformin modifies clofarabine – induced DNA damage in CEM and THP1 cells treated with metformin alone, clofarabine alone and metformin combined with clofarabine with concentrations indicated (Figure 4.12). DNA laddering was not observed in both CEM and THP1 cells treated with metformin alone or co-treated with clofarabine (Figure 4.12).

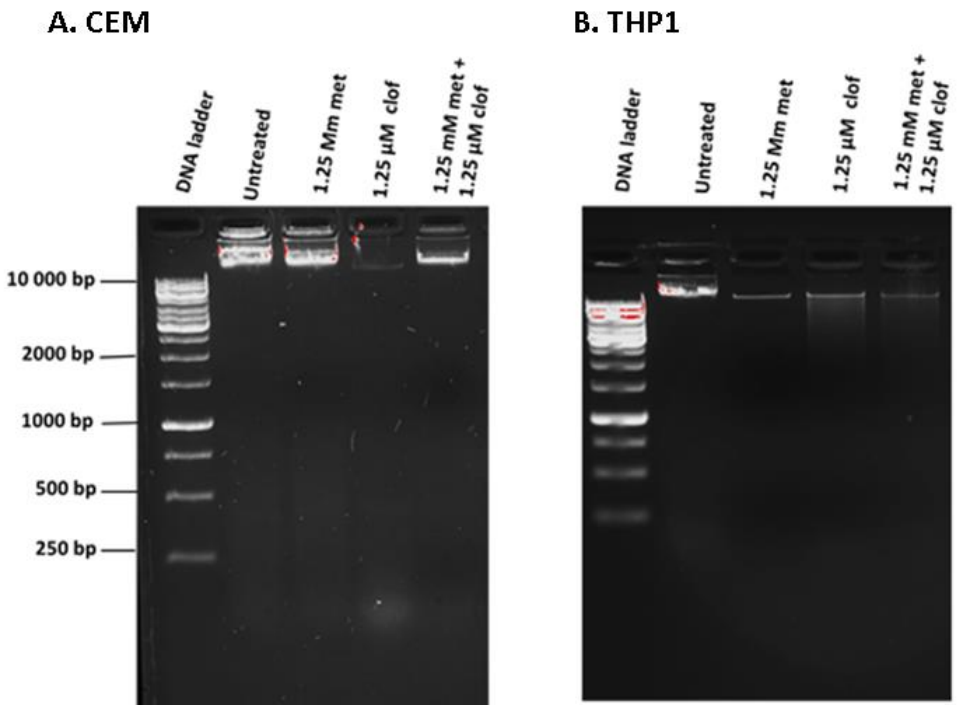

Figure 4.12: Metformin does not upregulate clofarabine-induced DNA fragmentation. CEM cells were treated for 72 hours and THP1 for 24 hours. There was no pronounced DNA laddering induced by metformin or clofarabine in both cell lines.

5. Discussion

Metformin is a commonly used drug to treat type 2 diabetes (Gong *et al*., 2012). Metformin is an inexpensive drug with a well-established track record (Gong *et al*., 2012; Fortez *et al*., 2014; Whitburn *et al*., 2017). Several studies have demonstrated that metformin poses antitumor properties and it can play a major role in cancer prevention (Ben Sahra *et al.,* 2010). In addition, metformin poses low toxicity and it does not affect the growth, differentiation or survival of normal cells (Green *et al*., 2012; Shi *et al*., 2012). Metformin has been demonstrated to arrest cell proliferation and induce accumulation in G0/G1 phase of the cell cycle in OSCC cell lines (Damelin *et al*., 2014). There was a breakthrough in investigating the effect of metformin in leukaemia cell proliferation rate, which showed that metformin decreases cell proliferation rate of chemotherapy resistant human ALL ($10E_1$ –CEM a Bcl-2 expressing cell line, R5-CEM a 4-HPR resistant cells both derived from native human leukaemia CEM cell lines and native CEM cell line) in time and dosage dependent manner (Rodriguez-Lirio *et al*., 2015).

Given that metformin exhibited anti-proliferative activity against OSCC cell lines and has the ability to reduce cell proliferation rate of leukaemia cell lines, we investigated if metformin can inhibit cell proliferation in human ALL CEM and the AML THP1 cell lines. In addition, metformin concentrations used in our study have previously shown a promising effect on inhibition of cell proliferation and cytotoxicity with minimal toxicity and do not affect haematopoietic precursors (Rodriguez-Lirio *et al*., 2015). Therefore, the clinically attainable concentrations

were used in this study. Our findings indicated that metformin significantly inhibits leukaemia cell proliferation in a time and dosage dependent manner.

To determine whether metformin impairs leukemia cell proliferation rate or cell viability we used Floid® microscope imaging. Our findings indicated that lower metformin concentrations decreased cell division. However, higher metformin concentrations exhibited a decrease in cell viability. It is possible that higher exposure of metformin may exert cytotoxic effects that could be exploited for cancer therapy.

From the previous studies in our lab, metformin was shown to induce sensitivity of OSCC to gemcitabine using MTT cytotoxicity assay (Mynhardt *et al*., 2018). Gemcitabine is a nucleotide analogue of deoxycytidine used to treat pancreatic cancer and can be administered to haematopoietic cancer patients (Ayman *et al.,* 2016; Matsumoto *et al*., 2018). With the solid evidence that metformin can be applied synergistically with nucleoside analogues such as gemcitabine, we investigated leukaemia cell lines co-treated with metformin and clofarabine using XTT assay. From the study metformin induces sensitivity of ALL CEM cells to clofarabine cytotoxicity, in comparison to clofarabine alone. To our knowledge, this is the only study that has shown metformin enhances clofarabine cytotoxicity to ALL CEM cell lines. However, metformin exhibits no significant effect to clofarabine effectiveness against AML THP1 cells in comparison to cells treated with clofarabine alone. Moreover, THP1 cells co-treated with higher dosage of clofarabine and metformin exhibited resistance to clofarabine effectiveness in

comparison to clofarabine alone. Clofarabine targets inhibition of RNR synthesis which induces supply of dNTPs in the cell for DNA replication and the inhibition of RNR subsequently reduces dNTPs concentration. However, metformin has shown to activate ataxia telangiectasia mutated (ATM) kinase that induces salvage dNTPs synthesis pathways to aid DNA repair process (Smal *et al*., 2006). It is possible that AML THP1 cells co-treated with metformin and clofarabine utilise salvage pathway synthesised dNTPs to mediate DNA repair to recover from stress exerted by clofarabine. In addition, our laboratory has shown that metformin can induce resistance of OSCC cells to some forms of chemotherapeutic drugs such as 5-fluorouracil, cisplatin and mitomycin (Damelin *et* al., 2014; Mynhardt et al., 2018). Furthermore, microscopy imaging showed that metformin enhances sensitivity of CEM cells to clofarabine and this supports the XTT results. However, in THP1 metformin showed to induce sensitivity of THP1 cells to clofarabine which contradicts with the XTT assay. This might be due to metformin enhancing cell accumulation at the G1/G0 stage of the cell cycle promoting inhibition of cell proliferation.

In order to determine the mechanism behind this modification of clofarabine cytotoxicity by metformin, we looked at the expression level of nucleotide metabolism enzymes. The TS and TK-1 enzymes are responsible for dTMP synthesis required for DNA synthesis and repair (Hu and Chang, 2007). TS mediate de novo synthesis of dTMP and TK-1 mediate salvage synthesis of dTMP (Cresce *et al*., 2011). dTMP is highly accumulated in tumour environment (Cresce *et al*., 2011). In addition, proliferating cells including tumour cells have higher expression

level of TS and TK-1 than non-proliferating cells (Derenzini *et al*., 2002). Thus, both enzymes are indicated to play a great role in enhancing chemotherapeutic nucleoside analogues resistance in cancer cells, by targeting DNA proliferation and termination of DNA synthesis and repair.

Our findings indicated that in CEM cells, higher metformin concentrations enhance downregulation of TS and TK-1 in a time and dosage dependent manner, compared to untreated cells. Non-proliferating cells exhibit low level of TS and TK-1 compared to highly proliferating cells and this support the evident that metformin inhibits leukaemia cells proliferation presented by XTT assay and cell imaging. In addition, downregulation of TK-1 and TS enzymes reduces mediation of dTTP synthesis required for DNA synthesis and repair thus reducing chances of clofarabine resistance. There was no statistically significant difference in expression level of TK-1 in THP1 cell lines treated with metformin compared to untreated cells.

The dCK and dGK are kinases responsible for nucleoside analogues phosphorylation prior to cell entry. In leukaemia treated with clofarabine, dCK and dGK phosphorylates clofarabine into its monophosphate derivative. Clofarabine monophosphate is further phosphorylated downstream by other kinases to its active form clofarabine triphosphate. Deficiency of dCK and dGK is highly associated with the cause of clofarabine resistance in leukaemia cancer (Lotfi *et al*., 1999; Mansson *et al*., 2003). Previously, our findings showed metformin enhanced dCK expression level in OSCC cell lines; an increase in dCK

level significantly increased gemcitabine nucleoside analogue sensitivity in OSCC cell lines (Mynhardt et al., 2018). Thus we investigated if metformin can regulate expression of dCK and dGK kinases in leukaemia cell lines.

In CEM cells we found that, metformin highly reduced expression level of both enzymes at 72 hours in 5 mM treatment compared to untreated cells, and also THP1 cells shows a decrease in dCK expression level in 2.5 mM metformin treated cells compared to untreated. A decrease in dCK and dGK expression level can enhance cancer resistance to clofarabine (Zhenchuk *et al.,* 2009). dCK and/or dGK deficiency in HL-60 and CCRF-CEM leukaemia cells has similarly shown resistance to other chemotherapeutic agents such as fludarabine, difluorodeoxcytidine and Ara C (Lotfi *et al*., 1999; Mansson *et al*., 2003). On the other, increased expression of dCK and dGK could possibly improve conversion of clofarabine to its active form. In our study however metformin-clofarabine co-treated CEM cells were more sensitive, whilst metformin-clofarabine co-treated THP1 were less sensitive, in comparison to clofarabine alone. This may imply that modifications in nucleoside metabolism are not responsible for the observed changes in cytotoxicity of clofarabine in the presence of metformin.

Apoptosis is a potent pathway that controls cell number in normal tissues and eliminates cells that can harm human survival (Makin and Hickman, 2002). Dysregulation of this process can lead to tumour progression (tumorigenesis) (Makin and Hickman, 2002). Apoptosis regulation requires series of proteins (Hall, 2010). Caspase-3 is one of the major caspase/protein responsible for apoptosis

progression (Dewson *et al*., 2010). The protein is regarded as a major executioner caspase and it can be regulated through extrinsic apoptotic pathway and/or intrinsic apoptotic pathway (Milner *et al*., 2002). Activated caspase-3 act by degrading multiple cellular proteins and it is also responsible for change in cell morphology (Milner *et al*., 2002). Moreover, caspase-3 also induces DNA fragmentation in cells undergoing apoptosis (Milner *et al*., 2002). We investigated if metformin induces cell death through apoptosis using capase-3 analysis by western blot technique. From our findings, apoptosis through caspase-3 activation in metformin treated CEM and THP1 cells was not evident. Suggesting that apoptosis is not the primary mechanism of leukaemia cell death in the metformin treatment. Moreover, previous findings from our lab demonstrated that metformin does not induce OSCC cell death through caspase 3 cleavage (Jivan *et al*., 2017). In addition, CEM cell line shows little inactive caspase-3 (35 kDa) with 5mM metformin at 72 hours treatment, this might be due to induction of cell death through other non-caspase-3 dependant mechanisms, which may have resulted in low cell numbers.

Furthermore, we investigated if metformin induces apoptosis via DNA fragmentation. We found no evidence of DNA fragmentation with metformin treated CEM and THP1 cells. Surprisingly, DNA fragmentation is not evident in gemcitabine positive control, this might be because gemcitabine stock used was too old, thus we used untreated cells as our technical control to construct a meaningful conclusion. We further investigated if metformin induces clofarabine-induced DNA fragmentation. There was no evident of DNA laddering from both

cell lines. This also supports the notion that metformin does not induce apoptosis but inhibits cell proliferation. Furthermore, this is in line with the evidence presented by Rodriguez-Lirio *et al,* (2015) that metformin does not induce apoptosis in CEM cell lines even in higher dosage 10 mM at 72 hours exposure (Rodriguez-Lirio *et al*., 2015). Metformin also showed to decrease CEM cell lines proliferation rate (Rodriguez-Lirio *et al*., 2015). In addition, from Rodriguez-Lirio *et al* (2015) study metformin showed to exert energetic stress in ALL cells by causing imbalance between PKCε and PKCδ kinases responsible for energy homeostasis and that leads to cell death to cells that can't cope with energy deficiency. Moreover, the study conducted by Renner *et al,* (2018) showed that even at 10 mM metformin for 48 hours, metformin exhibits no effect on cell proliferation nor apoptosis on THP1 cell lines.

In conclusion, our results demonstrate that metformin inhibits cell proliferation in both human leukaemia CEM and THP1 cell lines. Higher dose of metformin decreases expression level of TK-1, TS dCK and dGK, which is in line with the anti-proliferative activity of metformin. Interestingly, the combination of metformin and clofarabine was more cytotoxic than clofarabine alone in CEM but not THP1, where in the latter we observed a small increase in possible resistance. These findings should be further established using additional cell lines and in vivo as the impacts of metformin on clofarabine treatment could be very important in these two subtypes in that it could improve clofarabine in ALL but causes resistance to clofarabine in AML.

6. References

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