# An *in vitro* investigation into the anti-cancer potential of novel tetraoxane compounds for the treatment of chronic lymphocytic leukaemia

By

**Alma O'Reilly B.Sc.**

Supervised by:

**Dr. Mary Garvey**

**Dr. James Murphy**

Dept. Life Science, IT Sligo

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**Title:** An *in vitro* investigation into the anti-cancer potential of novel tetraoxane compounds for the treatment of chronic lymphocytic leukaemia

**Name:** Alma O'Reilly

**Student Number:** s00115662

**Supervisors:** Dr. Mary Garvey

Dr. James Murphy

**Declaration:** "I hereby declare that this project is entirely my own work and that it has not been submitted for any other academic award, or part thereof, at this or any other education establishment".

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Alma O'Reilly

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# Abbreviations

ALL - Acute Lymphocytic Leukaemia

AML - Acute Myeloid Leukaemia

ART - Artemisinin

ASH - American Society of Haematology

ATM - Ataxia telangiectasia mutated gene

ATP - Adenosine triphosphate

B - Bendamustine

BCR - B-cell receptor

BR - Bendamustine & Rituximab

BTK - Bruton tyrosine kinase

CIRS - Cumulative Illness Rating Scale

CLB - Chlorambucil

CLL- Chronic Lymphocytic Leukaemia

CLL-IPI - International prognostic index for patients with CLL

CML - Chronic Myeloid Leukaemia

CrCl - Creatinine clearance

Del17p - Deletion of chromosome 17p

Del13q14 - Deletion of chromosome 13q

Del11q23 - Deletion of 11p

DHA - Dihydroartemisinin

DMSO - Dimethyl sulphoxide

EBV - Epstein-Barr virus

ECOG - Eastern Cooperative Oncology Group

FC - Fludarabine & Cyclophosphamide

FCR - Fludarabine, Cyclophosphamide, & Rituximab

FDA - Food and Drug Administration

FISH - Interphase fluorescence in situ hybridisation

G - Obinutuzumab

GSH - Glutathione

GSSG - Oxidised Glutathione

HPLC – High-Performance Liquid Chromatography

$H_2O_2$ – Hydrogen Peroxide

Ibr – Ibrutinib

$IC_{10}$ - Concentration inducing 10% inhibition

$IC_{50}$ - Concentration inducing 50% inhibition

IGHV - Immunoglobulin heavy chain variable regions

IV - Intravenous

KIs - Kinase inhibitors

LCL - Lymphoblastoid cell line

LDH - Lactate dehydrogenase

Mono - Monotherapy

MTT - Thiazolyl blue tetrazolium bromide

MHC-1 - Major histocompatibility complex class 1

NCI - National Cancer Institute

O - Ofatumumab

ORR - Overall response rates

OS - Overall survival

PBMCs - Peripheral blood mononuclear cells

PFS - Progression free survival

Pl3Ks -Phosphatidylinositol 3 kinases

p53 - Tumour suppressor protein

R - Rituximab

RHH - Rituximab and hyaluronidase human

$RO^{\bullet}$ - Alkoxyl radicals

ROS - Reactive oxygen species

R/R - Relapsed/refracted

RT - Richter's transformation

t-AML/MDS - Therapy-related myeloid leukaemia/ myelodysplastic syndrome

TP53 - tumour protein 53

V - Venetoclax

ZAP70 - tyrosine kinase zeta-associated protein 70

$\beta_2$M - $\beta_2$-microglobulin

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# Abstract

This study aims to evaluate the therapeutic potential of five novel tetraoxane compounds and one novel tetraoxane-naphthalimide hybrid compound for chronic lymphocytic leukaemia. These novel compounds are predicted to deliver targeted toxicity to cancer cells with reduced activity on non-cancer, healthy tissue. This selectivity is expected due to a number of properties associated with tetraoxane compounds, including the affinity of the cleavage of the endoperoxide bridge by the elevated levels of iron associated with cancer cells, and the expected production of reactive oxygen species (ROS) by cancer cells on exposure to the cytotoxic compounds. The study focuses on Chronic Lymphocytic Leukaemia, as it the most prevalent type of leukaemia in adults in the western world. However, these compounds have the potential to be equally as effective against other cancer types.

The six test compounds, along with two reference compounds, Chlorambucil and Dihydroartemisinin, were evaluated on two leukaemia cell lines, HL-60 and MEC-1, and a non-cancer peripheral blood cell line, BL-2052. The anti-proliferation potential of the compounds on the leukaemia cell lines was determined and the selectivity of each compound toward the cancer cell lines over the non-cancerous BL-2052 cell line was established. Initial screening involved determination of compound $IC_{50}$ values, cell membrane permeability, and analysis of mechanism of cell death, via flow cytometric analysis.

The results show promising anti-cancer properties of all six compounds, with $IC_{50}$ values in the low micromolar range. A comparative study of the selectivity of the compounds for leukaemic cells shows compound selectivity, with all six novel tetraoxanes less

potent against the non-cancer cell line than HL-60s. The novel compounds showed higher activity towards both leukaemic cell lines analysed in comparison to the currently utilised chemotherapeutic drug Chlorambucil. These findings show potential for these novel compounds in the endless search to provide more effective treatment options for patients while reducing adverse effects. However, further investigation of these tetraoxane compounds is required to determine their full potential.

# Dissemination List

## Publications

- Chronic Lymphocytic Leukemia: A Review of Front-line Treatment Options, With a Focus on Elderly CLL Patients (2018) **O'Reilly A**, Murphy J, Rawe S, Garvey M. *Clin Lymphoma, Myeloma Leuk*. 2018:1-8. doi:10.1016/j.clml.2018.02.003.
- An In vitro evaluation of Novel Tetraoxane Compounds against acute promyelocytic leukaemia and chronic lymphocytic leukaemia (2018) **O'Reilly A.**, Murphy J., Rawe S., Garvey M. (submitted to Leukemia Research Journal, August 2018)

## Conferences

- Poster presentation at the Blood Cancer Network Ireland "Advances in translational and early clinical research in blood cancers" symposium (2017), held at Trinity Translational Medicine Institute (TTMI), St James's Hospital, Dublin. 'The Leukaemia Therapeutic Potential of Novel Hybrid Compounds and the Potential for Personalised Treatment'. **O'Reilly, A.**, Dr. Hodgson, A., Rawe, S., Murphy, J., and Garvey, M.
- Poster presentation and elevator pitch at the Annual IT Sligo Postgraduate Research Conference (2017), held at Institute of Technology Sligo. 'Determining the Leukaemia therapeutic potential of a number of novel hybrid compounds and the potential for personalised treatment evaluation'. **O'Reilly, A.**, Dr. Hodgson, A., Rawe, S., Garvey, M., and Murphy, J.
- Oral presentation at the Biomedical Postgraduate Research Conference (2017), held at Institute of Technology Sligo. 'An Investigation into the Anti-Cancer Properties of Modified Chemotherapeutics on Leukaemia Cells'. **O'Reilly, A.,** Dr. Hodgson, A., Rawe, S., Murphy, J., and Garvey, M.
- Oral presentation at the Annual IT Sligo Postgraduate Research Conference (2018), held at Institute of Technology Sligo. 'Determining the Leukaemia Therapeutic Potential of Novel Tetraoxane Compounds'. **O'Reilly, A.** Dr. Hodgson, A., Rawe, S., Garvey, M., and Murphy, J.

# 1. Introduction

## 1.1. Cancer

In Ireland, one person is diagnosed with cancer every three minutes, resulting in at least one mortality every hour from the disease (Marie Keating Foundation 2017). With the incidence of cancer on the rise, it is predicted that by the year 2020, 1 in 2 people will be diagnosed with cancer in their lifetime. Cancer is the second biggest killer in Ireland, accounting for approximately 30% of deaths each year, second only to heart disease, which accounts for 31% of all deaths nationwide (Irish Cancer Society 2017b). In 2014, Ireland had the third highest mortality rate for cancer-related deaths out of all EU member states (European Commission 2017).

Cancer can originate almost anywhere in the human body and occurs as a result of accumulated genetic mutations (Ponder 1992). While a cell can possess hundreds or even thousands of mutations, typically only between two to eight are the driving forces behind the progression of cancer (Vogelstein et al. 2013). These genetic alterations may be point mutations such as G12V Ras, deletions, inversions amplifications or large-scale rearrangements, as seen with the BCR-ABL fusions involving chromosome 9 and 22 (Sever & Brugge 2015). Such mutations allow for over-proliferation of cells, along with escaping mechanisms essential for homeostasis. The progression of cancer is a complex collaboration between the tumour cells and their microenvironment, with cancer cells developing a number of well-defined characteristics needed for survival and invasion (Sever & Brugge 2015).

There are six cancer hallmarks identified for the development of cancer cells which help rationalise the complex process of tumour development. These hallmarks, detailed by

Hanahan and Weinberg (2011), comprise the six biological capabilities accomplished during the multistep development of malignant tumours (Hanahan & Weinberg 2011). These hallmarks are sustaining growth signalling, evading growth suppressors, resisting apoptotic cell death, enabling replicative immortality, inducing angiogenesis and activating tissue invasion and metastasis (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011). Since being comprised, two new hallmarks have been included as enabling processes, genome instability and tumour-promoting inflammation. These hallmarks are critical for acquiring the initial six cancer hallmarks along with two additional hallmarks; reprogramming of energy metabolism and avoiding immune destruction (Hanahan & Weinberg 2011). The majority of these processes are due to the disruption of cellular signal transduction pathways, such as the Ras-ERK and PI3K-Akt signalling pathways (Sever & Brugge 2015).

### 1.1.1. Leukaemia

Approximately 10% of all cancers are blood cancers. At present leukaemia is the fourth most prevalent cancer in Ireland, with over 1,900 people diagnosed per year (Irish Cancer Society 2016). There is a continued need for the development of new and innovative treatment options for this fatal disease. Blood cancer is a broad term used to classify any cancers that affect cells of the blood and/or organs where blood cells develop such as the bone marrow and lymphatic system (BCNI 2015). In blood cancer, the normal development process of blood cells is interrupted with cells becoming unregulated and abnormal and subsequently preventing the normal cells from functioning properly (ASH 2015). The three main types of blood cancer are leukaemia, lymphoma and myeloma.

Leukaemia is a cancer of the white blood cells, being derived from the bone marrow and is one of the most common types of blood cancer (Mayo Clinic 2017). There are four main types of leukaemia, which make up 80% of all leukaemias: Chronic Lymphocytic Leukaemia (CLL), Acute Lymphocytic Leukaemia (ALL), Chronic Myeloid Leukaemia (CML) and Acute Myeloid Leukaemia (AML)(National Cancer Registry Ireland 2010). The name of each cancer provides two pieces of information about that specific disease, the nature of the disease, either acute or chronic, and the cell type it affects. Acute leukaemia occurs when young cells lose control of the rate of division and divide uncontrollably, these white blood cells are not fully developed and do not function properly. This form of the disease progresses rapidly and is usually very aggressive (Mayo Clinic 2016). Chronic leukaemia occurs when old cells, which are no longer needed, do not die and over time they build up in the blood and bone marrow, crowding out normal blood cells. This form of the disease usually develops gradually and is usually less aggressive than acute leukaemia (American Cancer Society 2016c). In the bone marrow two families of cells are produced, myeloid cells and lymphoid cells. Myeloid cells include erythrocytes, thrombocytes and some leukocytes (monocytes and granulocytes), typically cells of the innate immune system response. Lymphoid cells include all other types of leukocytes (lymphocytes) and play a predominant role in the adaptive immune system (Irish Cancer Society 2017a).

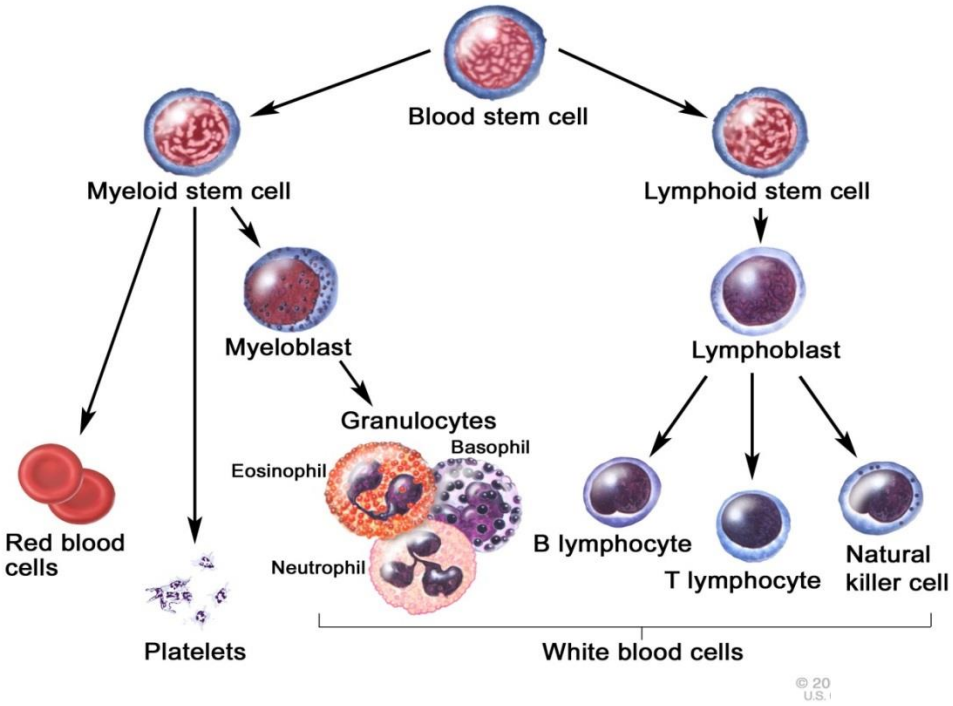



**Figure 1: Development and classification of different blood cell types from stem cells.** (National Cancer Institute 2017b)

## 1.2. Chronic Lymphocytic Leukaemia

CLL is the most common type of Leukaemia in the western world (Ghia et al. 2007). It is most common in adults over the age of 50 and accounts for almost 40% of all leukaemias (National Cancer Registry Ireland 2010). The median age of diagnosis for CLL is 70 years, with a higher incidence rate in males; average new diagnosis per 100,000 persons being 6.4 for males and 3.3 for females (National Cancer Institute 2017a).

This disease is characterised by a relentless accumulation of small mature CD5+ B lymphocytes in the blood, bone marrow and secondary lymphoid organs, lymph nodes and spleen (Ghia et al. 2007). These cells do not mature properly and are unable to carry out their immunological function in fighting infection as normal lymphocytes would. Normally functional B-lymphocytes are responsible for the production of both protective innate and adaptive antibodies, making them a critical part of the immune

system (Lebien & Tedder 2008). CLL cells survive longer than they are needed, eventually building up in the blood and crowding out healthy, functioning cells (American Cancer Society 2016c). This form of leukaemia originates in the bone marrow and can lead to the development of lymphadenopathy (enlargement of the lymph nodes), hepatomegaly (enlargement of the liver) and splenomegaly (enlargement of the spleen) (Bain 2015).

CLL is a heterogeneous disease, with one-third of patients developing a form of CLL which progresses so slowly they never require treatment (Irish Cancer Society 2012). However, other patients develop a faster, more aggressive form of the disease, which needs treatment to prolong patient survival and quality of life (Leukemia and Lymphoma Society n.d.). The cause of CLL is unknown, with no evidence that exposure to radiation, smoking, diet or viral infection causes the disease (Irish Cancer Society 2012). Some factors which may increase a person's risk of developing CLL include: family history; risk is 2-4 times higher in first relatives of a CLL patient, age; 90% of CLL cases are diagnosed in patients over the age of 50, gender, and race; CLL is common in white populations but rare in Asian and American Indian populations (Irish Cancer Society 2012; Cancer.Net 2016).

### 1.2.1. Detection of CLL

The International Workshop on CLL (IWCLL) outlines the criteria for diagnosis of CLL. A monoclonal B lymphocyte cell count of over 5000 cells/µl for a minimum of three months, along with the Immunophenotype co-expression of surface markers CD5, CD19, CD20 and CD23, detected by flow cytometry being required for positive detection of CLL (Hallek et al. 2008; Montserrat & Rozman 1995).

### 1.2.2. Staging of CLL

The clinical staging system developed in 1977, known as the 'Binet system', is the most common staging system used in Europe for CLL (Rai & Jain 2011; American Cancer Society 2016b). This system measures the number of white blood cells in the blood, the enlargement of lymph nodes in the neck, armpits, or groin, along with any enlargement of the spleen or liver (Irish Cancer Society 2012). The development of anaemia (low red blood cells) or thrombocytopenia (low platelets) is also taken into account.

The 3 stages of CLL are as follows:

- **Stage A:** (Low Risk) High white blood cell count, less than 3 enlarged glands.
- **Stage B:** (Medium Risk) High white blood cell count, 3 or more enlarged glands. Spleen or liver may be enlarged.
- **Stage C:** (High Risk) High blood cell count, anaemia and/or low platelets. 3 or more enlarged glands and spleen, or liver may be enlarged.

This staging system for CLL, developed over 40 years ago, is reliable for initial identification of patients requiring treatment and those who only require observation as initial disease management. However, the heterogeneous nature of CLL is not factored into this staging system and therefore cannot determine the clinical course the disease is likely to undergo. As noted by Rai and Jain in 2011, some patients developed extremely aggressive forms of the disease and died within 2 to 3 years of diagnosis, despite receiving treatment, while other CLL patients could live for many years and die from unrelated causes, decades after diagnosis (Rai & Jain 2011). The vast diversity of this disease has become better understood over the last four decades, and the need for

introducing a new staging system, including identification of prognostic factors for CLL, is much more apparent than when the Binet system was first developed.

### 1.2.3. Prognostic Factors of CLL

There have been many studies performed which show the stratification of CLL, according to gene mutation status of the immunoglobulin heavy chain variable regions (IGHV) and the identification of genetic abnormalities by interphase fluorescence in situ hybridisation (FISH). Such studies aim to give a more accurate opportunity for predicting the clinical course of individual patients' disease, even in early-stage CLL (Parikh et al. 2016; Rai & Jain 2011). An international prognostic index for patients with CLL (CLL-IPI) was created by the CLL-IPI working group with the aim of allowing a more targeted system for the management of CLL patients in clinical practice and clinical trials. This prognostic index combines genetic, biochemical and clinical parameters to identify four prognostic subgroups which had significantly different overall survival (OS) rates after five years; low risk (90.5-96.0%), intermediate risk (75.5-83.2%), high risk (57.9-68.8%) and very high risk (12.5-34.1%) (The International CLL-IPI working group 2016). The database collected by the CLL-IPI group was used to identify five independent prognostic factors; tumour protein 53 (TP53) status, IGHV mutational Status, serum β2-microglobulin concentration, clinical stage, and age.

The TP53 gene, a protein-coding gene, is located on the short arm of chromosome 17 at region 17p13 (Lodé et al. 2010) This gene encodes a tumour suppressor protein (p53) which responses to cellular stress. The protein regulates the expression of target genes leading to cell-cycle arrest and induces apoptosis, programmed cell death, as a means of protecting against mutations and the development of cancerous cells (Vogelstein et al. 2000). TP53 mutations have been identified in almost all types of cancers of the

varying organs and tissues of the body. Disruption to TP53 occurs in a significant number of CLL patients due to either the deletion of the short arm of chromosome 17 (del17p) or a mutation of TP53 or both (Rivlin et al. 2011; Rossi et al. 2009). Del17p is a well-established poor prognostic factor for CLL, associated with aggressive disease progression, short median progression-free survival (PFS), resistance to chemotherapeutic agents such as Chlorambucil and Fludarabine, and short OS (Dicker et al. 2009). The recognition of the link between the loss of TP53 function and such poor prognostic factors has only been established in recent years. In approximately 80% of del17p CLL cases, the remaining TP53 allele is also mutated, and only a fraction of CLL patients (approx. 5%) have been identified with TP53 mutations without del17p. Small sample sizes and heterogeneity of patient groups have made it difficult to define the role of TP53 mutations in the disease (Dufour et al. 2013; Zenz, Eichhorst, et al. 2010). However, in the past decade, a number of studies have concluded that TP53 mutations, regardless of del(17p), are associated with significantly poorer overall response rates (ORR), shorter PFS and OS (Zenz, Eichhorst, et al. 2010; Gonzalez et al. 2011; Rossi et al. 2009; Dicker et al. 2009).

The mutational status of the IGHV is defined by sequencing CLL cells and comparing to the germ-line sequence. If there is a difference of ≥2% to the corresponding germ-line sequence the disease is given a mutated status (Rai & Jain 2016). Mutated CLL is associated with good prognosis of slow progression and longer OS, while un-mutated CLL (<2% deviation from germ-line) has a more aggressive clinical course. A systematic review, conducted by Parikh *et al.*, in 2016, showed a PFS range of 1-5 years for un-mutated CLL, which was significantly shorter than that of mutated CLL (9.2 to 18.9 years) (Parikh et al. 2016).

Serum $\beta_2$-microglobulin ($\beta_2$M) concentration is a biochemical parameter which the CLL-IPI group identified as another independent prognostic factor. $\beta_2$M is a small protein molecule synthesised by most nucleated cells and is normally released from cells into bodily fluids such as blood and urine (Seo et al. 2016). The protein is essential for the formulation of major histocompatibility complex class 1 (MHC-1) proteins, which play a vital role in adaptive immunity (Wieczorek et al. 2017). Elevated levels of serum $\beta_2$M are found with many haematological malignancies and are a well-established and simple predictor of CLL progression (Delgado et al. 2009). CLL patients with elevated levels of $\beta_2$M serum (≤3·5 mg/L vs. >3·5 mg/L) have been shown to take a similar disease course to that of un-mutated IGHV CLL, with regards to OS, according to the data analysed by the CLL-IPI group (The International CLL-IPI working group 2016).

The clinical staging system (Binet A vs. Binet B-C) and the age of patients at diagnosis are also identified as independent parameters in the prognostic index for categorising patients by the CLL-IPI working group. The two age groups, which provide significant prognosis indications, were determined as over 65 years and least than or equal to 65 (The International CLL-IPI working group 2016).

FISH a method used for identifying genomic aberrations, in CLL it has helped determine a number of cytogenetic abnormalities with prognostic relevance. Chromosomal aberrations, which are seen in over 80% of CLL patients, can be a major indication of the clinical course of the disease for individual patients (Rai & Jain 2016). Along with Del17p, a number of other chromosomal abnormalities have been identified which provide important indications of CLL disease progression and survival. These include, but are not limited to; a deletion of chromosome 13q (Del13q14), deletion of 11p (del11q23), and trisomy of 12q (an extra chromosome 12) (Döhner et al. 2000). Del13q14, which is

observed in approximately 55% of patients, is seen as a good risk factor, indicating slow disease progression and good response to chemotherapy. Patients with Del13q14, as a sole aberration, have the longest median survival time out of the named genetic subgroups, with a study by Döhner et al. giving a median survival time of 11.1 years (Döhner et al. 2000). Trisomy 12q is seen as an intermediate prognostic factor, with a median OS of approximately 114 months, however some uncertainty still surrounds this aberration (Zenz, Mertens, et al. 2010; Bulian et al. 2017). Del11q23 is a poor prognostic factor, which occurs in approximately 18% of patients, associated with aggressive disease and short OS times (median= 79 months)(Döhner et al. 2000). Deletion of the Ataxia Telangiectasia Mutated (ATM) gene, located on the long arm of chromosome 11, results in loss-of-function of TP53 resulting in resistance to cytotoxic drugs (Dighiero & Hamblin 2008).

Other genetic markers which have been found to play a role in indicating the clinical course which the disease is likely to undergo, include expression of the cell surface marker CD38 and the protein tyrosine kinase zeta-associated protein 70 (ZAP70). Elevated levels of CD38 and ZAP70 expression correlate with unmutated CLL and have been shown to independently predict poor prognosis and an aggressive disease course (Chiorazzi et al. 2017; Crespo et al. 2003; Hallek et al. 2008).

### 1.2.4. Treatment of CLL

There is no cure for CLL, however there are a number of therapeutic options currently in use. The most common treatment for CLL is chemotherapy, other treatment options available include active monitoring, steroid therapy, combination therapy and stem cell transplantation (Irish Cancer Society 2012).

Active monitoring is sufficient for stage A patients who do not experience any symptoms of the disease. These patients, also referred to as treatment-naïve patients, receive regular blood tests for monitoring disease progression, with many people not developing symptoms for many years, and some never requiring active treatment for the disease. For patients with worsening blood cell counts, or who develop symptoms, clinical intervention is required (Cancer.Net Editorial Board 2012).

When treatment is required for CLL patients chemotherapy is often the best course of action, however the patient's age and general health must be considered when a course of treatment is being decided. Chemotherapy involves the use of powerful cytotoxic drugs to destroy or control the growth of cancer cells by inhibiting cell division and cell proliferation (American Cancer Society 2016a). These drugs are usually administered intravenously (IV) into the bloodstream or via oral drug delivery in tablet form (Cancer.Net Editorial Board 2012). Typically these drugs are non-selectively potent and so they also destroy or damage healthy non-cancerous cells in the body, leading to many side effects, including hair loss, mouth sores, loss of appetite, nausea and vomiting (American Cancer Society 2016a). Chemoimmunotherapy is now the current standard of treatment for CLL patients in general good health. Fit patients under the age of 65 are recommended fludarabine, cyclophosphamide, and rituximab (FCR) as first-line treatment, while patients over 65 are typically recommended bendamustine and rituximab (BR). The prevalence of CLL in older people leads to a number of additional factors which must be taken into account when picking a course of treatment, and many older patients with comorbidities are unsuitable for intense chemoimmunotherapy.

As older CLL patients can differ dramatically in physiological aging and pathological conditions (comorbidities and geriatric syndromes), various treatment options must be

available which can cater for both patient-related and disease-related risk factors. Due to an age-related decline in hematopoietic stem cells, which are necessary for the production of new blood cells by the body, chemotherapy-related myelotoxicities are more frequent in older patients with CLL. This is a high-risk factor as resulting infections or anaemia could negatively affect a patient's current comorbidities and lead to treatment-related morbidity or mortality (Eichhorst, Hallek, et al. 2016).

## 1.3. Review of Current Frontline treatment Options for Elderly CLL Patients

### 1.3.1. Monoclonal Antibodies

Anti-CD20 antibodies are a group of compounds which are added to chemotherapy regimens to provide a patient with chemoimmunotherapy, a treatment option which has become the gold standard of treatment for CLL patients fit for treatment.

#### *1.3.1.1. Rituximab*

Since the approval of rituximab in 1997, the type I monoclonal anti-CD20 antibody has been used in the treatment of numerous illnesses, such as follicular B-cell lymphoma, aggressive lymphoma and CLL (Robak, Lech-Maranda, et al. 2010). Its mode of action involves binding to the CD20 antigen on the surface of B-cells. Although some studies showed it to be effective as a monotherapy (Hainsworth et al. 2003), it was found the antibody had a much greater effect when used in combination with other chemotherapy agents (Keating et al. 2005). However, the chemotherapeutic agent which rituximab is combined with must be considered carefully, as the adverse effects of some treatment options are not tolerable by older patients or patients with comorbidities.

FCR is the gold standard of treatment for CLL patients under the age of 65, with otherwise good health and prognostic factors and who have not received previous

treatment. Some older patients with good health and favourable prognostic factors may be suitable for FCR; however, for most patients over 65 and patients with comorbidities, FCR is poorly tolerated. Myelosuppression, a side effect of therapy, causes a decrease in the function of the bone marrow, which leads to low red blood cells, white blood cells, and platelets. It is one of the leading side effects which lead to the discontinuation of FCR treatment (Wiestner 2016). Myelosuppression and its complications following FCR treatment is more frequent in older patients, meaning a different course of treatment with less side effects is more favourable for this group (Balducci 2003).

The CLL10 study

Patients who are not suitable for FCR as frontline treatment, due to age and/or comorbidities, have other treatment options available which are better tolerated. The CLL10 study, a comparative study conducted by Eichhorst *et al. (*2016), investigated FCR vs. BR as a first-line treatment option for advanced CLL patients and found BR to be a better-tolerated treatment option for older CLL patients with similar efficacy (Eichhorst, Fink, et al. 2016)**.** This study consisted of 561 treatment-naïve patients with active CLL and in good physical health. The patients were split into two groups, the FCR-group (282 patients) and the BR-group (279 patients). The age range of the study was 33-81 years, with a median age of 61.5, and patients with *del17p* were excluded. (Eichhorst, Fink, et al. 2016). As expected, the FCR-group experienced significantly longer progression-free survival (PFS), with a median of 57.6 months in comparison to a PFS of 42.3 months for the BR group (Cheung et al. 2017). Interestingly, when the PFS was analysed by splitting the population into two groups by age, 'under 65' group and 'over 65', a difference was noted. In the younger age group, a significant difference was seen in median PFS for the two arms, 38.5 months (BR) compared to 53.6 months (FCR). However, when the 'over

65' group was analysed, there were no significant difference found(Eichhorst, Fink, et al. 2016). Taken this into account, along with the fact that FCR treatment was found to be more toxic in the elderly patient group, with 71% of patients experiencing grade 4 adverse effects (resulting in life-threatening consequences and/or hospitalization (National Cancer Institute 2009)), in comparison to 41% in the BR group and a higher occurrence of therapy-related myeloid leukaemia/ myelodysplastic syndrome (t-AML/MDS) in the older patient group in the FCR arm of the study, BR is a much better front-line chemoimmunotherapy option than FCR for elder CLL patients not suitable for FCR (Eichhorst, Fink, et al. 2016; Love 2014a; Love 2014b).

Rituximab and Hyaluronidase Human

A new development in the treatment of CLL with FCR has recently been approved by the U.S. Food and Drug Administration (FDA). In June 2017, rituximab and hyaluronidase human (RHH) was approved for the treatment of three blood cancers; follicular lymphoma, diffuse large B-cell lymphoma and CLL (FDA 2017). Human hyaluronidase is an endoglycosidase, an enzyme that cleaves specific internal glycosidic linkages of oligosaccharides and polysaccharides leading to a release of oligosaccharides (Yamamoto 2015). It increases the rate of dispersion and absorption of drugs which are co-administered by subcutaneous injection. The approval of this new product means rituximab can be administered in 5-7 minutes, a greatly reduced time in comparison to the standard IV administration which takes several hours RHH can be used for both treatment-naïve and previously treated CLL patients in combination with FC (FDA 2017; Stenger 2017). Unfortunately, to date, there is no clinical information available on bendamustine in combination with RHH.

#### *1.3.1.2. Obinutuzumab*

Obinutuzumab (G), a glycoengineered type II anti-CD20 monoclonal antibody, also referred to as GA101, has shown great promise for elderly CLL patient with comorbidity and was proved by the FDA in November 2013 in combination with chlorambucil for the treatment of CLL (Lee et al. 2014); Food and Drug Administration 2013). Obinutuzumab showed increased direct cytotoxicity, along with higher antibody-dependent cellular cytotoxicity (Owen & Stewart 2015). It was proven to have superior antitumor activity than rituximab in preliminary studies, giving complete tumour remission and increased overall survival (OS) (Mossner et al. 2010). Numerous clinical trials were conducted to determine the safety and effectiveness of obinutuzumab in recent years. Phase I and phase II clinical trials to evaluate the effectiveness of obinutuzumab as a monotherapy, showed promise for heavily pre-treated CLL patients. These trials showed obinutuzumab as a more effective monotherapy than rituximab and ofatumumab, as it proved to have a higher efficiency of B-cell depletion (Cartron et al. 2014; Morschhauser et al. 2009).

The CLL11 Study

A phase III clinical trial, CLL11 study compared treatment options of G in combination with chlorambucil (G+CLB), against Chlorambucil alone (CLB) and Chlorambucil plus Rituximab (R+CLB). This trial consisted of 589 patients with previously untreated CLL. For stage 1, the patients were split into three groups, at a 2:2:1 ratio (238 patients in the G+CLB group, 233 patients in the R+CLB group and 118 patients in the CLB group). Stage 2 was comprised of 192 additional patients, randomly grouped into either the G+CLB or R+CLB arm (Lee et al. 2014; U.S. National Library of Medicine 2017f). The median age of the trial population was 73, and co-existing comorbidities was part of the inclusion criteria (total Cumulative Illness Rating Scale (CIRS) >6 and/or creatinine clearance (CrCl)

of <70ml/min) (U.S. National Library of Medicine 2017f). A recent update on this study shows that G+CLB almost doubled the PFS median in comparison to the R+CLB combination, extending this time to 29.2 months, in comparison to 15.4 months (Goede et al. 2015). G+CLB According to Dr. Carolyn Owens, in many parts of Canada, BR is the preferred treatment option when tolerable. However, for unfit older patients, not fit for such aggressive treatment, the G+CLB combination is now a commonly used regimen in most treatment centres (Owen 2017). Albeit, it must be noted that there has been no clinical trial for the comparison of BR and G+CLB conducted to date.

The GREEN Study

An ongoing phase IIIB clinical trial called the GREEN study, is comparing the safety of as obinutuzumab as a monotherapy (G-Mono) or in combination with different chemotherapies in both untreated and relapsed/refracted (R/R) patients (i.e., patients no longer responding to treatments). The combination treatment option analysed were: G+ fludarabine and cyclophosphamide (FC) for fit patients only (i.e., CIRS ≤6 and CrCl ≥70ml/min), G+CLB for unfit patients only (CIRS >6 and CrCl <70ml/min) or G+B for any patient (Stilgenbauer et al. 2017). The study is currently active, with an estimated completion date of October 2018 (U.S. National Library of Medicine 2017a). However, the results of the primary analysis of the trial were presented at the 59$^{th}$ annual meeting and exposition of the American Society of Haematology (ASH) in December 2017.

The trial population is 971 patients, split into three patient groups: 339 fit; 291 unfit and 341 R/R, with a median age of 66 years. The initial report concluded that toxicities were ‘manageable and no new safety signals were identified' (Stilgenbauer et al. 2017). The median observation time for the study to date was 24.5 months. The most frequent adverse effects reported across all treatment options were neutropenia (58.4%), pyrexia

(32%), thrombocytopenia (31.2%), nausea (27.8%) and anaemia (23.7%), with no significant difference in the three patient groups. 80.3% of patients suffered grade 3 or higher adverse effects, with neutropenia, thrombocytopenia, anaemia, and pneumonia being the most frequent. A similar frequency of grade 3 and higher adverse effects were experienced by all three patient groups. However, serious adverse effects (neutropenia, pneumonia and febrile neutropenia) had a higher occurrence in the unfit patient group (58.8%) vs. 43.7% in the fit patient group (Stilgenbauer et al. 2017).

A comparison of treatments showed the lowest death rate in the G+FC patient group (4.7%) followed by 7.8% in the GB group, 7.9% for thee G+CLB and 8.7% in the G-Mono group. However, it must be noted That the G+FC patient group experienced the highest rate of adverse effects (87.6%) over the other treatment groups (G-Mono: 75.4%, G+CLB: 76.3%, & GB: 79.7%). There was also a significantly higher occurrence of grade 3 or higher adverse effects with special interest for infection in the G+FC group (70.5%) than other treatment groups (G-Mono: 49%, G+CLB: 53% and GB: 52.6%) (Stilgenbauer et al. 2017).

With the initial findings of the study showing acceptable safety data which was in line with that of previously reported G-based treatments; the forthcoming results should help determine the best obinutuzumab treatment combinations for patients of different fitness groups and also for R/R patient groups.

#### *1.3.1.3. Ofatumumab*

Ofatumumab (O), also known as HuMax-CD20, is a fully human type I anti-CD20 monoclonal antibody which targets a distinct small-loop epitope on the CD20 molecule (Wierda et al. 2010). Preliminary studies showed higher levels of cytotoxicity with

ofatumumab over rituximab (Pawluczkowycz et al. 2009). Early clinical trials focused on the effectiveness of ofatumumab as a single-agent therapy for R/R patients. These trials concluded the ofatumumab was a well-tolerated, effective treatment for patients with R/R CLL with poor prognosis (Coiffier et al. 2008; Wierda et al. 2010). After another clinical trial, conducted by Lemery *et al.*, which analysed ofatumumab as a treatment for CLL patients refracted to fludarabine and alemtuzumab the FDA granted accelerated approval of ofatumumab for R/R CLL patients (Lemery et al. 2010).

The COMPLEMENT Study

A Phase III clinical trial (COMPLEMENT-1) conducted by Hillmen, *et al*., in 2015 looked at ofatumumab in combination with Clb (O+CLB) vs. CLB alone in the treatment of treatment-naive CLL patients. The study contained 447 patients with active CLL who were unsuitable for fludarabine-based treatment; the median age of the trial was 69. The O+CLB group showed significantly longer PFS at 22.4 months, while the CLB group was 13.1 months. Although adverse effects were more frequent in the O+CLB arm of the study (50% vs. 43% CLB alone), this trial found that front-line treatment with O+CLB, of elderly patients and patients with comorbidities, was an important treatment option for those not suitable for more intense regimens (Hillmen, Robak, et al. 2015).

The results of another phase III clinical trial, COMPLEMENT-2, looked at ofatumumab in combination with FC (O+FC) vs. FC alone for treatment of relapsed patients, has been reported by Robak *et al.* (2017). The study, consisting of 365 patients, with a median age of 61.5, showed that with the O+FC arm of the study the median PFS of patients was significantly improved, 28.9 months for O+FC versus 18.8 months for FC alone (Robak et al. 2017). 74% of patients experienced grade 3 or higher adverse effects in the O+FC group vs. 69% for the FC group. The study concluded that O+FC combination therapy

was of manageable safety, with increased PFS (Robak et al. 2017; U.S. National Library of Medicine 2017h). Interestingly, a reduced occurrence of thrombocytopenia and anaemia were seen in the O+FC treatment option over FC (Grade 3 or higher thrombocytopenia: 14% vs. 25%, and anaemia (all grades): 20% vs. 30% respectively), indicating ofatumumab may help to prevent myelosuppression (Robak et al. 2017).

Although there is no direct comparative study, as of yet, of O+FC and FCR therapy, an indirect comparison of the COMPLEMENT-2 trial against another phase III trial of previously treated CLL patients which were treated with either FCR or FC (population size: 552, median age: 62.5) indicates that grade 3 and higher adverse effects were comparable in the 2 treatment options (Complement trial: O+FC= 74%, FC= 69%; FCR vs. FC trial: FCR= 80% and FC= 74%)(Robak et al. 2017; Robak, Dmoszynska, et al. 2010). However, a direct comparison trial of O+FC vs. FCR is needed to confirm these suggestions and would help to determine which treatment option is more efficient and tolerable. If ofatumumab was found to reduce the risk of myelosuppression in FC treatment, this could give more elderly patients a chance of receiving FC-based therapy.

### 1.3.2. Small Molecules

Three novel agents have recently been approved for treatment of CLL in the U.S., two kinase inhibitors (KIs), ibrutinib and idelalisib, and the *Bcl-2* inhibitor venetoclax. There were no guidelines on the superiority of the three compounds, until a recent study conducted by Mato and colleagues (2017). This study, which included 683 patients, identifies ibrutinib as superior to idelalisib. Where KIs were not effective, venetoclax appeared superior to chemoimmunotherapy combinations. Also, treatment with venetoclax after ibrutinib failure was recommended as a superior treatment option to idelalisib (Mato et al. 2017).

#### *1.3.2.1. Ibrutinib*

Ibrutinib, is a Bruton tyrosine kinase (*BTK)* inhibitor, was originally approved by the FDA in 2014 for the treatment of CLL patients, who had received at least one other treatment and for patients with *del17p.* It was granted further approval by the FDA as a first-line treatment of CLL in March 2016 (AbbVie Inc. 2016). The compound which is an effective treatment for patients with poor prognostic factors also shows promising results for the treatment of elder CLL patients (Farooqui et al. 2015; O'Brien et al. 2016; O'Brien et al. 2014).

This inhibitor acts downstream of the B-cell receptor (BCR) pathway, inhibiting *BTK*, a critical component of the BCR signalling pathway which is only essential for B-cells and therefore the inhibition of this kinase is not fatal (De Rooij et al. 2012). Both in vitro and in vivo, this *BTK* inhibitor reduced the ability of microenvironment-induced survival and proliferation of CLL cells (De Rooij et al. 2012). Ibrutinib as a front-line therapy for CLL patients with the unfavourable *del17p* show more effective responses than reported for FCR treatment and is now the front-line standard treatment for this patient group (Farooqui et al. 2015; Edenhofer & Stilgenbauer 2017). It is also an alternative treatment option to chemoimmunotherapy for elderly patients (Byrd et al. 2015; Burger et al. 2015).

<u>The RESONATE Study</u>

RESONATE, a phase III clinical trial investigated the use of ibrutinib (Ibr) or O as a treatment option for pre-treated CLL patients unsuitable for chemoimmunotherapy. Inclusion criteria for the study were patients who received at least one previous treatment, which resulted in short remission time due to one of the following: aged over 70, coexisting illnesses or has *Del17p* CLL. The median age was 67 with a study

population of 391 patients. The findings of this study showed Ibr to be superior to O in all sub-groups analysed, including R/R patients, patients with *del17p* and patients >65. Thereby, concluding Ibr as a monotherapy is an excellent treatment option for CLL patients unsuitable for immunochemotherapy (Byrd et al. 2014).

Another phase III clinical trial (RESONATE-2) conducted by Burger *et al.* in 2015 showed Ibr to be a superior treatment regimen to CLB in elderly patients. The study, involving 269 CLL patients over 65, had a median age of 73. This study showed Ibr achieved significantly longer PFS than CLB; at a median follow up of 18.4 months PFS medians were not reached vs. 18.9 months, respectively. OS for Ibr was significantly improved over that of CLB, at 24 months the OS rates were 98% and 85%, respectively (Burger et al. 2015). A follow-up study on the trail by Barr *et al.*, showed an increased PFS for Ibr over CLB, at 24 months 89% vs. 34% respectively (Barr et al. 2016). It has been proven Ibr as a monotherapy is a treatment option without the use of traditional chemotherapy drugs which can provide a first-line treatment for elderly patients not suitable for FCR treatment.

An indirect comparison of ibrutinib as a monotherapy and BR therapy was conducted from the results of two phase III clinical trials, the RESONATE and HELIOS trials, by Hillmen *et al.* in 2015. The RESONATE trial (discussed above) had a study population of 391 patient, with a median age of 67 (Byrd et al. 2014). The HELIOS trial, Which included a population of 578 patients and had a median age of 63.5 years, investigated Ibr in combination with BR (Ibr+BR) vs. BR alone as a treatment regimen for previously treated patients (Chanan-Khan et al. 2016). As these two trials had variable populations, including patient characteristics (such as age) and exclusion of high-risk factor groups such as *Del17p* in the HELIOS trial, the group used patient-level data from both studies

to complete the cross-comparison. This cross-comparison of the two trials suggested single-agent Ibr was a superior treatment option to BR treatment and that the combination regimen of Ibr+BR had comparable results for PFS and OS in comparison to Ibr alone (Hillmen, Fraser, et al. 2015). However, a direct comparison Ibr monotherapy vs. Ibr+BR combination therapy is needed to determine the superiority of the two regimens.

Notably, in the HELIOS trial, the combination treatment of Ibr+BR in patients suitable for BR therapy showed significantly improved outcomes with no new safety signals and a manageable safety profile, suggesting that Ibr+BR could be a superior treatment regimen for elderly patients eligible for BR therapy (Chanan-Khan et al. 2016).

#### *1.3.2.2. Idelalisib*

Idelalisib is an orally available, highly specific and reversible kinase inhibitor which targets the phosphatidylinositol 3 kinases (*Pl3Ks*) (Lannutti et al. 2011). Pl3Ks is essential for the activation, proliferation, migration, and survival of B cells, along with their homing and retention in lymphoid tissue (Burger & Okkenhaug 2014). Idelalisib, also known as zydelig, is shown to be an effective treatment option for patients with R/R CLL, even in patients with poor prognosis factors (Brown et al. 2014). A phase II clinical study, reported in 2014 by Zelenetz *et al.*, of idelalisib as a monotherapy for previously untreated elderly patients (>65) showed encouraging results with a manageable safety profile (Zelenetz et al. 2014). Another phase II trial by O'Brien *et al.* in 2015 showed promising results for a combination regimen of idelalisib plus rituximab for treatment-naïve elderly patients (median age: 71). In this trial overall response rate (ORR) for patients with a *del17p* or *TP53* mutation was 100%, and an ORR for unmutated immunoglobulin heavy-chain variable genes (IGHV), another poor progression factor,

was 97%. After a 36 month period, the PFS rate was 83% (O'Brien et al. 2015). The advantage of this combination in comparison to either agent as a monotherapy is a shortened duration of lymphocytosis (high lymphocyte count), along with improved PFS times, response rates and OS (Furman et al. 2014). These findings showed excellent potential for idelalisib as a treatment regimen for elderly patients and patients with poor prognostic factors.

However, further investigations have raised concern about idelalisib, and it is not thought to be an unsafe monotherapy for treatment-naïve patients. Idelalisib was approved by the FDA in July 2014 for combination treatment with rituximab in relapsed or refractory CLL but not for uses as a first-line treatment option for CLL (National Cancer Institute 2014). However, since the approval by the FDA, at least six clinical trials involving idelalisib were stopped due to severe adverse effects and toxicity leading to death (FDA 2016a). In March 2016, the FDA announced they were reviewing findings of clinical trials and alerted healthcare professionals of "increased rates of adverse effects" of idelalisib (Inman 2016). At present, the FDA is reviewing the results of the clinical trials and has warned of the increased rates of adverse events, including death (FDA 2016a).

#### *1.3.2.3. Venetoclax*

Venetoclax (V), also referred to as ABT-199, is an anti-apoptotic *Bcl-2* inhibitor which received accelerated US FDA accelerated approval in 2016 for the treatment of relapsed *del17p* CLL, as it is proven to be highly effective for the treatment of R/R CLL (FDA 2016b)(Stilgenbauer et al. 2016). Venetoclax has been proven to be highly active in patients with poor progression factors such as the *del17p*, with preclinical data showing the ability of the inhibitor to kill CLL cells while sparing healthy T-cells, granulocytes, and platelets (Khaw et al. 2014). *Bcl-2* is a pro-survival protein; its function is to inhibit the

actions of pro-apoptotic proteins such as *BAX/BAK*. When *Bcl-2* is inhibited in the cell, the activation of such pro-apoptotic proteins are triggered. The *Bcl-2* protein is known to be critical for B-cell survival, while proteins such as *Bcl-$x_L$* are more important for the survival of other lymphocytes such as T-cells and granulocytes. As a result of this, venetoclax is selective to B-cells. However, the compound is as potent on non-CLL B-cells as CLL B-cells (Khaw et al. 2014). Venetoclax has been shown to produce promising results both as a monotherapy and in combination with rituximab (Stilgenbauer et al. 2016; Seymour et al. 2017).

Although venetoclax has been shown to be an efficient treatment for R/R CLL patients, the uses of the *Bcl-2* inhibitor as in a treatment regimen for elderly CLL patients is not clear. There are currently a number of clinical trials underway assessing the efficacy and safety of venetoclax as a monotherapy and in combination therapies (V+BR for R/R and previously untreated patients, G+V vs. G+CLB for previously untreated patients, V+G+Ibr in R/R and previously untreated patients, V+Ibr in treatment-naïve patients) (U.S. National Library of Medicine 2018).

The CLL14 Trial

One such trial, the CLL14 study, which as a focus on treatment-naïve CLL patients with coexisting medical issues, is a phase III study looking at the efficacy and safety of V+G combination therapy vs. G+CLB. This trial is currently active and as 445 participants enrolled (U.S. National Library of Medicine 2017b). Although there are no results published to date, a run-in safety phase was conducted. This phase consisted of 13 patients with a median age of 75; it found no initial safety risks and the CLL14 trial was opened in August 2015 (Fischer et al. 2015). Anticipated results from this study, along with the numerous other clinical trials actively investigating the safety and efficiency of

V as both a monotherapy and in combination therapies, will help advice healthcare professionals on the uses of these regimens for elderly patients with active CLL.

| Trial Name | Treatments | No. of patients | Median Age (Years) | Patient Group | Superior Treatment for >65 years |
|---|---|---|---|---|---|
| CLL10 (Eichhorst, Fink, et al. 2016) | FCR vs. BR | 561 | 61.5 | Untreated CLL; *Del17p* excluded | BR *[1] |
| CLL11 (U.S. National Library of Medicine 2017f; Goede et al. 2015) | G+CLB, CLB, R+CLB | 589 | 73 | Untreated CLL; coexisting health issues | G+CLB |
| GREEN(Stilgenbauer et al. 2017) | G-Mono, G+FC, G+CLB, GB | 971 | 66 | R/R CLL & Untreated CLL | Ongoing *[2] |
| COMPLEMENT-1 (Hillmen, Robak, et al. 2015) | O+CLB, CLB | 447 | 69 | Untreated CLL; not suitable for fludarabine-based treatment | O+CLB |
| COMPLEMENT-2 (Robak et al. 2017) | O+FC, FC | 365 | 61.5 | R/R CLL | N/A*[3] |
| RESONATE (Byrd et al. 2014) | Ibr, O | 391 | 67 | R/R CLL | Ibr |
| RESONATE-2 (Barr et al. 2016) | Ibr, CLB | 269 | 73 | Untreated CLL; 65 years & older | Ibr |
| HELIOS (Chanan-Khan et al. 2016) | Ibr+BR, BR | 578 | 63.5 | R/R CLL | N/A*[4] |
| CLL14 (U.S. National Library of Medicine 2017b) | V+G, G+CLB | 445 | Not reported yet | Untreated CLL; coexisting health issues | Ongoing *[5] |

[1] Although low median age, >65s results were analysed
[2] Estimated completion date: October 2018(U.S. National Library of Medicine 2017a)
[3] Due to low median age, not suitable for determining superior regimen for >65s.
[4] Due to low median age, not suitable for determining superior regimen for >65s.
[5] Estimated completion date: September 2021 (U.S. National Library of Medicine 2017c)

**Table 1: Summary of important clinical trials for determination of superior treatment regimens for elderly CLL patients.**

### 1.3.3. Upcoming Clinical Trials

According to the U.S National Library of Medicine, there are currently almost 200 clinical trials recruiting CLL patients (U.S. National Library of Medicine 2017d). These trials are investigating numerous novel treatment options and new combinations of currently approved therapies with the purpose of further improving the progress of CLL treatment. Unfortunately, there is a common misrepresentation of the CLL population in a large number of clinical trials. While the median age of diagnosis for the disease is 70, with many patients not needing immediate treatment, the average age in many clinical trials fall below 65 years (Eichhorst, Fink, et al. 2016; Robak et al. 2017; Chanan-Khan et al. 2016), with some studies setting the age criteria at 18-70 years, therefore preventing patients over 70 participating in the trial (U.S. National Library of Medicine 2017g). Along with age, exclusion criteria for many studies prohibit the recruitment of patients with common health issues which are representative of the overall CLL population. The ECOG (Eastern Cooperative Oncology Group) performance status is a scale, from 0-5, used for measuring a patient's level of function and their ability to conduct daily tasks (ECOG-ACRIN Cancer Research Group 2017). For a large number of clinical trials, an ECOG level of 0-2 is acceptable for inclusion in the study, where 2= 'Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours' (ECOG-ACRIN Cancer Research Group 2017). Patients with an ECOG level =/>3 (3= 'Capable of only limited self-care; confined to bed or chair more than 50% of waking hours') are often excluded from participation in clinical trials, even though a high proportion of CLL patients may fall into this ECOG level (U.S National Library of Medicine 2017; U.S. National Library of Medicine 2017e). A study on small cell lung cancer, which looked at the uptake and tolerance of chemotherapy on

patients 75 and older, found that 39% of patients aged 75-79 were categorised as ECOG level 3 or 4 (Fisher et al. 2012).

### 1.3.4. Need for Novel Treatment Options

Although there has been significant progress made in the field of CLL treatment in the past two decades, CLL remains an incurable disease. The available options for patients with poor prognostic factors and elderly patients with comorbidities are growing rapidly, with better-tolerated therapies and more effective outcomes. Unfortunately, elderly patients and patients with co-existing comorbidities, which account for a high percentage of the CLL population, are still significantly under-represented in the field of clinical research. This leaves many unanswered questions and insecurities when choosing the most effective and best-tolerated regimens for patients who fall into these categories. The overall CLL population needs to be better represented in clinical trials with guidelines, for ensuring trial participants are chosen as a true reflection of disease characteristics, needed to provide more accurate information on treatment regimens.

It is therefore critically important to develop new drug therapy options which are more selective for cancerous cells over non-cancer cells. Such drugs will reduce the severity of side effects experienced by patients. Improving patient morbidity is essential in such situations where persons are undergoing regular and prolonged chemotherapy. The novel compounds under investigation in this project may offer such potential due to the mechanism of action proposed for these tetraoxanes. The affinity of the cleavage of the endoperoxide bridge by elevated levels of intracellular iron, associated with cancer cells, and the expected production of reactive oxygen species (ROS) on exposure to these cytotoxic compounds are expected to provoke selectivity for cancer cells. Cancer cells typically have higher levels of ROS than non-cancer neighbouring cells, making them

more susceptible to elevated levels of oxidative stress, therefore inducing apoptosis (Lai et al. 2013).

To date, there is a lack of published data with the uses of non-cancerous cell lines for the analysis of compound selectivity. However, such comparative studies can provide vital information, in the early stages of drug screening, about compound sensitivity for cancer cells, and therefore help in the endless search for identifying new, effective treatments with minimal adverse side effects. Previous studies, conducted for different cancers, have shown the use of non-cancerous immortalised cell lines for predicting the effects of a compound on healthy, non-cancerous tissues *in vivo* (Žegura et al. 2008; Mandalapu et al. 2016).

## 1.4. Compound Information

### 1.4.1. Novel Tetraoxanes

In the early 1990s, 1,2,4,5-tetraoxanes were first investigated, by Vennerstorm *et al*., as potential alternative antimalarial endoperoxides (Vennerstrom et al. 1992). These compounds, containing two endoperoxide functional groups, have been found to be more stable, and often more potent, than their 1,2,4-trioxane counterparts and also have excellent anti-malarial properties (O'Neill et al. 2010; Opsenica et al. 2008). These analogues of ART, a well-established anti-malaria drug, have also been found to possess anti-proliferative activity, in sub-micromolar levels, in a number of cancer cell lines, including leukaemia (Opsenica et al. 2008; Copple et al. 2012). Although the mechanism of action of endoperoxide-containing compounds results in activity selective towards cancer cells, offering more targeted effect and fewer side effects than many existing chemotherapy agents, the compounds remain under-investigated as potential anticancer agents. These compounds are inexpensive to make, easy to synthesise, and proven to be highly active, and therefore offer excellent promise as potential anti-cancer agents.

The exact mechanism of tetraoxanes is still unclear. However, reactive oxygen species (ROS) are believed to be involved in the anti-malarial properties of tetraoxanes, with alkoxyl ($RO^{\bullet}$) radicals thought to play an important role in the anti-malarial and anti-cancer activity of the compounds(Kumura et al. 2009; Opsenica et al. 2006). ROS generating agents possess exception potential as anti-cancer agents as cancer cells are known to have naturally higher ROS levels than neighbouring non-cancer cells (Zitka et al. 2012). Due to this fact, cancer cells are more susceptible to oxidative stress than normal cells, with an increase in ROS less tolerable by cancer cells, triggering apoptosis. This phenomenon can be utilised for selectively targeting cancer cells, with many studies

already proven manipulation of cancer cells' ROS levels to be fatal (Troyano et al. 2001; Englert & Shacter 2002).

Although the activity of tetraoxanes, as both anti-malarial and anti-cancer compounds, is presumed to be similar to that of ART, the mode of action of tetraoxanes has been proven to differ in relation to its anti-malaria properties (Kumura et al. 2009). However, *Mercer et al.* demonstrated the endoperoxide structure, within the chemical structure of ART, is responsible for both the pharmacological and toxic properties associated with the compound. The mechanism of apoptotic cell death induced by endoperoxides via ROS generation, DNA degradation and mitochondrial depolarisation has been demonstrated in numerous cell lines (Mercer et al. 2007). These endoperoxide compounds are structurally different from ART derivatives, containing two endoperoxide bridges in the tetraoxane structure (see figure 2). The structural formulae of the five tetraoxane compounds analysed in this work can be found in table 2.

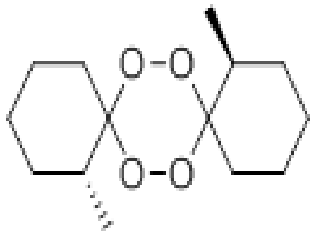



**Figure 2: Chemical structure of a 1,2,4,5 tetraoxane synthesised by Vennerstorm et al.** (Vennerstrom et al. 1992)**.**

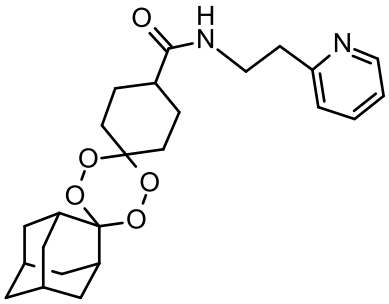
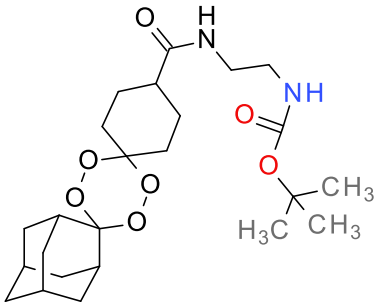
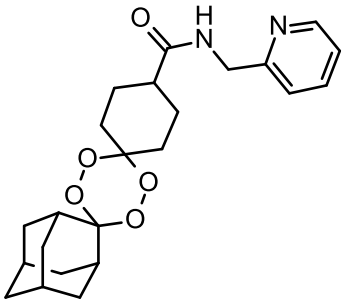
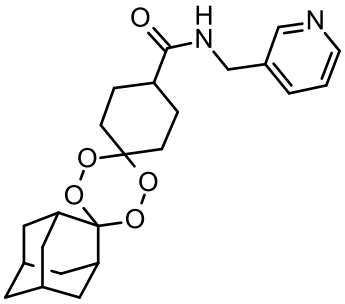
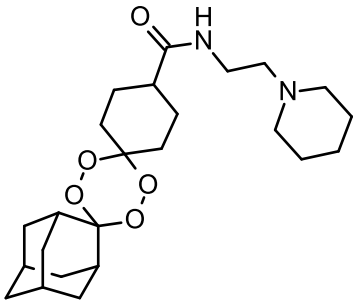
| | |
|---|---|
|  <br>**RR5**<br>Chemical Formula: $C_{24}H_{32}N_2O_5$<br>Molecular Weight: 428.52 |  <br>**SR9**<br>Chemical Formula: $C_{24}H_{38}N_2O_7$<br>Molecular Weight: 466.57 |
|  <br>**RR6**<br>Chemical Formula: $C_{23}H_{30}N_2O_5$<br>Molecular Weight: 414.49 |  <br>**SR10**<br>Chemical Formula: $C_{23}H_{30}N_2O_5$<br>Molecular Weight: 414.49 |
|  <br>**RR7**<br>Chemical Formula: $C_{24}H_{38}N_2O_5$<br>Molecular Weight: 434.57 | |

**Table 2: Table of structural formulae, chemical formulae and molecular weight of novel tetraoxanes compounds**

### 1.4.2. Novel Tetraoxane-Naphthalimide Hybrid

Hybrid compounds are compounds synthesised by the linking of two different and independently active 'parent' compounds to create one novel compound with dual activity. They have been described as chemical entities with two or more structural domains containing different biological functions and the ability to exert dual activity (Meunier 2008). The aim of developing a hybrid compound is to achieve synergy of multiple modes of actions, utilising the unique modes of actions relative to each parent drug, leading to greater potency (Kennedy 2012). Hybrids present several advantages as chemotherapeutic agents, including augmented potency, resilience to drug resistance, better selectivity, enhanced cellular uptake and improved bioavailability (Müller-Schiffmann et al. 2012). Hybrid compounds have the ability to target several sites simultaneously resulting in amplification of potency, hence being effective at lower doses making them an attractive anti-cancer agent (Müller-Schiffmann et al. 2012). An important benefit of hybrid compounds is their ability to overcome development of resistance by virtue of their multimodal drug action. Enhanced safety and efficacy has been previously demonstrated in hybrid compounds in comparison to the respective parent drugs (Das et al. 2010). The use of hybrid compounds has been successfully employed in the treatment of many diseases including cancer, neurodegenerative disorders and malaria (Decker 2011).

Previous studies, conducted by this research group, evaluated a library of hybrid compounds containing an endoperoxide linked to a DNA-targeting naphthalimide on both tumour and non-tumour cells. These compounds were found to be selectively toxic against cancer cells, with one such compound showing 11-fold more potency for prostate cancer cell line PC-3 versus a non-tumour prostate cell line (PNT1A) (Murray

2017). These findings prove the potential of endoperoxide-naphthalimide compounds as potential chemotherapeutic agents. The hybrid compound analysed here, *MK121*, (synthesised by Dr Sarah Rawe, DIT) is a conjugation of a DNA intercalating naphthalimide moiety, and an endoperoxide-containing moiety. The ultimate goal of this consolidation is to synergise the potency of the DNA intercalating agent with the iron-activated selectivity associated with endoperoxides. MK121 is a non-cleavable tetraoxane hybrid, synthesised by the covalent linking of a tetraoxane moiety with a naphthalimide via an amide coupling.

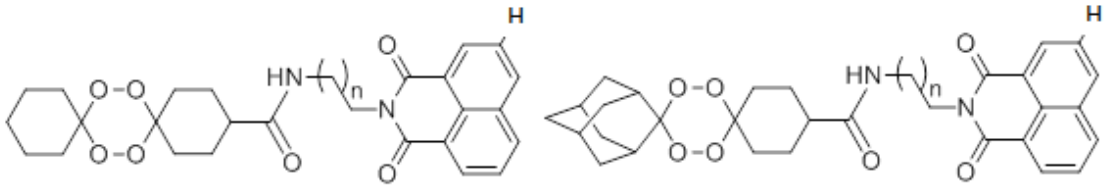



**Figure 3: Chemical structure of hybrid compound MK121, linker length n=2.**

### *Naphthalimides*

Naphthalimides are a class of drugs first developed as anticancer drugs in 1973 by Braña and colleagues who designed the first series of 3-nitronaphthalimides to be tested (Braña & Ramos 2001; Llombart et al. 1992). These compounds are DNA-intercalating agents which have high cytotoxicity activity against a range of tumour cells. These compounds work by binding to the DNA between the base pairs of the double-stranded helix, along with inhibition of topoisomerase-II, an enzyme essential for DNA synthesis(Kamal et al. 2008; Ott et al. 2008). Naphthalimides play an imperative part in the development of novel chemotherapeutic agent and are utilised in this project in the synthesis of the novel tetraoxane-naphthalimide compound analysed.

Mitonafide was one of the first naphthalimide derivatives to reach clinical trials, and although showed promising antitumour activities, neurotoxicity associated with the compound lead to discontinuation of the clinical trials at stage II (Kamal et al. 2008). The nitro-group of mitonafide is thought to be responsible for the toxic side-effects of the compound (Ott et al. 2008; Llombart et al. 1992). Since then numerous compounds have been synthesised with the aim of retaining the potency of these DNA-intercalating compounds with heightened selectivity to reduce the adverse effects associated with the parent compounds.

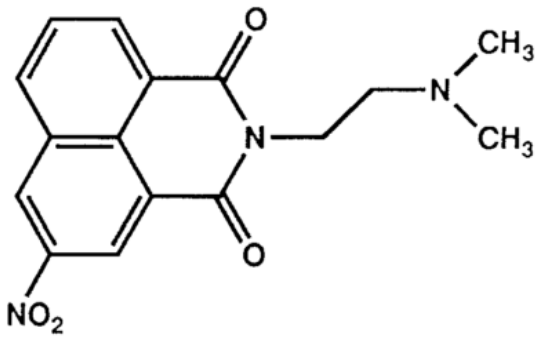



**Figure 4: Chemical structure of Mitonafide**

### 1.4.3. Artemisinin and Dihydroartemisinin

The naturally occurring 1,2,4-trioxane, artemisinin (ART), isolated from the sweet wormwood plant *Artemisia annua L.*, is a powerful anti-malarial drug which has been used for the treatment of malaria since 1973 with minimal side effects (Krishna et al. 2004; Lai & Singh 1995). Dihydroartemisinin (DHA), also known as Artenimol, is the main metabolite of ART derivatives and is one of the more effective analogues for malaria (Lu et al. 2008). ART, along with its semi-synthetic derivatives such as Artemether and Artesunate have been found to possess anti-cancer properties. The anti-cancer potential of ART derivatives, reported by Sun et al. in 1992, on a number of cells lines, found

significant activity against P388 (the murine leukaemia cell line) and SMMC-7721 (a hepatoma cell line) while having decreased effects on non-cancer cell lines. These findings suggested ART, and its derivatives had specific selectivity for cancer cells (Sun et al. 1992). Since then numerous studies have since been conducted which demonstrate the efficacy of these compounds as anti-cancer agents and the modes of actions of the compounds (Woerdenbag et al. 1993; Efferth et al. 2003). After exposure to DHA, cancer cells undergo a rapid induction of apoptosis, indicating Artemisinin and its derivatives selectively kill cancer cells in vitro (Singh & Lai 2004). Also as ART has been used for many years as an anti-malarial drug, it is known to be a safe drug, with minimal side effects even when administered at high concentrations (Lai & Singh 1995). Due to the poor-bioavailability and limited availability of ART, and its semisynthetic derivatives, this has prompted a vast development of alternative next-generation endoperoxide-based compounds.

ART is a sesquiterpene lactone which contains an endoperoxide group (O-O) (see figure 5). The peroxide within the 1,2,4-trioxane ring is crucial to give ARTs the basis for its antimalarial properties (Krishna et al. 2004). DHA and its other analogues also contain an endoperoxide group. When this endoperoxide bond comes into contact with iron, a reaction occurs causing free radicals to be formed (Brown 2006).

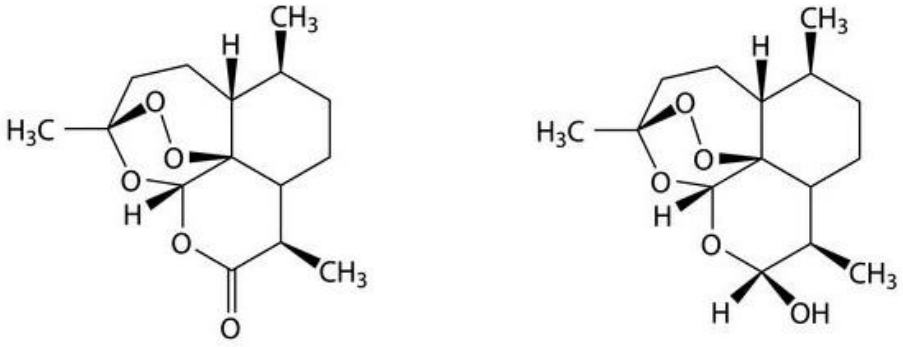



**Figure 5: Chemical structure of ART and DHA, respectively.**

Over the last two decades, both preclinical studies and clinical studies have provided strong evidence that ART, and its derivatives, contain potential anticancer properties (Mijatovic et al. 2008; Krishna et al. 2008). The anti-cancer properties of Artesunate were tested, in vitro, on 55 cancer cell lines by the U.S. National Cancer Institute (NCI). The results showed Artesunate inhibits and kills a range of cancer cells originated from different tissue types, with the compound showing to be most active on leukaemia cells (Efferth et al. 2001). In a study conducted by Lu et al. (2008) they determined DHA inhibited proliferation of a variety of tumour cells originating in different tissue types, effectively inducing apoptosis in HL-60 leukaemia cells (Lu et al. 2008). It was also found that DHA induced apoptosis is iron-dependent, which is a useful property as this makes DHA more effective on cancer cells than non-cancerous cells. ART-derived compounds are iron-dependent which means the iron-rich cancer cells are more susceptible to cytotoxic effects from these compounds (Li et al. 2016). This means not only do ART, and its derivatives have the ability to inhibit proliferation in a range of cancer cells, they are also much less toxic to non-cancer cells, making them a desirable compound for cancer treatments.

In the presence of iron, the endoperoxide bridge is reduced in the cytoplasm, causing the formation of toxic-free radicals or reactive oxygen species (ROS). The radicals then oxidise lipids, cause damage to membranes and proteins and cause DHA damage, all of which forces the cell to undergo apoptosis (Nam et al. 2007; Singh & Lai 2004; Crespo-Ortiz & Wei 2012). As cancer cells have significantly larger amounts of intracellular free iron, they are more abundant in iron than healthy cells, making them more susceptible to cytotoxicity of ART and its derivatives (Lai et al. 2013). Apoptosis, autophagy and cell cycle arrest are three of the main pathways which mediate the actions of ART and DHA, with the generation of ROS suggested as the central event responsible for the cytotoxic effects of ARTs (Bhaw-Luximon & Jhurry 2017). Nonetheless, some uncertainty still remains about the mechanisms of action of endoperoxides, with multimodal mechanisms thought to be accountable for the cytotoxicity and capability of evading drug resistance (Kennedy 2012).

### 1.4.4. Chlorambucil

Chlorambucil (CLB), an alkylating agent derived from nitrogen mustard, was the main treatment choice for CLL up to the late 1990's. Nitrogen mustards are an important class of alkylating agents, compounds which prevent DNA replication by the attachment of an alkyl group (Warwick 1963), which play a vital role in cancer therapy (Saha et al. 2013). The phenylbutyric acid, CLB, was first synthesised in 1953, with it being established as a treatment for CLL shortly after (Begleiter et al. 1996). Although new, more effective treatment options have been developed since the late 1990's (O'Reilly et al. 2018), CLB still plays a vital role in CLL treatment for elderly patients and patients of low tolerability to more intense treatments (Jaglowski & Jones 2011).

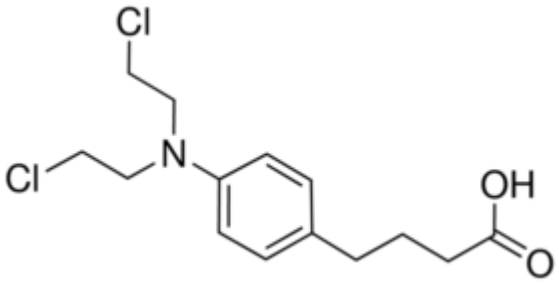



**Figure 6: Chemical Structure of Chlorambucil**

CLB is a bifunctional alkylating agent; this means the agent binds to both strands of the DNA double helix, creating an interstrand crosslink, which prevents the DNA strand from separating which in turn will prevent the cell from replicating and may induce apoptosis or necrosis due to restricting the repairing ability of the cell (Colvin 2002). CLB has also been demonstrated to bind to RNA, proteins and membranes, consequently inhibiting protein synthesis, enzyme activity and disrupting signalling processes (Begleiter et al. 1996; Povirk & Shuker 1994).

Although the easy application and relatively few complications of CLB mean it remains a good choice of therapy for elderly patients, low remission rates and resistance to the agent are two significant limitations of the alkylating agent. A clinical trial conducted by Rai *et al.* in 2000 on 509 patients, resulted in partial remission for 33% of patients in the CLB treatment arm (group size= 181 patients), with only 4% achieving complete remission (Rai et al. 2000). Furthermore, the median remission period and PFS time was only 14 months, indicating the short treatment-free periods achieved for patients on CLB therapy. The mechanisms of CLB resistance have been investigated for many years, with numerous mechanisms implicating the activity of CLB as an effective alkylating agent. Elevated levels of antioxidants such as glutathione and detoxifying enzymes known as glutathione s-transferases have been shown to reduce the effectiveness of

CLB caused by binding of the compound with glutathione, resulting in reduced agent binding to DNA, RNA, proteins and membrane (Tew 1994; Begleiter et al. 1996). Other modes of resistance are likely to include a change in the delivery of CLB to cells, alterations in DNA repair activities and modifications to apoptosis pathways (Reed 1997; Tan et al. 1987; Panasci et al. 2001).

## 1.5. Aims and Objectives

This project aims to determine the anti-cancer potential of a number of novel tetraoxane compounds for the treatment of chronic lymphocytic leukaemia. These compounds may also have a wider application across a broad range of other cancer types. Due to the high incidents of leukaemia, with it ranked in the top 10 most commonly diagnosed invasive cancers (average 2015-2017), it was selected for the screening and analysis of these novel tetraxoane compounds (National Cancer Registry Ireland 2017). Leukaemia also provides an ideal cell model for cytotoxic screening, with easy access to primary patient samples and high patient numbers for accumulation of samples, for the potential of further anaylsis involving the assessment of these compounds on primary leukaemia cells.

The objective of this project is to evaluate the small panel of novel tetraoxane compounds on a range of relevant commercial cell lines, to determine the anti-proliferation potential of each compound along with its selectivity for cancer cells over non-cancerous cell lines. To achieve this, the six novel compounds will be evaluated on two cancer cell lines, HL-60 and MEC-1, along with one non-cancer peripheral blood cell line, BL2052. The potency of the compounds toward the two cancer cell lines, in comparison to that of the non-cancer peripheral blood cells line, will be analysed to determine the selectivity of the compounds and to identify a therapy window to assess suitability of the compounds as chemotherapy agents. The compounds with the widest therapeutic window, i.e. the highest non-cancer/ cancer ratio, are predicted to be the more promising therapeutic drugs, with less adverse effects. This is a comparative study with two reference compounds, Chlorambucil and Dihydroartemisinin, analysed

alongside the novel compounds to provide relevant and relatable information for comparing the potential of the novel compounds to routinely used clinical drugs.

A range of assays are utilised to orchestrate this analysis in accordance with the biocompatibility ISO 10995 recommendations. These include the routinely used colorimetric-based assays Thiazolyl Blue Tetrazolium Bromide (MTT) and Lactate Dehydrogenase (LDH), along with flow cytometric analysis for the detection of the cell death pathway induced by the compounds. A range of assays for the detection of cytotoxicity were utilised and compared for the accurate determination of $IC_{50}$ concentrations for the compounds screened. A preliminary stability study is also performed on the six novel tetraoxanes to evaluate compound stability, after storage at -80°C for a 9-month period.

# 2. Experimental Methodology

## 2.1. Cell Line Information

### 2.1.1. MEC-1

The MEC-1 cell line (DSMZ no.: ACC 497) originated from the peripheral blood of a 61-year-old Caucasian male with B-cell CLL who was undergoing prolymphocytoid transformation (Stacchini et al. 1999; DSMZ n.d.). The cell line, which was established in 1993 and has an approximate doubling time of 40 hours (Drexler 2010).This cell line is one of the best characterised CLL cell lines and is commonly used as a proxy for primary CLL analysis (Thurgood et al. 2017). The MEC-1 cells are a representation of CLL with poor prognosis, as they carry the del17p mutation and is known to be resistant to chemotherapeutic agents (Stacchini et al. 1999).

Although this cell line has been derived from a patient with CLL, the cells are Epstein-Barr virus (EBV) transformed and therefore differ from primary CLL cells as they actively proliferate and are not in the $G_0$ phase of the cell cycle, as are more than 99% of primary CLL cells in circulation (Stacchini et al. 1999; Andreeff et al. 1980). However, the cell line is a good indicator for preliminary screening of novel anti-cancer agents and will provide a good indication of the efficacy of the novel compounds analysed in this study.

The MEC-1 cells are round, polymorphic cells which grow mainly in suspension, with a small number of cells becoming slightly adherent. They also form into small aggregates in media.

### 2.1.2. HL-60

The HL-60 cell line, kindly gifted from Prof. Orla Howe (Dublin Institute of Technology (DIT), Ireland), was derived from a 36-year-old Caucasian female patient with acute

promyelocytic leukaemia (American Type Culture Collection (ATCC) 2014). These cells have a doubling time of 20-45 hours, depending on the sub-line (Drexler 2010).

As this cell line originating from a form of acute leukaemia, the cells have a high doubling rate and are easily grown in medium, making it an ideal cell line to work with for initial studies for leukaemia treatment. The extensive literature on the in-vitro analysis of HL-60 cells makes it a popular cell line for evaluating the potential of the novel compounds investigated in this study. Studies on the well-established HL60 cells allowed for initial screening of the compounds with reference to a widely published cell line. However, as this study is focused on CLL, it is necessary also to investigate the potency of the novel compounds on cells derived from CLL, to give a more accurate indication of the anti-proliferation potential of the compounds on CLL patient samples.

HL-60 cells are single round cells which grow in suspension. All sub-lines of HL-60 cells display myeloblastic or promyelocytic morphology, 'large, blast-like cells with characteristic large, rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules' (Birnie 1988). This morphology is displayed in approximately 90-95% of the cell culture, with the remaining resembling the morphology of mature myeloid cells (Birnie 1988).

### 2.1.3. BL-2052

The BL-2052 cell line originated from the peripheral blood of a 65-year-old Caucasian male. This individual was diagnosed with Mesothelioma (a form of lung cancer) however no hematopoietic disorders were detected and therefore the cell line derived from the peripheral blood of this patient is classed as non-cancerous (ATCC 2014; ATCC 2013). This cell line was EBV transformed and was used as a proxy for healthy non-tumour

blood samples for the purpose of determining the therapeutic-window of the novel compounds analysed in this study.

The cell type of this cell line is B-lymphoblast, a type of b lymphocyte which was stimulated by an antigen and is enlarged or an immature B-cell which can develop into a mature cell (National Library of Medicine n.d.). LCLs grow in clusters and present a typical rosette morphology (Hussain et al. 2012).

## 2.2. Cell Culture Parameters

HL-60 and MEC-1 cells were cultured RPMI 1640 medium (Sigma-Aldrich, cat. No. R8758, Ireland) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, cat. no. F7524, Ireland) and 1% penicillin-streptomycin (Sigma-Aldrich, cat. No. P4333, Ireland). BL-2052 cells were cultured RPMI 1640 medium (Sigma-Aldrich, cat. No. R8758, Ireland) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate (Sigma-Aldrich, cat. No. S8636, Ireland) All cell lines were maintained at 37$^{o}$C, 5% $O_2$, and 5% $CO_2$ in a fully humidified atmosphere in a Thermo 3110 $CO_2$ Incubator (Thermo Fisher Scientific, UK).

## 2.3. Compound Preparation and Addition

The six novel compounds were synthesised by Dr Sarah Rawe at the School of Chemistry and Pharmaceutical Science, DIT, Ireland. All characteristic analysis was performed by her team at DIT, and is therefore not included in this study. The six novel compounds, along with the two reference compounds, DHA and CLB, were dissolved in 100% dimethyl sulphoxide (DMSO) to the following concentrations: 50mM (novel tetraoxanes RR5, RR6, RR7, SR9, SR10); 40mM (hybrid tetraoxane MK121; 100mM (reference compounds DHA and CLB). All stocks were divided into 20µl aliquots and stored at -80 $^{o}$C. On the day of each experiment, an aliquot of each compound was thawed and further dissolved in RPMI medium to a final concentration of 2X final desired concentration in 0.2% (v/v) DMSO before addition to cells. Cells were exposed to a range of concentrations for a set time period. DMSO concentration remained constant at 0.1% at all concentration ranges.

Cells were counted using a Z2 Particle analyser (Beckman Coulter, FL, USA) and seeded into 96-well plates (Sarstedt, cat. no. 83.3924.500, Ireland) in 100µl fresh medium at the

following seeding densities: 40,000 cells/well (HL60), 100,000 cells/well (MEC-1 & BL-2052). After a 24 hour period, 100µl of either fresh medium, 0.2% (v/v) DMSO in fresh medium (vehicle control), 400µM $H_2O_2$ (positive control), or 2X final concentration of compound, in 0.2% (v/v) DMSO and fresh medium, was added to the appropriate triplicate wells.

## 2.4. MTT Reduction Assay

Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich, cat. No. M2128, Ireland) is a routinely used colorimetric-based assay which measures metabolic activity of cells as a measure of proliferation and cytotoxicity (Riss & Moravec 2004). This assay is widely used for the determination of $IC_{50}$ values of compounds. The principle of this assay is the ability of viable, metabolising cells to reduce the water-soluble yellow tetrazolium salt to a violet-blue water-insoluble formazan precipitate (Schulze-Osthoff 2008). Once solubilised in an appropriate solvent, such as DMSO, the absorbance of formazan was spectrophotometrically measured at a wavelength of 584nm on a FLUOstar Optima 96-well plate reader (BMG Labtech, UK). The absorption of the dissolved formazan correlates to the number of metabolically active cells (Ulukaya et al. 2008).

Data from this assay was used to generate the $IC_{50}$ value for each compound at a 24 hour time point. Briefly, after the 24hr incubation with compound, 20µl MTT (5mg/ml), was added to each well and incubated for 2 hours (final MTT conc. on cells 0.5mg/ml). The 96-well plate was then centrifuged for 5 minutes at 157g at 4°C in a Jouan MR23i centrifuge (ThermoFisher, MA, USA) and the supernatant removed. 200µl DMSO was added to each well and incubated at room temp in the dark for 20 minutes. Absorbance was read at 584nm on an Optima plate reader.

After a preliminary screen of the compounds, the following concentration ranges were chosen for determination of $IC_{50}$ values for HL-60 cell line: 0-25µM (RR5, RR6, RR7, SR9, SR10), 0-5µM (MK121 & DHA), and 0-100µM (CLB). For MEC-1 and BL-2052 cells higher concentrations were required: 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0-40 µM (MK121) and 0-100 µM (DHA & CLB).

## 2.5. Lactate Dehydrogenase Assay

Lactate Dehydrogenase (LDH) is a cytoplasmic enzyme which is present in all cells, and its release into cell culture medium is used as a marker for the determination of cell lysis and necrosis (Riss & Moravec 2004; Schulze-Osthoff 2008). The Pierce LDH cytotoxicity assay kit (Bioscience, cat. No. 88954, Ireland) is a colorimetric method for determination of cell plasma membrane damage and leakage of LDH into the cell culture media (Vanden Berghe et al. 2013). LDH present in the cell culture medium converts lactate to pyruvate via the reduction of Nicotinamide Adenine Dinucleotide (NAD+) to NADH (reduced form). NADH is then used to reduce a tetrazolium salt to a red formazan product which can be read on a spectrophotometer at a wavelength of 485nm. The amount of formazan produced is directly proportional to the quantity of LDH in the cell culture media(Thermo Scientific 2014).

Cells were exposed to the same concentration ranges used for the MTT assay (see Section 2.4.) for each compound, 0.1% DMSO (vehicle control/spontaneous LDH release control) or maximum LDH release control (lysis buffer) in triplicate. After a 24hr incubation period, the 96-well plate was centrifuged using the Jouan MR23i centrifuge at 157g for 5 minutes at 4°C. 50µl cell culture medium was removed and added to a new 96 well plate. 50µl of reaction mixture was added to each plate mixed and then incubated at room temperature in the dark for 30 minutes. 50µl of stop solution was

added, and absorbance was read on the 96-well plate reader at 485nm. Percentage cytotoxicity was determined by subtracting the absorbance of the spontaneous LDH release control (vehicle control, 0.1% DMSO) from the absorbance of each sample, and expressing this value as a percentage of total LDH activity.

$$\%\ Cell\ Lysis = \frac{Compound\ treated\ LDH\ activity - spontaneous\ LDH\ control}{max.\ LDH\ control} X100$$

## 2.6. Flow Cytometry Analysis

### 2.6.1. Cell Viability Determination

Forward-angle light scatter is used as a measure of cell size, while side scatter represents the internal complexity of the cell (Brown & Wittwer 2000). On this basis, it is possible to distinguish between dead cells and live cells efficiently, with viable cells characterised by increased forward scatter and reduced side-scatter. Dead/apoptotic cells will exhibit high side scatter with decreased forward scatter (Preobrazhensky et al. 2001). However, this economic method of cell viability determination is not currently utilised for cell viability testing. In this study, the uses of forward scatter and side-scatter analysis, without the uses for vital dye, is investigated as a cell viability assay and compared to the routinely used MTT assay.

Briefly, after 24 hours of exposure to compound concentrations, cells were transferred to 0.5ml eppendorfs and centrifuged for 5 minutes at 120g at 4°C using the MIKRO 200R Centrifuge (Hettich Zentrifugen, Germany). The supernatant was removed and cells were re-suspended in 200µl 1X Binding buffer, each sample was then run on the BD Accuri C6 Flow Cytometer (BD Bioscience, Ireland), and gated according to viable/dead cells.

### 2.6.2. Apoptosis Detection

Early characteristics of apoptosis include the loss of asymmetry in the plasma membrane. In healthy, non-apoptotic cells, phospholipid phosphatidylserine (PS) is located on the inner leaflet of the membrane. However, with apoptotic cells, on the other hand, PS is translocated from the inner leaflet to the outer leaflet of the membrane, exposing it to the external cellular environment; this can be used as a marker of the apoptosis pathway (EBioscience 2016). Apoptosis can be measured efficiently by the using fluorescent dyes such as Annexin-V FITC and Propidium Iodide (PI). Annexin V is a $Ca^{2+}$ dependent phospholipid-binding protein with a high affinity for PS and binds to exposed PS on apoptotic cell membranes (EBioscience 2016). For flow cytometry analysis Annexin V can be attached to a fluorescent dye, such as FITC, and retain its high affinity for PS, therefore serving as a sensitive probe for analysis of cells undergoing apoptosis(Hingorani et al. 2011). Although cells in early stages of apoptosis have experienced PS translocation, membrane integrity is not lost until later stages of cell death occur. Annexin V is used in conjunction with a vital stain such as PI, as this dye is not permeable to live cells and therefore is only taken up by dead cells or cells in the later stages of apoptosis, where there has been a loss of membrane integrity (Invitrogen 2006).

The FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, cat. no. 556547, Ireland), was used as per protocol. Briefly, after exposure to compounds ($IC_{50}$ concentration as determined by MTT) flow cytometric analysis was performed at three time points; 12 hours, 24 hours and 48 hours. Cells were transferred to 0.5ml eppendorfs and centrifuged for 5 minutes at 120g at 4°C using the MILRO 200R Centrifuge. Supernatant was removed and 100µl 1X binding buffer added. 5µl FITC and 5µl PI dye was added to

appropriate tubes and incubated at room temperature, in the dark, for 15 minutes. 100µl of 1X binding buffer was added and samples were run on the BD Accuri C6 Flow Cytometer. For analysis, viable cells were identified as both Annexin V-FITC and PI negative (FITC-/PI-), whereas cells in early-stage apoptosis were Annexin V-FITC positive but PI negative (FITC+/PI-). Cells which are in late stage apoptosis or dead are positive for both Annexin V-FITC and PI (FITC+/PI+)and necrotic cells were detected as PI positive, Annexin V negative (FITC-/PI+) (Hingorani et al. 2011).

### 2.7. Statistical Analysis

MTT and LDH graphs and analysis were generated by nonlinear regression using GraphPad Prism version 5.03; standard curves were generated from the mean of triplicate experiments ± standard deviation (SD). Two-tailed unpaired t-test was used to identify statistically significant differences between non-cancer cell line (BL-2052) $IC_{50}$ values and HL-60 $IC_{50}$ values, with a p-value of less than 0.05 indicating statistical significance. One-tailed paired t-test was used to identify statistically significant differences between compound activity after 9 months of storage, p-value of less than 0.05 indicating statistical significance.

Flow cytometric data were processed using Microsoft Office Excel, results are expressed as mean ± SD. Two-tailed unpaired t-tests were used to identify statistically significant differences between vehicle control data and compound data for flow cytometry, with a p-value of less than 0.05 indicating statistical significance.

# 3. Results

## 3.1. Cell Viability- $IC_{50}$ Determination

### 3.1.1. Comparison of Three Cell Lines

Determination of $IC_{50}$ values for each compound provides an indication of the potent anti-cancer properties of the compounds. All six novel compounds showed evidence of promising anti-cancer properties on both leukaemic cell lines, MEC-1 and HL-60. The $IC_{50}$ concentration range for the five tetraoxane compounds on MEC-1 was between 22.7-47.8µM, with DHA also falling within this range (39.2µM). The hybrid compound MK121 showing excellent potency on this known-to-be resistant cell line, with the lowest $IC_{50}$ concentration of all eight compounds analysed, at 4.7 µM (see table 3). CLB was the least potent on the CLL cell line, at the 24 hour time point, giving an $IC_{50}$ value of 85.1µM. The $IC_{50}$ concentrations, for the HL-60 cell line, ranged from 6.0µM to 9.0µM for the five novel tetraoxane compounds. MK121, the tetraoxane hybrid compound, again presented the most potency of all six tetraoxanes with an $IC_{50}$ concentration of 2.6µM (table 3). These concentration ranges show promise as the $IC_{50}$ value for CLB on the same cell line was 29.6µM. DHA was the most potent out of the eight compounds for HL-60s with an $IC_{50}$ value of 0.9 µM. For the non-cancer cell line, BL-2052, $IC_{50}$ concentrations ranged from 8.8µM to >50 µM for the five novel tetraoxane compounds. MK121 IC50 concentration was calculated at 5.6 µM, showing less potent than the two leukaemia cell lines analysed.

All six novel tetraoxanes showed greater potency against HL-60 versus MEC-1, with RR5 having the biggest decrease in potency, with a 6-fold difference. Disappointingly, four of the five novel tetraoxanes analysed had a higher $IC_{50}$ concentration for the non-cancer cell line BL-2052 than MEC-1, with RR6 being the only compound showing possible

selectivity for MEC-1. MK121, the hybrid compound showed higher potency on MEC-1, however a statistically significant difference was not reached (p=0.2498, unpaired two-tailed t-test). Nevertheless, compounds ranked in order of selectivity, for HL-60 cells over BL-2052, indicate promising potential for all six novel compounds, with higher or equal selectivity to the currently used CLB drug (see table 4).

| $IC_{50}$ (μM) | HL-60 | MEC-1 | BL-2052 |
|---|---|---|---|
| DHA | 0.9(±0.04) | 39.2(±3.8) | 16.6(±0.9) |
| MK121 | 2.6(±0.4) | 4.7(±1.2) | 5.6(±1.0) |
| SR9 | 6.7(±0.1) | 22.7(±1.7) | 8.8(±3.4) |
| RR7 | 6.0(±0.7) | 33.3(±3.0) | 9.2(±0.3) |
| RR5 | 7.4(±0.9) | 45.5(±0.7) | 36.8(±2.0) |
| RR6 | 7.5(±1.9) | 37.6(±1.3) | >50 |
| SR10 | 9.0(±1.4) | 47.8(±4.6) | 30.2(±5.8) |
| CLB | 29.6(±7.3) | 85.1(±5.9) | 38.9(±3.7) |

**Table 3: $IC_{50}$ values calculated for MEC-1, HL-60, and BL2052 cells, respectively, from dose-response curves from MTT assay. Reference compounds are highlighted in grey. Table ranked in order of potency for HL-60 cell line.**

| Compound | Ranking | Non-cancer/ cancer ratio |
|---|---|---|
| DHA | 1 | 18.4 |
| RR6 | 2 | >6.7 |
| RR5 | 3 | 5.0 |
| SR10 | 4 | 3.4 |
| MK121 | 5 | 2.1 |
| RR7 | 6 | 1.5 |
| SR9 | 7 | 1.3 |
| CLB | 8 | 1.3 |

**Table 4: Compound selectivity. Non-cancer/cancer ratios were generated using $IC_{50}$ values for BL-2052 and HL-60 cell lines (Table 3). Higher non/cancer/cancer ratios indicate better cancer cell selectivity. Compounds are ranked (1-8) in order of highest selectivity.**

## MEC-1 Cell Line Analysis

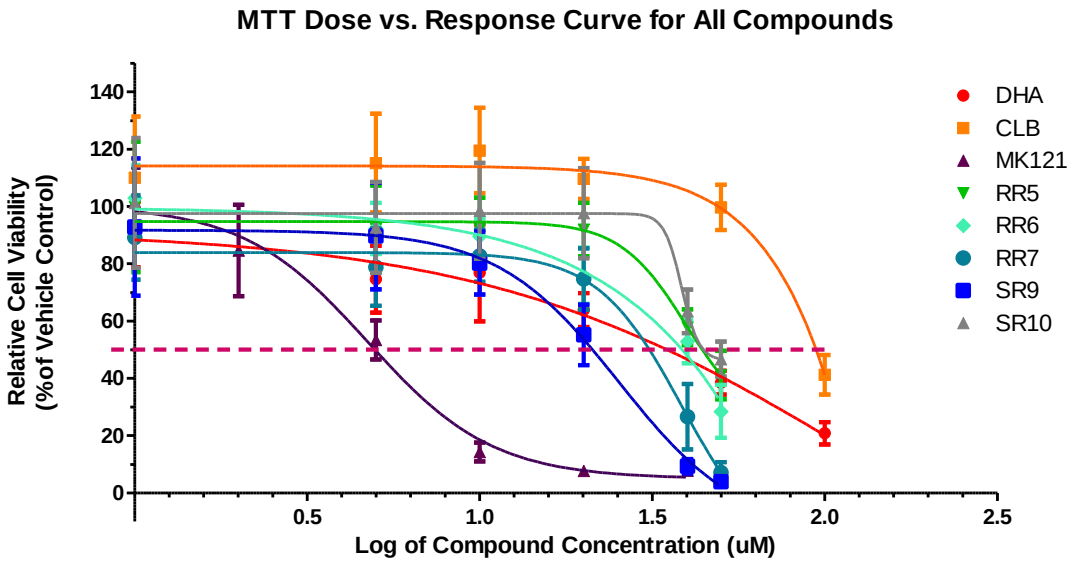



**Figure 7: Cell viability analysis of MEC-1 cells after exposure to compounds at the following concentration ranges; 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0-40 µM (MK121) and 0-100 µM (DHA & CLB). MEC-1 cells seeded at 100,000 cells/well, treated with compound for 24 hours and analysed using MTT Assay. Dose-response curve for all compounds constructed from absorbance @ 584nM and expressed as percentage viability of vehicle control. All data is representative of triplicate experiments (±SD).**

## HL-60 Cell Line Analysis

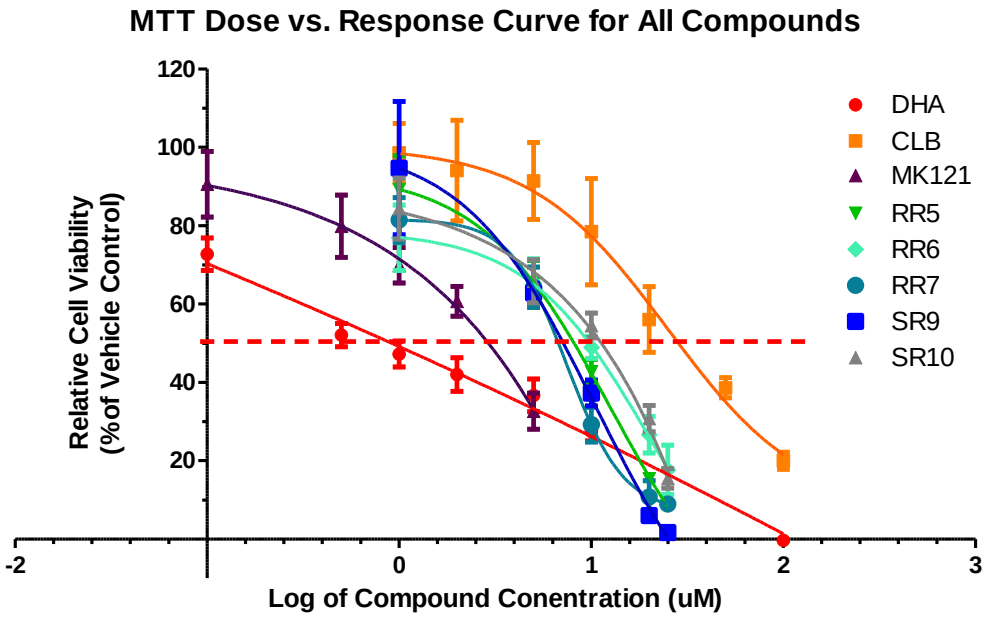



**Figure 8: MTT cell viability analysis of HL-60 cells after exposure to compounds at the following concentration ranges; 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0.1-5uM (MK121), 0.1-100uM (DHA) and 1-100uM (CLB).HL-60 cells seeded at 40,000 cells/well, treated with compound for 24 hours and analysed using MTT Assay. Dose-response curve for all compounds constructed from absorbance @ 584nM and expressed as percentage viability of vehicle control. All data is representative of triplicate experiments (±SD).**

**BL2052 Cell Line Analysis**

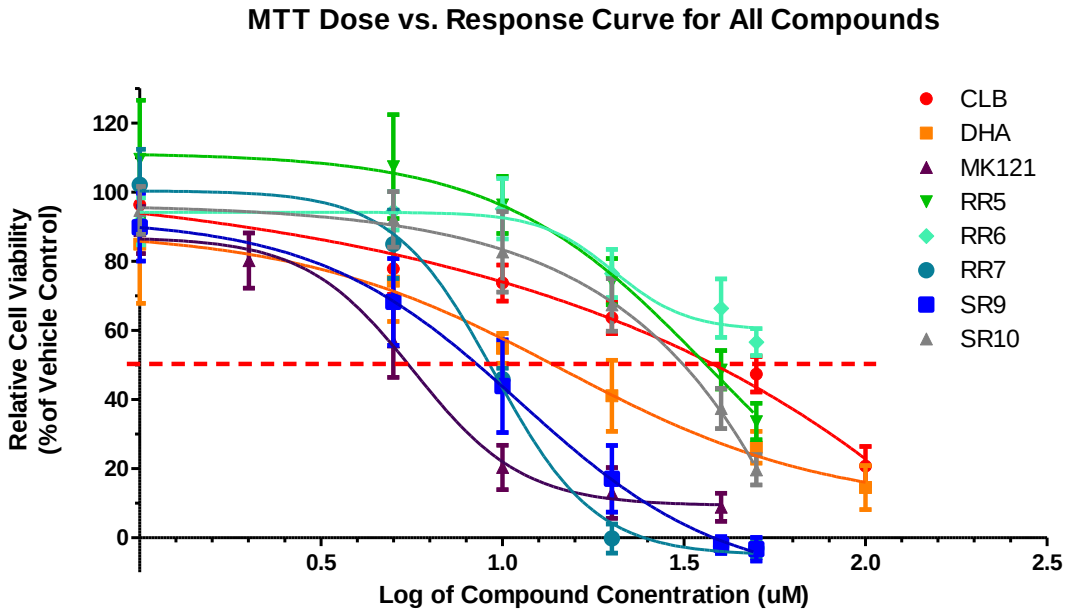



**Figure 9: Cell viability analysis of BL-2052 cells after exposure to compounds at the following concentration ranges; 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0-40 µM (MK121) and 0-100 µM (DHA & CLB). BL-2052 cells seeded at 100,000 cells/well, treated with compound for 24 hours and analysed using MTT Assay. Dose-response curve for all compounds constructed from absorbance @ 584nM and expressed as percentage viability of vehicle control. All data is representative of triplicate experiments (±SD).**

### 3.1.2. Flow Cytometric $IC_{50}$ Analysis- Cell Count (HL-60s)

A comparative study of cell count performed by flow cytometric analysis versus MTT for determination of $IC_{50}$ values was conducted. As seen in table 5 all $IC_{50}$ concentrations obtained via flow cytometric analysis were higher than the MTT assay. The variation observed between the $IC_{50}$ values obtained by the two methods is not unexpected as different end-points were analysed. However, the order of potency for all eight compounds screened was identical, therefore either method can be utilised for cell viability determination.

| HL-60 | MTT | | Flow- Cell Count | |
|---|---|---|---|---|
| | $IC_{50}$ (µM) | SD (µM) | $IC_{50}$ (µM) | SD (µM) |
| DHA | 0.9 | ±0.04 | 1.2 | ±0.6 |
| MK121 | 2.7 | ±0.4 | 3.7 | ±0.9 |
| RR7 | 6.0 | ±0.7 | 9.2 | ±1.8 |
| SR9 | 6.7 | ±0.1 | 10.6 | ±2.6 |
| RR5 | 7.4 | ±0.9 | 11.3 | ±0.6 |
| RR6 | 7.5 | ±1.9 | 12.9 | ±1.1 |
| SR10 | 9.0 | ±1.4 | 14.9 | ±2.6 |
| CLB | 29.6 | ±7.3 | 32.6 | ±2.6 |

**Table 5: $IC_{50}$ values calculated for HL-60 cells for all compounds from dose-response curves for MTT and Cell count experiments preformed on using flow cytometry, ranked in order of potency. Reference compounds are highlighted in grey.**

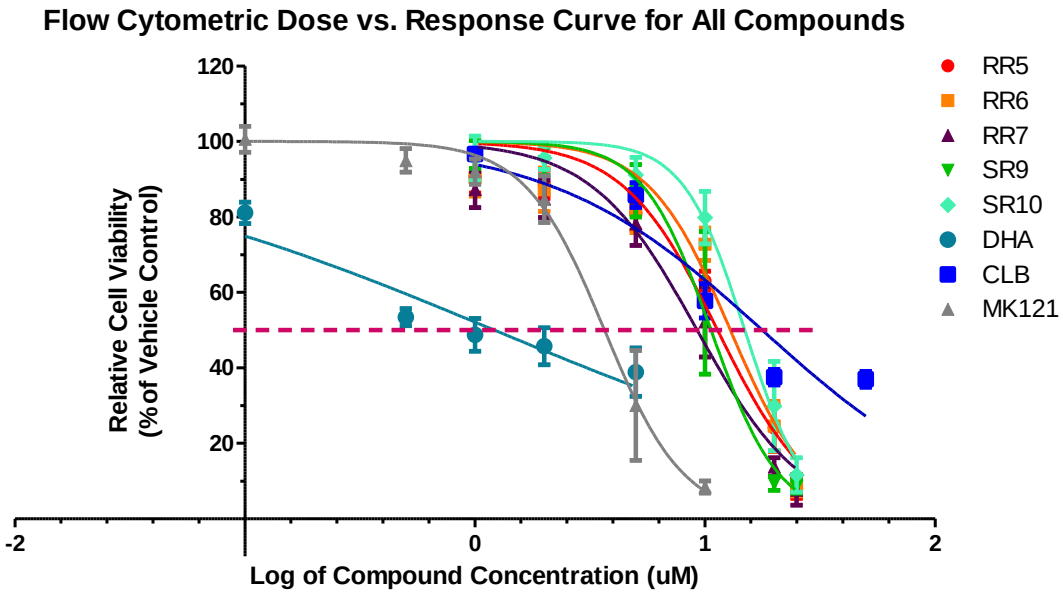



**Figure 10: Flow cytometric cell viability analysis of HL-60 cells after exposure to compounds at the following concentration ranges; 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0.1-5uM (MK121), 0.1-100uM (DHA) and 1-100uM (CLB). HL-60 cells seeded at 40,000 cells/well, treated with compound for 24 hours and analysed using flow cytometric cell count. Dose-response curve for all compounds constructed from counted viable cells and expressed as a percentage of vehicle control. All data is representative of triplicate experiments (±SD).**

## 3.2. Determination of Plasma Membrane Damage - LDH Assay

LDH present in the cell culture medium can be measured to determine the extent of plasma membrane damage, as a marker of irreversible cytotoxicity. In this study, this assay was used to determine the extent of plasma membrane damage induced by each compound after a 24-hour period. The $IC_{10}$ value was calculated, due to the low levels of LDH detected (see Figure 11). These results were compared to $IC_{10}$ values calculated from the MTT assay preformed on MEC-1 cells. For all compounds analysed, the $IC_{10}$ values were higher in the LDH assay, therefore indicating that the plasma membrane is not likely to be the primary target of these novel compounds at the concentration range analysed. However, results indicate that some irreversible damage is caused to the cells, with the hybrid compound again showing most potency.

As these results suggest plasma membrane damage was unlikely to be the main target for cytotoxicity caused by these novel compounds, the LDH assay was not carried out on further cell lines.

| MEC-1 | LDH Assay | | MTT Assay | |
|---|---|---|---|---|
| | $IC_{10}$ (µM) | SD (µM) | $IC_{10}$ (µM) | SD (µM) |
| MK121 | 3.7 | ±1.3 | 2.0 | ±0.8 |
| SR9 | 17.3 | ±2.0 | 11.0 | ±1.7 |
| DHA | 23.0 | ±3.4 | 2.3 | N/A |
| RR5 | 26.0 | ±2.2 | 18.8 | ±6.1 |
| SR10 | 33.4 | ±6.2 | 24.1 | ±9.5 |
| RR7 | 36.4 | ±1.6 | 14.0 | N/A |
| RR6 | 41.4 | ±3.1 | 8.9 | ±1.6 |
| CLB | 98.4 | ±1.3 | 59.3 | ±5.9 |

**Table 6: $IC_{10}$ values calculated for MEC-1 cells for all compounds from dose-response curves from LDH assay and MTT assay, respectively. Table ranked in order of potency ranked in order of potency as determined from LDH assay. N/A indicates $IC_{10}$ undetermined. Reference compounds are highlighted in grey.**

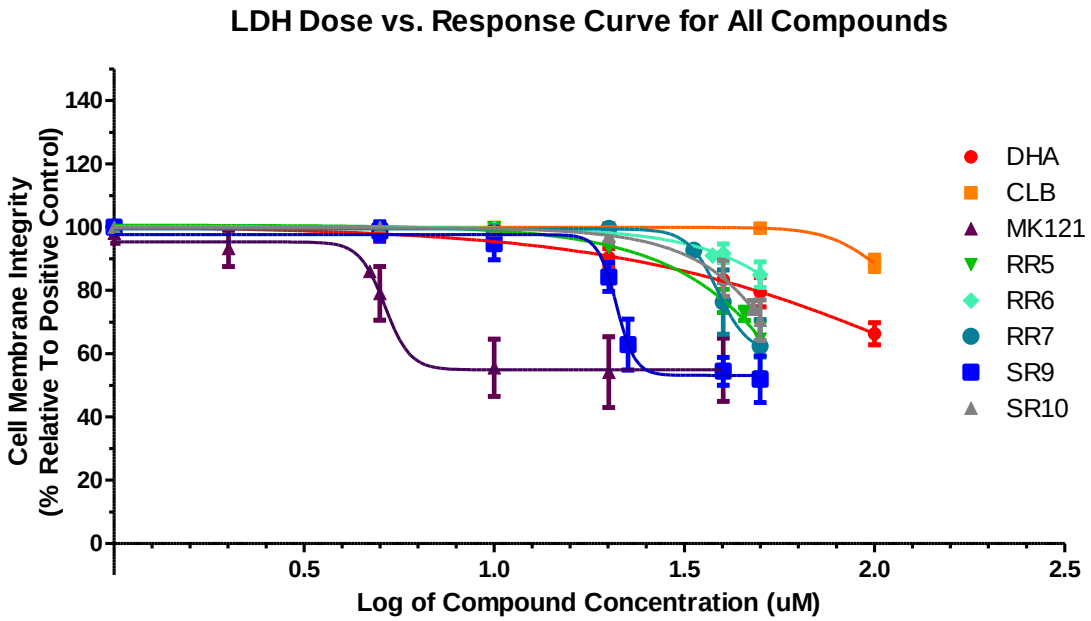



**Figure 11: Cell membrane integrity analysis of MEC-1 cells after exposure to compounds at the following concentration ranges; 0-50 µM (RR5, RR6, RR7, SR9, SR10), 0-40 µM (MK121) and 0-100 µM (DHA & CLB). MEC-1 cells seeded at 100,000 cells/well, treated with compound for 24 hours and analysed using LDH Assay. Dose-response curve for all compounds constructed from absorbance @ 485nM and expressed as percentage cell membrane integrity relative to lysis control. $IC_{10}$ concentrations determined as 90% relative membrane integrity. All data is representative of triplicate experiments (±SD).**

## 3.3. Flow Cytometric Analysis: Apoptosis Detection of Cell lines

MEC-1 and HL-60 cells were exposed to all eight compounds, at the $IC_{50}$ concentrations determined from the MTT assays, for various time periods, to indicate the time frame in which each compound induced cytotoxic effects on the cell line. It is important to consider the overall cell numbers analysed in each sample, for the compounds, to give a better indication of cell numbers in each population. This gives a clearer overview of the cell population and growth rate during exposure. To achieve this, the number of cells in each population was expressed as a percentage of the total number of cells analysed in the vehicle control population. This provides more relevant results for comparison of each sample to the vehicle control analysed in each assay. These results can be seen in figure 12 and figure 13 for MEC-1 and HL-60, respectively. The graphs illustrate the reduction of cell number in the live, apoptotic and death cell populations over the course of time.

For the MEC-1 cell line, a decrease in live cell numbers can be noted across all three time points for all compounds screened (Figure 12). The hybrid compound, MK121 has the most immediate effect, with approximately 55% live cells after the first 12 hours of exposure. However, over the 24 and 48-hour time points this population is only further reduced by 10%. The remaining tetraoxane compounds reduced cell numbers in the live cell population gradually over the 48-hour timeframe. All compounds showed a much greater effect on the HL-60 cell line, with the $IC_{50}$ concentrations showing a reduction of more than 50% by the 24 hour time point. Further effects of their cytotoxic properties can be observed by 48 hours, with less than 20% viable cells in all cell populations analysed (Figure 13). The cytotoxicity induced in the 24 to 48 hour timeframe is notably greater on the HL-60 cell line in comparison to MEC-1.

**MEC-1 Cell Line Analysis**

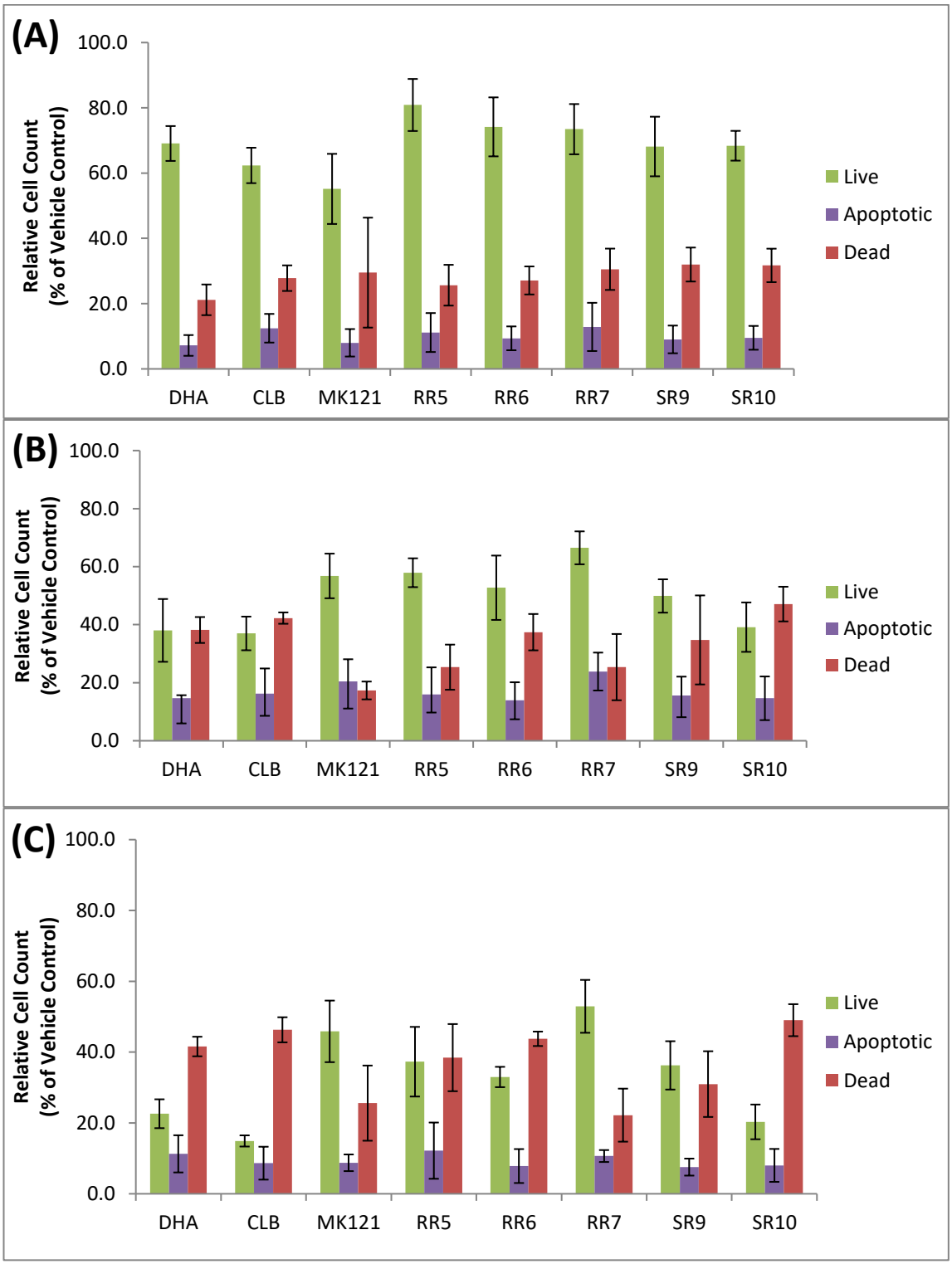



**Figure 12: Flow cytometry analysis of MEC-1 cells after exposure to compounds at $IC_{50}$ concentrations (as determined by MTT). MEC-1 cells were seeded at 100,000 cells/well, treated with compound for various time periods (A=12Hr, B=24Hr & C=48Hr) and analysed using the Annexin-V apoptosis kit. Flow cytometric data expressed as percentage cell count relative to total cells present in the vehicle control; live (FITC-/PI-), apoptotic (FITC+/PI-), dead via apoptosis (FITC+/PI+) and necrotic (FITC-/ PI+). All data is representative of triplicate experiments (±SD).**

**HL-60 Cell Line Analysis**

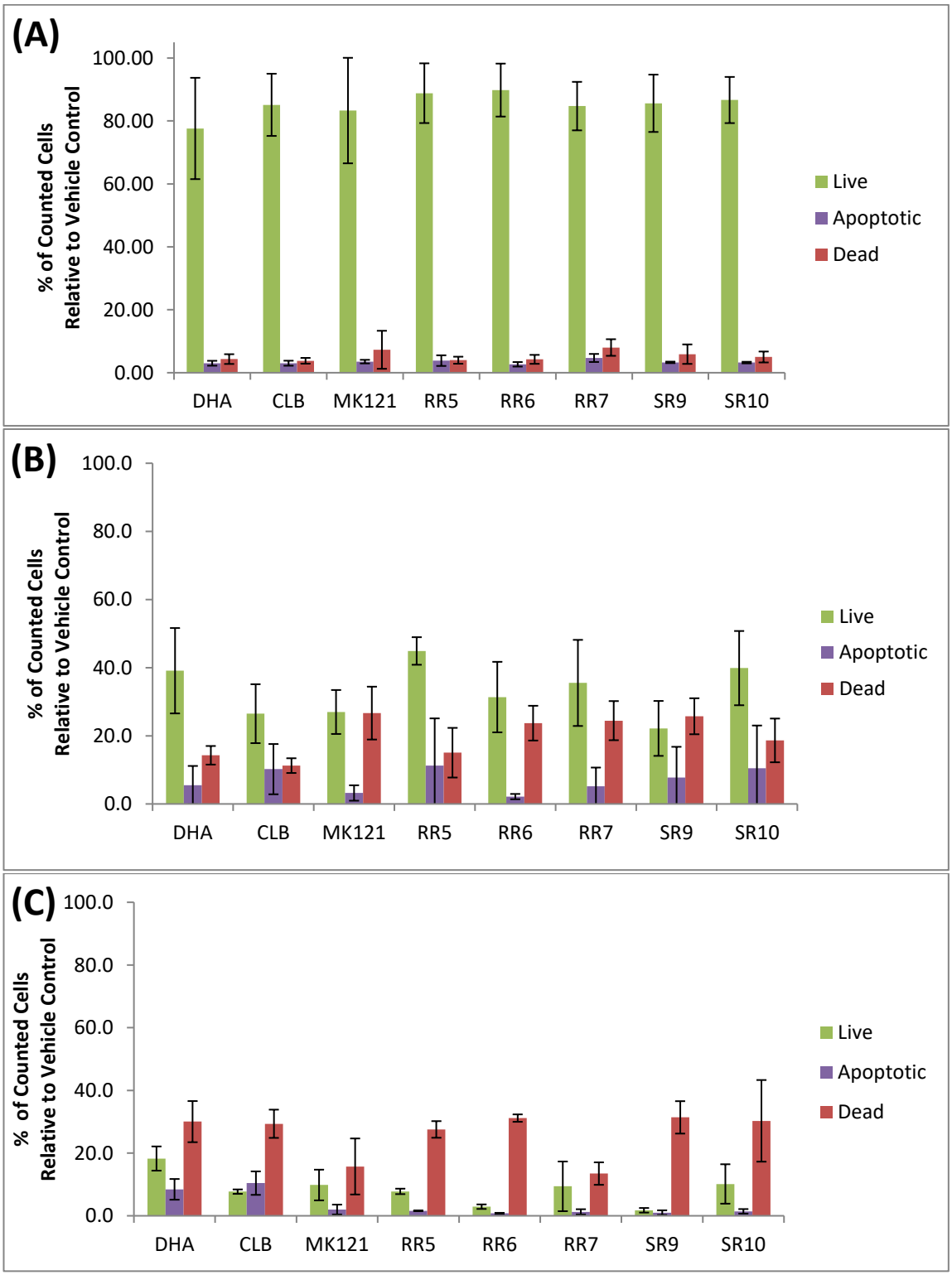



**Figure 13: Flow cytometry analysis of HL-60 cells after exposure to compounds at $IC_{50}$ concentrations (as determined by MTT). HL-60 cells were seeded at 40,000 cells/well, treated with compound for various time periods (A=12Hr, B=24Hr & C=48Hr) and analysed using the Annexin-V apoptosis kit. Flow cytometric data expressed as percentage cell count relative to total cells present in the vehicle control; live (FITC-/PI-), apoptotic (FITC+/PI-), dead via apoptosis (FITC+/PI+) and necrotic (FITC-/ PI+). Data is representative of triplicate experiments (±SD), except compounds RR5, RR6 and MK121 are represented in duplicate.**

## 3.4. Compound Stability – MTT $IC_{50}$ Re-evaluation (HL-60s)

Re-evaluation of novel compounds' $IC_{50}$ concentrations was performed after 9 months to determine compound stability and degradation. Aliquots of compounds, dissolved in 100% DMSO, were stored for 9 months at -80°C before they were re-evaluated for anti-proliferation activity using MTT on HL-60s. Statistically significant degradation of all six novel compounds was noted (P≤0.05, paired one-tailed t-test), with RR6 showing the highest level of degradation with over 4-fold reduction in activity (see table 7). There was a 2 fold increase in the $IC_{50}$ values of the remaining five compounds. These findings demonstrate the importance of compounds stability testing and in the case of this study, the significance of using freshly dissolved compound solutions for relevant and accurate screening of compounds.

| $IC_{50}$ (µM) | Nov 2017 | Aug 2018 | P value |
|---|---|---|---|
| MK121 | 2.6(±0.4) | 4.9(±0.5) | 0.0281 |
| RR5 | 7.4(±0.9) | 14.1(±1.4) | 0.0023 |
| RR6 | 7.5(±1.9) | 33.1(±4.5) | 0.0084 |
| RR7 | 6.0(±0.7) | 12.1(±1.3) | 0.0026 |
| SR9 | 6.7(±0.1) | 11.2(±1.4) | 0.0125 |
| SR10 | 9.0(±1.4) | 19.9(±0.7) | 0.0017 |

**Table 7: $IC_{50}$ values calculated for novel compounds before and after nine months storage @ -80 degrees in 100% DMSO, from dose-response curves from MTT assay. Concentrations calculated on HL-60 cell line at the following concentration ranges: 0-50µM (RR5, RR6, RR7, SR9, SR10) AND 0-40µM (MK121). Paired, one-tailed t-test, statistically significant p<0.05.**

# 4. Discussion

## 4.1. Incubation Parameters

To date, with *in vitro* studies, one parameter which is frequently overlooked is the oxygen levels in which cells are maintained. Routine cell culturing is typically conducted in $CO_2$ incubators which are maintained at 37°C, 5% (v/v) $CO_2$ and 95% humidity, the oxygen level in these incubators is atmospheric oxygen (21% (v/v) or 160mm Hg). However, the physiological oxygen levels of cells *in viv*o is much lower, ranging from 13% to 3% depending on the location of the cells (McKeown 2014). An $O_2$ level of 5% (40mm Hg) has been previously established as the physiological $O_2$ levels available to blood cells *in vivo* (Chapple et al. 2016; Atkuri et al. 2007). Numerous studies have been conducted to investigate the effect of various $O_2$ levels, such as atmospheric $O_2$ (21% or 160mm Hg) and $O_2$ levels which cells are naturally exposed to *in-vivo*, on both commercial cell lines and primary cell cultures. These studies show the importance of using the correct $O_2$ level to closer mimic that of *in-vivo* conditions (Chapple et al. 2016; Keeley et al. 2017; Murray et al. 2014; Atkuri et al. 2005). Our research group previously reported a significant increase in efficacy of Artesunate when evaluated at oxygen levels representative of *in vivo* conditions, in comparison to atmospheric oxygen levels routinely used for *in vitro* analysis (Murray et al. 2014).

All cell culture conducted throughout this study was maintained at the physiologically relevant $O_2$ level of 5% (v/v), a representative of normoxic oxygen levels of human venous blood (Atkuri et al. 2007). This was achieved using an oxygen controlled Thermo 3110 $CO_2$ Incubator.

## 4.2. $IC_{50}$ Detection Method Comparison

Two methods for the determination of $IC_{50}$ values were performed and compared using the HL-60 cell line. MTT, a routinely used assay for the identification of $IC_{50}$ concentrations was compared to a less utilised method of cell count using the Accuri C6 flow cytometer. As mentioned in Section 2.6.1., the flow cytometer has the ability to analyse single cells to identify viability of an overall population of cells using light scatter. As seen in Section 3.1.2. Table 5, the $IC_{50}$ values determined for each compound, using the two methods, were comparable with marginally higher concentrations obtained for all compounds using the flow cytometry technique. A difference between the $IC_{50}$ values was expected as the two methods analysis different endpoints. The endpoint assessed with the MTT assay is the reduction of the yellow tetrazolium salt by dehydrogenase in viable, metabolic cells, therefore measuring the metabolic activity of the cell population (Stockert et al. 2012). Flow cytometry uses light scatter as a detection method for cell viability. Changes in cell size and density affect the light scatter detected, this property is utilised as a classification method for detection of viable and non-viable cells within a cell population. Increased cellular density and a reduction in cell size are characteristic properties of the apoptotic cell death pathway (Pietkiewicz et al. 2015). A live, viable cell is recorded irrespective of how metabolically active it is via this method, whereas with the MTT assay viable but less active cells register a lower MTT reading than viable, actively metabolising cells. Both methods derived the same order of potency for all eight compounds, therefore either method could be utilised for the determination of $IC_{50}$ concentrations. For this study, the MTT assay was employed for $IC_{50}$ determination of the remaining cell lines, as it is a more efficient method which is better recognised, and fits the guidelines of the biocompatibility ISO recommendations.

### 4.3. Anti-Proliferation Potential of Novel Tetraoxanes

Cell cytotoxicity evaluation was performed in according's with the ISO international standards, which requires that two tests be chosen for determination of cytotoxic effects induced by test compounds. The two assays used were MTT, a measure of inhibition of cell growth, and LDH for evaluation of cell lysis and membrane integrity. For this study two reference compounds were used as a comparison to the novel compounds to help identify the anti-cancer potential of each compound relative to *a)* a compound currently used in routine treatment of CLL (CLB) and *b)* an endoperoxide compound proven to have anti-cancer properties (DHA).

From the six novel compounds analysed in this study the most potent compound is the tetraoxane-naphthalimide hybrid, MK121. For the MEC-1 cell line, the $IC_{50}$ concentration for MK121 was 8-fold and 17-fold more potent than DHA and CLB, respectively, with all six of the tetraoxanes showing good potency against the MEC-1 cell line. This cell line, derived from a CLL patient with poor-prognostic factors, inherits the del(17p) deletion associated with poor response to conventional chemotherapy agents (Stacchini et al. 1999). Although the concentration of DHA which gave 50% inhibition of HL-60 cells was lower than that of the novel compounds screened at a concentration of 0.9µM (table 3), the tetraoxane compounds still showed promising activity in relation to the currently used chemotherapeutic agent CLB on HL-60s, with CLB presenting as the less potent compound tested.

It is not unexpected that all the compounds investigated in this study were more sensitive to HL-60s over the MEC-1 cell line. HL-60 cells have been proven to be highly sensitive to other endoperoxide-containing compounds, while the MEC-1 cell line inherits the del17p mutation, associated with resistance to chemotherapeutic drugs

(Efferth et al. 2001; Stacchini et al. 1999). As mentioned previously (see Section 1.2.3.), the del17p mutation is associated with the disruption of the TP53 gene, responsible for the encoding of the pivotal p53 tumour suppressor protein. This protein plays a key role in regulating signalling pathways such as cell cycle arrest, DNA repair and apoptosis (Rivlin et al. 2011). The absence of this master regulator in the MEC-1 cell line may explain the significant reduced cytotoxic effects of these tetraoxane compounds on the cell line.

Screening of compounds on the non-cancer cell line, BL-2052, was carried out to evaluate the potential therapeutic window of each agent. Unfortunately, in comparison to the 'hard-to-treat' MEC-1 cell line, only one of the five novel tetraoxane compounds, RR6, were significantly less potent on BL-2052 (see Section 3.1.1. table 3). MK121 presented more potent on MEC-1 but the therapeutic window did not reach a statistically significant difference ($p=0.2498$, unpaired two-tailed t-test). Although these results are discouraging, the proven resistance of CLL with a Del17p mutation must be considered, and therefore the potential of these compounds to selectively treat other forms of leukaemia should not be ruled out. In comparison to HL-60s, all compounds screened, except SR9 and CLB, are significantly less toxic on BL-2052 ($p<0.05$, unpaired two-tailed t-test). Interestingly, CLB, the routinely used leukaemia therapeutic drug, was significantly more toxic on the non-cancerous cell line than MEC-1, with $IC_{50}$ concentrations of 38.9µM and 85.6µM, respectively, with no significant difference in the toxicity of BL2052 versus HL-60 ($p=0.1128$, unpaired two-tailed t-test). Known side-effects of CLB include, bone marrow suppression, peripheral neuropathy and hepatotoxicity (GlaxoSmithKline 2006). Novel compounds with more selectivity to

cancer cells hold potential to reduce the adverse effects associated with currently used therapeutics such as CLB.

Conversion of HL-60 and BL-2052 $IC_{50}$ values to a non-cancer/cancer ratio provides an indication of compound selectivity. Interestingly, five out of the six novel compounds show selectivity superior to CLB (RR6, RR5, RR7, SR10 and MK121), with SR9 presenting an equal therapeutic window (Section 3.1.1., table 4). With compounds showing higher potency and more selectivity than the currently utilised leukaemia drug CLB, there is excellent promise for the uses of these compounds for more effective treatments with less adverse effects. The reason for the higher selectivity of the novel compounds is yet to be determined, and while the exact mechanism of tetraoxanes is still unclear, reactive oxygen species (ROS) are believed to be involved in the anti-cancer properties of tetraoxanes (Kumura et al. 2009; Opsenica et al. 2006). Cancer cells are known to have naturally higher ROS levels than non-cancer cells, making cancer cells more susceptible to oxidative stress (Zitka et al. 2012). Therefore, an increase in ROS levels within cancer cells is less tolerable and can induce apoptosis at lower levels than non-cancer cells. With this knowledge ROS levels can be used as an effective target for cancer cells, with many studies haven already proven this phenomenon (Troyano et al. 2001; Englert & Shacter 2002). It is also possible that elevated levels of intracellular iron, naturally present in cancer cells, causes an increase in the endoperoxide cleavage associated with tetraoxane compounds (Lai et al. 2013).

Disruption of cell membrane integrity was analysed using the LDH assay. From the results obtained $IC_{10}$ concentrations were generated. These results indicate that, within the range analysed for determination of $IC_{50}$ by MTT, plasma membrane damage is unlikely to be not a primary target for the compounds screened. It is however

noteworthy that MK121 shows a significantly higher level of lysis induced at lower concentrations than all other compounds (see Section 3.2., table 6). These finding may suggest the hybrid compound, has a different mode of action to the other compounds analysed. This was to be expected as hybrid compounds are synthesised with the aim of synergising multiple modes of actions to utilise the unique modes of actions relative to each parent drug, leading to greater potency (Kennedy 2012).

### 4.4. Indication of Cell Death Pathway

To identify the pathway of cell death induced by the six novel compounds analysed in this study, apoptosis detection was executed with the use of the Annexin-V apoptosis detection kit. With the MEC-1 cell line, a significant decrease in viable cells is present for MK121, SR9 and SR10 ($p<0.05$ two-tailed, unpaired t-test), after 12 hours, with small but not statistically significant reductions evident with the remaining tetraoxanes (see Section 3.3., figure 12). For HL-60 cells, no significant toxicity is detectable at the 12 hour time point (Section 3.3., figure 13). By 24 hours, a statistically significant reduction in viable cells is detected across all compounds, for both cell lines ($p<0.05$, unpaired two-tailed t-test). By 48 hours, with HL-60 cells have a higher percentage of dead cells were detected than viable cells. MEC-1 cells were also inhibited further by the 48-hour time point for all compounds screened. However, the effectiveness of the novel compounds is significantly less against MEC-1 than HL-60 cells during the 24-48 hour time period. These results imply compound activity after 24 hours and indicate that longer exposure times could reduce the $IC_{50}$ concentrations further, depending on the cell line tested.

The detection of apoptotic cells by flow cytometry, along with low levels of membrane damage detected by LDH, indicate the cell death pathway induced by these novel compounds is likely to be apoptosis, rather than necrosis. The detection of cell shrinkage

and intracellular organelle condensation, characteristics of apoptosis, by light scatter via flow cytometry also provides added assurance of apoptotic cell death (Edinger & Thompson 2004). This is the cell death pathway that was expected to be induced by these novel tetraoxane compounds, as numerous studies conducted on DHA and other tetraoxane compounds showed apoptotic cell death (Opsenica et al. 2000; Handrick et al. 2010).

### 4.5. Compound Stability

An important analysis parameter for all novel compounds is compound stability. Stability testing is used to evaluate expiry dates, and a beyond-use date. Evaluation of the compounds stability in solution of 100% DMSO was performed using MTT. Re-evaluation of $IC_{50}$ values after a 9 month period of storage at -80°C indicates a significant reduction of compound activity for all six novel compounds (see Section 3.4., table 7). Notable reduction in compound activity suggests degradation of compound structure, which will have a significant effect of reproducibility of results. The significantly reduced activity of these novel compounds over this period is surprising, as tetraoxane compounds are generally well known to be thermodynamically stable (Opsenica et al. 2008; Kumar et al. 2010). However, DHA has been previously shown to be highly unstable under various test conditions. A study conducted by Parapini *et al.* in 2015 confirmed a loss in biological activity and chemical degradation of DHA was pH, time and temperature dependent (Parapini et al. 2015). These findings show that compound stability testing is critical to ensure accurate potency testing of novel compounds.

# 5. Conclusion

All six novel compounds have been proven to possess excellent anti-proliferation properties, with $IC_{50}$ concentrations significantly lower than CLB. The novel tetraoxane compounds also showed superior selectivity toward the leukaemic cell line HL-60 over the non-cancerous cell line analysed BL-2052. However, when BL-2052 was compared to MEC-1 the findings were not as auspicious. Nevertheless, the findings in this study suggest potential for these novel tetraoxane compounds, in the search for new therapeutic drugs, with five out of six showing most selectivity for cancer cells than the currently used drug CLB. The higher potency and superior selectivity of these compounds for HL-60s, when compared to the currently used chemotherapeutic CLB, present the possibility of more effective treatment agents, with the potential of drastically reduced adverse side effects. This study presents the potential of these never-before-tested compounds in the never ending search for better therapeutic drugs, to help vastly improve patient quality of life and morbidity during a prolonged and arduous undertaking.

Membrane damage was not indicated as a primary target for cytotoxicity, with all compounds showing characteristic properties of apoptotic cell death, via flow cytometric analysis. Evidences of significant compound degradation while dissolved in solution of 100% DMSO, stored at -80 degrees, indicates the need for further compound stability testing to be performed on the compounds, with caution needed when analysing compounds after a period of storage. While RR6 showed the best selectivity for cancer cells, it must be considered a possibility this result is contrived by compound degradation, as a 4-fold decrease in RR6 activity after 9 months storage was detected.

Further analysis is required to confirm the selectivity of the novel compounds to cancer cells and determine the exact mechanism of actions for these novel tetraoxane compounds. This will provide valuable information on the potential of these compounds as chemotherapeutic agents. While all six compounds showed potential as novel therapeutic agents, making it difficult to rule out any at this stage, hybrid compound MK121, along with tetraoxanes RR5, RR6 and SR10, displayed superior selectivity over CLB.

# 6. Proposed Future Work

Further investigation into compound stability is essential these novel tetraoxanes, with HPLC highly recommended to generate a stability profile. A variety of tests need to be utilised to gain important information of the mechanism of actions behind the novel compounds. ROS is believed to be the primary inducer of apoptotic cell death associated with endoperoxides. Total ROS, total Glutathione (GSH) and the ratio of oxidised GSH (GSSG) to GSH provide a measure of the levels of ROS in cells after exposure to compounds. ATP determination for distinguishing between cytostatic and cytocidal effect is also recommended. This assay would provide additional information on the lethality of compounds. DNA damage, another biochemical hallmark associated with ART-induced apoptosis, needs to be analysed for relative expression of markers for single-stranded and double-strand DNA repair. Mitochondrial DNA damage analysis could also be performed as this is a particularly sensitive early marker for DNA damage.

Primary leukaemia cell analysis would provide more relative information on the effectiveness of the compounds *in vivo.* It would also be useful to conduct a comparative study of the non-cancer cell line BL-2052 against healthy non-cancerous primary B-cells to determine the reliability of using an immortalised commercial non-cancer cell line for selectivity studies. It would also be a recommendation to test the novel tetraoxanes on a liver cell line, as this could provide vital information on compound selectivity and possible side effects linked to such compounds.

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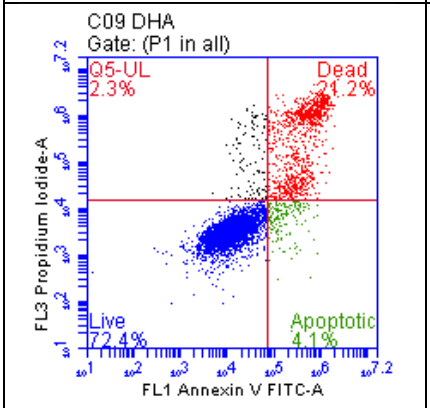
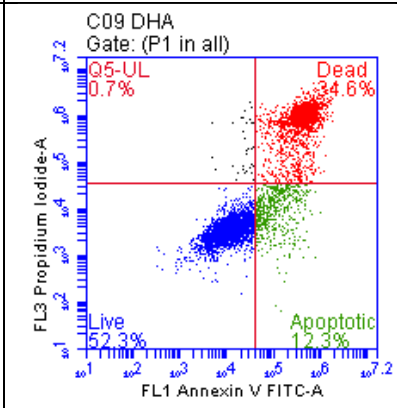
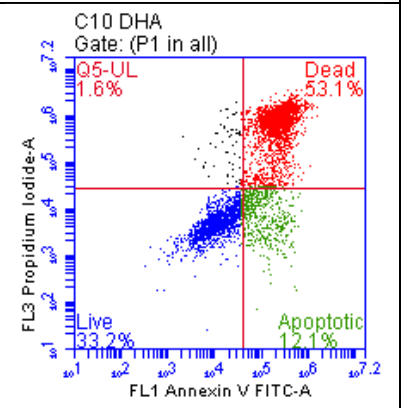
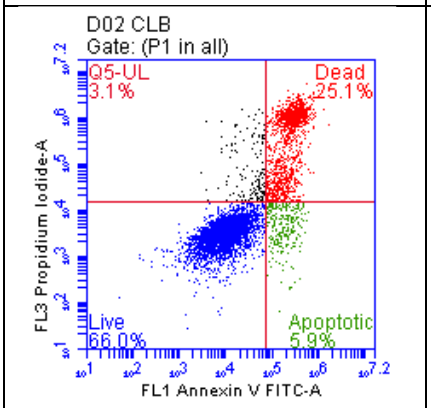
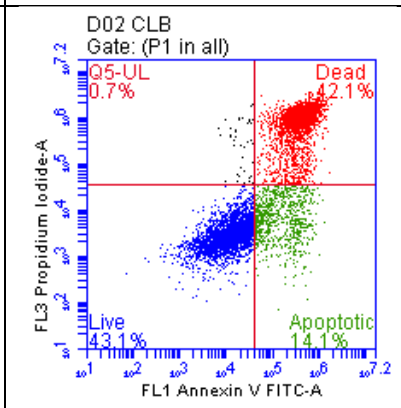
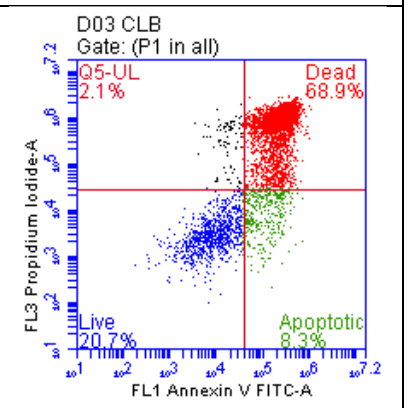
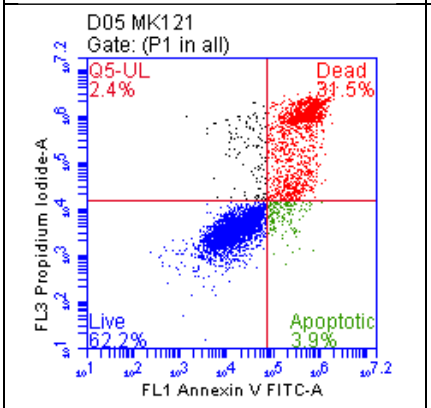
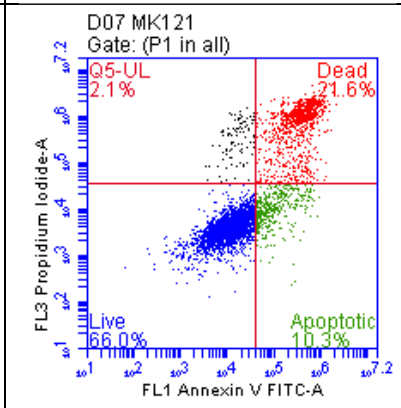
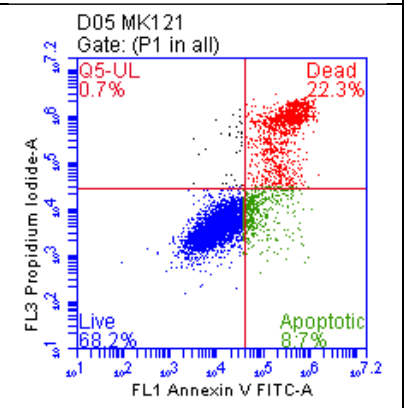
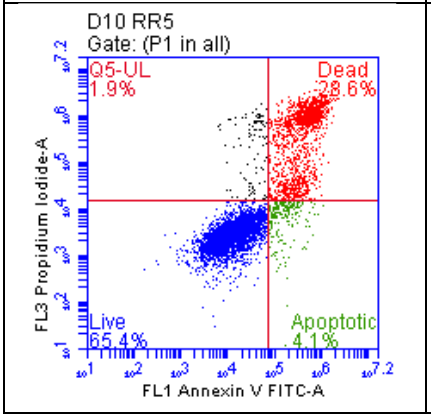
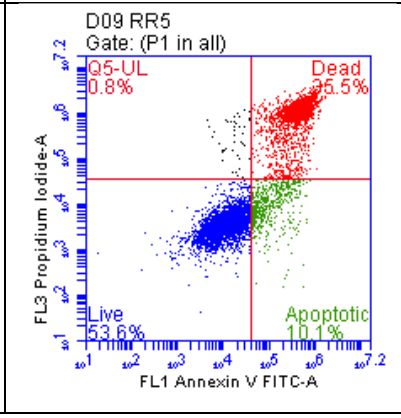
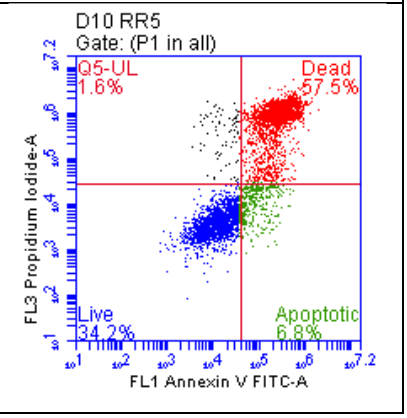
# 8. Appendices

## 8.1. Flow Cytometric Analysis:

### 8.1.1. Apoptosis Detection of MEC-1 at various time points

| 12 Hours | 24 Hours | 48 Hours |
|---|---|---|
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

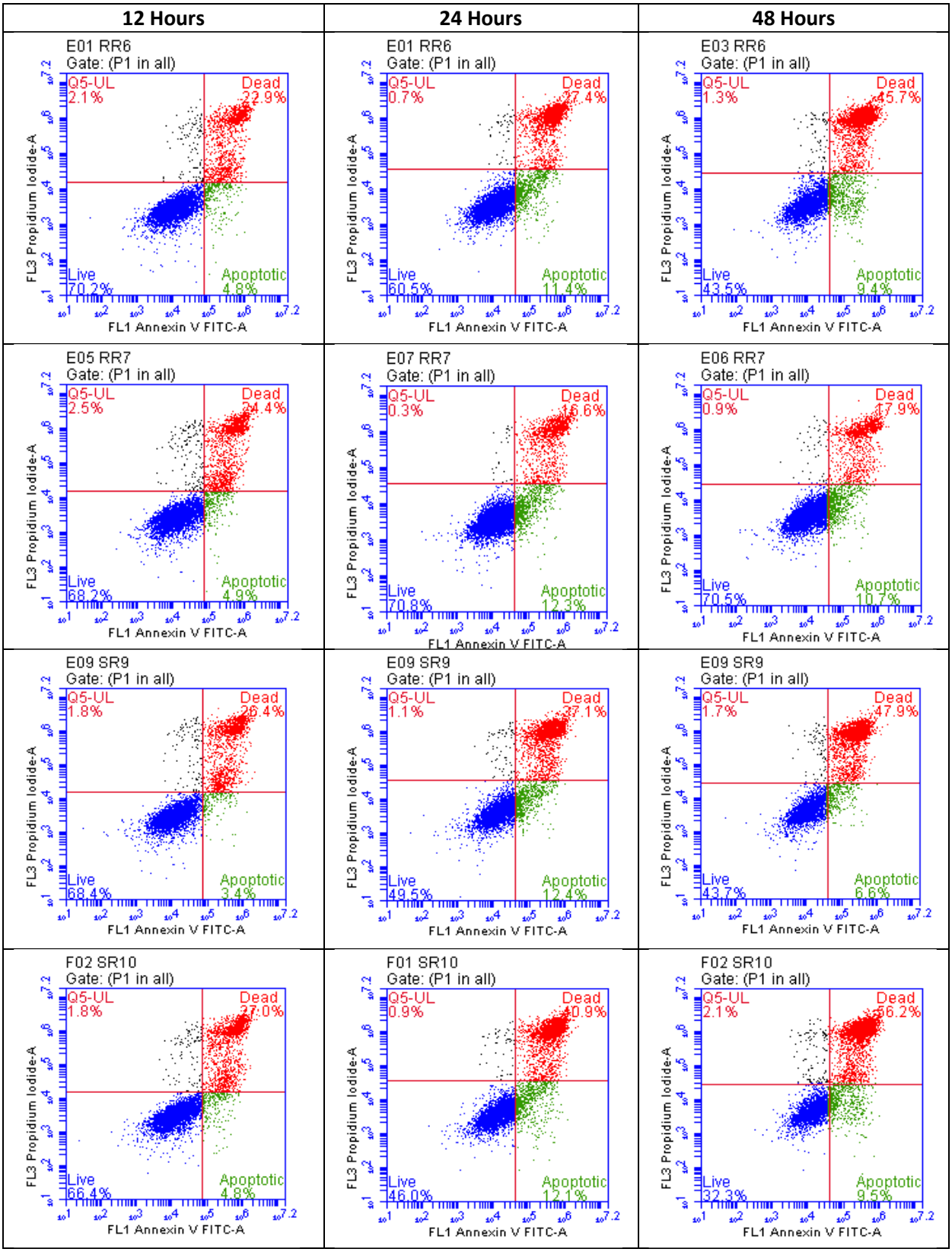
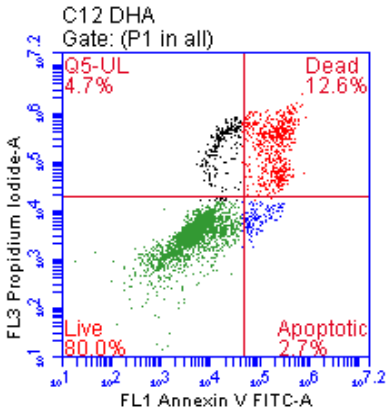
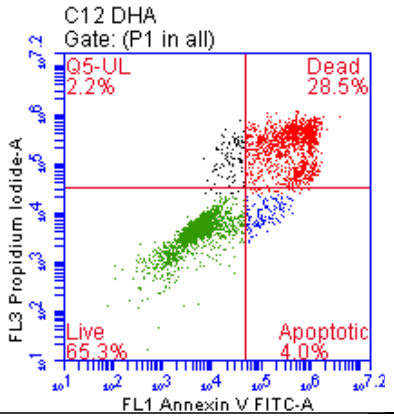
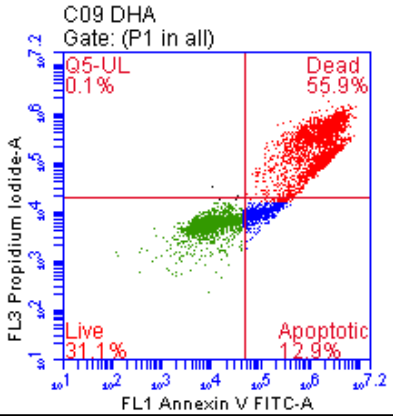
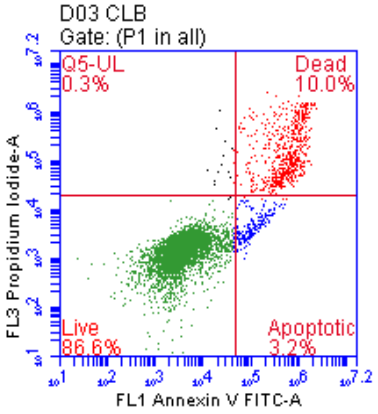
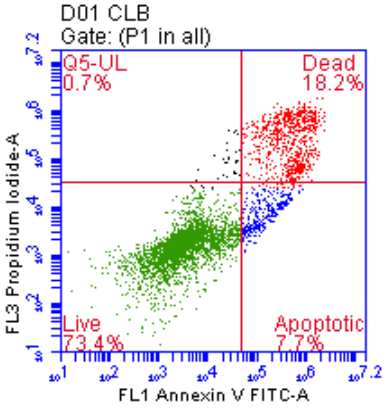
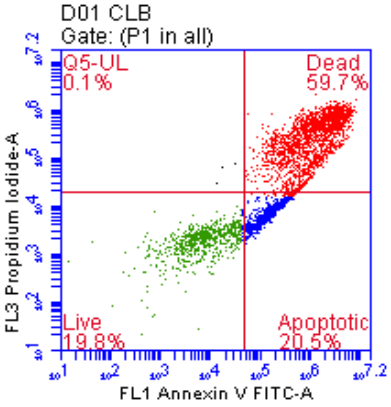
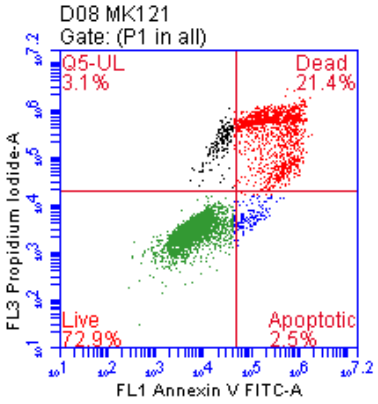
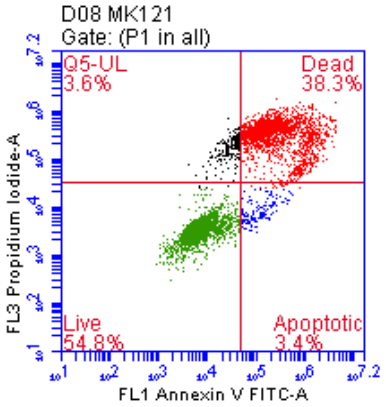
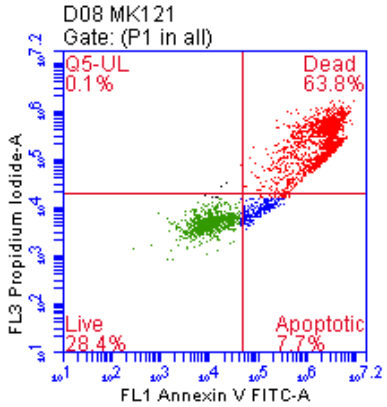
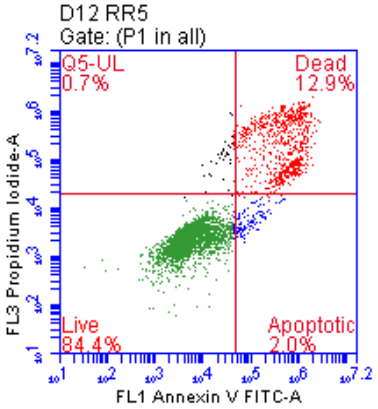
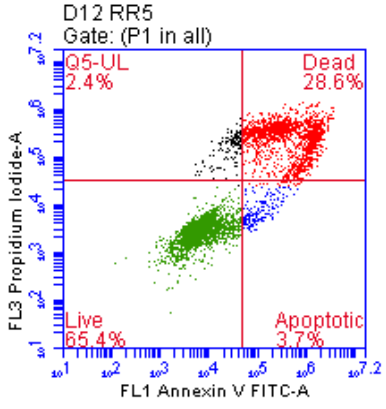
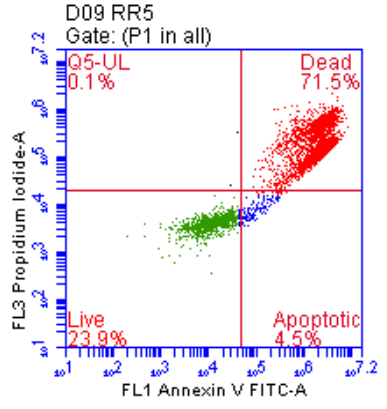



**Table 8: Dot plot of FITC-Annexin V/PI flow cytometric results of MEC-1 cells after various time points (12, 24 and 48 hours) of exposure to $IC_{50}$ concentrations of each compound (as determined by MTT assay). MEC-1 cells were seeded at 100,000 cells/well, treated with compounds and analysed at various time points using the Annexin-V apoptosis kit. A total of 10,000 events were counted for each sample and gated to exclude background/debris. Appropriate controls were used to gate the relative cell population. The lower left quadrants of each plot (Live) represents viable cells per sample (FITC-/PI-), the lower right quadrant (Apoptotic) shows FITC+/PI- cell population and the upper right quadrant shows late apoptotic/dead cells (FITC+/PI+). One representative experiment out of three is shown for each time point.**

### 8.1.2. Apoptosis detection of HL-60 at various time points

| 12 Hours | 24 Hours | 48 Hours |
|---|---|---|
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

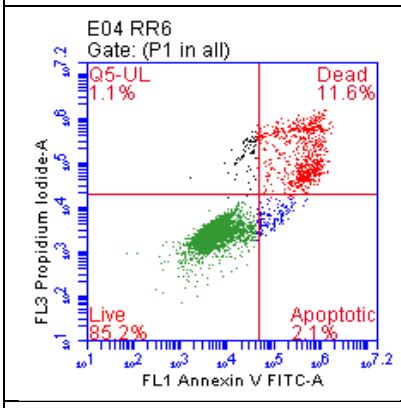
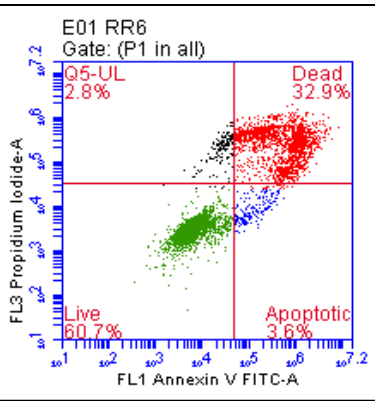
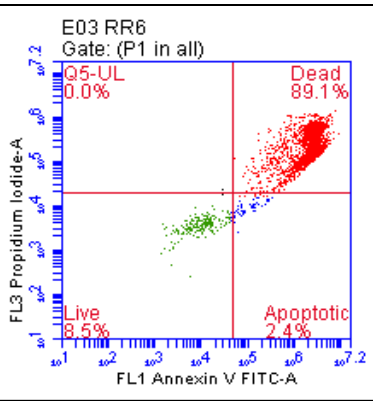
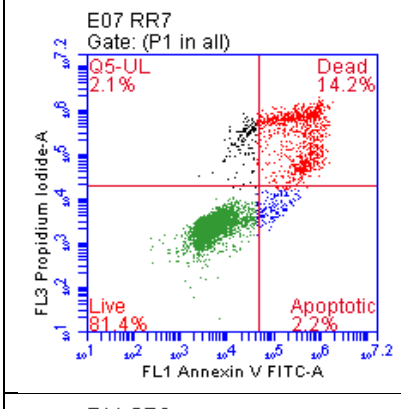
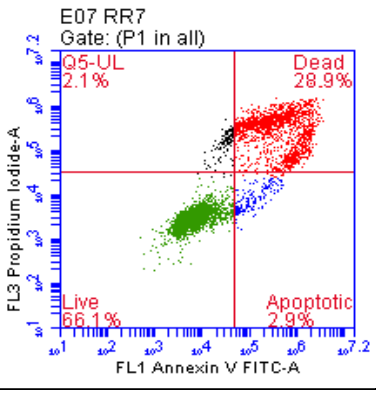
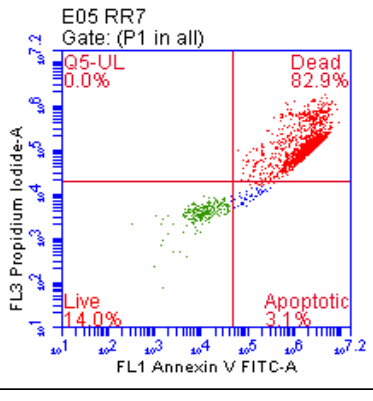
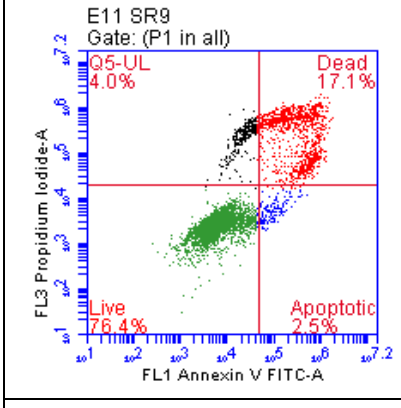
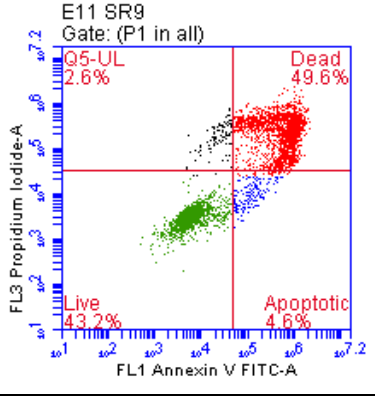
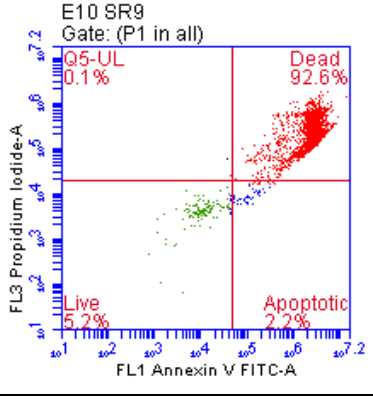
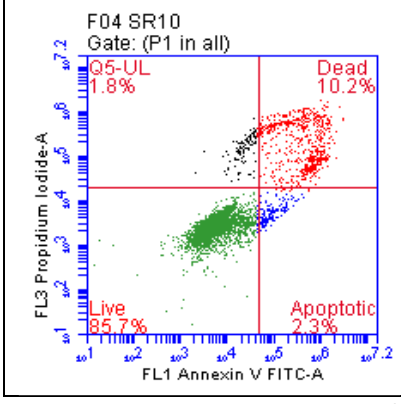
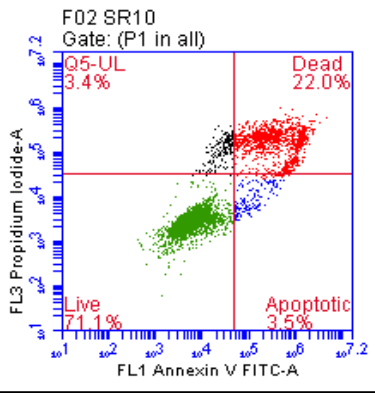
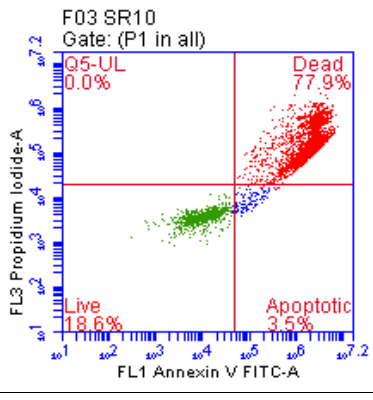
| 12 Hours | 24 Hours | 48 Hours |
|---|---|---|
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

**Table 9: Dot plot of FITC-Annexin V/PI flow cytometric results of HL-60 cells after various time points (12, 24 and 48 hours) of exposure to $IC_{50}$ concentrations of each compound (as determined by MTT assay). HL-60 cells were seeded at 40,000 cells/well, treated with compounds and analysed at various time points using the Annexin-V apoptosis kit. A total of 10,000 events were counted for each sample and gated to exclude background/debris. Appropriate controls were used to gate the relative cell population. The lower left quadrants of each plot (Live) represents viable cells per sample (FITC-/PI-), the lower right quadrant (Apoptotic) shows FITC+/PI- cell population and the upper right quadrant shows late apoptotic/dead cells (FITC+/PI+). One representative experiment out of two is shown for each time point.**