

**PHLORIDZIN DOCOSAHEXAENOATE INDUCES
CYTOTOXIC EFFECTS IN HUMAN LEUKEMIC CELLS**

by

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For my beloved mum and dad ♥

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ABSTRACT

Polyphenols have potential as pharmacological adjuvants to overcome some clinical challenges that arise in leukemia management. The efficacy of polyphenols can be enhanced by acylation with fatty acids. In this study, the cytotoxic effects of docosahexaenoic acid-acylated phloridzin, known as phloridzin docosahexaenoate (PZ-DHA), was studied in two human leukemic cell lines, K562 and Jurkat cells. PZ-DHA significantly reduced the viability and ATP levels of the leukemic cells. PZ-DHA-induced cell morphological changes and apoptotic cell death was further studied by evaluating DNA fragmentation, lactate dehydrogenase (LDH) release, and caspase activation. PZ-DHA was also found to selectively kill Jurkat cells, while sparing normal murine T-cells. Furthermore, interferon-induced phosphorylation of STAT3 protein was downregulated in PZ-DHA-treated Jurkat cells. PZ-DHA also suppressed the proliferation of Jurkat cells xenotransplanted in zebrafish embryos. Both *in vitro* and *in vivo* findings from this study demonstrate the efficacy of PZ-DHA as a novel potential therapeutic agent for leukemia.

LIST OF ABBREVIATIONS USED

ABL1	Abelson murine leukemia viral oncogene homolog 1
AIF	Apoptosis inducing factor
AKT	Protein kinase B
ALA	α -linoleic acid
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of Variance
APS	Ammonium persulfate
ARF	Alternate reading frame product of the p16INK41 protein
ATP	Adenosine triphosphate
ATRA	All-trans-retinoic acid
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
CAD	Caspase-activated DNases
Caspase	Cysteine-dependent aspartate-directed protease
CR	Complete remission
DFF	DNA fragmentation factor
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
EGCG	Epigallocatechin gallate
EGF	Epidermal Growth Factor
EPA	Eicosapentaenoic acid
FBS	Fetal Bovine Serum
G-CSF	Granulocyte colony-stimulating factor
GLUT	Glucose transporter-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hpf	Hours post fertilization
hpi	Hours post injection
hpt	Hours post treatment
HRP	Horseradish peroxidase
HTLV-1	Human T-cell lymphotropic virus type 1
ICAD	Inhibitor of caspase-activated DNases
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
Ki-67	Antigen Ki-67 associated with cell proliferation
LDH	Lactate dehydrogenase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

MTT	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
MYC	Myelocytomatosis viral oncogene homolog
NF- κ B	Nuclear Factor-KappaB
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PIAS	Protein inhibitors of activated STAT
PML	Promyelocytic leukemia
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonyl fluoride
PS	Phosphatidylserine
PT	Phloretin
PTP	Protein tyrosine phosphatases
PUFA	Polyunsaturated fatty acid
PZ	Phloridzin
PZ-DHA	Phloridzin docosahexaenoate
RAR α	Retinoic acid receptor alpha
RLU	Relative luminescence units
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGLT	Sodium-dependent glucose transporters
SH2	Src homology 2
SHP-1	Src homology 2 domain tyrosine phosphatase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription
STS	Staurosporine
TAE	Tris-Acetate-EDTA
T-ALL	T-cell acute lymphoblastic leukemia
TEMED	Tetramethylethylenediamine
TSLP	Tymic stromal-derived lymphopoietin
TYK2	Tyrosine kinase 2
VEGF	Vascular Endothelial Growth Factor

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CHAPTER 1: INTRODUCTION

1.1 PREAMBLE

Leukemia represents a group of heterogeneous malignant diseases caused by the overproduction of leukocytes in the bone marrow. Presumably, a series of distinct genetic mutations that prevents normal differentiation and proliferation of hematopoietic cells causes biological and clinical heterogeneity (Landau et al., 2014), hence inducing distinct types and subtypes of leukemia. Recent advances in studying the biology and genetic landscape of leukemia has led to more subset-specific therapies. While the overall prognosis and survival rate of leukemic patients have markedly improved, drug resistance and disease relapse are often associated with treatment failure (Hochhaus et al., 2002; Klumper et al., 1995). Ultimately, intensifying drug dose leads to intolerable adverse effects, which emphasizes the need of translating molecular mechanisms into the development of novel drugs that are safe and effective for leukemia management.

One of the most commonly dysregulated pathways in hematological malignancies is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway (Furqan et al., 2013b; Lin et al., 2000; Ward et al., 2000). JAK/STAT signaling plays a critical role in transmitting extracellular signals from the surface membrane into the nucleus to activate transcription of genes involved in cell proliferation and survival. Acquired mutations that cause constitutive ligand/receptor binding or oncogenic tyrosine kinase activity, leading to aberrant activation of JAK and STAT proteins, are seen in all types of leukemia (**Table 1**). Novel JAK and STAT inhibitors are progressively making their way into the arsenal of therapeutics for cancer (Fagard et al., 2013; Furqan et al., 2013b; Hayakawa et al., 2013; Quintás-Cardama et al., 2010).

However, the search for a specific and selective, yet potent, anti-JAK/STAT agent remains a challenging goal.

Phytochemicals in plant-based food provide numerous health benefits, including protection against cancer (Kandaswami et al.; Ren et al., 2003; Rupasinghe, 2013; Russo et al., 2010). Dietary phytochemicals interfere with the multistep process of carcinogenesis, which includes tumour initiation, promotion, malignant conversion, and progression. These phytochemicals are capable of not only reducing the adverse side effects of chemotherapy but also increasing the efficacy of conventional chemotherapeutics. Recently, our research team reviewed the use of phytochemicals in targeting JAK/STAT signaling pathway (Arumuggam et al., 2015). Many phytochemicals reportedly show anti-cancer effects in malignant cells through suppression of JAK/STAT signaling. However, therapeutic efficacy of phytochemicals is limited in humans due to poor intestinal absorption and low metabolic stability (Crespy et al., 2001; Manach et al., 2004).

In efforts to increase the bioavailability of phloridzin (PZ), a major phenolic glucoside largely found in apples, our research group synthesized a derivative of PZ called phloridzin docosahexaenoate (PZ-DHA) through an enzyme catalyzed acylation of PZ with docosahexaenoic acid (DHA), an omega-3 fatty acid found in fish oil (Ziaullah et al., 2013). The esterification reaction of PZ with DHA is beneficial to both compounds; as the modification not only could improve the bioavailability of PZ, but it also increases the stability of the unsaturated fatty acid, DHA. Strikingly, PZ or DHA alone shows anti-proliferative effects against cancer cells *in vitro* and *in vivo* (Kato et al., 2007, 2002; Nelson and Falk, 1993a).

This research aims to study the cytotoxic effects of PZ-DHA in two human leukemic cell lines, Jurkat (T-cell acute lymphoblastic leukemia) and K562 (chronic myeloid leukemia). The putative mechanism of action of PZ-DHA is elucidated through investigating its antagonistic activity against JAK/STAT signaling. The efficacy of PZ-DHA is also tested using *in vivo* model employing zebrafish xenotransplanted with Jurkat and K562 cells.

1.2 LEUKEMIA

Leukemia is a commonly diagnosed hematological neoplasm that affects the blood-forming, or hematopoietic cells, in the bone marrow. Leukemia ranks as the ninth cause of cancer-related deaths worldwide in 2012, accounting for 150,000 deaths in men and 114,000 deaths in women (Ferlay et al., 2015). It was estimated that 6200 Canadians were diagnosed with leukemia, and 2700 Canadians died of this disease in 2015 (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015). Leukemia is also the most common cancer in children. In Canada, leukemia accounted for 32% of all childhood cancers (ages 0-14) and 26% of all childhood cancer deaths between 2006 and 2010 (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015).

Leukemia, literally means "white blood" in Greek, is mostly associated with an excessive production of immature leukocytes in the bloodstream (Braun and Anderson, 2007). Under normal physiological conditions, the hematopoietic stem cells develop into leukocytes that provide defense against infections or foreign invaders. When leukemia occurs, abnormal leukocytes disrupt hematopoiesis, and hence suppress the production and functionality of healthy leukocytes and other blood cells such as erythrocytes and platelets. Over time, leukemic cells overcrowd the bone marrow and blood vessels, and

spread into distal organs including lymph nodes, spleen, liver, brain, and spinal cord (Braun and Anderson, 2007). Unlike solid tumours, which begin in major organs and eventually spread into the blood, leukemia begins in the bone marrow and ultimately affects various parts, including the immune, lymphatic, and circulatory systems in the body.

Cellular heterogeneity in hematological malignancies contributes to four major types of leukemia: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). Leukemia is primarily classified based on cellular differentiation, either lymphoid or myeloid, and cellular maturity, either acute or chronic (Rytting, 2015). Lymphoid leukemia interferes with the maturation of lymphoid cells that differentiate into B cells and T cells, whereas myeloid leukemia disrupts the maturation of myeloid cells into monocytes, erythrocytes, granulocytes, and platelets. Acute leukemia consists of immature, undifferentiated leukocytes called blast. Blast cells are non-functional and they proliferate rapidly. Chronic leukemia arises from clonal expansion involving relatively mature cells, and the disease often progresses gradually (Zaharieva et al., 2013).

1.2.1 Causes of leukemia

The causes of leukemia remain unclear, however, risk factors include congenital genetic disorders, genetic predisposition, leukemogenic carcinogens exposure, and virus infection. For example, children with genetic disorders like Down's syndrome or Fanconi anemia, an autosomal recessive disorder, are more likely to develop AML or ALL than other children (Auerbach and Allen, 1991; Fong and Brodeur, 1987; Xavier and Taub, 2010). Leukemia is often associated with irreversible genetic damage such as non-

random chromosomal translocations (Aplan, 2006), inversions, deletions, or additions, which result in the formation of fusion oncogenes. One of the common translocation that occurs between chromosome 9 and chromosome 22, also known as Philadelphia chromosome, is found in more than 90% of patients with CML (Kurzrock, 2003; Lozzio and Lozzio, 1975). Previous studies also show that exposure to carcinogens such as to benzene, radiation, or ethylene oxide increases the likelihood of developing leukemia (Hogstedt, 1979; Levine and Bloomfield, 1992). Certain viruses are also known to cause leukemia. The human T-cell lymphotropic virus type 1 (HTLV-1) is often associated with adult T-cell leukemia, a highly aggressive form of peripheral T-cell neoplasm (Matsuoka, 2005).

1.2.2 Leukemia management

Ongoing research studies and scientific discoveries have led to tremendous progress in treating leukemia (Greaves, 2016). The common types of treatment include chemotherapy, targeted therapy, radiation therapy, stem cell transplant, surgery, and combination therapy. Although chemotherapy has been the principal mode for killing cancer cells, undesirably it also affects normal cell types in the human body that leads to life-threatening side effects (Chabner and Roberts, 2005).

Recently, targeted therapy has been a revolutionary mode of treatment, especially in treating patients with CML, converting the disease from a fatal to manageable condition. Imatinib mesylate is an example of drug used in targeted therapy for CML and sometimes in ALL management. Imatinib, a small-molecule tyrosine kinase inhibitor, selectively targets BCR-ABL tyrosine kinase that presents in patients with Philadelphia chromosome-positive (Deininger and Druker, 2003). The Philadelphia chromosome is a

genetic abnormality consisting of a reciprocal translocation that occurs when the *ABL1* gene on chromosome 9 juxtaposed onto the *BCR* gene on chromosome 22, coding for the oncogenic kinase BCR-ABL. All-trans-retinoic acid (ATRA) is another effective drug used in targeted therapy to treat patient affected with acute promyelocytic leukemia (APL), a subtype of AML. APL is associated with another chromosomal translocation involving *RAR α* gene on chromosome 17, often with *PML* gene on chromosome 15. ATRA targets the hybrid proteins with altered functions resulting from the fusion of *RAR α* and *PML*, and induces terminal differentiation of immature leukemic promyelocytes into mature granulocytes, after which the differentiated malignant cells undergoes normal apoptosis (Degos and Wang, 2001).

The treatment for ALL cases, including pediatric T-cell ALL (T-ALL) patients typically consists of three stages known as induction, consolidation, and maintenance (Takeuchi et al., 2002). The remission induction therapy, mostly using aggressive chemotherapeutic agents like doxorubicin, is targeted at eradicating most ALL blast cells for prolonged survival. Doxorubicin is a DNA intercalating agent that inhibits the progression of the topoisomerase II enzyme, thereby preventing DNA replication and ultimately inhibiting protein synthesis (Tacar et al., 2013). Patients in complete remission (CR) then receive consolidation or intensification therapy, followed by maintenance or continuation therapy. The overall survival of pediatric ALL patients has increased over the past years, where now the CR rate exceeds 90 % and event-free survival (EFS) rate is about 85 % (Pui et al., 2009). Risk-stratification and leukemia management have provided more tailored therapy and improved overall survival of patients. However, drug

resistance, suboptimal response, or intolerance towards side-effects are challenges that have been constantly seen in patients undergoing cancer treatment.

Although targeted therapy has reduced cytotoxicity towards normal cells, the application of most drugs including ATRA, imatinib or second-generation tyrosine kinase inhibitors, such as dasatinib and nilotinib, is still limited due to emergence of resistance and intolerance (Bhamidipati et al., 2013; Druker et al., 2006; Gruber et al., 2012). In efforts to combat resistance and alleviate adverse side effects, current research in leukemia management is focused on exploring the efficacy and potency of novel drug candidates that could be used to target prominent signaling pathways involved in leukemogenesis. One of the major signal transduction pathways that has aberrant activation in leukemia is JAK/STAT signaling pathway (Furqan et al., 2013b; Vainchenker and Constantinescu, 2013; Ward et al., 2000). The possibility and necessity of exploring JAK/STAT pathway as a therapeutic target in leukemia treatment is emerging (Ferrajoli et al., 2006; Martinez-Lostao et al., 2005; Mughal et al., 2014).

1.3 JAK/STAT SIGNALING PATHWAY

Since its discovery in the 1990s (Schindler et al., 1992; Velazquez et al., 1992), the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has been extensively studied (Aaronson and Horvath, 2002; Heim, 1991; O'Shea et al., 2011, 2002; Rawlings et al., 2004; Stark and Darnell, 2012). The STAT proteins are a family of latent transcription factors in the cytoplasm (Schindler et al., 1992), which are activated by JAK proteins through phosphorylation of tyrosine residues. The intracellular JAK/STAT signaling cascade is crucial for efficient transduction of various extracellular signals to the nucleus, which regulates critical physiological processes such as

hematopoiesis, embryonic development, immunity, cell growth, and apoptosis. Although initially the JAK/STAT pathway was found to be the principal mechanism for interferon-induced signaling, many different cytokines including interleukins (IL), growth factors (for instance, EGF and PDGF), hormones (such as erythropoietin, thrombopoietin, and prolactin), and other polypeptides were later identified as ligands for the JAK/STAT signaling pathway (Darnell Jr., 1997; Darnell et al., 1994).

The studies using mammalian cells have established a canonical receptor-ligand coupled JAK/STAT signaling pathway (**Figure 1**). The JAK/STAT pathway is a direct mechanism to translate extracellular signals into transcriptional responses (Darnell Jr., 1997; Darnell et al., 1994; Mitchell and John, 2005; Schindler and Darnell, 1995; Watowich et al., 1996). Most cytokine receptors associated with STATs form stable association with JAKs because they do not have intrinsic tyrosine kinase activity. JAKs bind specifically to intracellular domains of cytokine receptor chains. Upon ligand binding to the receptor, JAKs become activated and further phosphorylate the intracellular tyrosine residues of the receptor, creating docking sites for the src homology 2 (SH2) domains of STATs. STATs are recruited to the receptor, and activated by tyrosine phosphorylation, leading to their homo- or heterodimerization and subsequent translocation to and retention in the nucleus. Once in the nucleus, STAT dimers bind to specific regulatory elements to activate or repress transcription of target genes encoding functional proteins such as cyclins, p21, c-Myc, Bcl-2, Mcl-1, VEGF, survivin, and many others. The JAK/STAT signaling pathway is also involved in self-renewal and continued maintenance of the stem cell population (Kiger et al., 2001).

There are seven known members in mammalian STAT family, namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Many studies have shown elevated expression of STATs, particularly STAT1, STAT3, and STAT5, in both tumour cell lines and primary patient samples (Bowman et al., 2000; Bromberg, 2002; Denley, 2008; Garcia et al., 2001; Hernandez-Vargas et al., 2011; Slattery et al., 2013; Vainchenker and Constantinescu, 2013; Weber-Nordt et al., 1996). The activated signaling of these major cancer-promoting STATs is detected in hematological malignancies and solid tumours, including breast (Borgés et al., 2008; Hernandez-Vargas et al., 2011), prostate (Kroon et al., 2013; Tam et al., 2007), pancreatic (Denley, 2008), colorectal (Slattery et al., 2013; Spano et al., 2006) and brain (Jain et al., 2012) cancers.

The four members of the JAK family are JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK 2). Apart from the cytokine-induced JAK phosphorylation, STATs proteins can also be activated by oncogenic tyrosine kinases depending on the receptor and cellular content (Bowman et al., 2000). Non-receptor tyrosine kinases that can directly phosphorylate STATs are cytoplasmic kinases such as c-Src and Abl. IL-6 activation of its cognate receptor, gp130, is a potent STAT3 activator in cancer (Boulton et al., 1995). Additionally, the c-Met receptor responding to hepatocyte growth factor (HGF), activates STAT3 signaling (Boccaccio et al., 1998; Zhang et al., 2002). Activated growth factor receptors with intrinsic tyrosine kinase activity, such as EGF receptor and PDGF receptor, also directly phosphorylate STAT proteins.

The JAK/STAT signaling pathway is guarded by three major classes of negative regulators. JAKs can be negatively regulated by suppressor of cytokine signaling (SOCS) proteins and protein tyrosine phosphatases (PTPs). A well-studied PTP is SRC homology

2 (SH2) domain-containing protein-tyrosine phosphatase called SHP-1 (David et al., 1995; Klingmüller et al., 1995; Mackenzie et al., 2013). STATs can be negatively regulated by PTPs in the cytoplasm or nucleus, and by protein inhibitors of activated STAT (PIAS) proteins. PTPs have the simplest mechanism, in which they dephosphorylate activated JAKs or the cytokine receptors (Simoncic et al., 2002). SOCS protein act in a negative feedback loop, where activated STATs stimulate SOCS expression and, in turn, SOCS bind to STATs, JAKs, or the receptors to suppress the signaling pathway (Krebs and Hilton, 2001). PIAS proteins, however, bind to phosphorylated STATs dimers and prevent them from recognizing DNA-binding site (Shuai, 2000). Dysfunctional negative regulators of JAK/STAT pathway are also commonly detected in cancer.

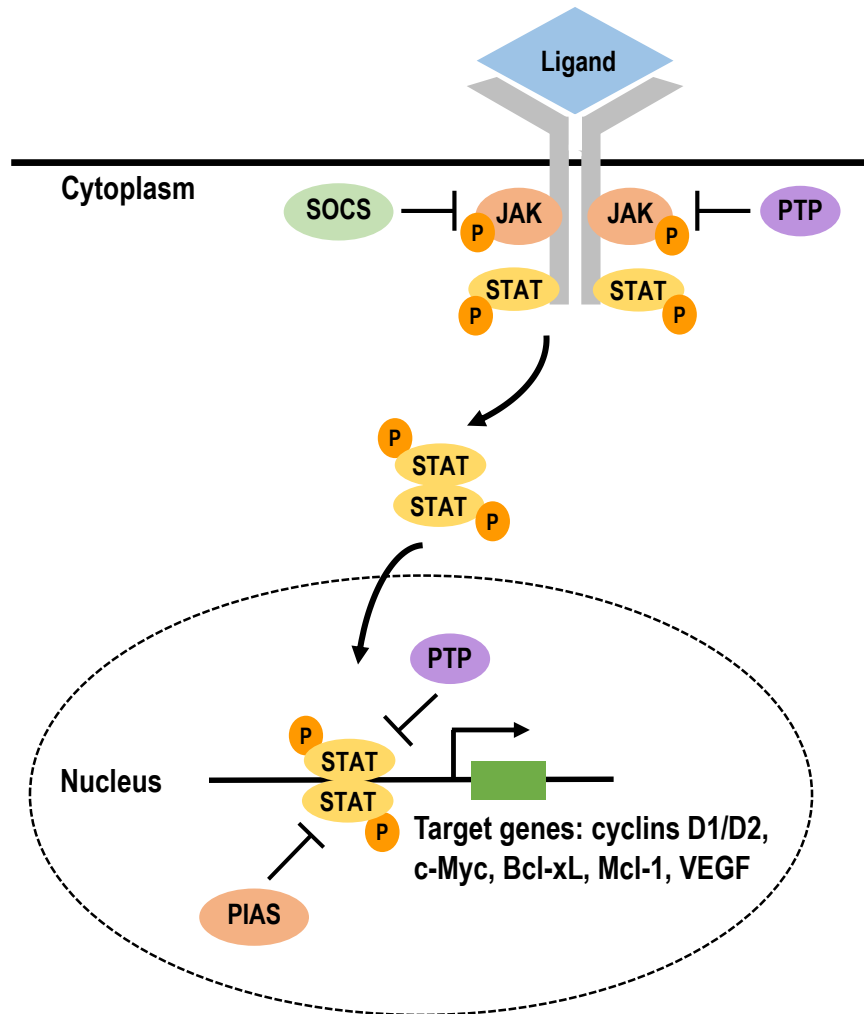


Figure 1. The canonical JAK/STAT signaling pathway.

A schematic representation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway. The activation of JAKs after ligand-receptor coupling stimulates phosphorylation of STATs, followed by STAT dimerization and translocation into the nucleus to activate gene transcription. The JAK/STAT signaling pathway is negatively regulated by suppressor of cytokine signaling (SOCS), protein tyrosine phosphatases (PTPs), and protein inhibitors of activated STAT (PIAS) proteins.

1.3.1 Dysregulation of JAK/STAT signaling in leukemia

The JAK/STAT signaling pathway is often aberrantly activated in hematological malignancies (**Table 1**), reflecting the critical role of JAK and STAT proteins in hematopoiesis. Many members of the large cytokine receptor superfamily that transmit signals via JAK/STAT pathway, including erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic stromal-derived lymphopoietin (TSLP), interleukins, and interferons, are key regulators for blood formation and immune responses (Ihle et al., 1995; Springuel et al., 2015; Watowich et al., 1996). In the event of dysfunctional JAK or STAT activation, for example, through mutations that cause constitutive ligand/receptor coupling or abnormal tyrosine kinase activity, aberrant JAK/STAT signaling stimulates the proliferation and survival pathways of leukemic cells.

The IL7R-JAK pathway is disrupted in 27.7% of T-ALL cases, with somatic mutations found in the IL-7 cytokine receptor, JAK1, JAK3, and STAT5 (Vicente et al., 2015). Besides, a mutation called JAK2 Δ IREED, caused by deletion in the pseudokinase domain of JAK2, was detected in a patient with Down syndrome and B-cell ALL (Malinge et al., 2007). STAT proteins are also often targets of mutations in leukemia. Recurrent activating mutations of STAT3 and STAT5 have been found in large granular lymphocytic leukemia (Andersson et al., 2013). In addition, oncogenic cytoplasmic kinases could also activate STAT signaling independent of JAK activation. For instance, constitutive activation of STATs were seen in hematopoietic cell line transformed by Bcr/Abl oncogene (Carlesso et al., 1996). Similarly, STAT1, STAT3, and STAT5 were

constitutively phosphorylated without the presence of IL-3 in a myeloid progenitor cell line, 32Dc13, transformed with v-src oncogene (Chaturvedi et al., 1997).

Table 1. Activated JAKs and STATs in leukemia.

Type of leukemia	Activated JAKs/STATs	Ref.
T- ALL	JAK1, JAK3, STAT1, STAT3, STAT5	(Migone et al., 1995; Takemoto et al., 1997)
ALL	STAT1, STAT5, JAK2	(Gouilleux-Gruart et al., 1996; Meydan et al., 1996)
AML	STAT1, STAT3, STAT5	(Ghoshal Gupta et al., 2008; Gouilleux-Gruart et al., 1996; Hayakawa et al., 1998; Weber-Nordt et al., 1996)
CLL	STAT1, STAT3	(Frank et al., 1997)
CML	STAT5	(Shuai et al., 1996; Weber-Nordt et al., 1996)
Erythroleukemia	STAT5	(Chrétien et al., 1996)
Megakaryocytic leukemia	JAK2, STAT5	(Liu et al., 1999)
Large granular lymphocyte leukemia	STAT3	(Epling-Burnette, 2013; Koskela et al., 2012; Liang et al., 2016)

The formation of fusion proteins associated with JAK is also detected in leukemic patients. A translocation between chromosome 9 and 12 gives rise to a fusion protein called TEL-JAK2, in which JAK associates with TEL, an oncogene coding for a member of the E26 leukemic virus-induced ETS transcription factor family (Lacronique et al.,

2000, 1997). The TEL–JAK2 fusion was first found in a child with T-ALL. Oligomerization of Tel-JAK led to the ligand-independent, constitutive activation of tyrosine kinase activity, rendering an increase in activated STAT levels in IL-3-dependent Ba/F3 hematopoietic cell line (Lacronique et al., 2000). This oncoprotein is also associated with pre-B-cell ALL (B-ALL) and atypical CML (Peeters et al., 1997). A novel SSBP2-JAK2 fusion gene resulting from chromosomal rearrangement is also found in patients with pre-B-ALL (Poitras et al., 2008). Additionally, mutations in negative regulators of JAK/STAT signaling such as PTPN2 and CD45, JAK-regulating phosphatases, are seen in T-ALL cases in combination with JAK mutations (Kleppe et al., 2010; Porcu et al., 2012).

1.3.2 JAK and STAT inhibitors in leukemia management

Given the potential of the JAK/STAT pathway as an effective drug target, current research interest has shifted towards the development of novel JAK/STAT inhibitors as an approach to suppress the activation of tumour-promoting genes (Darnell, 2012, 2002; Fagard et al., 2013; Furqan et al., 2013a; Miklossy et al., 2013; Mughal et al., 2014). There is overwhelming evidence that inhibition of JAK/STAT pathway leads to apoptosis of cancer cells and/or synergistically enhances the potency of chemotherapeutic drugs (Aoki et al., 2003; Dorritie et al., 2014; Iwamaru et al., 2007; Nelson et al., 2011; Redell et al., 2011; Siddiquee and Turkson, 2008; Xiong et al., 2008). However, while being cytotoxic to cancer cells, another factor that has to be considered before introducing JAK/STAT inhibitors as potential therapeutics is to demonstrate their lack of toxicity to normal cells.

Several studies suggest that inactivation of STAT could be tolerated adequately by normal cells. STAT1-deficient mice did not show developmental defects, however they were highly susceptible to viral diseases (Durbin et al., 1996). Similarly, loss of STAT3 in various adult murine cells, including T-cells, rendered relatively normal cells with a partial defect in proliferation (Akira, 2000). However, mice bearing a STAT3-mutant allele die during early embryogenesis (Takeda et al., 1997), suggesting that STAT3 suppression in adult tissue may not cause lethality. Another study shows that STAT5-deficient mice demonstrated relatively normal development, although disruption of prolactin- and IL-induced signaling caused impaired mammary gland development and T-cell responses, respectively (Matsumoto et al., 1999). Taken together, these findings suggest that JAK/STAT signaling may not be essential, as over time normal cells become capable of utilizing alternate signaling pathways, which makes them tolerant to the inhibition of any one pathway (Bromberg, 2002). By contrast, malignant cells are more dependent on specific overactivated pathways for their growth and survival; therefore, targeted JAK/STAT inhibitors may cause selective toxicity towards leukemic cells.

Some promising work involving JAK/STAT inhibitors in leukemia have been published in leading scientific journals of high impact implying the importance and necessity of this area of research. A novel STAT inhibitor, OPB-31121, was found to selectively inhibit activation of STAT3 and STAT5, and to suppress growth in various hematopoietic malignant cells while sparing normal human cord blood cells (Hayakawa et al., 2013). Strikingly, OPB-31121 was also shown to alleviate resistance to an upstream kinase inhibitor by inhibiting STAT signaling. It has also been shown that pimozone, a selective STAT5 inhibitor, induced apoptosis in CML cells resistant to kinase

inhibitors, while enhancing inhibitory effects of drugs such as imatinib or nilotinib (Nelson et al., 2011). Another interesting finding shows that fludarabine, a DNA-intercalating agent commonly used in CLL treatment, also suppresses STAT1 mRNA and protein expression (Frank et al., 1999), which may underlie an additional mechanism of anti-leukemic drug.

Furthermore, there is also strong evidence that JAK inhibitors have proven effective in suppressing leukemic cell growth. A small molecule inhibitor of JAK, AG490, suppresses JAK2 activation and inhibit proliferation of ALL cells *in vitro* and *in vivo* without affecting normal cells (Meydan et al., 1996). Another study shows that TG101348 suppresses JAK2, STAT3, and STAT5 phosphorylation, therefore reducing viability of mast cell leukemia cells (Lasho et al., 2010). TG101348 also showed synergistic effect in the presence of dasatinib. Similarly, in addition to inhibiting proliferation and inducing apoptosis in AML cells, a JAK2-selective inhibitor TG101209 reduced tumour growth and significantly prolonged survival of mice bearing leukemia cells (Lo et al., 2013). Collectively, the efficacy of JAK/STAT inhibitors in suppressing leukemic cell growth and survival has been delineated in numerous preclinical studies. Several JAK/STAT pathway inhibitors have also made their way into clinical trials (**Table 2**).

Despite significant promise in pre-clinical studies, only a limited number of JAK inhibitors have been suitable for clinical development. To date, ruxolitinib is the only potent JAK2 inhibitor that was approved by the US Food and Drug Administration (FDA) for treating patients with myelofibrosis (MF), a myeloproliferative disorder

(Deisseroth et al., 2012). Thus, development of novel agents targeting JAK/STAT signaling pathway remains an area of active research.

Table 2. Clinical trials with JAK/STAT inhibitors.

Drug	Target	Type of leukemia	Clinical trial phase	Ref.
Ruxolitinib phosphate (NCT02092324)	JAK1 JAK2 STAT5	Chronic neutrophilic leukemia or atypical CML	Phase 2- Active	(Fleischman et al., 2013; Gallipoli et al., 2014; Quintás-Cardama et al., 2010)
Ruxolitinib (NCT01712659)	JAK2 STAT5	Adult T-cell leukemia	Phase 2- Active	(Zhang et al., 2015)
Auranofin (NCT01419691)	JAK1 STAT3	CLL	Phase 2- Not recruiting	(Fiskus et al., 2014; Kim et al., 2007; Nakaya et al., 2011)
Pacritinib (NCT00719836)	JAK2	Advanced myeloid leukemia	Phase 1/2- Completed	(Hart et al., 2011a, 2011b)
Pacritinib (NCT02532010)	JAK2 STAT3 STAT5	AML	Phase 2- Active	(Hart et al., 2011b; Marrin et al., 2014)
AT9283 (NCT01431664)	JAK2	Relapsed and refractory acute leukemia	Phase 1- Completed	(Foran et al., 2014; Tanaka et al., 2010)
Pravastatin (NCT00107523)	STAT1	AML	Phase 1- Completed	(Zhou et al., 2009)
Lestaurtinib (NCT00079482)	JAK2 JAK3	AML	Phase 2- Completed	(Hexner et al., 2008; Smith et al., 2004)

1.4 PHYTOCHEMICALS AND JAK/STAT PATHWAY

1.4.1 Plants as source of anticancer agents

Plants synthesize an abundance of secondary metabolites, also known as phytochemicals, for promoting survival through protection and defense against environmental stressors (Williams et al., 1989). These phytochemicals have been thoroughly exploited for medicinal and pharmacological applications over the past decades. Hundreds of peer-reviewed articles have reported evidence supporting the roles of phytochemicals as antioxidant, anti-inflammatory, antimicrobial and/or anticancer agents. Understanding the molecular targets and putative mechanisms of action of phytochemicals may lead to discovery of novel drugs or new possibilities for the development of functional food and natural health products.

The use of phytochemicals as chemotherapeutics is evident (Gordaliza, 2008; Kinghorn et al., 2009; Mann, 2002; Ram and Kumari, 2001; Ren et al., 2003). Notably, over the time frame between early 1950s and 2014, 49% of clinically-used anticancer drugs were either natural products or naturally derived therefrom (Newman and Cragg, 2016). The natural compounds that are plant-derived can be primarily characterized into four classes such as vinca alkaloids, epipodophyllotoxins, taxanes, and camptothecins (Balunas and Kinghorn, 2005). Strikingly, vinca alkaloids and epipodophyllotoxin derivatives are widely used for treating hematological malignancies. The vinca alkaloids, vincristine and vinblastine, are anti-mitotic agents that exhibit cytotoxicity through binding to tubulin, thus inhibiting mitosis and causing cell-cycle arrest (Cutts, 1961). Vincristine sulfate (Oncovin®) and vinblastine sulfate (Velban®) were the first plant-derived anticancer agents to be approved by the U.S. Food and Drug Administration

(FDA), in 1963 and 1965, respectively (Lucas et al., 2010). Similarly, etoposide (VePesid®) and Teniposide (Vumon®), which are podophyllotoxin derivatives that inhibit topoisomerase II, received FDA approval in 1983 and 1993, respectively (Kinghorn, 2015; van Maanen et al., 1988). These drugs are used to treat various cancers including leukemias and lymphomas.

As reviewed by Katrin Sak, on-going research studies show that plant-based compounds help to alleviate drug resistance and adverse side effects (Sak, 2012); thus, enhance the overall efficiency of clinically-viable chemotherapeutics. A group of polyphenols have been shown to synergistically increase the cytotoxicity of doxorubicin and etoposide in lymphoid leukemia cells (Mahbub et al., 2015). Besides uncovering potent drugs or adjuvant agents, studying the biological activities of natural compounds and their derivatives also increases the likelihood of discovering novel tumour survival and tumouricidal pathways (Lucas et al., 2010). Once the candidates undergo successful preliminary screening and shortlisting based on their cytotoxic effects, the mode of action will be further investigated. Therefore, even if natural compounds do not reach clinical trials, the learning curve undoubtedly provides tremendous insight into the biology of cancer and drug discovery.

1.4.2 Phytochemicals targeting JAK/STAT signaling in leukemia

Many promising studies have reported the ability of phytochemicals to significantly inhibit tumour growth *in vitro* and *in vivo* animal models (Alhosin et al., 2015; Baur and Sinclair, 2006; Gali-Muhtasib et al., 2006; Juárez, 2014; Li-Weber, 2009; Majeed et al., 2014; Wang et al., 2014; Yuan et al., 2015). These phytochemicals often interfere with the molecular signal transduction pathways of cancer cells that respond to

various external stimuli, disfavoring tumour formation by suppressing uncontrolled cell proliferation, differentiation, anti-apoptosis, and angiogenesis. As was noted earlier, one of the major pathways that play a pivotal role in regulating cell growth and survival is the JAK/STAT signaling pathway. Interestingly, many phytochemicals have been shown to exhibit anticancer effects via inhibition of JAK/STAT signaling as we reviewed recently (Arumuggam et al., 2015). **Table 3** summarizes the intervention of selected phytochemicals in modulating JAK/STAT signaling specifically in leukemic cells.

Table 3. Effects of plant-derived agents on JAK/STAT signaling in leukemia.

Natural derivatives	Type of leukemia	Cell line	Key findings	Ref.
Apigenin	Promyelocytic leukemia	HL-60	- decreased phosphorylation of JAK2 and STAT3	(Ruela-de-Sousa et al., 2010)
	Erythroleukemia	TF1	- decreased phosphorylation of JAK2, STAT3, STAT5	
Cryptotanshinone	CML	K562	- inhibits JAK2 and STAT3 activation - regulated expression of SHP-1	(Jung et al., 2013)
Cucurbitacin B	CML	K562	- inhibited STAT3 activation - showed growth inhibition	(Chan et al., 2010)
Curcumin	Adult T cell leukemia	MT-2, HuT102, SLB-1	- decreased JAK and STAT phosphorylation - induced of growth-arrest and apoptosis	(Rajasingh et al., 2006)

Natural derivatives	Type of leukemia	Cell line	Key findings	Ref.
Erythrina suberosa	Promyelocytic leukemia	HL-60	- inhibited STAT3, STAT5, and STAT6 phosphorylation	(Kumar et al., 2013)
Icariside	CML	K562, primary CML cells	- downregulated JAK2 and STAT3 activation - prolong lifespan of NOD-SCID nude mice bearing K562 cells	(Zhu et al., 2011)
Icariside II	AML	U937	- inhibited JAK2 and STAT3 activation - enhanced the expression of SHP-1	(Kang et al., 2012)
Resveratrol	Myeloid leukemia	HEL, SEL-2	- comparable to ruxolitinib - suppressed activation of JAK1, JAK2, Tyk2, STAT3, and STAT5	(Espinoza et al., 2013)
Tanshinone IIA	CML	K562	- inhibits JAK2 and STAT5 activation - augmented synergy with imatinib - regulated expression of SHP-1 and SHP-2	(Jung et al., 2013)

As reviewed earlier, the JAK/STAT signaling pathway is an important player in regulating transcription of genes involved in maintaining normal cellular functions. Therefore, constitutive activation of JAK/STAT signaling triggers perturbations, such as downregulation of cell death and cell cycle, leading to leukemogenesis. In recent years, researchers have established a link between natural compounds and suppression of the JAK/STAT signaling pathway. **Table 3** shows that a reasonable number of phytochemicals from two distinct groups, namely flavonoids and terpenes, exhibit promising inhibitory effects on JAK and STAT activation, which causes apoptosis and inhibition of leukemic cell proliferation. This suggests that exploiting novel phytochemicals or their derivatives might accelerate the process of discovering a potent and persistent JAK or STAT inhibitor. Besides, further exploring the modulation of flavonoids or terpenes on JAK/STAT pathway might enable a rapid elucidation of their putative mechanism of action.

1.5 A NOVEL PLANT DERIVATIVE: PHLORIDZIN DOCOSAHEXAENOATE

1.5.1 Phloridzin

Flavonoids are a large group of polyphenols that consist of flavonols, flavones, flavanones, flavanols (catechins), isoflavones, anthocyanidins, and chalcones (Lackie, 2010). Phloridzin (PZ) or phlorizin (also known as phloretin-2'-O-glucoside) (**Figure 2a**), a flavonoid that belongs to the dihydrochalcones, was first isolated by French chemists in 1835 from the bark of apple trees (Ehrenkranz et al., 2005). PZ is also found in different tissues such as apple peel, pulp, seeds, and leaves (Escarpa and González, 1998; Gosch et al., 2010). A study reported that PZ content varies between 3-303 mg/kg among 67 different types of apple, in which Starkrimson variety had the highest level of

PZ (Wojdyło et al., 2008). Although apple is an abundant source, PZ is also present in other plant species including lettuce, strawberry, pomegranate, and cranberry (Gosch et al., 2010).

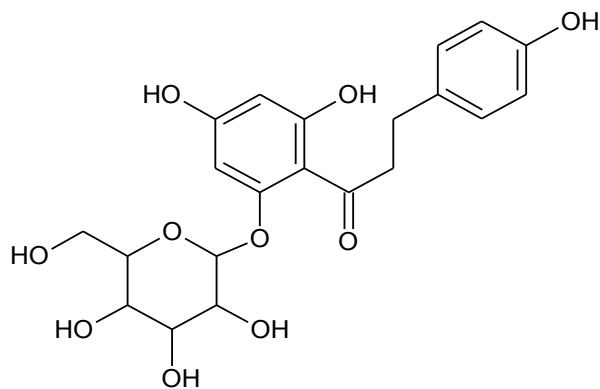
Initially, a German physician observed that PZ-injected dogs produced renal glycosuria (von Mering J., 1886). Glycosuria is the excretion of glucose into urine; this phenomenon is uncommon in healthy individuals as the plasma glucose that is filtered in kidney glomerulus is normally reabsorbed. The principal mode of action of PZ was later associated with the inhibition of sodium-dependent glucose transporters (SGLTs), which consequently blocks intestinal glucose absorption and promotes glycosuria (Ikumi et al., 2008; Masumoto et al., 2009; Panayotova-Heiermann et al., 1995; Vick et al., 1973). The ability of PZ to inhibit glucose reabsorption into the plasma has been intertwined in treating diabetes by attempting to block glucose entry into the cells (Lu et al., 2012; Najafian et al., 2012; Rossetti et al., 1987; Zhao et al., 2004).

PZ was identified as a potent SGLT inhibitor; however, further development of PZ as an anti-diabetic agent was challenged due to its non-selective activity, poor bioavailability and low metabolic stability. PZ inhibits both SGLT1 and SGLT2 that are mainly expressed in the gastrointestinal tract and kidneys, respectively. Blocking SGLT1 disrupts the intestinal transport of glucose, causing gastrointestinal side effects such as diarrhea and dehydration (Chao and Henry, 2010; Kasahara et al., 2001). Like most flavonoids, PZ also has poor intestinal absorption; hence low bioavailability (Crespy et al., 2001; Manach et al., 2004). Apart from that, PZ is highly susceptible to lactase-phloridzin hydrolase mediated cleavage of the glycosidic linkage (Day et al., 2000), which produces its corresponding aglycone phloretin (PT) (**Figure 2b**). Besides being a

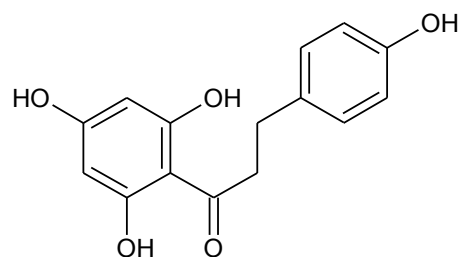
weaker inhibitor of glucose reabsorption (Chan and Lotspeich, 1962), PT also blocks the facilitated glucose transporter-2 (GLUT2), which is expressed in essentially all cells, causing undesirable effects (Ehrenkranz et al., 2005; Wheeler and Hinkle, 1981).

Many research studies, therefore, have been involved in the search for a promising PZ derivative that could eliminate these disadvantages. Fortunately, PZ lead to the discovery of many selective SGLT2 inhibitors including dapagliflozin that has been recently approved by FDA for glycemic control in adults with type 2 diabetes (Bonner et al., 2015; List et al., 2009; Syed et al., 2015).

(a) Phloridzin



(b) Phloretin



(c) Docosaheptaenoic acid

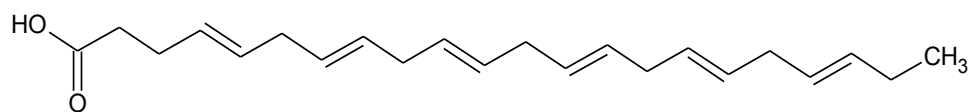


Figure 2. Chemical structures of (a) phloridzin, (b) phloretin, and (c) docosaheptaenoic acid.

Phloridzin: $C_{21}H_{24}O_{10}$, 436.41 g/mol; phloretin: $C_{15}H_{14}O_5$, 274.27 g/mol;

docosaheptaenoic acid: $C_{22}H_{32}O_2$, 328.57 g/mol.

1.5.2 Anticancer effects of phloridzin and its aglycon phloretin

The ability of phloridzin (PZ) and phloretin (PT) to inhibit glucose reabsorption suggested that they could be further explored for potential anticancer effects. However, only limited studies have been conducted due to the bioavailability issues concerning PZ and PT. PZ and PT have been shown to significantly block glucose transport into rat mammary adenocarcinoma and methylcholanthrene-induced stage 3 transitional cell bladder carcinoma cells both *in vitro* and *in vivo* experiments (Nelson and Falk, 1993b). Nelson and Falk also demonstrated that PZ and PT significantly inhibited tumour cell growth in mice subcutaneously transplanted with mammary adenocarcinoma and bladder carcinoma (Nelson and Falk, 1993a).

Recently, another study showed that PT and its derivatives had significant cytotoxic effects against one or several human cancer cell lines, including A549 lung carcinoma, Bel-7402 hepatoma carcinoma, HepG2 hepatoma carcinoma, and HT-29 colon carcinoma (Qin et al., 2015). PZ showed relatively weaker anticancer effects compared to other derivatives, which could possibly be due to its glycoside moiety that causes poor penetrability. PT has also been shown to induce apoptosis in HepG2 cells *in vitro* and *in vivo* models through inhibiting GLUT2-mediated glucose transport (Wu et al., 2009). PT-induced apoptosis was detected in B16 mouse melanoma 4A5 cells, human leukemia HL-60 cells, colon cancer HT-29 cells, gastric cancer BGC823 cells, and human hepatoma carcinoma SMMC-7721 cells (Kobori et al., 1999, 1997; Park et al., 2007; Wang et al., 2012). Pre-treating cells with extracellular glucose reversed the apoptotic effects on B16 melanoma cells showing that PT-induced cytotoxicity could be due to inhibition of glucose transport into the cells (Kobori et al., 1997). Besides, PT has

also been shown to enhance the chemotherapeutic effect of paclitaxel in mice transplanted with HepG2 cells (Yang et al., 2009). Min et al., (2015) found that PT exhibited significant cytotoxic effects on A549 non-small cell lung carcinoma cells through upregulation of p38 MAPK and JNK1/2 pathways.

1.5.3 Acylation of flavonoids with fatty acids

As mentioned previously, most flavonoid glycosides have limited bioavailability due to poor intestinal absorption and low metabolic stability. Numerous attempts have been made to increase the systemic bioavailability of flavonoids, including efficient nano-delivery using microemulsion methods (Malik et al., 2012; Shen et al., 2011; Solanki et al., 2012), methylation to block free hydroxyl groups in flavonoids (Thilakarathna and Rupasinghe, 2013; Wen and Walle, 2006), and enzymatic acylation with fatty acids to increase penetrability into the cells (Mellou et al., 2006; Salem et al., 2010; Stevenson et al., 2006; Ziaullah et al., 2013). The enzymatic acylation, commonly performed using immobilized lipase B (Novozym 435®) from *Candida antarctica*, is preferred over chemical acylation due to its regioselectivity, simple one-step reaction, and relatively high conversion yields (Danieli et al., 1997; Kontogianni et al., 2001; Ziaullah et al., 2013). Regioselectivity protects functional hydroxyl groups that are present in flavonoids.

Flavonoids are very commonly modified with long chain fatty acids. Other than increasing the lipophilicity of flavonoid glucosides, and hence promoting enhanced bioavailability (Figueroa-Espinoza and Villeneuve, 2005; Salem et al., 2010; Stevenson et al., 2006), the acyl donor long chain fatty acids also exhibit significant health benefits (Kinsella, 1986; Ruxton et al., 2004). Polyunsaturated fatty acids (PUFAs) are associated

with reduced risk of developing cardiovascular diseases and diabetes (Gray et al., 2013; von Schacky and Harris, 2007). Furthermore, the brain is enriched with PUFAs, which participate in signal transduction, acting as precursors to many bioactive metabolites that regulate neurotransmission, cell survival, and neuroinflammation (Bazinet and Layé, 2014; Fotuhi et al., 2009). Therefore, unsaturated fatty acids also play a pivotal role in preventing brain disorders.

Despite the promising health benefits of unsaturated fatty acids, their potential as a functional food ingredient is limited due to high susceptibility to oxidation. Oxidation causes quality deterioration in foods, resulting in low nutritional value and unfavourable flavor (Wang et al., 2008). Two major oxidation reactions which occur in lipid-containing food is auto-oxidation and photo-oxidation, of which auto-oxidation is more common (Bradley and Min, 1992). Photo-oxidation is caused by exposure to light and can be efficiently controlled by storing food in dark. Auto-oxidation occurs in the presence of oxygen and is initiated by the interaction of highly reactive radicals with unsaturated fatty acids, causing damage to the cell membrane, and it may give rise to end-products that could be mutagenic or carcinogenic (Mylonas and Kouretas, 1999).

Therefore, esterification of a flavonoid glycoside with an unsaturated fatty acid is mutually advantageous to both compounds, as the modification not only improves the flavonoid bioavailability, but it also increases the stability of the unsaturated fatty acid. In efforts to increase the metabolic stability and bioavailability of the potential anticancer agent phloridzin (PZ), Ziaullah et al., (2013) synthesized six novel derivatives of phloridzin (PZ) through acylation with various long chain unsaturated and saturated fatty

acids using lipase B. Currently, our group has been actively studying the anticancer effects of the PZ ester synthesized through acylation with docosahexaenoic acid (DHA).

1.5.4 Docosahexaenoic acid and its anticancer properties

Docosahexaenoic acid (DHA) (**Figure 2c**) is an omega-3 (ω -3) polyunsaturated fatty acid (PUFA) with a 22-carbon long chain and six double bonds. The human body converts essential fatty acid α -linoleic acid (ALA) to eicosapentaenoic acid (EPA), which is the precursor for DHA. However, the conversion rate is normally inadequate to provide DHA sufficiency (Bistran, 2003); hence, DHA is obtained directly from food sources, especially fish oil. DHA is a significant structural component of the phospholipid membranes, and is found abundantly in brain, retina and sperm (Lin et al., 1993; Neuringer et al., 1986).

The health benefits of DHA has been extensively studied. DHA plays an important role in the development of the nervous system in infants. Poor DHA levels in maternal milk has been associated with an increased risk of delayed or limited neural and visual development (Innis, 2007). A significant correlation has been shown between DHA intake and improved memory, which emphasises the importance of DHA in research pertaining to Alzheimer's disease (Hashimoto et al., 2015; Yurko-Mauro et al., 2010). Dietary intake of DHA also shows cardio-protective effects. In spontaneously hypertensive male rats, DHA was found to suppress ischaemia-induced cardiac arrhythmias and disrupt hypertension developments (McLennan et al., 1996).

In addition, fish oil consumption is linked with low incidence of cancer (Augustsson et al., 2003; Caygill et al., 1996). The anticancer effects of fish oil are often attributed to its abundant content of DHA. Dietary DHA was found to be the primary

tumour-suppressing fatty acid in athymic mice bearing human colon carcinoma (Kato et al., 2007, 2002). Schley et al., (2005) reported that DHA is more potent than EPA in inhibiting the proliferation of human breast carcinoma MDA-MB-231 cells, and the study also postulated that the possible mechanism of action is through suppressing the Akt/NF- κ B cell survival pathway. Another study showed that DHA strongly inhibited cell growth and induced cell cycle arrest in mouse mammary carcinoma 4T1 cells and human breast carcinoma MCF-7 cells through downregulation of Wnt/ β -catenin signaling. Furthermore, a fish oil-supplemented diet significantly suppressed the growth of 4T1 mouse mammary carcinoma in an animal model (Xue et al., 2014). Cytotoxic effect of DHA has also been seen in lung cancer cells, where DHA induces apoptosis via increasing the levels of MAP kinase phosphatase-1 (MKP-1) (Serini et al., 2008). Besides, DHA is also an effective adjuvant agent as it synergistically enhances the efficacy of numerous chemotherapeutic drugs both *in vitro* and *in vivo* studies (Siddiqui et al., 2011).

1.5.5 The emergence of phloridzin docosahexaenoate

As mentioned above, the docosahexaenoic acid (DHA)-acylated phloridzin (PZ), also known as phloridzin docosahexaenoate (PZ-DHA) (**Figure 3**), is emerging as a potent anticancer agent. In 2013, Ziaullah et al., (2013) synthesized a series of long chain fatty acids-acylated derivatives of PZ using lipase B. Six long chain fatty acids, such as saturated fatty acid, stearic acid; monounsaturated fatty acid, oleic acid; and polyunsaturated fatty acids, linoleic acid, α -linolenic acid, eicosapentaenoic acid, and DHA, were used to synthesize their corresponding esters. Among all PZ esters, PZ-DHA showed the greatest inhibition of tyrosinase activity (Ziaullah et al., 2013). The inhibition

of tyrosinase, an oxidase that catalyses melanin formation, is crucial in the treatment of dermatological disorders, including hyperpigmentation and melanoma.

In another study, Nair et al., (2014) investigated the cytotoxic effects of the novel PZ esters in comparison with PZ, aglycone PT, and the corresponding fatty acids against human hepatoma HEPG2 cells, human breast carcinoma MDA-MB-231 cells, and human acute monocytic leukemia THP-1 cells. Strikingly, fatty acid esters of PZ selectively inhibited the growth of cancer cells, while sparing normal human and rat hepatocytes. In the preliminary screening study, PZ-DHA showed the strongest selective inhibitory effects against cancer cells compared to the other esters, and therefore was chosen for further exploration. Gene expression profiles obtained from PZ-DHA-treated HepG2 cells associated PZ-DHA with the downregulation of anti-apoptotic genes, growth factor receptors and downstream signaling, cell cycle machinery, and epigenetic regulators. A recent study has also shown the ability of PZ-DHA to inhibit the production of pro-inflammatory biomarkers in lipopolysaccharide-induced inflammation in THP-1 differentiated macrophages (Sekhon-Loodu et al., 2015).

The anti-proliferative effects of PZ-DHA was further evaluated in triple negative breast cancer MDA-MB-231 cells both *in vitro* and *in vivo* (Fernando, 2014). PZ-DHA caused time- and dose-dependent cytotoxicity in breast cancer cells, while low cytotoxicity was detected in human mammary epithelial cells. Moreover, DNA fragmentation and caspase 3/7 activation confirmed PZ-DHA-induced apoptosis. PZ-DHA also significantly reduced tumour growth in non-obese diabetic severe combined immune-deficient (NOD-SCID) mice bearing breast cancer cells.

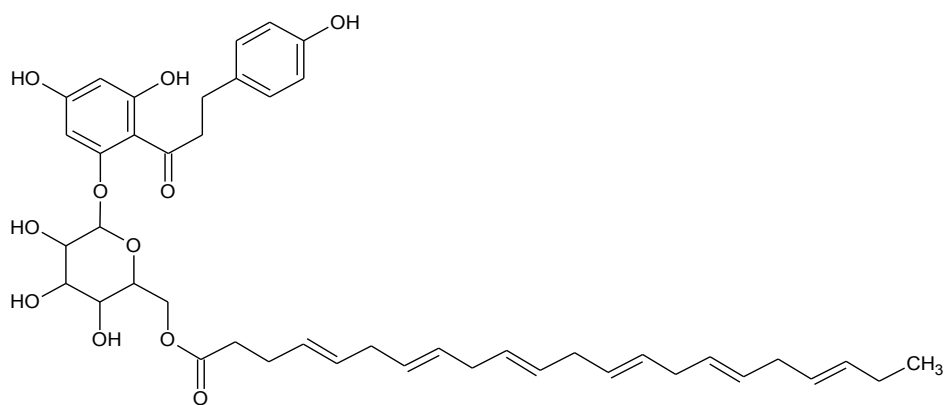


Figure 3. Chemical structure of phloridzin docosahexaenoate.

Phloridzin docosahexaenoate: $C_{43}H_{54}O_{11}$, 746.88 g/mol.

1.6 ZEBRAFISH AS A MODEL FOR LEUKEMIA

Zebrafish is a robust model to study normal and perturbed development processes. A high degree of genetic conservation in hematopoiesis between zebrafish and humans strongly suggests that zebrafish can be used as a tool for leukemia studies (Song et al., 2004). Strikingly, the canonical JAK/STAT signaling pathway is also conserved in zebrafish (Hou et al., 2002). Although zebrafish are phylogenetically more distant from humans than rodents are, their physiology is well conserved as they share 82% of disease-associated genes and metabolic pathways with humans (Lieschke and Currie, 2007).

In addition to high genetic similarity with mammals, the zebrafish has distinct advantages over other animal models. Zebrafish allows large-scale screening to study phenotypic effects of small molecules, and perhaps no other organism is better suited for similar phenotyping (Zon and Peterson, 2005). Besides, the transparent nature of zebrafish embryos coupled with advanced *in vivo* imaging techniques facilitate tumour growth studies without having to dissect the animals (Zon and Peterson, 2005). For improved visualization of tumour engraftment, White et al., (2008) created the *casper* double pigment mutant zebrafish line. Additionally, the zebrafish is genetically-tractable, hence, it allows advanced genetic manipulation that renders mutant and transgenic lines for disease-modeling, using a variety of methods including antisense technology through morpholino oligonucleotides (Nasevicius and Ekker, 2000), microinjection of mRNA (Yuan and Sun, 2009), and the clustered regularly-interspaced short palindromic repeats (CRISPR) nuclease system (Jao et al., 2013).

Other technical advantages are also often associated with the zebrafish model. For instance, zebrafish are small, and can be bred, reared, and maintained easily. Zebrafish become reproductively mature at approximately three months of age, and produce large numbers of offspring per mating pair. Rapid external fertilization of eggs enables easy access for genetic manipulation and imaging (White et al., 2013). In addition to being a cost-effective alternative to rodents, the relatively short duration for completing *in vivo* studies make the zebrafish model well-suited for developmental and toxicological studies.

1.6.1 Zebrafish xenotransplantation

Xenotransplantation refers to the transfer of living cells, tissues or organs between species. Despite the presence of human disease orthologues in zebrafish, human tumours may not be fully represented in the zebrafish model due to certain exceptions. For example, zebrafish do not have the *INK4a/ARF* tumour suppressor gene, which may restrict the mimicking of tumour microenvironment for *in vivo* studies (Sharpless, 2005). Herein xenotransplanting human cancer cells into zebrafish becomes a useful technique.

The first xenotransplantation of human cells into zebrafish occurred in 2005, where zebrafish embryos were successfully engrafted with human metastatic melanoma cells (Lee et al., 2005). The yolk sac close to the duct of Cuvier was established as a suitable site for injection due to its size and ease of transplantation. The standard temperature for incubating zebrafish is 28 °C, whereas the standard temperature for culturing cells is 37°C. Haldi et al., (2006) reported that incubation of xenotransplanted embryos at 35 °C compensates for both the zebrafish embryogenesis and the growth of injected tumour cells. Fluorescent tracking dye can be used to label and track well-

characterized cell lines or primary cells (Bentley et al., 2015; Corkery et al., 2011; Haldi et al., 2006).

Using zebrafish as a xenograft model is often advantageous than mammalian systems. Although the mouse as a host will provide an evolutionary-similar environment, it is often difficult to perform *in vivo* imaging following xenotransplantation in mice. Furthermore, both the use of immune-permissive mice and engraftment heterogeneity that requires a large number of animals increase the costs of animal studies (Konantz et al., 2012). An important feature of zebrafish that enables efficient xenotransplantation is that the zebrafish embryos lack a fully functional adaptive immune system until 4 weeks post-fertilization, therefore, unlike immune-competent mice, zebrafish do not require immunosuppression prior xenotransplantation (Lam et al., 2004). With the help of advanced *in vivo* imaging, a time frame of 7 days post-injection is sufficient to visualise cancer cell proliferation, migration, and response to a range of treatments in real time (Haldi et al., 2006).

Since the zebrafish is small, a low number of cells (about 50-200) is sufficient to induce tumour formation. Furthermore, drug responses can be studied by directly adding the drug of interest to the water, which requires minimal time and resources. In addition, the small size of zebrafish is also significant for preclinical assessment as it reduces the amount of drug required for each experiment (Haldi et al., 2006). Following treatment, the embryos can be enzymatically dissociated to single cell suspensions for quantification and further analysis (Corkery et al., 2011). The ease and cost-effectiveness of experiments, together with the unique imaging advances, make the zebrafish model a prominent tool to study tumour progression and drug responses.

1.7 HYPOTHESIS

Phloridzin docosahexaenoate (PZ-DHA), a structurally modified derivative of phloridzin (PZ), will show selective cytotoxicity to human leukemic cells *in vitro* and in an *in vivo* model employing zebrafish xenografts. PZ-DHA will suppress the activation of the JAK/STAT pathway in human leukemic cells.

1.8 RESEARCH OBJECTIVES

- (a) To study the cytotoxic effects of phloridzin docosahexaenoate (PZ-DHA) in human T-cell acute lymphoblastic leukemia (T-ALL) Jurkat cells and human chronic myeloid leukemia (CML) K562 cells, relative to parent compounds, phloridzin (PZ) and docosahexaenoic acid (DHA); aglycone of PZ, phloretin (PT); and conventional drugs, doxorubicin and imatinib.
- (b) To study the selective cytotoxicity of PZ-DHA using normal murine-derived T cells and bone marrow cells.
- (c) To elucidate the putative mode of action by investigating JAK and STAT protein expression levels in PZ-DHA treated leukemic cells.
- (d) To validate the anti-proliferative properties of PZ-DHA *in vivo* using a zebrafish xenograft model.

CHAPTER 2: MATERIALS AND METHODS

2.1 ANTIBODIES

The antibodies used in this study include anti-pSTAT1 (1:2000), anti-pSTAT3 (1:4000), anti-pSTAT5 (1:2000), anti-pJAK2 (1:2000), anti-p-c-Abl (1:1000), anti-pSrc (1:2000), anti-STAT1 (1:2000), anti-STAT3 (1:4000), anti-STAT5 (1:2000), and anti-JAK2 (1:2000) from Cell Signaling Technology through New England Biolabs (Whitby, ON). Anti-actin from Sigma-Aldrich (Oakville, ON) and goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate from Abcam Inc (Toronto, ON) were kindly provided by Dr. Catherine Too (Department of Biochemistry & Molecular Biology, Dalhousie University).

2.2 CELL LINES

Two human leukemic cell lines and normal mouse cells were used in this study. T-cell acute lymphoblastic leukemia (T-ALL) Jurkat cells and chronic myeloid leukemia (CML) K562 cells were kind gifts from Dr. Catherine Too (Department of Biochemistry & Molecular Biology, Dalhousie University).

Murine T-cells and bone marrow cells were kindly provided by Simon Gebremeskel from Dr. Brent Johnston's laboratory (Department of Microbiology & Immunology, Dalhousie University). The cells were isolated from 8- to 12-week-old female C57/BL6 mice purchased from Charles River laboratories (*Wilmington, MA*). T-cells were derived from mouse spleens, and bone marrow cells were isolated from mouse femur and tibia.

2.3 CHEMICALS AND REAGENTS

RPMI-1640 medium, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), trypan blue stain, phenazine methosulfate (PMS), phloretin (PT), interferon alpha-2b (IFN α -2b), IGEPAL[®] CA-630, sodium deoxycholate, sodium dodecyl sulfate (SDS), phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail, sodium orthovanadate, phloridzin (PZ), imatinib mesylate, and doxorubicin were purchased from Sigma-Aldrich (Oakville, ON). Docosahexaenoic acid (DHA) and staurosporine were purchased from Nu-Chekprep (Elysian, MN) and Biovision Incorporated (Milpitas, CA), respectively. PZ-DHA, PZ, DHA, PT, imatinib mesylate, and doxorubicin were dissolved in DMSO for 40 μ M stock solutions and stored at -80 °C. The stock concentration for staurosporine (STS) was 107.17 μ M. Kits used in this study were purchased from Promega (Madison, WI) unless otherwise stated.

2.4 CELL CULTURE CONDITIONS

Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. K562 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Both cell lines, cultured in suspension, were maintained at 37 °C in a humidified incubator with 5% CO₂. Cells were subcultured at 1×10^6 cells/ml by addition or replacement of fresh medium in T-75 flasks. For cryopreservation, complete growth medium supplemented with 5% v/v DMSO was used, and cells were stored in liquid nitrogen. Cell number was determined using a hemocytometer based on the ability of viable cells to exclude trypan blue staining.

2.5 CELL VIABILITY ASSAYS

2.5.1 MTS assay

Cell viability was measured using MTS assay, which is a colorimetric method comprising a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS) (Cory et al., 1991). MTS is bio-reduced into a formazan product by NAD(P)H-dependent dehydrogenase enzymes found in metabolically active cells. The amount of formazan produced is measured by absorbance at 490 nm, which is directly proportional to the number of live cells in the culture.

The MTS assay was performed using commercially available CellTiter 96® Aqueous MTS reagent powder (Promega, Madison, WI) and PMS (Sigma Aldrich, Oakville, ON). According to the manufacturer's protocols, Jurkat and K562 were seeded in 96-well plates at a density of 3.5×10^4 cells/well, 5×10^3 cells/well, respectively. Cells were then treated with vehicle (0.25% DMSO) or test compounds (PZ-DHA, PZ, DHA, PT, imatinib, and doxorubicin) at various doses such as 10, 25, 50, 75, and 100 μ M for 12, 24, and 48 h at 37 °C. At the end of each time-points, MTS/PMS solution (final concentrations: MTS, 333 μ g/ml; PMS, 25 μ M) was added into each well and incubated for 2.5 h at 37 °C. The absorbance at 490 nm was measured using the Infinite® 200 PRO multimode microplate reader (Tecan Trading AG, Switzerland). Background absorbance values were obtained from culture medium supplemented with treatment conditions in the absence of cells and were subtracted from their respective cells-containing counterparts.

Relative cell viability (%) was calculated as: $[\text{absorbance}_{\text{test compounds}} / \text{absorbance}_{\text{vehicle}}] \times 100$. Each experiment was performed in four replicates and repeated three times independently.

2.5.2 ATP assay

Adenosine 5'-triphosphate (ATP) is required for cell survival. ATP levels significantly correlate with cell number and viability (Kangas et al., 1984). When cells lose membrane integrity, they lose the ability to synthesize ATP, and endogenous ATPases deplete any remaining ATP rapidly (Riss et al., 2015). The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) is a single-reagent method of determining metabolically active cells based on the levels of ATP present. Besides providing reagent that contains detergent to lyse cell membrane and ATPase inhibitors to stabilize the ATP released from lysed cells, the assay also contains luciferin (substrate) and luciferase (enzyme) that provides measurable bioluminescence signal in the presence of ATP.

Following manufacturer's protocol, Jurkat and K562 cells were seeded in opaque-walled 96-well plates at a density of 3.5×10^4 cells/well and 5×10^3 cells/well, respectively. Cells were then treated with vehicle (0.25 % DMSO) or test compounds (PZ-DHA, PZ, DHA, PT, imatinib, and doxorubicin) at 100 μM for 24 h at 37 °C. The plates were then equilibrated at room temperature for 30 min before the addition of an equal volume of CellTiter-Glo® Reagent into each well. The contents were mixed for 2 min on an orbital shaker to induce cell lysis. The plates were incubated at room temperature for 10 min to stabilize signal and luminescence was recorded using the Infinite® 200 PRO multimode microplate reader (Tecan Trading AG, Switzerland). ATP

levels were measured relative to vehicle-treated controls. Each experiment was performed in three replicates and repeated three times independently.

2.6 MORPHOLOGY ASSESSMENT USING PHASE CONTRAST MICROSCOPY

The distinct morphological changes of apoptosis has been extensively described (Elmore, 2007; Häcker, 2000). In brief, the characteristics include cell shrinkage, chromatin condensation, membrane blebbing, and apoptotic body formation. Jurkat and K562 cells were seeded in 24-well plates at a density of 1.4×10^5 cells/well and 2.5×10^4 cells/well, respectively. Cells were then treated with 50 and 100 μM of test compounds (PZ-DHA, PZ, DHA, and PT) or vehicle (0.25 % DMSO) for 24 h at 37 °C. The morphology of cells was observed under an inverted phase contrast Nikon Eclipse E 100 microscope (Nikon, Mississauga, ON) and images were captured at 100 \times magnification using Infinity digital microscopy camera (Lumenera corporation, Ottawa, ON). Photographs are representative of three independent experiments.

2.7 DNA FRAGMENTATION ASSAY

In most cases, DNA fragmentation is considered a biochemical hallmark of apoptosis (Bortner et al., 1995). During apoptosis, endonucleases are activated to cleave internucleosomal DNA, thus generating multiple DNA fragments that can be detected as a DNA ladder following agarose gel electrophoresis. A demonstrable presence of DNA laddering has generally been regarded as a distinguishing feature of cell death by apoptosis (Yeung, 2002).

Jurkat and K562 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and 3×10^5 cells/well, respectively. Cells were then treated with 50 or 100 μM of test compounds (PZ-DHA, PZ, DHA, and PT), or vehicle (0.25 % DMSO) for 24 h at

37 °C. Total DNA was extracted from cells using the GenElute Mammalian Genomic DNA MiniPrep kit (Sigma-Aldrich, St. Louis, MO). Briefly, treated cells were lysed and transferred to binding columns. After several washes, DNA was eluted and stored at 4 °C. For detection of DNA fragmentation, the extracted DNA samples were electrophoresed in a 1.2% agarose gel containing 0.5 µg/mL GelRed™ stain (Biotium Inc., Hayward, CA) in Tris-Acetate-EDTA (TAE) buffer, pH 8.3. After electrophoresis, the gel image was captured using the Gel Doc 100 system (Bio-Rad, Mississauga, ON).

2.8 LACTATE DEHYDROGENASE (LDH) ASSAY

Apoptosis or necrosis-induced cell death can be further studied by quantifying the release of lactate dehydrogenase (LDH) into extracellular space due to loss of membrane integrity. LDH assay measures cell death using a coupled two-step reaction. In the first step, LDH catalyses the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses the newly-formed NADH and H^+ to catalyse the reduction of a tetrazolium salt (INT) to highly-colored formazan which can be quantified by measuring absorbance at 490 nm.

LDH activity was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) according to the manufacturer's protocol. Jurkat and K562 cells were seeded in 24-well plates at a density of 3.5×10^4 cells/well and 5×10^3 cells/well, respectively. Cells were then treated with vehicle (0.25 % DMSO) or test compounds (PZ-DHA, PZ, DHA, PT) at 50 or 100 µM for 24 h at 37 °C. An additional control for measuring maximum LDH release was prepared by adding lysis buffer (9% Triton X-100) to positive control wells 45 min before the intended treatment time-point. After centrifugation at $300 \times g$ for 10 min, the supernatant was transferred to a fresh

plate, followed by addition of equal amount of CytoTox 96[®] Reagent. The enzymatic reaction was stopped by adding 1 M acetic acid after 30 min incubation at room temperature, protected from light, and absorbance at 490 nm was measured. The percent cytotoxicity was expressed as: [experimental LDH release/maximal LDH release] ×100%.

2.9 LUMINESCENT ASSAY FOR CASPASE 3/7 DETECTION

The activation of caspase 3 and 7 enzymes were measured using caspase-Glo[®] 3/7 assay kit (Promega, Madison, WI). The members of the cysteinyl aspartate-specific proteinases (caspase) family, especially caspase 3, play key effector roles in apoptosis in mammalian cells (Nicholson and Thornberry, 1997; Porter and Jänicke, 1999). This assay uses aspartic acid-glutamic acid-valine-aspartic acid (DEVD) tetrapeptide sequence-containing luminogenic substrate, which shows selectivity for both caspase 3 and 7 (Bayascas et al., 2002). Briefly, upon caspase 3/7, the DEVD peptide is cleaved, and produces aminoluciferin that reacts with luciferase to generate luminescent signal.

Following manufacturer's protocol, Jurkat and K562 cells were seeded in opaque-walled 96-well plates at a density of 3.5×10^4 cells/well and 5×10^3 cells/well, respectively. Cells were then treated with vehicle (0.25 % DMSO) or test compounds (PZ-DHA, PZ, DHA, PT, imatinib, and doxorubicin) at 100 μ M for 12 h at 37 °C. Staurosporine (1 μ M) was used as positive control. The plates were allowed to equilibrate to room temperature prior to adding 100 μ L of Caspase-Glo[®] 3/7 Reagent to each well containing 100 μ l of treated cells and no-cells counterpart. The contents were gently mixed using a plate shaker for 30 seconds, and incubated for 2 hr at 37 °C. Luminescence was measured on the Infinite[®] 200 PRO microplate reader (Tecan, Switzerland).

2.10 FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

Flow cytometric identification of apoptotic and necrotic cells was performed with Annexin V/ propidium iodide (PI) double staining method (Vermes et al., 1995). Annexin V is a member of the Annexin family of calcium-dependent intracellular proteins that binds to phosphatidylserine (PS). PS is normally only found on the intracellular leaflet of the plasma membrane in normal healthy cells, but during early apoptosis, membrane asymmetry is lost and PS translocates to the outer leaflet. Hence, fluorochrome-labeled Annexin V can be used to specifically target and identify apoptotic cells. PI is used in conjunction with Annexin V to differentiate between apoptotic and necrotic cells. PI is a fluorescent dye that binds to DNA by intercalating between the bases. Early apoptotic cells will exclude PI, while late stage apoptotic cells and necrotic cells will stain positively, due to the loss of membrane integrity that allows PI to readily bind to DNA.

Jurkat, K562, murine T-cells, and murine bone marrow cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Cells were then treated with PZ-DHA (25, 50, and 75 μ M) or vehicle (0.25 % DMSO) for 24 h at 37 °C. Treated cells were stained with Annexin V/PI using Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. Briefly, cells were washed with $1 \times$ PBS and subjected to centrifugation at $200 \times g$ for 5 min. Cell pellets were then resuspended in premixed Annexin-V-FLUOS labeling solution. After 15 min incubation at room temperature, flow cytometric analysis was performed using a FACSCalibur instrument (BD Bioscience, Mississauga, ON).

2.11 WESTERN BLOT ANALYSIS

2.11.1 Preparation of total cell lysates

Jurkat and K562 cells were seeded in T-25 flasks at a density of 4×10^6 cells. Cells were then treated with 10, 15, and 30 μM of PZ-DHA or vehicle (0.08 % DMSO) for 24 h at 37 °C. Jurkat cells were stimulated with IFN α -2b at a final concentration of 5×10^4 units/ml for 1 hr at 37 °C. After the desired time period, cells were harvested and washed once with $1 \times$ PBS. The cell pellets were resuspended in ice-cold RIPA lysis buffer solution ($1 \times$ PBS containing 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS; and freshly added 1 mM PMSF, 1 mM sodium orthovanadate, and protease inhibitor cocktail). Cells were disrupted by passing through a 21-gauge needle and additional PMSF (10 $\mu\text{g}/\text{ml}$) was added. The cells were incubated for 50 min on ice and then centrifuged at 13,000 rpm for 20 min at 4°C to remove cell debris. The supernatant, which contained cellular protein, was collected and stored at -20°C until further analysis.

2.11.2 Protein assay

Protein concentrations of total cell lysates were determined using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON), which is based on a dye-binding method previously described by Bradford (Bradford, 1976). Briefly, the Bradford assay is a colorimetric method based on the absorbance shift of Coomassie Brilliant Blue G-250 dye, a dye that is predominantly in the doubly protonated red cationic form (470 nm) and converts to a stable unprotonated blue form (595 nm) upon binding to proteins.

Bovine serum albumin (BSA) standard (2 mg/ml) was used to construct a standard curve that falls in the linear range of 0.2 $\mu\text{g}/\text{ml}$ to 0.8 $\mu\text{g}/\text{ml}$. Samples were diluted at a ratio of 1:4 with sterile water. One part of dye reagent concentrate was diluted

with 4 parts of distilled water. Five millilitres of dye reagent was then added to 100 μ l of the standards and samples prepared in duplicates. Absorbance was measured at 595 nm using Eppendorf® Biophotometer. The values from the samples were plotted against the standard curve to obtain the total protein concentration of each sample.

2.11.3 Polyacrylamide gel electrophoresis

Protein samples were diluted with 50 % (v/v) of 3 \times sample buffer (188 mM Tris chloride of pH-6.8, 3% SDS, 30% glycerol, 0.01% bromophenol blue, and 15% β -mercaptoethanol). The protein samples (20 μ g/lane) were incubated for 5 min at 95°C, and then loaded next to Precision Plus Protein™ Standards (Bio-Rad, Mississauga, ON, Canada). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The polyacrylamide gels contained a 4% stacking component (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate (APS), and 0.05% tetramethylethylenediamine (TEMED)) and a 10 % resolving component (0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% APS, and 0.05% TEMED). Protein samples were resolved at 200 V in SDS-PAGE running buffer (0.02 M Tris-HCL, pH 8.3, 0.2 M glycine and 0.1% SDS).

2.11.4 Western blot

Following SDS-PAGE, resolved proteins were transferred onto BioTrace™ NT nitrocellulose membranes (Pall Life Sciences, Pensacola, FL) in Western transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol), at 100 V for 2 h at 4°C or 30 V overnight at 4°C. Nitrocellulose membranes were blocked in 10% nonfat milk (w/v) in Tris-buffered saline-Tween 20 solution (TBST; 0.02 M Tris-HCl, pH 7.6, 0.2 M NaCl, 0.05% Tween 20) for 1 h at room temperature or overnight at 4°C. The nitrocellulose

blots were then incubated with primary antibody prepared in 10% nonfat milk in TBST (0.05% Tween 20) for 2 h at room temperature overnight at 4°C. The blots were washed 3 times with TBS for 15 min each time. The blots were then incubated with secondary antibody prepared in 10% nonfat milk in TBST (0.05% Tween 20) for 1 h. The same washing cycle was repeated. Immunoreactive signals were detected using ImmunoStar™ WesternCTM Chemiluminescence (Bio-Rad, Mississauga, ON) and visualized by exposure to X-ray film.

2.12 DRUG EFFICACY IN *IN VIVO* ZEBRAFISH XENOGRAFT

2.12.1 Zebrafish (*Danio rerio*) husbandry

Zebrafish and facilities were provided by Dr. Jason Berman (IWK Health Centre, Halifax, NS). The fish were maintained, bred and developmentally staged according to standard protocols (Westerfield, 2000). Zebrafish were maintained in 28.5 °C water at pH 6-8, and exposed to light for 14 hours. *Casper* zebrafish were employed in this study. *Casper* is a translucent mutant line that allows rapid monitoring and *in vivo* imaging of tumor progression (White et al., 2008). The embryos were collected, and incubated at 28.5 °C in egg water (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂ and 0.16 mM MgSO₄). Use of zebrafish in this study was approved by the Dalhousie University Animal Care Committee (Protocol 15-126).

2.12.2 Cell labeling

Prior to xenotransplanting leukemic cells into *casper* embryos, K562 and Jurkat cells were labelled with a fluorescent tracking dye. Cells were cultured to approximately 80% confluency in T-75 flask, harvested, and washed with 1 × PBS. Cells were centrifuged for 5 min at 100 × g, and washed twice with their respective complete growth

medium. Cells were re-suspended at a concentration of 10 million cells per ml in $1 \times$ PBS with a 5 $\mu\text{g}/\text{ml}$ final concentration of CellTracker™ Orange CMTMR dye (Life Technologies, Burlington, ON, Canada). The suspension was incubated for 20 min at 37 °C and then washed once in PBS/FBS in a ratio of 1:10 (FBS: PBS) before resuspending in complete growth medium for injection into embryos.

2.12.3 Xenotransplantation

Xenotransplantation was carried out as described by Corkery et al., (2011) (**Figure 4**). *Casper* embryos at 48-hour post fertilization (hpf) were anesthetized with Tricaine (ethyl 3-aminobenzoate methanesulfonate salt, MS-222, Sigma-Aldrich) at a final concentration of 200 $\mu\text{g}/\text{mL}$ prior to injection. A manual PLI-100 microinjector (Warner Instruments, Hamden, CT) was used to load the cell suspension into a pulled capillary needle for embryo injection. Embryos were arrayed in a six lane agarose plate prepared in a 42 medium size (10 cm) Petri dish. Approximately 50-100 cells were injected into the yolk sac of each embryo. Following injection, embryos were kept at 28 °C for 1 h and then re-located to a 35 °C incubator for the duration of the experiments. At 24 h post injection (hpi), embryos were screened for the presence of a fluorescent cell mass within the yolk site. Positive embryos were isolated for experiments.

2.12.4 Live Cell Imaging and Cell Growth Quantification

A group of 4-6 embryos, arrayed in a 6-well plate, were imaged and analyzed at 24, 48, 72, and 96 hpi to ensure successful engraftment and proliferation of leukemic cells in zebrafish embryos. Live cell imaging was done using an inverted Axio Observer Z1 microscope equipped with a Colibri LED light source (Carl Zeiss, Westlar, Germany) and an AxioCam Rev 3.0 CCD camera and Axiovision Rel 4.0 software (Carl Zeiss

Microimaging Inc). Both brightfield and accompanying fluorescent images were captured for analysis.

Cell growth was quantified by dissociating embryos with uniform fluorescent cell mass at the site of injection at 24, 48, 72, and 96 hpi. For each time-point, a group of 15-20 embryos was euthanized by Tricaine overdose. Embryos were then transferred to microtube containing 1 ml of $1 \times$ PBS. To this suspension, 54 μ l of pre-warmed collagenase (Sigma-Aldrich, Oakville, ON, Canada) at 100 mg/ml was added. Embryos were incubated at 37 °C for 10 min, then physically dissociated through pipetting up and down 10 times at 5 min intervals until embryos were visibly dissociated. Collagenase activity was then halted by addition of 200 μ l of FBS, followed by centrifugation at $300 \times$ g for 5 min. The pellets were washed in 1 ml of PBS/FBS solution, and finally resuspended again in 10 μ l of PBS/FBS per embryo. The single cell suspension was imaged using the inverted Axio Observer Z1 microscope, and analyzed using a semi-automated macro (Image J computer software, NIH, Bethesda, MD) where relative fluorescent cell numbers per embryo was determined.

2.12.5 *In vivo* cell proliferation assay

Cell proliferation assay is similar to growth quantification described in Section 2.12.4. Briefly, a group of 15-20 positively injected embryos were divided into two groups; one sacrificed at 24 hpi, and the other maintained with presence (PZ-DHA) or absence (0.25 % DMSO) of treatment and sacrificed at 96 hpi. Cell number determined at 24 hpi is the base-line number of leukemia cells prior to treatment. PZ-DHA was added directly to the fish water, and embryos were incubated at 35 °C until 96 hpi. At indicated time-point, embryos were dissociated into single-cell suspension and quantified.

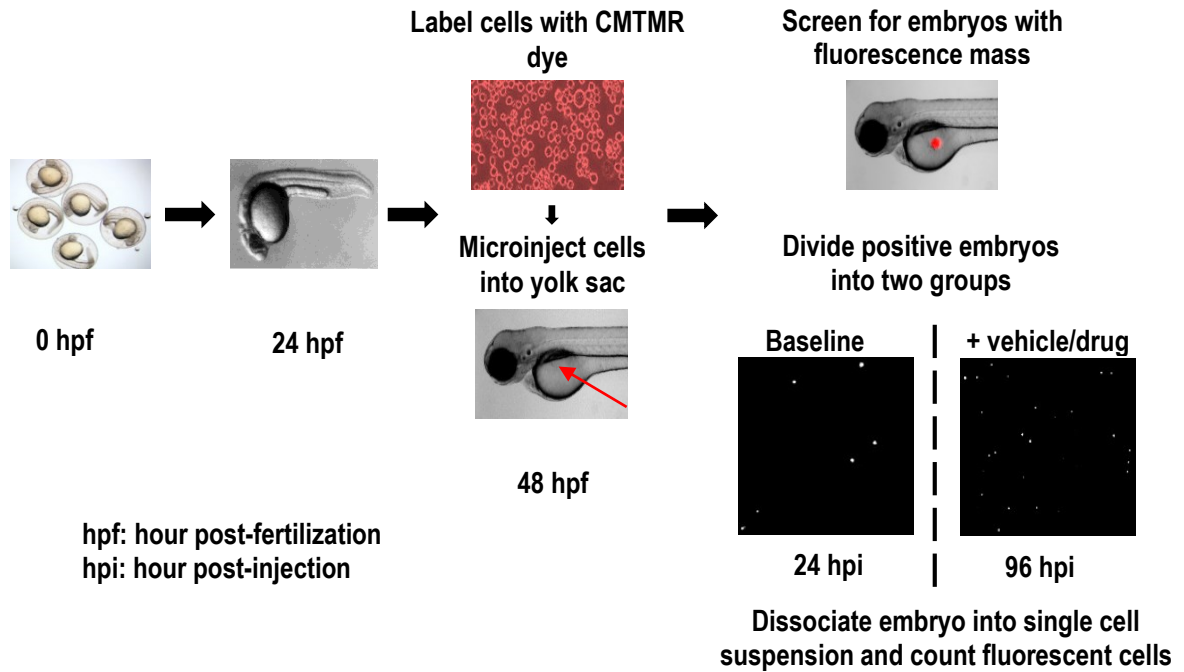


Figure 4. Schematic representation of *in vivo* human leukemia proliferation assay using zebrafish xenotransplantation.

Fluorescently-labelled human leukemic cells are microinjected into the yolk sac of 24 h post-fertilization (hpf) *casper* embryos. Embryos are screened for consistent fluorescent mass at the site of injection, and treatments begin at 24 hours post-injection (hpi), and last for 72 h. The average number of fluorescent cells is quantified following enzymatic dissociation of embryos to a single cell suspension at baseline (24 hpi) and 96 hpi. Fold increase is determined by dividing cell count at 96 hpi by cell count at 24 hpi.

Modified from Corkery et al., (2011).

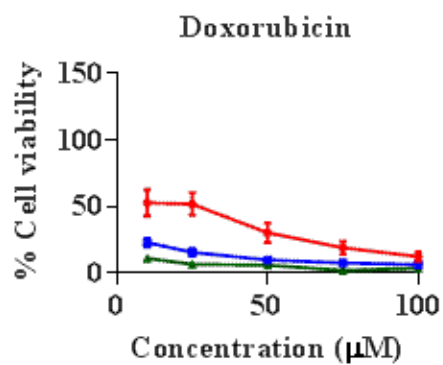
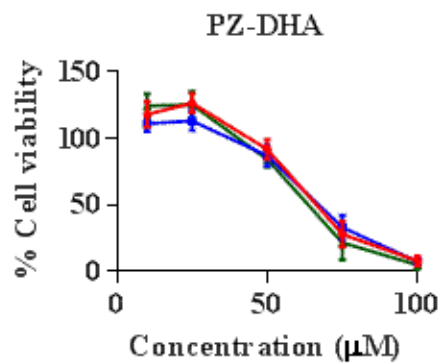
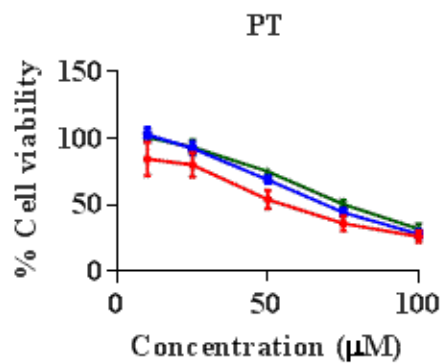
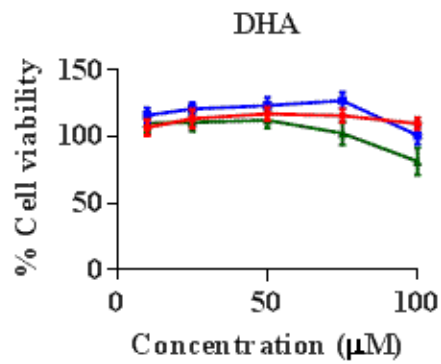
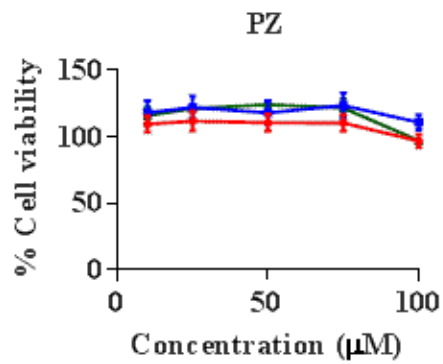
2.13 STATISTICAL ANALYSIS

All the data were analyzed statistically using Minitab 16 at 5%, 1% or 0.1% significance level. Results were expressed as mean \pm SEM of three independent experiments conducted in triplicates or quadruplicates. Differences among means were analysed using 2-sample Student's *t*-test or one-way analysis of variance (ANOVA) using Tukey's or Dunnett's tests as the multiple means comparison method where appropriate. Differences were considered statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001.

CHAPTER 3: RESULTS

3.1 PZ-DHA (NOT PZ OR DHA) IS CYTOTOXIC TO JURKAT AND K562 CELLS.

To evaluate the putative anti-proliferative activity of PZ-DHA, MTS assay that measures metabolic activity as an indication of cell viability was performed. The inhibitory effect of PZ-DHA relative to its parent compounds (PZ and DHA), and aglycone of PZ (PT) was evaluated against human T-cell acute lymphoblastic leukemia (Jurkat) and human chronic myeloid leukemia (K562) cell lines. Doxorubicin and imatinib were used as positive drug controls for Jurkat and K562 cell lines, respectively. Cell lines were treated with increasing concentrations (10, 25, 50, 75, 100 μM) of compounds/drugs at 12, 24, and 48 h time-points. PZ-DHA and aglycone PT caused a reduction in the viability of both Jurkat and K562 cells in a dose-dependent manner whereas, both parent compounds PZ and DHA were not effective at reducing viability, with the exception that DHA caused a marked inhibitory effect on Jurkat and K562 cells at the highest treatment concentration (100 μM) at the 48 h time-point (**Figure 5 and Figure 6**). As expected, the control drugs showed both dose- and time-dependent decreases in cell viability upon treatment. The IC_{50} values of PZ-DHA, PT, and control drugs for respective cell lines were determined using Graphpad Prism software and tabulated as **Table 4**.



—●— 12 h —●— 24 h —●— 48 h

Figure 5. PZ-DHA and aglycone PT reduce viability of Jurkat cells in a dose-dependent manner.

Viability of Jurkat cells upon treatment with PZ-DHA was evaluated using MTS assay. Cells were seeded at a density of 3.5×10^4 cells/well. Cells were then treated with 10, 25, 50, 75, or 100 μM of PZ-DHA, PZ, DHA, PT, and doxorubicin for 12, 24, and 48 h at 37 °C. Data are mean value of three independent experiments performed in quadruplicates. Mean \pm SEM ($n = 12$).

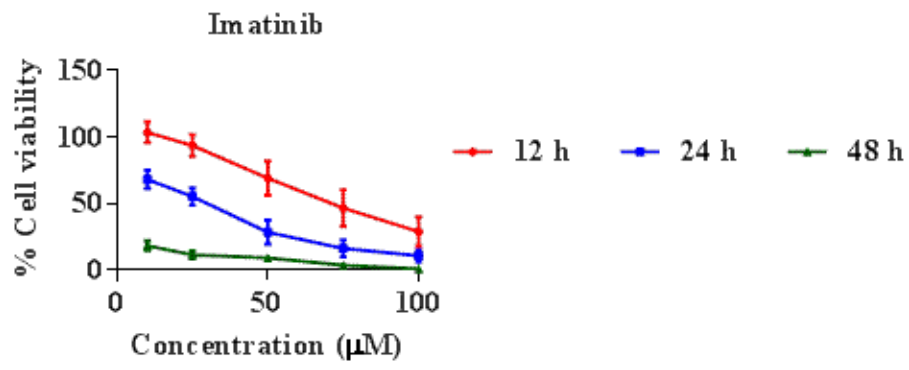
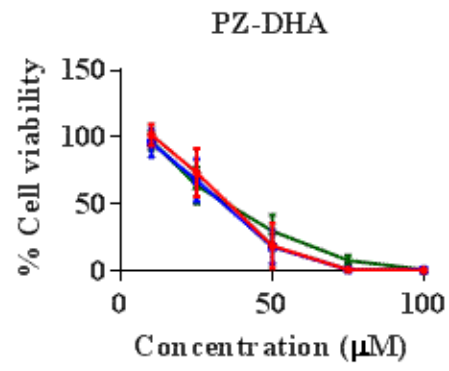
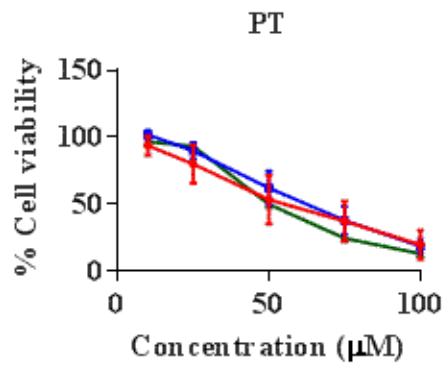
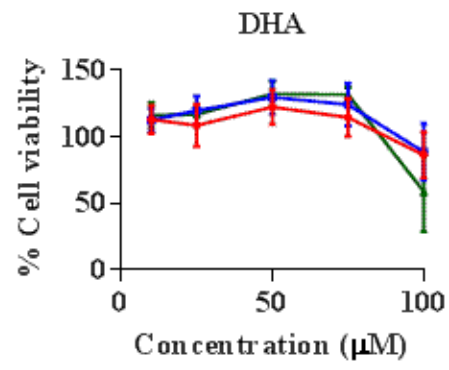
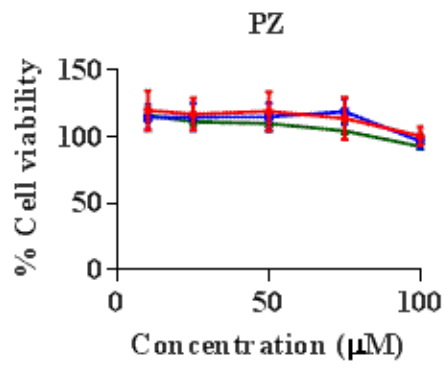


Figure 6. PZ-DHA and aglycone PT reduce viability of K562 cells in a dose-dependent manner.

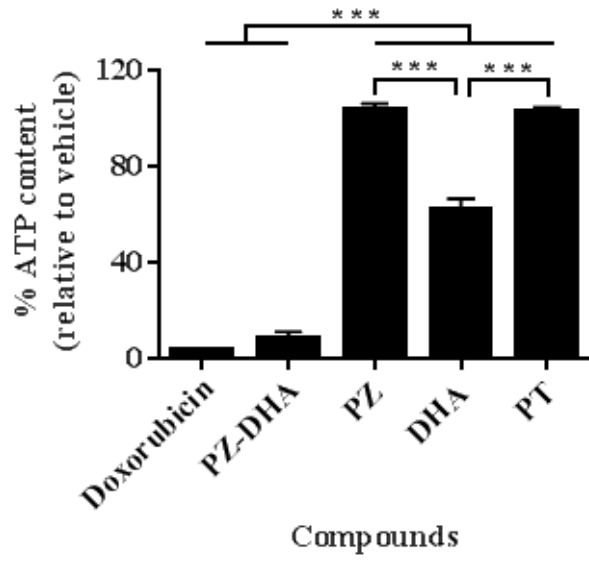
Viability of K562 cells upon treatment with PZ-DHA was evaluated using MTS assay. Cells were seeded at a density of 5×10^3 cells/well, respectively. Cells were then treated with 10, 25, 50, 75, or 100 μM of PZ-DHA, PZ, DHA, PT, and imatinib for 12, 24, and 48 h at 37 °C. Data are mean value of three independent experiments performed in quadruplicates. Mean \pm SEM ($n = 12$).

Table 4. IC₅₀ values determined by non-linear regression of MTS assay.

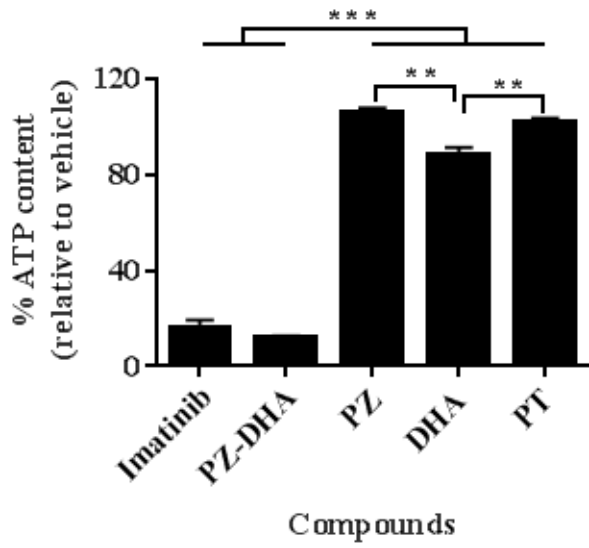
Compound /drug	Cell line	IC ₅₀ values (μM)		
		12 h	24 h	48 h
PZ-DHA	Jurkat	67.03	67.37	63.07
	K562	32.57	30.01	33.04
PT	Jurkat	53.40	66.47	75.50
	K562	52.97	60.22	51.33
Doxorubicin	Jurkat	16.19	1.508	0.269
Imatinib	K562	70.27	23.37	1.497

3.2 PZ-DHA-TREATED JURKAT AND K562 CELLS HAVE LOW CELLULAR ATP LEVELS.

ATP has been widely used as a valid marker for determining viable cell number. The luminogenic ATP assay is considered more sensitive and precise than other viability assays including the MTS assay (Dawson et al., 2010; Riss et al., 2015). The percent viability obtained using these assays are comparable, however, certain exceptions do exist (Chakrabarti et al., 2000; Wang et al., 2010). ATP levels were measured in Jurkat and K562 cells treated with vehicle (0.25 % DMSO) or 100 μ M of test compounds (PZ-DHA, PZ, DHA, PT, doxorubicin, and imatinib) for 24 h. As expected, significant reduction in ATP levels were detected in Jurkat and K562 cells treated with 100 μ M of PZ-DHA or positive controls (doxorubicin and imatinib) (**Figure 7**). Although aglycone PT showed reduced cell viability in MTS assay, no significant change in ATP levels were observed in PT-treated Jurkat and K562 cells. DHA-treated Jurkat and K562 cells showed significantly lower ATP levels than PZ- and PT-treated cells.



(a)



(b)

Figure 7. PZ-DHA causes significant reduction of ATP levels in (a) Jurkat and (b) K562 cells.

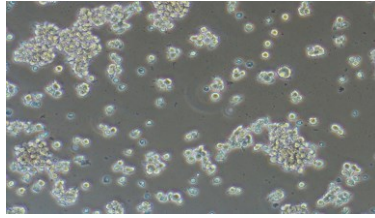
The change in ATP levels as an indication of cell viability was measured using CellTiter-Glo® Luminescent Cell Viability Assay upon treatment with 100 μ M of test compounds or vehicle (0.25 % DMSO) for 24 h. Data shown are mean \pm SEM of three independent experiments conducted in quadruplicates. Statistical analysis was performed using one-way ANOVA and the differences among means were compared using Tukey's test.

P < 0.01, and *P < 0.001.

3.3 PZ-DHA INDUCES EXTENSIVE MORPHOLOGICAL FEATURES OF APOPTOSIS.

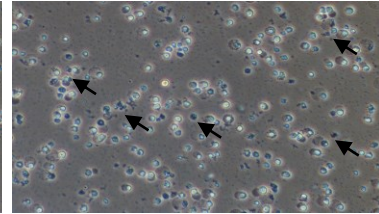
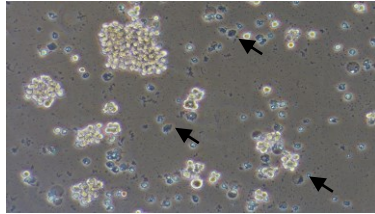
Both MTS and ATP assays showed significant reduction in the viability of PZ-DHA-treated Jurkat and K562 cells. Further studies were conducted to investigate whether reduced cell viability was caused by inhibition of cell proliferation or induction of apoptosis. A series of typical morphological changes, such as shrinkage of cells, membrane blebbing, and fragmentation into apoptotic bodies, is often associated with apoptosis. The morphology of Jurkat and K562 cells treated with 50 and 100 μ M of test compounds or vehicle (0.25 % DMSO) for 24 h were detected using phase contrast microscopy at 100 \times magnification. Both PZ-DHA-treated Jurkat and K562 cells displayed typical apoptotic morphology, with membrane blebbing and formation of apoptotic bodies being evident (**Figure 8** and **Figure 9**, respectively).

Vehicle

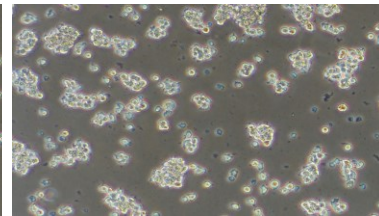
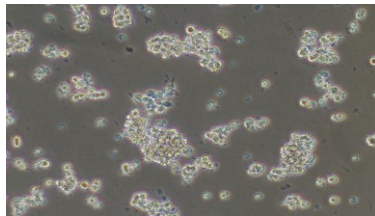


DMSO (0.25 %)

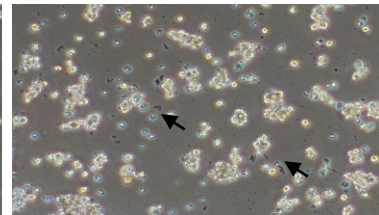
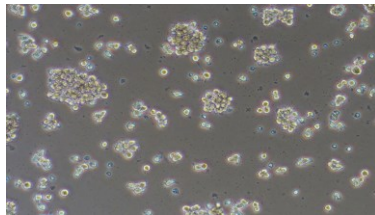
PZ-DHA



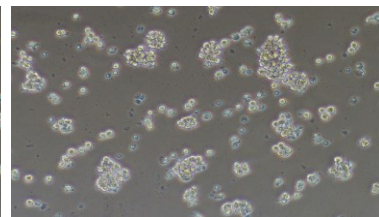
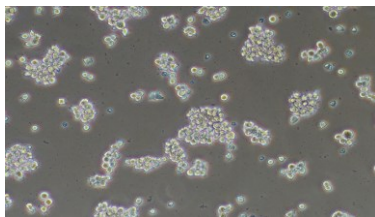
PZ



DHA



PT



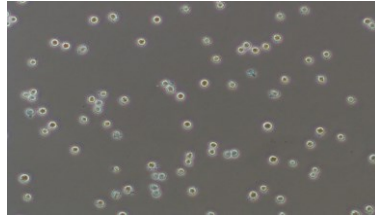
50 μM

100 μM

Figure 8. PZ-DHA-treated Jurkat cells exhibit extensive apoptotic features.

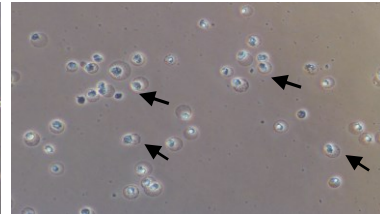
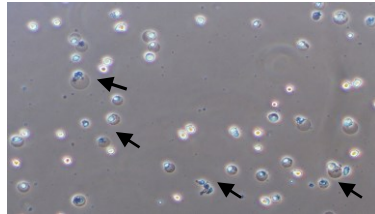
Cells were treated with 50 and 100 μ M of PT, PZ, DHA, PZ-DHA and vehicle for 24 h and photographed with Nikon Eclipse E 100 phase contrast microscope equipped with Infinity digital microscopy camera at \times 100 magnification using phase contrast microscopy. Photos are representative of three independent experiments. Arrows indicate cells that show morphological alterations.

Vehicle

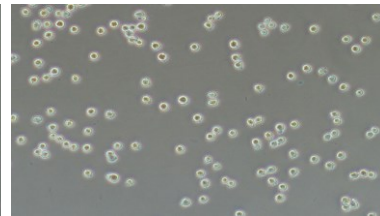
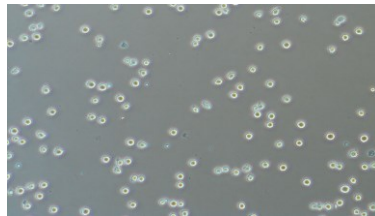


DMSO (0.25 %)

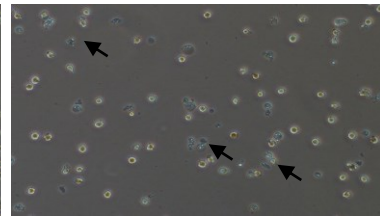
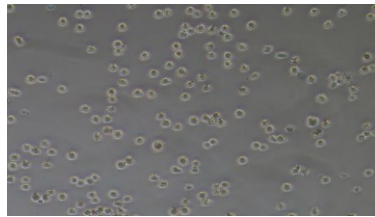
PZ-DHA



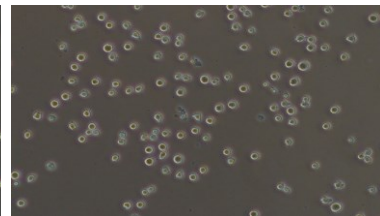
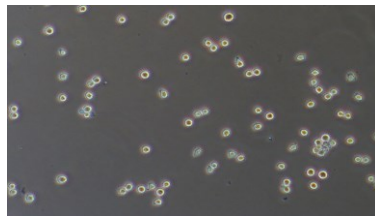
PZ



DHA



PT



50 μM

100 μM

Figure 9. PZ-DHA-treated K562 cells exhibit extensive apoptotic features.

Cells were treated with 50 and 100 μ M of PT, PZ, DHA, PZ-DHA and vehicle for 24 h and photographed with Nikon Eclipse E 100 phase contrast microscope equipped with Infinity digital microscopy camera at \times 100 magnification using phase contrast microscopy. Photos are representative of three independent experiments. Arrows indicate cells that show morphological alterations.

3.4 PZ-DHA CAUSES CELL DEATH BY INDUCING APOPTOSIS IN JURKAT AND K562 CELLS.

The extensive morphological changes in PZ-DHA-treated Jurkat and K562 cells suggested apoptotic cell death. This was further confirmed by conducting an appropriate apoptotic assay. Cells undergoing early apoptosis and late apoptosis/necrosis were detected using flow cytometry analysis based on Annexin V/propidium iodide (PI) dual staining method. Annexin V binds to phosphatidylserine indicative of early apoptosis; whereas PI is a membrane-impermeable DNA stain that is associated with late (end-stage) apoptosis or necrosis. Jurkat and K562 cells were treated with vehicle (0.25 % DMSO) or 100 μ M of test compounds (PZ-DHA, PZ, DHA, PT) for 12 h. Representative cytograms were obtained using FCS Express 5 Flow Cytometry software (De Novo) (**Figure 10**). In comparison to vehicle, PZ-, DHA-, or PT-treated Jurkat and K562 cells, the percent of early apoptotic and/or late apoptotic cells were markedly increased following treatment with 100 μ M of PZ-DHA for 12 h.

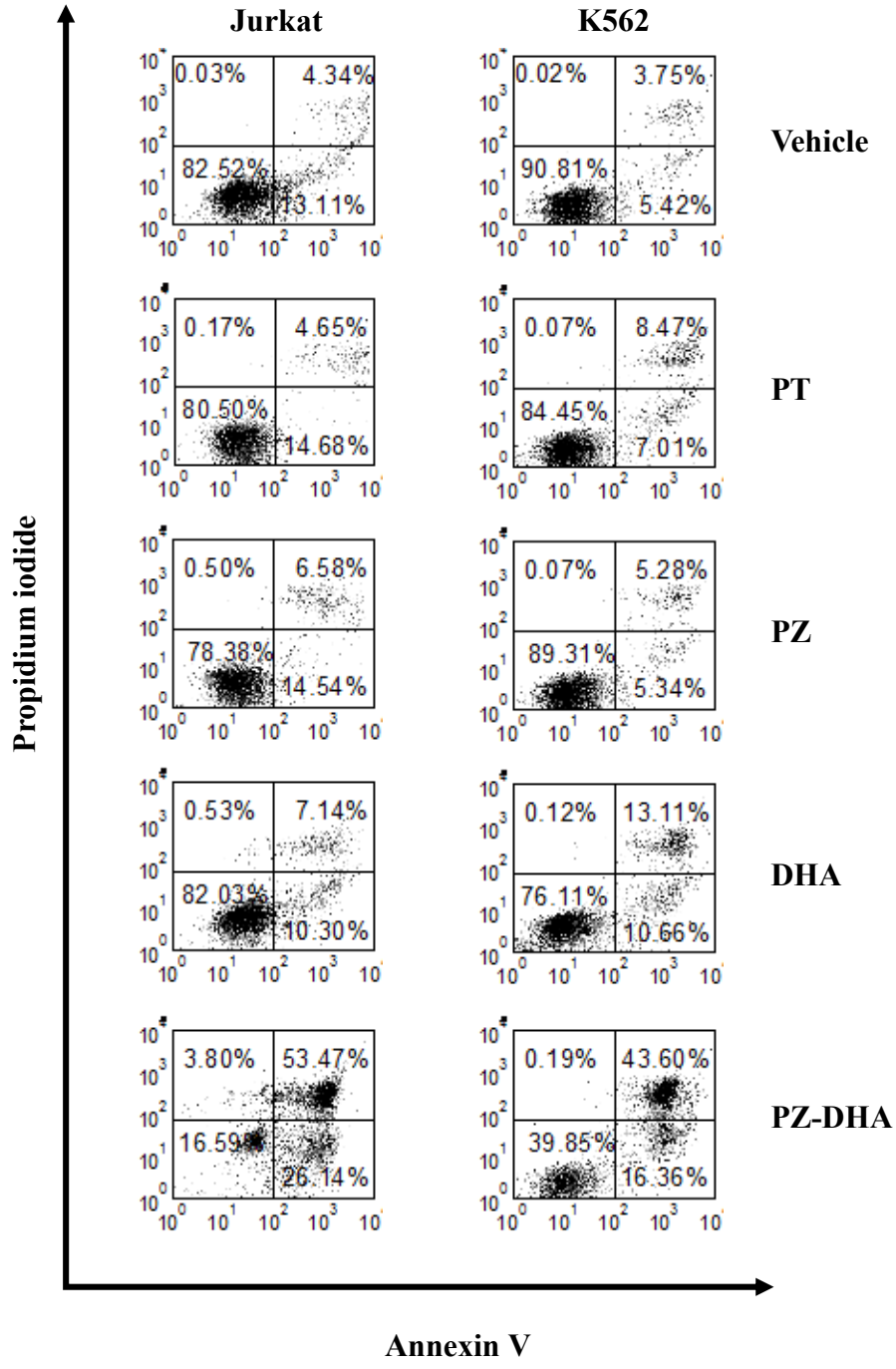


Figure 10. PZ-DHA markedly induces apoptosis in Jurkat and K562 cells in comparison to PT, PZ, and DHA.

Flow cytometry analysis with Annexin V/PI dual staining was used to detect apoptosis following treatment with vehicle (0.25% DMSO) or test compounds (PT, PZ, DHA or PZ-DHA at 100 μ M) for 12 h. The lower left quadrant (Annexin V-/PI-) represents viable cells, the lower right quadrant (Annexin V+/PI-) represents early-stage apoptotic cells, the upper left and right quadrants (Annexin V-/PI+ and Annexin V+/PI+) represents late-stage and/or necrotic cells. Cytograms are representative of one of two independent experiment.

3.5 CELL DEATH IN JURKAT CELLS IS ASSOCIATED WITH INTERNUCLEOSOMAL DNA FRAGMENTATION.

Apoptotic cell death was further evaluated by investigating the ability of PZ-DHA to induce apoptotic DNA fragmentation. When cells undergo apoptosis, chromosomal DNA is cleaved into multiples of internucleosomal 180-bp fragments, rendering DNA laddering that can be detected on agarose gel electrophoresis (Wyllie, 1980). Both Jurkat and K562 cells were treated with vehicle (0.25 % DMSO) or 100 μ M of test compounds (PZ-DHA, PZ, DHA, PT, imatinib, and doxorubicin) and for 24 h. DNA was extracted from treated cells and samples were run on a 1.2% agarose gel containing GelRedTM stain. Gel was then imaged under UV light. In treated Jurkat cells, PZ-DHA- and DHA-induced apoptotic DNA laddering effect (**Figure 11a**). Doxorubicin (control drug for Jurkat cells) did not show a DNA ladder. Interestingly, a DNA ladder was not detected in PZ-DHA-treated K562 cells (**Figure 11b**). However, the control drug for K562 cells, imatinib, did show the presence of an apoptotic DNA ladder.

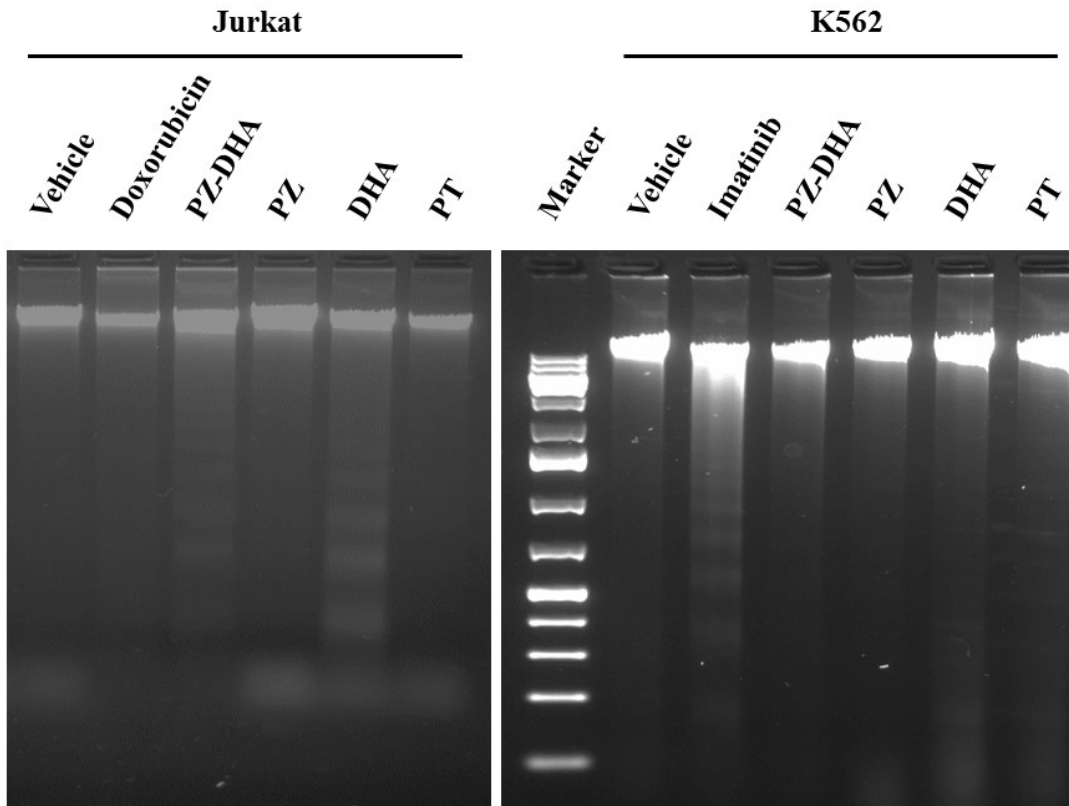
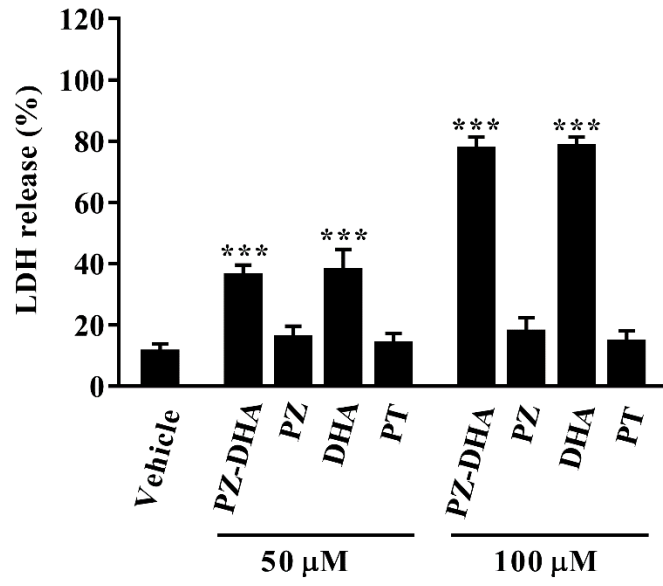


Figure 11. PZ-DHA-treated Jurkat cells show apoptotic DNA ladder.

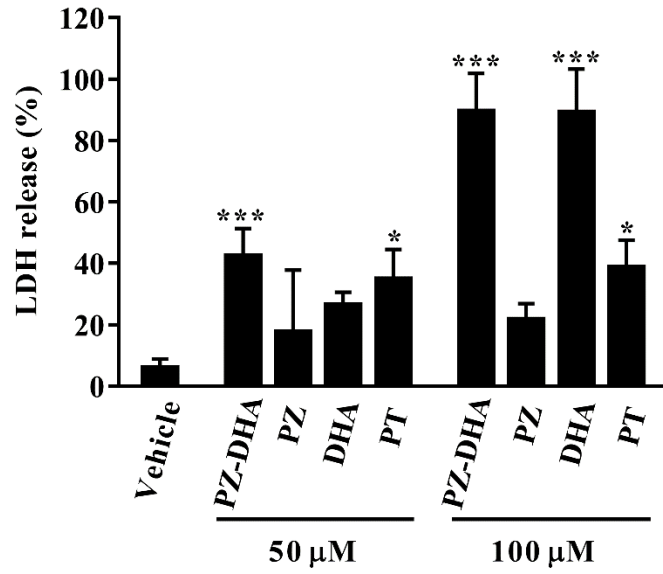
Both (a) Jurkat and (b) K562 cells were treated with vehicle (0.25% DMSO) or test compounds (100 μ M) for 24 h, followed by DNA extraction and UV visualization after electrophoresis on a 1.2% agarose gel. Marker: 100-base pair ladder molecular weight standard. Photos are representative of three independent experiments.

3.6 PZ-DHA STIMULATES THE RELEASE OF LACTATE DEHYDROGENASE IN JURKAT AND K562 CELLS.

To further investigate the differential response of PZ-DHA-induced DNA fragmentation in treated Jurkat and K562 cells, lactate dehydrogenase (LDH) assay was performed. LDH assay is an indicator of cell membrane integrity, and thus a measurement of cell death primarily caused by necrosis or late-stage apoptosis in response to external stimuli. LDH release was measured from Jurkat and K562 cells that were treated with either vehicle (0.25 % DMSO) or 50 and 100 μM of test compounds (PZ-DHA, PZ, DHA, and PT) for 24 h at 37 °C. **Figure 12** shows that LDH release into the culture medium was significantly elevated following treatment with either 50 or 100 μM PZ-DHA in Jurkat and K562 cells when compared with vehicle-treated cells. The data obtained were comparable with previously conducted experiments and may suggest that PZ-DHA causes post-apoptotic necrosis in human leukemic cell lines. PZ-DHA and DHA- induced LDH release in Jurkat and K562 cells were statistically equivalent, with the exception that 50 μM DHA-induced LDH leakage in Jurkat cells was not significantly different from vehicle-treated cells.



(a)



(b)

Figure 12. PZ-DHA significantly increases the release of lactate dehydrogenase in Jurkat and K562 cells compared to vehicle control.

Cell death induced upon treatment with vehicle (0.25 % DMSO) or test compounds (50 and 100 μ M) for 24 h was further confirmed by measuring LDH release from the cytosol of damaged (a) Jurkat and (b) K562 cells into the culture medium. Data shown are mean \pm SEM of three independent experiments conducted in quadruplicates. Statistical analysis was performed using one-way ANOVA and the differences among means were compared with the vehicle using Dunnett's test. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.7 PZ-DHA-INDUCED CELL DEATH MAY NOT REQUIRE CASPASE ACTIVATION.

Following promising experimental data showing PZ-DHA induces cytotoxicity in human leukemic cells, the ability of PZ-DHA to induce caspase activation was further evaluated. Caspases are frequently activated during apoptosis and are associated with the typical hallmarks of apoptosis such as formation of apoptotic bodies and DNA fragmentation. To investigate whether PZ-DHA stimulates caspase activation, the participation of caspase 3 and 7 in cleaving a profluorescent substrate was measured. The amount of fluorescent product formed is proportional to the amount of caspase 3/7 present in the sample. Caspase activities were measured in Jurkat and K562 cells treated with either vehicle (0.25 % DMSO) or 100 μ M of test compounds (PZ-DHA, PZ, DHA, or PT) for 12 h. Staurosporine (STS), which induces apoptosis via caspase 3, was used as a positive control. Unexpectedly, PZ-DHA-treated Jurkat and K562 cells did not show significant caspase 3/7 activation after 12 h post treatment when compared to vehicle-treated cells (**Figure 13**). STS induced caspase 3/7 activation. Among the test compounds, DHA-treated Jurkat and K562 cells showed significant caspase 3/7 activation, which was similar to STS.

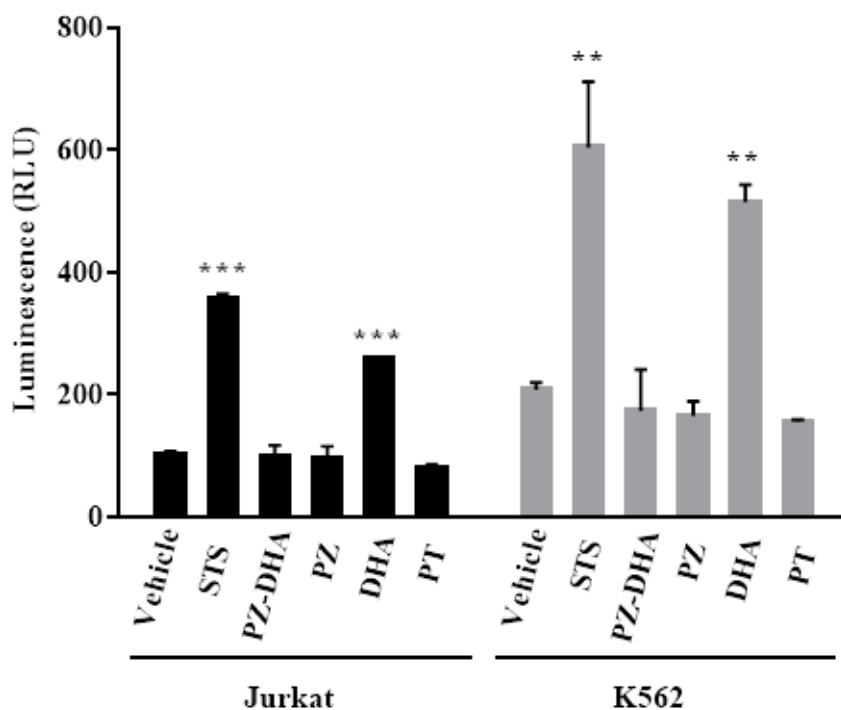
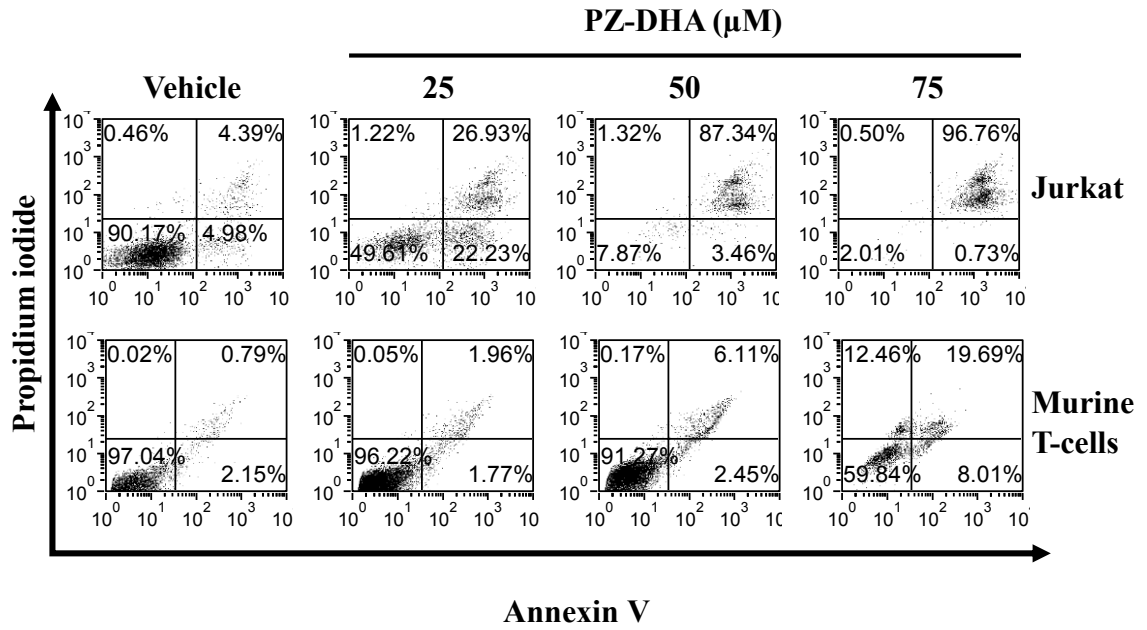


Figure 13. PZ-DHA may not require caspase activation to induce cell death in Jurkat and K562 cells.

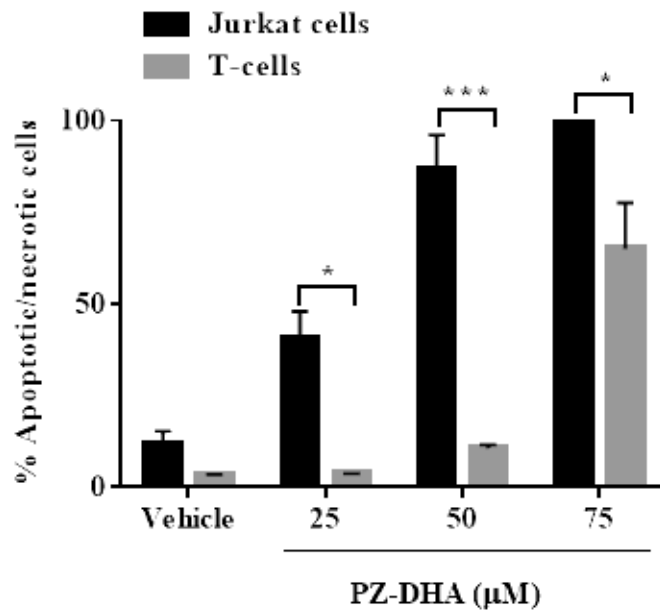
Caspase activation was measured in cells treated with either vehicle (0.25 % DMSO) or 100 μ M of test compounds for 12 h. Data shown are mean \pm SEM of three independent experiments conducted in quadruplicates. Statistical analysis was performed using one-way ANOVA and the differences among means were compared with the vehicle using Dunnett's test. ** $P < 0.01$, and *** $P < 0.001$. RLU = Relative luminescence unit.

3.8 THE CYTOTOXIC EFFECT OF PZ-DHA IS SELECTIVE TOWARDS JURKAT CELLS.

To determine whether PZ-DHA selectively kills leukemic cells, cell death caused by PZ-DHA was further evaluated in Jurkat and K562 cells in comparison with their normal cell counterparts, murine T-cells and murine bone marrow cells, respectively. Flow cytometric analysis using Annexin V/PI dual staining method was used to evaluate PZ-DHA-induced apoptotic/necrotic effect at concentrations of 25, 50, and 75 μM for 24 h. **Figure 14a** and **15a** show representative cytograms obtained from the data analysis performed using FCS Express 5 Flow Cytometry software (De Novo). The bar graphs shown in **Figure 14b** and **15b** (derived from respective a's) show sum of early apoptotic events (Annexin V⁺, PI⁻) and late apoptotic or necrotic events (Annexin V⁺, PI⁺; Annexin V⁻, PI⁺). PZ-DHA has significant minimal inhibitory effects on normal murine T-cells at concentrations that were effective at inducing cell death in Jurkat cells (**Figure 14**). However, murine bone marrow cells were not resistant to PZ-DHA at any tested concentrations as shown in **Figure 15**.



(a)

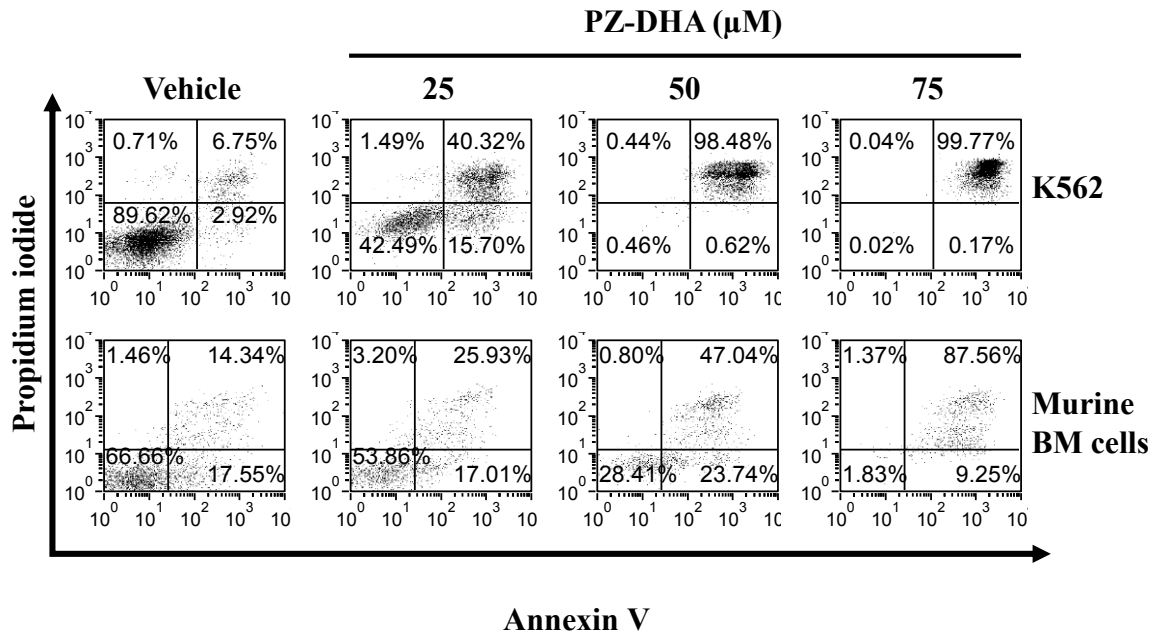


(b)

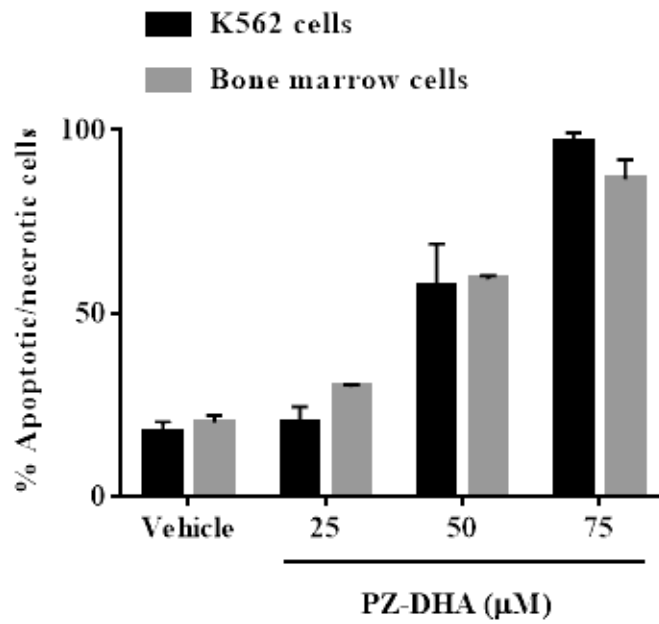
Figure 14. PZ-DHA selectively induces apoptosis in leukemic cells with minimum inhibitory effects on normal murine T-cells.

Flow cytometry analysis with Annexin V/PI dual staining was used to detect apoptosis in Jurkat cells and normal murine T- cells treated with different concentrations of PZ-DHA for 24 h. (a) Cytograms are representative of one of three independent experiments.

(b) Bar graphs (derived from A) shows the sum of % early apoptotic events (Annexin V⁺, PI⁻) and late apoptotic or necrotic events (Annexin V⁺, PI⁺; Annexin V⁻, PI⁺). Statistical analysis was performed using one-way ANOVA and the differences among means were compared with the vehicle using Tukey's test. *P < 0.05, **P < 0.01, and ***P < 0.001.



(a)



(b)

Figure 15. PZ-DHA induces apoptosis in both K562 cells and normal murine bone marrow cells.

Flow cytometry analysis with Annexin V/PI dual staining was used to detect apoptosis in K562 cells and normal murine bone marrow cells treated with different concentrations of PZ-DHA for 24 h. (a) Cytograms are representative of one of three independent experiments. (b) Bar graphs (derived from A) show the sum of % early apoptotic events (Annexin V⁺, PI⁻) and late apoptotic or necrotic events (Annexin V⁺, PI⁺; Annexin V⁻, PI⁺). Statistical analysis was performed using one-way ANOVA and the differences among means were compared with the vehicle using Tukey's test. *P < 0.05, **P < 0.01, and ***P < 0.001. BM: Bone marrow.

3.9 PZ-DHA SHOWS SPECIFIC INHIBITION OF STAT3 PHOSPHORYLATION IN JURKAT CELLS.

To further elucidate the putative mechanism of action of PZ-DHA, the effects of PZ-DHA on STAT1, STAT3, STAT5, and JAK2 activation in Jurkat and K562 cells were investigated. Jurkat cells constitutively express phosphorylated STAT1; however, STAT3 and STAT5 activation requires stimulation by interferon alpha-2b (IFN α -2b). In contrast, K562 cells shows constitutive activation of STAT3 and STAT5, but require inducible-phosphorylation of STAT1. Jurkat and K562 cells were treated with increasing concentrations of PZ-DHA (10, 15, 30 μ M) or vehicle (V) for 24 h prior to incubation with IFN α -2b for 1 hr. The expression of phospho-STATs was measured in relative to their respective STAT proteins using Western blot. β -actin was used as a loading control. PZ-DHA selectively inhibits the inducible-phosphorylation of STAT3 at 30 μ M, while not affecting the activation of STAT1 or STAT5 in Jurkat cells (**Figure 16**). No significant variation was observed in STAT1, STAT3, and STAT5 activation levels in PZ-DHA-treated K562 cells. Both Jurkat and K562 cells do not constitutively activate JAK2, and JAK2 activation was not inducible by IFN α -2b.

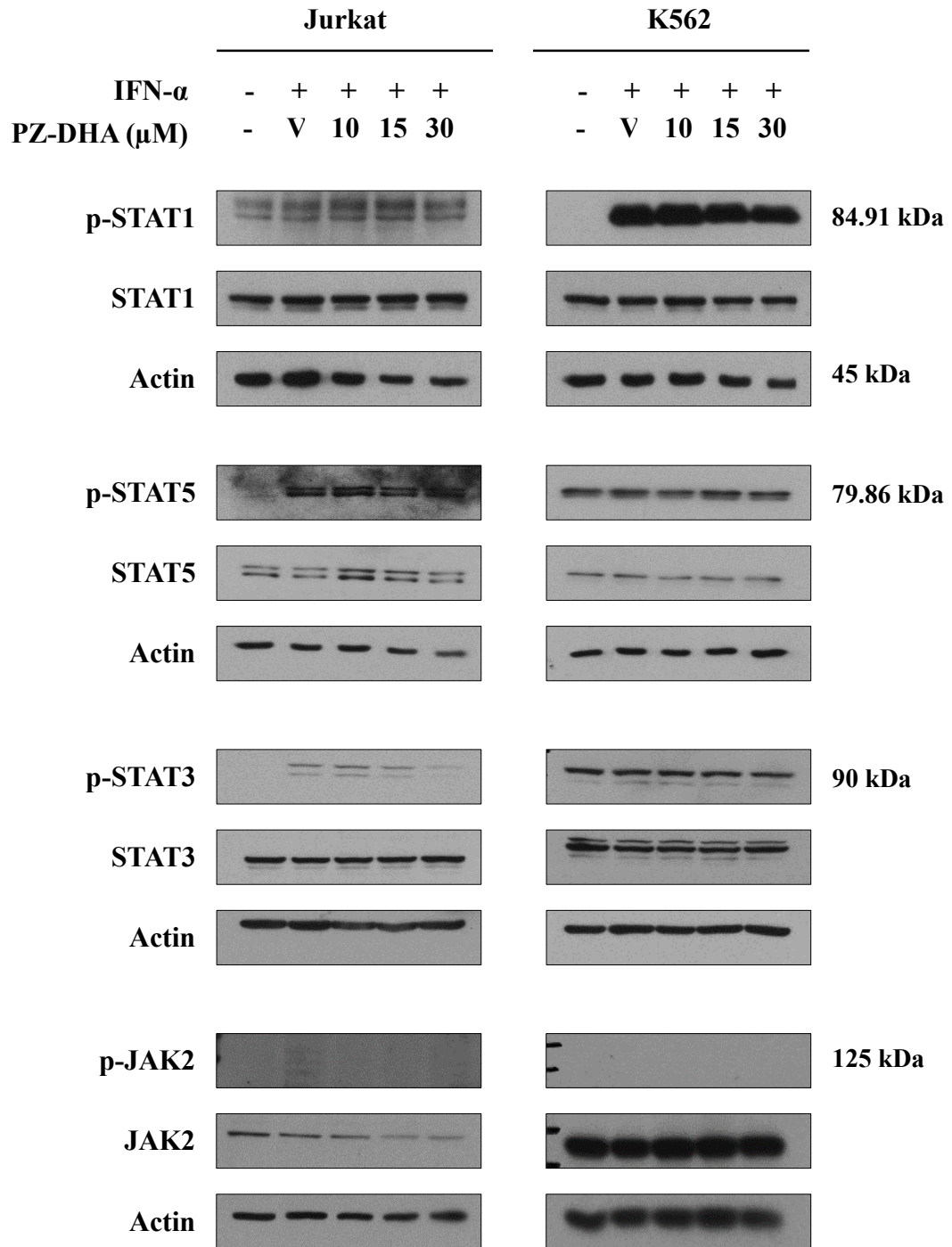


Figure 16. PZ-DHA suppresses inducible phosphorylation of STAT3 protein in Jurkat cells.

Cells were treated with indicated concentrations of PZ-DHA or vehicle (V) for 24 h before 1 h IFN α -2b incubation. Total cell lysates were collected and resolved by SDS-PAGE. The effects of PZ-DHA on phospho-STAT levels in relative to STAT expression in Jurkat and K562 cells were measured using Western blot assay. The respective bands were detected by chemiluminescence. β -actin was used as a loading control. Representative of three independent experiments.

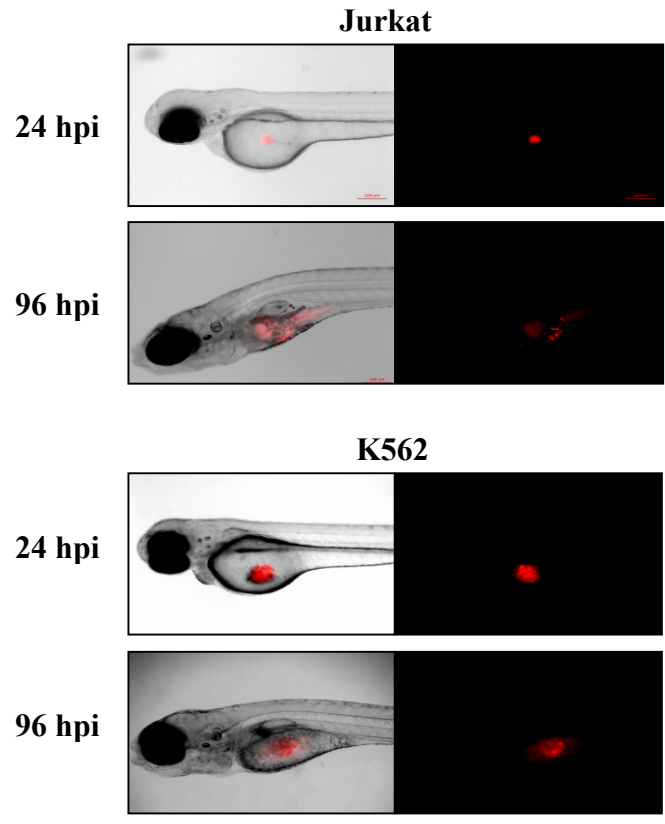
3.10 PZ-DHA INHIBITS JURKAT CELL GROWTH IN ZEBRAFISH XENOGRAFTS.

Testing of drug efficacy *in vivo* is an important aspect of drug discovery. Following the promising inhibitory effects of PZ-DHA against human leukemic cells *in vitro* cell-based assays, the anti-proliferative effect of PZ-DHA was further assessed in the zebrafish. The Berman lab has previously developed zebrafish human leukemia xenotransplantation models using both Jurkat and K562 cell lines (Bentley et al., 2015; Corkery et al., 2011). Using similar xenotransplantation techniques, approximately 50-100 fluorescently-labelled Jurkat or K562 cells were injected into *casper* zebrafish embryos (as described in **Figure 5**).

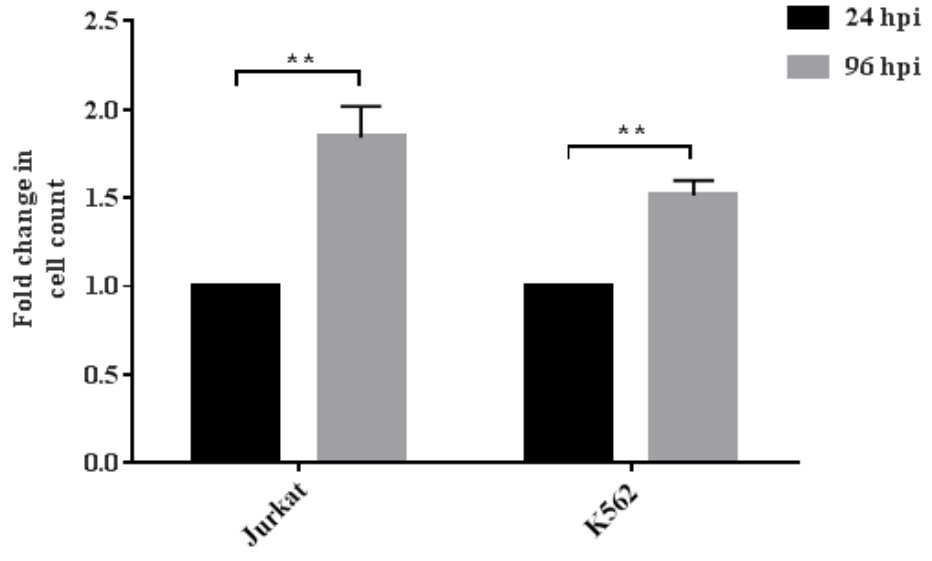
Figure 17a shows representative brightfield and fluorescent images of zebrafish embryos transplanted with either Jurkat or K562 cells at 24 h and 96 h post injection (hpi). To ensure successful engraftment of leukemic cells prior to drug treatment, a group of 20 embryos were dissociated at respective time points and the number of fluorescent cells were quantified. The fold increase in cell number was determined by dividing average fluorescent cell count at 96 hpi by average fluorescent cell count at 24 hpi and depicted as a bar graph (**Figure 17b**).

Jurkat and K562 xenotransplanted embryos were monitored in the presence of vehicle (0.375 % DMSO) or PZ-DHA at concentrations of 50 and 75 μ M for 72 h. Representative brightfield and fluorescent images of embryos injected with Jurkat and K562 cells from untreated and treated groups were shown in **Figure 18a** and **Figure 19a**, respectively. The proliferation rate of Jurkat and K562 cells in treated embryos were determined by quantifying fluorescent cells at 24 hpi (denoted 0 h post treatment (hpt) or

baseline) and at 96 hpi (72 hpt) following treatment with either vehicle or PZ-DHA. Jurkat xenotransplanted embryos exposed to 75 μ M of PZ-DHA showed significant reduction in cell growth compared to vehicle-treated control embryos (**Figure 18b**). However, no significant difference was observed between untreated and treated embryos xenografted with K562 cells (**Figure 19b**).



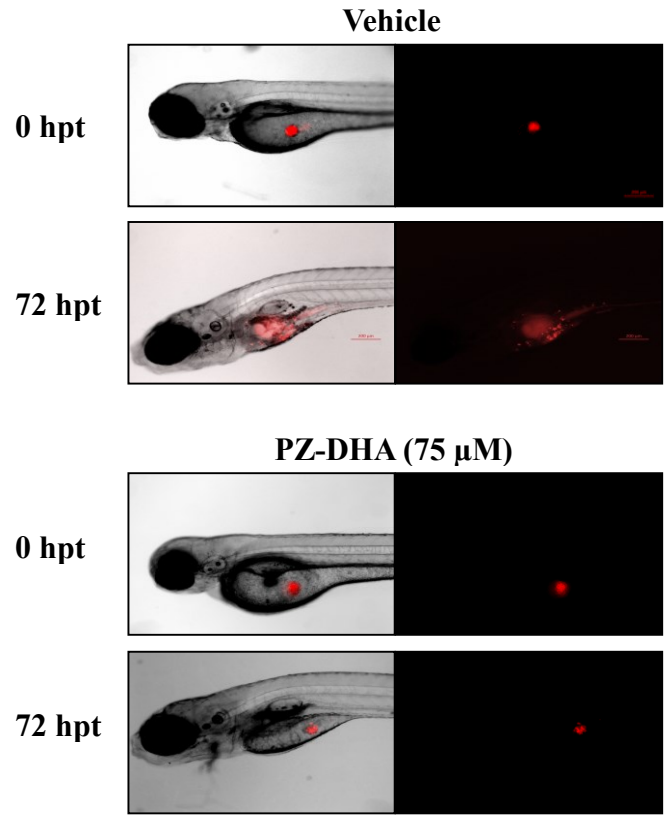
(a)



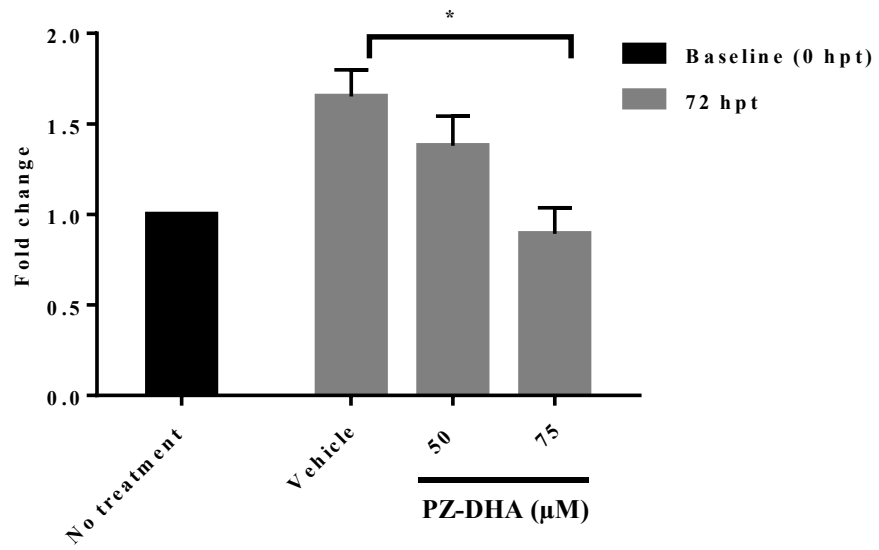
(b)

Figure 17. Jurkat and K562 human leukemic cells display significant fold increase in cell count following xenotransplantation in *casper* embryos.

(a) Representative brightfield and fluorescent images ($5 \times$ magnification) of zebrafish embryos injected with Jurkat and K562 cells at 24 and 96 h post injection (hpi). Images were taken using an inverted Axio Observer Z1 microscope equipped with a Colibri LED light source and an Axiocam Rev 3.0 CCD camera. (b) Bar graph depicts the number of fluorescent cells counted after enzymatically dissociating embryos to a single cell suspension at 24 and 72 hpi. Fold increase was determined by dividing cell count at 72 hpi by cell count at 24 hpi. Data represent mean \pm SEM (n=3). Statistical analysis was performed using two-tailed *t*-test. ****P < 0.01.**



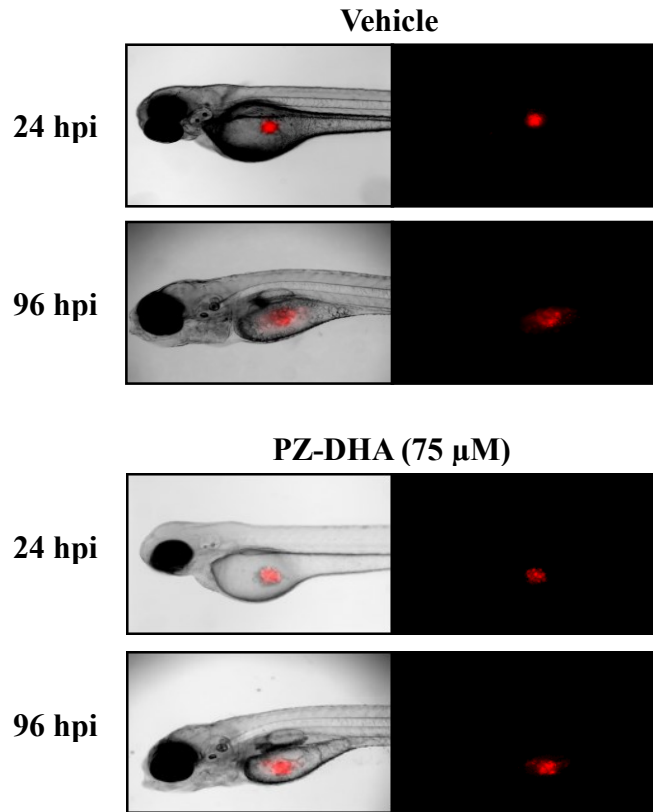
(a)



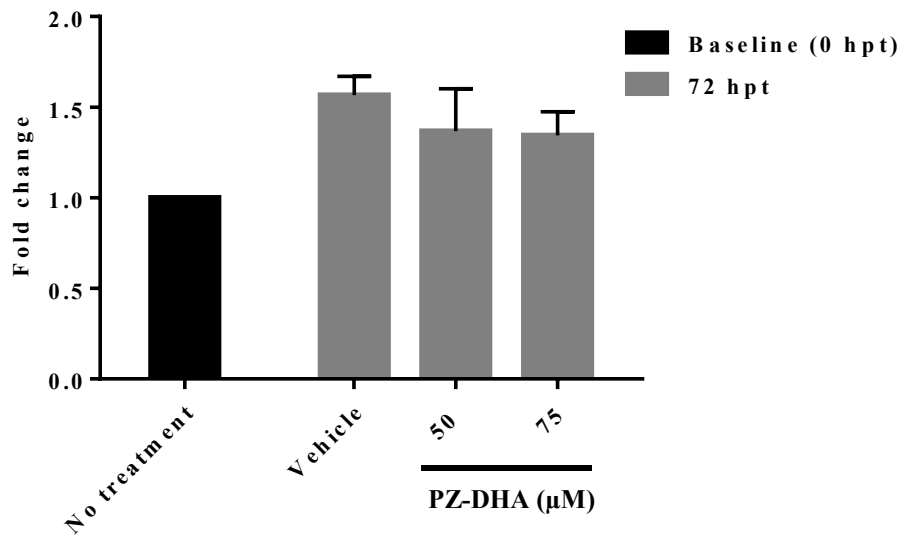
(b)

Figure 18. PZ-DHA suppresses *in vivo* proliferation of Jurkat cells in xenotransplanted embryos.

(a) Representative brightfield and fluorescent images ($5 \times$ magnification) of zebrafish embryos injected with fluorescently labeled Jurkat cells and monitored in the presence of vehicle (0.375 % DMSO) or PZ-DHA at concentrations of 50 and 75 μ M. (b) The effect of PZ-DHA on Jurkat cell proliferation was quantified by dissociating untreated and treated groups of embryos at baseline (0 hpt) and 72 hpt. Bar graph depicts fold change in cell number at 72 hpt relative to baseline. Data represent mean \pm SEM (n=3). Statistical analysis was performed using ANOVA and the differences among means were compared using Tukey's test. *P < 0.05.



(a)



(b)

Figure 19. PZ-DHA does not affect K562 cell proliferation in xenotransplanted embryos.

(a) Representative brightfield and fluorescent images ($5 \times$ magnification) of zebrafish embryos injected with fluorescently labeled K562 cells and monitored in the presence of vehicle (0.375 % DMSO) or PZ-DHA at concentrations of 50 and 75 μ M. (b) The effect of PZ-DHA on K562 cell proliferation was quantified by dissociating untreated and treated groups of embryos at baseline (0 hpt) and 72 hpt. Bar graph depicts fold change in cell number at 72 hpt relative to baseline. Data represent mean \pm SEM (n=3). Statistical analysis was performed using ANOVA and the differences among means were compared using Tukey's test.

CHAPTER 4: DISCUSSION

The cytotoxic effect of PZ-DHA has been previously tested in human hepatoma HepG2 cells, human breast carcinoma MDA-MB-231 cells, and human acute monocytic leukemia THP-1 cells (Fernando, 2014; Nair et al., 2014). This study was focused on further investigating the cytotoxicity of PZ-DHA on two well-established leukemic cancer cell lines: the Jurkat cell line initially derived from the peripheral blood of a 14 year old boy with T-cell acute lymphoblastic leukemia (T-ALL), (Schneider et al., 1977) and the K562 cell line initially derived from a 53-year old female with chronic myeloid leukemia (CML) in blast crisis (Lozzio and Lozzio, 1975). Previous studies have reported differential cytotoxic responses of flavonoids in leukemic cells (Mahbub et al., 2013; Moghaddam et al., 2012; Watanabe et al., 2012). Therefore, it is important and necessary to study whether PZ-DHA-induced cytotoxicity is selective towards specific types of leukemia. **Table 5** summarizes the key findings in this study.

Table 5. Summary of the PZ-DHA-induced cytotoxic effects.

Methods	Conditions	Description	Cell lines	
			Jurkat	K562
MTS assay	10, 25, 50, 75, and 100 μ M at 12, 24, 48 h	Dose-dependent reduction in cell viability	✓	✓
ATP assay	100 μ M at 24 h	Reduction of cellular ATP levels	✓	✓
Phase contrast microscopy	50 and 100 μ M at 24 h	Extensive morphological features of apoptosis	✓	✓
Flow cytometry analysis	100 μ M at 12 h	Induction of early apoptosis and late apoptosis/necrosis	✓	✓
	25,75, 100 μ M at 24 h	Protection of normal cell counterparts	✓	✗
DNA fragmentation	100 μ M at 24 h	Presence of a DNA ladder	✓	✗
LDH release	50 and 100 μ M at 24 h	LDH presence in media	✓	✓
Caspase activation	100 μ M at 12 h	Detection of caspase activity	✗	✗
Western blot	10, 15, and 30 μ M at 24 h	Inhibition of STAT3 activation	✓	✗
<i>In vivo</i> human leukemia proliferation assay	75 μ M at 72 h	Reduction of cell growth in xenotransplanted embryos	✓	✗

4.1 PZ-DHA CAUSES DOSE-DEPENDENT REDUCTION IN THE VIABILITY OF HUMAN LEUKEMIC CELLS.

The first set of experiments was performed to assess the effect of PZ-DHA on the viability of Jurkat and K562 cells. Cell viability was determined using (1) the MTS assay that measures metabolic activity based on tetrazolium salt reduction by NAD(P)H-dependent dehydrogenases in viable cells (Cory et al., 1991), and (2) the ATP assay that detects the presence of ATP as an indicator of cell viability, where cells lose the ability to synthesize ATP and endogenous ATPases degrade any remaining ATP within minutes after loss of membrane integrity (Kangas et al., 1984). Using the MTS assay, the effect of PZ-DHA on the viability of Jurkat and K562 cells was studied relative to its parent compounds (PZ and DHA), aglycone of PZ (PT), and positive controls (doxorubicin or imatinib) at increasing doses of 10, 25, 50, 75, and 100 μM for 12, 24, and 48 h (**Figure 5** and **Figure 6**, respectively). Strikingly, PZ-DHA and aglycone PT decreased the viability of both Jurkat and K562 cells in a dose-dependent and time-independent manner (at indicated time-points). The IC_{50} values following 24 h treatment with PZ-DHA were 67.3 μM and 30 μM for Jurkat and K562 cells, respectively (**Table 4**). However, the parent compounds PZ and DHA had no effect on cell viability, with the exception that DHA caused marked decrease in the viability of Jurkat and K562 at the highest treatment concentration (100 μM) at 48 h (**Figure 6**). The positive controls for Jurkat and K562 cells (doxorubicin and imatinib, respectively) exhibited dose- and time-dependent reduction in cell viability and the IC_{50} values were comparable with previous studies (Ferraro et al., 2003; Inaba et al., 2009).

The cell viability obtained using the MTS assay was then compared with the ATP detection method. As expected, ATP levels in Jurkat and K562 cells were significantly reduced following treatment with the positive controls or PZ-DHA at 100 μ M for 24 h (**Figure 7**). Surprisingly, aglycone PT did not significantly reduce ATP levels in treated cells, whereas DHA treatment caused significantly less ATP levels than aglycone PT. These results were inconsistent with the effects of PT and DHA on cell viability detected using the MTS assay. The MTS assay is sometimes associated with limitations such as low sensitivity and chemical interference; the ATP assay is considered more precise in predicting viable cell number (Dawson et al., 2010; van Tonder et al., 2015; Wang et al., 2010). Previous studies have shown that phytochemicals interfere and directly reduce tetrazolium salt, leading to an overestimation of the number of viable cells, thereby masking their potential cytotoxicity (Bruggisser et al., 2002; Wang et al., 2010). However, the results obtained in this study were contradictory, since the MTS assay showed an overestimation of the anti-proliferative effect upon exposure to increasing concentrations of aglycon PT when compared to the percent viable cells obtained using ATP detection method. Similar results has been shown by Nair et al., (2014). A plausible explanation for this observation is that PT might interfere with the activity of NAD(P)H-dependent-dehydrogenases, therefore, leading to false positive experimental data.

Nair et al., (2014) has reported the absence of DHA-induced cytotoxicity on THP-1 cells, however, others have shown the inhibitory effect of DHA on the viability of a range of cancer cell lines using the MTS assay (Danbara et al., 2004; Ding et al., 2004; Fernando, 2014), which eliminates the concern that DHA might interfere with the MTS reagent leading to false positive results. To the best of my knowledge, no study has

reported on the viability of Jurkat and K562 cells following treatment with DHA using the MTS assay, however, DHA-induced cytotoxicity on Jurkat and K562 cells has been shown using other viability assays such as trypan blue dye exclusion and thymidine incorporation method (Dawson et al., 2010; Jones, 1998; Siddiqui et al., 2003, 2001). From a different perspective, two independent studies have investigated the cytotoxic effect of DHA on SiHa human cervical cancer cell line using MTT and MTS assays. Similar to the MTS assay, MTT is based on tetrazolium reduction method and was developed prior to the MTS assay. Surprisingly, significant dose-dependent DHA-induced cytotoxicity was reported in SiHa cells using the MTT assay (Jeong et al., 2014), whereas no cytotoxicity was detected using MTS assay (Ding et al., 2004). It was also noted that the surprising lack of rottlerin (a polyphenol)-induced inhibition of MCF-7 breast carcinoma cells using the MTT assay (Torricelli et al., 2008) was associated with its uncoupling action that accelerates oxidative chain, leading to an overestimation of cell number (Maioli et al., 2009). However, another independent study showed significant reduction in the viability of human pancreatic cancer stem cells following exposure to rottlerin and assessment by the MTT assay (Chen et al., 2014). All these experimental results and observations suggest caution and re-evaluation on the use of MTS to study PT- and DHA-induced cytotoxic effects on human leukemic cells.

4.2 PZ-DHA INDUCES CELL DEATH IN HUMAN LEUKEMIC CELLS.

Cell death induced by PZ-DHA was further evaluated by examining the morphological and biochemical hallmarks of apoptosis. The definition of apoptosis based on distinct morphological features detected by electron microscopy was introduced in 1972 with the pioneering work of Kerr and his group (Kerr et al., 1972). The onset of

apoptosis is characterised by morphological alterations such as shrinkage of cells and the nucleus, chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Elmore, 2007). Unlike apoptosis, necrosis is another major form of cell death triggered by toxic or traumatic stimuli often resulting in premature death (Kerr, 1971). Necrosis leads to loss of membrane integrity, leakage of cellular contents, and complete cellular and nuclear lysis (Ziegler, 2004). PZ-DHA-treated Jurkat and K562 cells showed distinct features of apoptosis such as cell shrinkage, membrane blebbing and formation of apoptotic bodies at treatment doses of both 50 and 100 μ M when observed under phase contrast light microscopy (**Figure 8** and **Figure 9**). At 100 μ M, K562 cells also displayed features of necrosis like complete rupturing of plasma membrane, but no formation of apoptotic bodies. These morphological alterations should be further confirmed with high-resolution imaging using electron microscopy.

To further study PZ-DHA mediated cell death, flow cytometry analysis using Annexin V/PI dual staining was performed to differentiate between apoptosis and necrosis. Annexin V is a calcium-dependent phospholipid-binding protein that binds phosphatidylserine (PS) with a high-affinity (Andree et al., 1990). PS is normally located on the inner leaflet of plasma membrane, but during early apoptosis plasma membrane loses asymmetry causing PS to be translocated to the outer leaflet and can be detected by Annexin V (Koopman et al., 1994). **Figure 10** shows cytograms representing PZ-DHA-induced apoptotic and necrotic cell death in Jurkat and K562 cells after 12 h treatment in relative to vehicle and parent compounds (PZ and DHA). For both Jurkat and K562 cells, the population undergoing late stage apoptosis/necrosis were higher than the population of cells undergoing early stage apoptosis.

Another hallmark of apoptosis is DNA fragmentation. Apoptotic cell death was further evaluated by investigating the ability of PZ-DHA to induce internucleosomal DNA fragmentation. In most cases, endonucleases are activated when cells undergo apoptosis and they cleave chromosomal DNA into multiples of internucleosomal 180-bp fragments. The detection of DNA fragmentation in cultured cells involves techniques such as nuclear DNA extraction and characterization of DNA laddering on agarose gel electrophoresis. In this study, PZ-DHA and DHA were found to induce DNA fragmentation in Jurkat cells upon treatment with 100 μ M concentration for 24 h (**Figure 11**). No DNA laddering effect was detected in PZ-DHA-treated K562 cells. However, the positive control, imatinib, showed the presence of DNA ladder in K562 cells as shown previously (Gottschalk et al., 2004; Okada et al., 2004). Although ambiguous, the differential DNA laddering effect in Jurkat and K562 cells is not surprising. Some studies argue that DNA fragmentation is not required for apoptosis (Falcieri et al., 1993; Yuste et al., 2001). The presence of endonuclease inhibitors such as aurintricarboxylic acid (ATA) and Zn^{2+} inhibited drug-induced DNA fragmentation, but apoptotic cell death was still detected in murine fibrosarcoma cell line and murine thymocytes, respectively (Cohen et al., 1992; Schulze-Osthoff et al., 1994). Noteworthy, treating HL-60 and K562 cells with same doses of triazoles derivatives induced DNA fragmentation in HL-60 cells, but not in K562 cells (Coulidiati et al., 2015).

Necrosis refers to cell death that is not programmed but rather accidental. Fragmented nuclei or DNA is not a common feature of necrotic cell death (Ziegler, 2004). Okada et al., 2004 claims that DNA fragmentation is not required when cell death is primarily caused by necrosis. The differential effect of DNA laddering in PZ-DHA-

treated Jurkat and K562 cells was further evaluated using lactate dehydrogenase (LDH) assay. LDH is a soluble cytosolic enzyme that is released into the surrounding culture medium predominantly during necrosis, and late-stages of apoptosis (Chan et al., 2013). Precisely, LDH assay is an indicator of cell membrane integrity, and thus a measurement of cell death primarily due to late-apoptosis or necrosis. Upon treatment with 50 or 100 μ M of PZ-DHA, both Jurkat and K562 cells showed significantly increased levels of LDH release into the culture medium compared to vehicle-treated cells (**Figure 12**). Since PZ-DHA-induced LDH release in Jurkat cells were similar to that in K562 cells, necrosis-mediated cell death could not be confirmed in K562 cells. However, the experimental results were consistent with ATP assay, phase-contrast microscopy, and Annexin/PI staining suggesting that PZ-DHA induces both apoptosis and necrosis in Jurkat and K562 cells. The increase of LDH release could be caused by secondary necrosis that occurs after apoptosis (Piret et al., 2004; Silva, 2010). Secondary necrosis or post-apoptotic necrosis occurs when massive apoptosis overwhelms the engulfment of apoptotic bodies by phagocytes, thereby resulting in leakage of the cell contents with induction of inflammatory responses that resembles necrosis (Vanden Berghe et al., 2010; Ziegler, 2004). Similarly, other studies have showed that cytotoxic agents can mediate cell death via both apoptosis and necrosis. For instance, *tert*-butyl hydroperoxide, camptothecin, and glutamate have been shown to induce both DNA fragmentation and LDH release simultaneously in human hepatoma HepG2 cells, human cervical carcinoma HeLa cells, and mouse hippocampal HT22 cells, respectively (Piret et al., 2004; Zare-Mirakabadi et al., 2012; Zhang et al., 2003).

4.3 PZ-DHA-INDUCED CELL DEATH MAY NOT REQUIRE CASPASE ACTIVATION.

The morphological alterations of apoptosis are primarily, but not exclusively, mediated by caspase activation (Nicholson, 1999). Irrespective of the route to caspase activation (either intrinsic or extrinsic), major effector caspases such as caspase 3, caspase 6, and caspase 7 are mostly responsible in cleaving substrates and triggering apoptosis (Taylor et al., 2008). DNA fragmentation is initiated by endonucleases called caspase-activated DNases (CAD), also known as DNA fragmentation factor (DFF) (Liu et al., 1997). In healthy cells, CAD is found in association with inhibitor of CAD (ICAD), which represses the endonuclease activity. However, during apoptosis, ICAD is cleaved by caspases, resulting in the activation of CAD followed by chromatin condensation, nuclear shrinkage and DNA fragmentation (Liu et al., 1997; Taylor et al., 2008).

In this study, PZ-DHA displayed many prominent features of apoptosis in Jurkat and K562 cells. Next, caspase activation was evaluated in PZ-DHA-mediated cell death. Surprisingly, no caspase activation was detected in either Jurkat or K562 cells treated with 100 μ M PZ-DHA for 12 h (**Figure 13**). Two plausible explanations are: (1) PZ-DHA-induced apoptosis may not be caspase-dependent or (2) caspase activation is an early event of apoptosis, therefore, a shorter exposure time (30 min - 3 h) may be required.

Although caspase activation is considered a key element in apoptosis as mentioned above, there are increasing evidence that cytotoxic agents can initiate apoptosis without relying on caspase activation (Belmokhtar et al., 2001; Candé et al., 2004; Chipuk and Green, 2005; Jäättelä and Tschopp, 2003; Lavoie et al., 1998; Tait and

Green, 2008). One obvious example is a broad spectrum caspase inhibitor, z-VAD-fmk, did not prevent DNA fragmentation and subsequent apoptosis in murine carcinogen-induced acute lymphoid leukemia cells (Belmokhtar et al., 2001). To confirm caspase-independent cell death, PZ-DHA-induced apoptosis should be re-evaluated in the presence of a caspase inhibitor. Moreover, in the case of a rapid and transient caspase activation, there are possibilities of mis-detecting caspase activation by choosing a non-optimal time point. A previous study reported that staurosporine-induced activation of caspase 3 increased with prolonged incubation and reached maximal activity by 8 h, after which the activity declined towards the baseline level (Demelash et al., 2004). Therefore, it is very critical to evaluate caspase activation not only in presence of a caspase inhibitor, but also at a range of different time points before concluding that PZ-DHA-induced cell death is independent of caspase activation.

4.4 CELL DEATH CAUSED BY PZ-DHA IS SELECTIVE TOWARDS JURKAT T-CELL LEUKEMIC CELLS.

In addition to demonstrating the cytotoxic effects of a novel therapeutic agent against malignant cells, it is equally important to test if the compound is cytotoxic to normal cells. An ideal drug is expected to show favourable toxicity profile at effective doses, while limited deleterious effects on normal tissues. The apoptotic and/or necrotic effect of PZ-DHA was further evaluated in Jurkat and K562 cells in comparison with their respective normal cell counterparts, murine T-cells and murine bone marrow cells by flow cytometric analysis using Annexin V/PI dual staining. The leukemic cell lines and normal murine cells were treated with increasing doses (25, 50, and 75 μ M) of PZ-DHA for 24 h. In comparison with their normal counterparts, the population of PZ-DHA-

treated Jurkat cells that underwent apoptotic or necrotic cell death was significantly higher at all treatment doses (**Figure 14**). In other words, PZ-DHA-induced cytotoxicity was selective towards Jurkat cells causing almost no toxicity to normal murine T-cells at concentrations of 25 and 50 μ M. However, PZ-DHA did not induce selective killing against K562 cells as their normal counterparts, murine bone marrow cells, were also equally affected (**Figure 15**). Although this may raise concern about the safety of PZ-DHA as a potential therapeutic agent, the cytotoxicity is cell-type specific; murine T-cells are resistant to PZ-DHA, while murine bone marrow cells are not. Therefore, further investigation into the mechanism of PZ-DHA-induced cell death on normal cells is necessary because if cell death is mediated by distinct mechanisms (or signaling pathways) in malignant and normal cells, then combination therapy might be able to attenuate PZ-DHA-induced undesirable effects without compromising its anticancer effects (Wang et al., 2004).

4.5 THE EFFECT OF PZ-DHA ON STAT3 PHOSPHORYLATION IS SPECIFIC.

JAK/STAT signaling is frequently overactivated in different types of leukemia. (Gouilleux-Gruart et al., 1996; Lin et al., 2000; Migone et al., 1995; Vainchenker and Constantinescu, 2013; Weber-Nordt et al., 1996). One of the main goals of this study was to determine whether PZ-DHA exerts its cytotoxic effect through abrogation of JAK/STAT signaling in human leukemic cells. Jurkat cells expressed constitutively activated STAT1 and induced-STAT3 and STAT5, while K562 expressed induced-STAT1 and constitutive activation of STAT3 and STAT5. Others have shown similar activation of STATs in Jurkat and K562 cells (Chan et al., 2010; Jones et al., 2005; Ren et al., 2015). Western blot analysis showed that PZ-DHA suppressed IFN α -2b- induced

phosphorylation of STAT in Jurkat cells, but had no effect on STAT1 and STAT5 phosphorylation. PZ-DHA did not inhibit activation of STAT3 in K562 cells that have constitutive phosphorylation of STAT3. Unlike PZ-DHA, other natural compounds or derivatives like ursolic acid (Pathak et al., 2007), curcumin (Bharti et al., 2003), and CCDO-Imidazolidine (Liby et al., 2006) inhibit both constitutive and induced-STAT3 phosphorylation suggesting that phospho-STAT3 suppression by PZ-DHA might be cell-specific; meaning, PZ-DHA may induce selective inhibition of STAT3 in Jurkat cells. This is probably because the survival dependency of STAT3 signaling in K562 cells might be different from Jurkat cells. In fact, independent of STAT3 phosphorylation. Noteworthy, STAT3 is essential for the regulation of T-cell activation and survival (Oh et al., 2011; Yang et al., 2007). Western blot analysis also showed that JAK2 is not constitutively activate nor activated by IFN α -2b in Jurkat and K562 cell lines. A study reported that IFN γ can induce JAK2 activation in Jurkat cells (Hart et al., 2011a).

4.6 HUMAN LEUKEMIC CELLS SHOW DIFFERENTIAL RESPONSE TO PZ-DHA *IN VIVO*

With regard to preclinical development of new therapeutic agents, drug efficacy is commonly validated in cell line-derived xenografts following successful *in vitro* screening and response assessment. In this study, the efficacy of PZ-DHA was further evaluated in an *in vivo* model employing zebrafish embryos. The zebrafish has emerged as a robust model for cancer studies due to conserved genetics, embryonic transparency and unique imaging, time-efficiency and cost-effectiveness, and ease of genetic manipulations (Amatruda and Patton, 2008; Payne and Look, 2009; Zon and Peterson, 2005). Pioneering studies conducted at the Berman lab have explored the feasibility of

injecting Jurkat and K562 cell lines into *casper* double pigment mutant zebrafish line and monitoring *in vivo* response in the presence of drug compounds. Previous studies have shown that xenografted cell lines successfully recapitulate *in vitro* drug responses in zebrafish model (Bentley et al., 2015; Pruvot et al., 2011; Selderslaghs et al., 2009).

Taking advantage of these previously developed models, the cytotoxic response of PZ-DHA on Jurkat and K562 cells was further tested in *in vivo* human leukemia proliferation assay using zebrafish xenotransplantation (**Figure 4**). Jurkat cell number increased from ~50 to ~90 cells per xenografted embryo (1.8 fold) and K562 cells increased from ~95 to ~150 cells (1.6 fold) after 72 h. The fold increase of Jurkat cells falls in the range between ~1.5 to ~3 as shown previously by Bentley et al., (2015). However, the fold increase for K562 cells seems to be markedly less than previously reported value (2.6) (Corkery et al., 2011). The doubling time of K562 cells is ~24 h (Rutherford et al., 1981), however, the proliferation rate of xenografted cells is normally slower and this discrepancy is attributed to *in vivo* stress caused by fluctuating environmental conditions such as temperature (Veinotte, 2012).

For studying drug efficacy, vehicle or PZ-DHA was directly added to the water and leukemic cell proliferation was monitored using fluorescent imaging and cell number quantification. Although PZ-DHA exhibited cytotoxicity against both Jurkat and K562 cells in *in vitro* studies, surprisingly only proliferation of Jurkat cells was suppressed in zebrafish embryos (**Figure 18**). Earlier studies showed that the zebrafish model is well-suited for studying the efficacy of anti-T-ALL drugs because its adaptive immune system is similar to that of humans (Meeker and Trede, 2008; Ren et al., 2015; Ridges et al., 2012). The *in vivo* findings suggest that the PZ-DHA-induced cytotoxic response in

human leukemic cell lines could vary in the presence of the biologic content of an entire organism. There is increasing evidence that the interaction between non-malignant stromal cells and tumor microenvironment may affect tumor cell response to therapeutics (McMillin et al., 2013). Stroma-induced resistance is, however, not common to all types of cancer; it may vary depending on the particular type of tumor or microenvironment, thereby proposing PZ-DHA as a novel and potent therapeutic agent selective for T-cell leukemia.

CHAPTER 5: CONCLUSION

Leukemia involves a group of neoplastic diseases that differs significantly from each other in terms of molecular and pathological features of malignant leukocytes. Recent biological insights and novel clinical applications have converted leukemia from a fatal to a more manageable condition. For instance, the emergence of imatinib mesylate as a targeted drug for chronic myeloid leukemia has improved the long-term survival of patients with a 8-year survival rate of approximately 88% (Miranda et al., 2016). However, disease persistence due to clinical difficulties such as treatment failure, adverse drug reactions, or therapeutic resistance remains a challenging problem. This underlies the importance and necessity of developing novel chemotherapeutic or adjuvant agents for leukemia management.

Many studies have shown the anticancer effects of phytochemicals, also known as secondary metabolites of plants; it is noteworthy that 49% of conventional chemotherapeutics are naturally-derived compounds (Newman and Cragg, 2012). In this study, the cytotoxic properties of a novel phytochemical derivative, phloridzin docosaheptaenoate (PZ-DHA), was studied in two distinct human leukemic cell lines, Jurkat (T-cell acute lymphoblastic leukemia) and K562 (chronic myeloid leukemia). In the efforts of increasing the lipophilicity of phloridzin (PZ) into human cells, PZ was enzymatically acylated with an omega-3 fatty acid, docosaheptaenoic acid (DHA) (Ziaullah and Rupasinghe, 2016; Ziaullah et al., 2013).

In the present study, the efficacy of PZ-DHA was tested using cell culture systems and *in vivo* model employing zebrafish xenografts. PZ-DHA significantly suppressed the proliferation of Jurkat and K562 cells in a dose-dependent manner. In contrast, an

absence of inhibitory effect was observed following treatments with either parent compound, PZ or DHA. Significant reduction of ATP levels in PZ-DHA-treated Jurkat and K562 cells further validated the cytotoxic effect of PZ-DHA.

To investigate whether PZ-DHA induced apoptotic cell death, a series of experiments testing the morphological and biochemical features of apoptosis was performed. Phase contrast microscopy showed extensive morphological alterations of apoptosis on PZ-DHA-treated cells, with cell shrinkage, membrane blebbing, and formation of apoptotic bodies being evident. At higher concentrations, morphological features of necrosis like membrane rupture and cell lysis were apparent. The presence of an apoptotic ladder due to DNA fragmentation was detected in Jurkat cells, not in K562 cells, following treatment with PZ-DHA. DNA fragmentation is cell-type specific and may not be required during apoptosis/necrosis-mediated cell death (Cohen et al., 1992; Couliadiati et al., 2015; Falcieri et al., 1993). Jurkat and K562 cells also showed increased levels of lactate dehydrogenase (LDH) release into the culture medium emphasising the disruption of cell membrane integrity upon treatment with PZ-DHA.

Although PZ-DHA displayed many prominent features of apoptosis, no caspase activation was detected in either Jurkat or K562 cells treated with PZ-DHA for 12 h. PZ-DHA-induced apoptosis may not be caspase-dependent, however, this has to be further confirmed by monitoring cell death in the presence of a caspase inhibitor or cell line lacking caspase expression. Besides that, caspase activation is an early event of apoptosis. To ensure caspase activity was not mis-detected during this study, the assay should be re-conducted at an early treatment exposure time, for example between 30 min and 3h.

Flow cytometry analysis using Annexin V/PI dual staining further confirmed PZ-DHA-induced cell death in Jurkat and K562 cells. It was also revealed that PZ-DHA selectively kills Jurkat cells, while sparing its normal cell counterpart, murine T-cells. However, both K562 cells and its normal cell counterpart, murine bone marrow cells, were affected by PZ-DHA. This may raise concern about drug safety and thereby toxicological profile of PZ-DHA should be further tested in an *in vivo* model. Noteworthy, the highest tested dose of PZ-DHA (75 μ M) did not induce toxic/adverse effects to the survival or behaviour of zebrafish embryos. Besides that, no adverse side effects, based on behavioral observations, food intake, and body weight measurements, was observed in NOD-SCID female mice intraperitoneally administered with 100 mg/kg PZ-DHA (Fernando et al., (2016). *Carcinogenesis. In press*). Current data shows that PZ-DHA-induced cytotoxicity is cell-type specific, therefore, more insight into the mechanism of cell death is also important because if cell death is mediated by distinct mechanisms in malignant and normal cells, then PZ-DHA could be an effective adjuvant agent in combination therapy.

JAK and STAT proteins are often overexpressed in many types of leukemia (Ferrajoli et al., 2006; Lin et al., 2000; Siddiquee and Turkson, 2008; Vainchenker and Constantinescu, 2013; Ward et al., 2000). Numerous phytochemical derivatives have been shown to induce cytotoxic effects against malignant cells via interrupting JAK/STAT signaling (Arumuggam et al., 2015; Chan et al., 2010; Espinoza et al., 2013). In this study, the suppressive effect of PZ-DHA on STAT proteins was investigated. PZ-DHA selectively inhibited IFN α -2b-induced STAT3 phosphorylation in Jurkat cells, while not affecting activation of STAT1 and STAT5. However, PZ-DHA did not

suppress the phosphorylation of constitutively activated STAT3 in K562 cells. This emphasizes the survival dependency of Jurkat cells on STAT3 activation, which can be selectively inhibited by PZ-DHA.

The efficacy of PZ-DHA was further evaluated in zebrafish xenograft model. Using previously established models in the Berman lab, Jurkat and K562 cells were injected into *casper* zebrafish embryos and cell proliferation was monitored in the presence of vehicle or PZ-DHA. Although PZ-DHA showed inhibitory effects against both Jurkat and K562 cells in *in vitro* studies, only Jurkat cell proliferation was suppressed in the *in vivo* model. This suggests that PZ-DHA-induced cytotoxic response is selective towards T-cell acute lymphoblastic leukemia in the presence of tumor-stromal microenvironment. Taken together, all *in vitro* and *in vivo* findings in this study have identified potential cytotoxic effects of PZ-DHA against human leukemic cells, more specifically, human T-cell acute lymphoblastic leukemia (T-ALL).

5.1 FUTURE STUDIES

This research has provided significant insight on the inhibitory effects of PZ-DHA on human leukemic cells using both *in vitro* and *in vivo* models. At higher concentrations, cell death induced by PZ-DHA was mediated by apoptosis and necrosis. Cell death should be further assessed in the presence of a caspase inhibitor. Cell cycle and cell proliferation could be halted following treatment with sub-cytotoxic concentrations of PZ-DHA. This can be evaluated by conducting a series of experiments consisting of Oregon Green cell proliferation assay, flow cytometry for cell cycle analysis using propidium iodide (PI) dye, and Western blot analysis to quantify expression of proteins involved in cell cycle regulation (cyclins and retinoblastoma) and proliferation (Ki-67).

This study provided preliminary data on the effects of PZ-DHA on STAT activation in human leukemic cells. Now, it is evident that PZ-DHA blocks STAT3 activation in Jurkat T-ALL cells. Therefore, further studies are required to investigate the effects of PZ-DHA on STAT3 dimerization and/or translocation into the nucleus. To gain better understanding on anti-JAK/STAT effects of PZ-DHA on T-ALL cells, it is also necessary to study the effect of PZ-DHA on the other members of JAK/STAT signaling, such as JAKs, suppressor of cytokine signaling (SOCS), protein tyrosine phosphatases (PTPs), and protein inhibitors of activated STAT (PIAS) proteins. It will also be interesting to study the effect of PZ-DHA on the expression of tumor survival genes activated by JAK/STAT signaling such as Bcl-xL, Bcl-2, Mcl-1, and VEGF.

Many plant derivatives synergistically potentiate the cytotoxic effects of chemotherapeutic drugs (Sak, 2012; Seeram et al., 2004). The potential use of PZ-DHA

as an adjuvant agent should be further evaluated by studying the synergistic effect of PZ-DHA when used in combination with imatinib and doxorubicin. Furthermore, the cytotoxic effect of PZ-DHA should also be explored using drug-resistant cell lines.

In this study, the *in vivo* response to PZ-DHA was tested in zebrafish embryos xenografted with Jurkat and K562 human leukemia cell lines. Prior to proceeding with higher animal models, it is worthwhile to study the effect of PZ-DHA in zebrafish embryos transplanted with primary patient samples. This model might provide new insight on the interaction of PZ-DHA with tumor cells in a clinically relevant time frame, evaluating the prospects of employing PZ-DHA in personalized patient treatment.

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