Evaluating Nutraceuticals for Selective Toxicity Toward Leukemia Stem Cells

by

Sarah-Grace Rota

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Pharmacy

Waterloo, Ontario, Canada, 2015

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Targeting leukemia stem cells (LSCs) is critical to improving the poor outcome of acute myeloid leukemia (AML) patients. Nutraceuticals (i.e., food derived bioactive compounds) provide a wealthy resource for novel anti-cancer, and specifically anti-AML drug discovery. With the advent of novel LSC cell lines, preliminary screening of these compounds against LSC-like cells can be achieved rapidly. To identify potential novel anti-LSC therapeutics, we created and screened a unique library consisting of 288 nutraceuticals in an MTS assay against TEX leukemia cells, a surrogate LSC line and K562, a control cell line which does not possess LSC activity. Here, we identified diosmetin, a flavonoid found in citrus fruits and various green plants, as a novel anti-LSC agent (EC_{50}: 6.0 ± 1.7μM). To confirm its activity, diosmetin (10μM) reduced clonogenic growth of primary AML cells (n = 4) with no effect on normal CD34 positive bone marrow derived stem cells (n = 3) observed in colony forming cell assays. A dose-response and time course analysis performed via the Annexin/PI assay and flow cytometry revealed that diosmetin induced apoptosis, as evidenced by the accumulation of ANN+/PI- cells. Apoptosis was further confirmed by a subG1 peak after performing cell cycle analysis.

Utilizing the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool, we determined that the estrogen receptor (ER) was a potential molecular target for diosmetin's anti-leukemia activity. To assess the role of estrogen receptors, we measured ERα and ERβ protein levels in diosmetin sensitive and insensitive cell lines. Interestingly, diosmetin sensitive cell lines display significantly elevated ERβ protein levels compared to diosmetin insensitive cells. However, this pattern was not observed

for ERα. Similar results were observed through quantitative PCR measures, as TEX cells displayed levels of ESR2 (ERβ) mRNA, with no observed levels of ESR1 (ERα) mRNA levels. The opposite results were observed in K562 cells. Through ER reporter assays, it was demonstrated that diosmetin acts as a partial agonist in ERβ reporter cells, increasing luciferase activity with increasing doses of diosmetin in ERβ reporter cells. Moreover, we find that caspase 8 but not caspase 9 is elevated following diosmetin treatment, consistent with the extrinsic pathway of apoptosis and our observed increased in TNF-α, similar to previous reports highlighting the link between ERβ agonists and cancer cell death. In summary, these studies highlight that estrogen receptors, specifically ERβ, is a novel LSC therapeutic target, and the potential role of nutraceuticals as promising compounds for future drug discovery endeavours.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. Paul Spagnuolo, for the opportunity to be apart of his team and for believing in my abilities. His continual guidance, patience and support were unparalleled. Dr. Spagnuolo provided a creative and spirited space to grow as a scientist and a professional, challenging us to aim higher, but also never allowing us to be afraid of failure, and for this I am truly grateful. I would like to thank my advisory committee members, Dr. Jonathan Blay and Dr. Praveen Nekkar, for their encouragement, feedback, and kindness throughout this program. I would like to acknowledge the collaborators who have contributed to the success of this thesis: Dr. Minden and Dr. Doxey. Many thanks to Sarah Rae for her assistance and patience when dealing with the many administrative requests; the graduate students are so lucky to have you. Special thanks to Dr. Cutler, who graciously provided his invaluable mentorship throughout my project. I would also like to acknowledge and thank Spencer Berg and Nawaz Ahmed, for both their friendship and contributions to the success of the project (but will never be formally acknowledged). To my lab family, Eric Lee, Leonard Angka, and Kevin the fish, thank you for your unconditional support and friendship. I am quite certain I would not have made it through this without you. I will hold our friendship forever near and dear to my heart. Thank you to the all the graduate students at the School of Pharmacy, I am thankful to have spent the last two years in such a friendly and supportive network. Special thanks goes out to Jessica, Sam, and Rabiya for always having an open door to share some laughs (and many tears). To Nick, thank you for loving and supporting me through these years and never letting me fall short of my potential. Thank you to my sisters, Carli and Bianca, for all of your encouragement, for

believing in me, and lifting my spirits when I needed it the most. Last but not least, thank you to my parents for their continual trust, love and support. It is through your love that I can achieve anything. My successes are yours.

This thesis was written in special memory of Dominic “Carlo” Ussoletti and Ken Mogg.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATIONS

α-toc	α-tocopherol
AF-1	Activating factor 2
AF-2	Activating factor 1
AML	Acute Myeloid Leukemia
ANN	Annexin V
APML	Acute promyelocytic leukemia
ATRA	All-trans-retinoic acid
CFC	Cell forming colony
CLL	Chronic lymphocytic leukemia
CML	Chronic Myeloid Leukemia
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBD	DNA binding domain
DCFH/DA	2',7'-Dicholorofluorescein diacetate
DD	Death domain
DEVD	Asp-Glu-Val-Asp
DISC	Death inducing signalling complex
Dios	Diosmetin
DMSO	Dimethyl sulfoxide
FADD	Fas-associated-death-domain
HTS	High throughput screening
HSC	Hematopoietic stem cell
E2	17β-Estradiol
EC_{50}	Half maximal effective concentration
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen Response Element
LBD	Ligand binding domain
LPS	Lipopolysaccharide

LSC	Leukemia stem cell
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium
NAC	N-Acetyl-cysteine
NHP	Natural health product
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
OD	Optical density
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction Real-Time Quantitative PCR (qPCR)
PDB	Protein data bank
PI	Propidium Iodide
PS	Phosphotidylserine
ROS	Reactive oxygen species
SERM	Selective estrogen receptor modulator
t-bid	Truncated form of Bid
TNF	Tumour necrosis factor
Z-VAD	Z-VAD-FMK; Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl] fluoromethylketone

CHAPTER 1: LITERATURE REVIEW

1.1. Drug discovery and leukemia stem cells

There are several hallmarks that distinguish cancerous cells from normal cells (i.e., uncontrolled proliferation, resisting cell death, evading anti-growth signals).[1] In leukemia, these distinguishing features can be the result of ineffective differentiation of stem cells, which produces heterogeneous progeny. The mechanisms behind this heterogeneity are not very well understood, however there is strong evidence that the development of leukemia is a hierarchical model, with stem cells at the origin.[2]

Current anti-cancer therapies decrease the bulk cancer cell population.[1] While this may create a dramatic response, long-term remission is unlikely if leukemia stem cells are not eliminated. In addition, standard chemotherapy induces cytotoxic effects in healthy cells, which have several negative consequences on patients. In the case of acute myeloid leukemia (AML), extremely poor patient outcome is associated with the failure of current therapy to target and eliminate leukemia stem cells (LSCs).[3] With such poor statistics of successful remission without relapse and death (i.e., less than 10%, 5-year survival rate for older adults), it is clear that the target for novel AML therapeutics should be focused on LSCs. With the advent of improved cell culture techniques, researchers now have the ability to take advantage of high-throughput screening to identify compounds with selective toxicity toward LSCs. Rapid screening of large compound libraries can be achieved, identifying new molecules *in vitro* which can be further evaluated *in vivo*.

1.2. Acute myeloid leukemia

AML is a hematopoietic malignancy characterized by an accumulation of immature myeloid cells, termed "blasts", in the marrow.[4] As a result, a disruption of hematopoiesis occurs, reducing the number of myeloblasts (i.e. blast cells) that become terminally differentiated into normal, functioning blood cells. AML is the most common myeloid leukemia and consists of many subtypes that share common characteristics. The prevalence of AML is approximately 3-8 in 100,000 people, rising to 17.9 in 100,000, in populations who are 65 years of age or older[4]. Although there have been recent advances in treatments for other haematological malignancies, poor outcomes for AML patients have remained constant for older populations.[5] About 15-20 percent of patients fail to achieve complete remission and, more than 40 percent of patients who do, will relapse within 2 years.[6] The prognosis is especially poor for patients over the age of 65, with a 2-year survival rate of less than 10 percent.[7] This is particularly concerning as nearly two thirds of AML patients are 65 years or older.[8,9] Lastly, AML accounts for 32% of cancer related deaths in children.[10]

1.2.1. Treatment

The main goal of AML therapy is to induce remission and avoid disease reoccurrence (i.e., patient relapse). Remission is defined as the presence of fewer than 5 percent blasts in the bone marrow together with the recovery of normal peripheral blood counts.[10] Typically all patients who are newly diagnosed with AML are recommended to start induction therapy. Traditionally, this includes cytarabine (Ara-C; a pyrimidine analog) and daunorubicin (an anthracycline) in a "3+7" regimen.[7,10,11] The 3+7 regiment consists of daunorubicin at 45mg/m^2 intravenously for three days, and cytarabine at

100mg/m^2 intravenously by continuous infusion for seven days.[10] Many side effects result from this treatment such as nausea, vomiting, haemorrhage, ataxia, and severe fatigue due to the highly cytotoxic nature of these chemotherapeutics. This hinders induction therapy treatment options for the majority of AML patients (i.e. those 65 years and older), as their tolerance to the cytotoxicity is much lower than that of a younger patient. This is a significant problem as it is estimated that only 30 percent of elderly patients are fit to undergo treatment beyond palliative care.[12] Although there have been efforts to reduce the cytotoxic side effects of current treatment standards through the use of other agents, there is no alternate treatment that surpasses the success rate of this induction therapy.[9]

Post induction therapy is generally needed following initial therapy, if remission has been achieved. Additional therapy is available depending on the ability of the patient to withstand further treatment. These options include: allogenic bone marrow transplantation from an human leukocyte antigen-matched donor, autologous bone marrow transplantation, or subsequent rounds of chemotherapy.

Flaws in current induction therapies are clear; long-term survival is rare due to the high rate of relapse. It is obvious that while the current standard of care may eliminate the bulk of the immature myeloid cells, it fails greatly in eliminating the cells that are responsible for maintaining the disease.

1.3. Leukemia stem cells

The hematopoietic system is an organized hierarchy maintained by rare hematopoietic stem cells (HSCs) that self-renew and differentiate to produce mature blood lineages.[4] Leukemia can occur when the regulatory signals for these processes become mutated or dysregulated. Leukemia is believed to maintain the hierarchical

model that is seen in normal hematopoiesis, with LSCs at the apex (Figure 1).[3] Evidence has shown the existence of LSCs, which are defined by functional attributes including the ability to instigate, maintain and serially propagate leukemia *in vivo* while retaining the capacity to differentiate into committed progeny that lack these properties.[4,13,14] The earliest studies supporting this claim observed a small population of cells (CD34+/CD38-) that were able to engraft into immune-compromised mice, whereas the bulk blasts were not capable of this.[15] Furthermore, the bulk mass has been characterized by limited proliferative ability, suggesting that there was a population of malignant progenitor cells that must be maintaining the disease.[4] Finally, long-term engraftment was observed with this cell population (CD34+/CD38-) in mouse xenograft models through serial transplantation[16], leading to the hypothesis that LSCs retain the ability to regulate self-renewal through conservation of self-renewal pathways.[10]

LSCs represent a very small portion of the AML cell population, accounting for only 0.1-1% of the blasts.[8,17] With the discovery of LSCs, morphological characterization has shown that this population expresses surface markers, which are unique from hematopoietic stem cells. For example, while LSCs lack the expression of CD90 (Thy-1; stem cell marker)[18], they have increased expression of CD34+/CD38- (stem cell marker/myeloid marker, respectively) phenotype.[4] Furthermore, LSCs posses unique antigens for receptors including T cell receptor markers, CD96[19] and TIM3[20], as well as interleukin 2 and 3 receptor markers, CD25[21] and CD123[22] respectively. In addition, antigens that are associated with cell-cell interactions are found in LSCs including CD44[8] involved in cell adhesion, as well as CD47[17], which has been associated with the

inhibition of phagocytosis as a way of LSC chemo-resistance. The novel LSC antigen, CLL-1, has also been identified.[23]

It is believed that AML is initiated and maintained by this rare population of LSCs, which evade current chemotherapy treatments (Figure 1) through some of their unique biological properties such as a quiescent state, multidrug resistant efflux pumps, and localized protection in the bone marrow.[10] Most of the current therapies for leukemia have been designed on general biological properties of malignant blast cells, such as rapid cell cycle activity.[17,24] However, since LSCs can be found in a quiescent state the use of these strategies may not effectively target the LSC population and consequently the disease may not be eradicated successfully.[25,26] The central role LSCs play in relapse and the refractory nature of AML highlights the critical need to develop new chemotherapeutic strategies to directly target the LSC population.

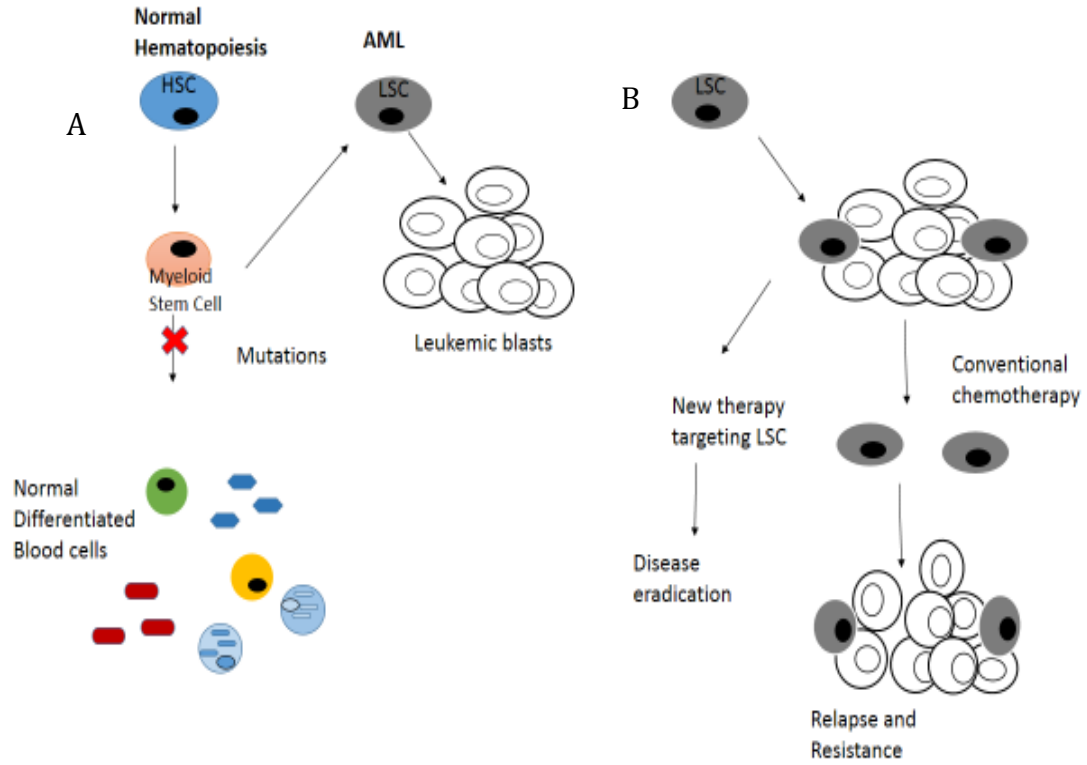


Figure 1. Leukemia stem cells (LSCs) are able to evade current chemotherapy treatment. (A) *Leukemia progression*; mutations occur at the progenitor stage, giving rise to the LSC. The LSCs produce myeloid blast cells that are unable to differentiate, leading to hematopoietic insufficiency. (B) *AML treatment*; chemotherapy is able to eliminate the bulk myeloid blasts, but is unsuccessful in eradicating LSCs. Relapse occurs due to the survival of LSCs, which are able to regenerate leukemic blasts and are subsequently resistant to chemotherapy. This highlights the need to discover compounds that target LSCs. Modified from Jordan, C. *The leukemic stem cell.* Best Pract. Res. Clin. Haematol. 2007.[27]

1.4. Programmed cell death

Programmed cell death (PCD) is an important terminal pathway for cells and is involved in a variety of biological events that include elimination of harmful cells, morphogenesis, and maintenance of tissue homeostasis.[28] Dysfunction of programmed cell death can lead to physiological implications, most notably the development of cancer and resistance to chemotherapy. Increasing evidence suggests that cancer therapy is related to, and can be improved by targeting a PCD pathway.[29] The ultimate goal of anti-cancer therapy is to kill cancer cells quickly and effectively. A hallmark of cancer in general is the ability to evade PCD, leading to uncontrolled proliferation, which may be a reflection of defective or absent cell death machinery.[30] In principle, both of these components represent a potential target for clinical intervention.

It is widely accepted that there are three major mechanisms of PCD; apoptosis, autophagy and programmed necrosis. Each of these display several phenotypes, affecting various intracellular organelles, membranes, and the cell nucleus. Autophagy is a catabolic process, which recycles organelles to enhance survival under conditions of scarcity or starvation.[31] It can be characterized by the generation of autophagosomes, large organelles that engulf various cellular components which fuse with lysosomes, to generate limiting materials.[28] However, in the presence of prolonged autophagy

signalling events, the cell may use autophagy as a mode of PCD.[31] Programmed necrosis is a passive process that was previously thought to be unregulated. Current studies, however, have shown evidence that necrosis results from the controlled interplay between several signalling pathways, independent from caspases or lysosomes.[28,32] Necrosis can include signs of regulated process such as mitochondrial dysfunction, enhanced generation of reactive oxygen species (ROS), nuclear degradation and ATP depletion.[28] Necrosis is characterized by early loss of integrity of the plasma membrane, allowing influx of extracellular fluid which causes swelling of the cell and its organelles.[32] Apoptosis is a genetically regulated process of cell death where cells systematically undergo a series of morphological changes and are consumed by surrounding cells.[32] Due to the relevance of apoptosis in cancer onset, this mechanism of PCD will be explained more thoroughly.

1.5. Apoptosis

Apoptosis acts as part of a quality control and repair mechanism by eliminating damaged or senescent cells. Phenotypic characteristics of apoptosis include cytoplasmic and chromatin condensation, membrane blebbing, and the formation of membrane bound apoptotic bodies.[28] The major biochemical features of apoptosis include activation of intracellular proteases, most notably the caspases, and internucleosomal DNA fragmentation.[32]

Although many genes are involved in the regulation of apoptosis, the key mediators are the caspases. Caspases are a group of aspartate-specific cysteine proteases, which cleave their substrates on the carboxyl side of the aspartate residue.[33,34] There are 14 known caspases, of which two-thirds have been shown to play a role in apoptosis.

Caspases are synthesized as procaspases, which through pro-apoptotic signals are digested by protease to form active caspases.[28] Apoptosis-related caspases can be divided into two groups, the initiator caspases (caspase 8, 9 and 10) and the effector caspases (caspases 2, 3, 6 and 7). It is the effector caspases that degrade multiple cell proteins and are responsible for the morphological changes that ultimately lead to cell death.[33] For example, caspases 3 and 7 are involved in the cleavage of the DNA repair protein, poly(ADP-ribose)ylation polymerase (PARP).[33] PARP is cleaved at the DEVD (Asp-Glu-Val-Asp) tetra-peptide site which is recognized by both caspase 3 and caspase 7, rendering PARP unable to perform DNA repair. Inactivation of PARP allows DNA fragmentation to occur, which is characteristic of all apoptotic pathways.[32] The activation of effector caspases is performed through initiator caspases by cleavage of specific aspartate residues that separate the small and large subunits.[35] The initiator caspases are auto-activated, however they are tightly regulated and often require assembly of multicomponent complexes under apoptotic conditions.[35]

Caspase activation can commit a cell to two major pathways, death receptor pathway (extrinsic pathway) or the mitochondrial pathway (intrinsic pathway). Factors that determine which death pathway is activated include the stage of the cell cycle, the type of apoptotic stimulus, and the stage of cellular activation.[32] Although the pathways are distinct, some evidence shows that they may be linked, influencing each other through involved molecules.[36]

The mitochondrial pathway is prompted by initiators, which include increased intracellular ROS, DNA damage, unfolded protein response, and the deprivation of growth factors.[37] Intracellular sensors indicate cell damage, ultimately leading to

activation of caspase 9, and subsequently leading to cell death (Figure 2A).[29] The intrinsic apoptotic cascade is regulated by the balance between pro-apoptotic (ex.Bim, Puma, Bid, Bax and Bak) and anti-apoptotic (ex. Bcl-2 and Bcl-xl) BCL2 family. Increased mitochondrial permeability occurs in response to initiating signals, promoting the release of pro-apoptotic proteins, such as cytochrome c, from the intermitochondrial membrane space into the cytosol.[32] Bax and Bak are Bcl-2 pro-apoptotic proteins which are critical players in this loss of mitochondrial membrane potential.[37] Cleavage of Bid to tBid inhibits the anti-apoptotic BCL2 proteins Bcl-2/Bcl-xl, liberating Bax and Bak.[32] Cytochrome c, when released, is a part of the apoptosome complex. This complex allows the activation of caspase 9 which then results in the proteolytic activation of the executioner caspases 3 and 7, leading to apoptosis.[38]

The death receptor pathway is activated at the cell surface through ligation of "death receptors", initiating apoptosis. Death receptors belong to the tumour necrosis factor (TNF) superfamily.[16] The most well characterized are Fas, DR3, DR4/5, TNF-R1 and TNF-R2[22]. Ligation of these receptors initiates a multi-protein cascade starting with the activation of caspase 8 or 10, followed by activation of caspase 3 and 7 (Figure 2B). Death receptors contain an intracellular death domain (DD), which upon ligand binding associates with an adaptor protein called Fas-associated-death-domain (FADD).[22] FADD interacts with procaspase 8 to form a complex at the receptor called the death inducing signalling complex (DISC).[23] Once assembled, DISC induces the activation of caspase 8, which in turn activates the downstream effector caspases. Crosstalk can also occur between the death receptor pathway and the intrinsic pathway, whereby caspase 8 cleaves

Bid.[22] The truncated form of Bid (tBid) is an inhibitor of the anti-apoptotic proteins found within the mitochondria.[22]

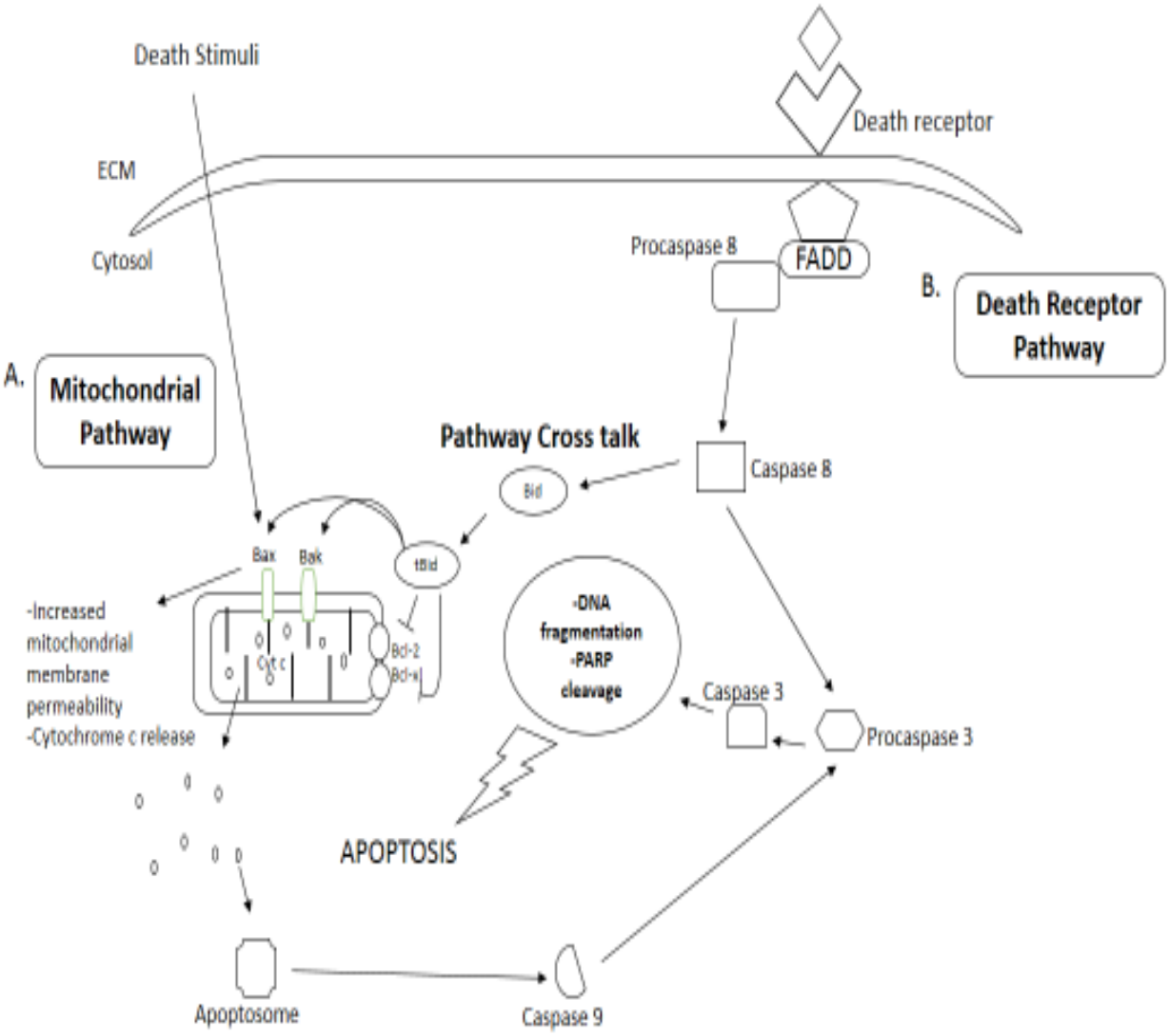


Figure 2. Pathways of apoptosis. (A) The mitochondrial (intrinsic pathway) involves the release of pro-apoptotic proteins leading to the activation of caspase 9. (B) The death receptor (extrinsic pathway) involves ligation of death receptors initiating activation of caspase 8. Both pathways eventually lead to the activation of caspase 3/7, and ultimately apoptosis. Modified from Hotchkiss et al. *Mechanisms of Cell death*. N Engl J Med. 2009[16].

1.6. High-throughput screening

High-throughput screening (HTS) is a drug discovery method as a systematic and unbiased approach to identify novel therapeutic agents.[39] HTS involves testing a high number of compounds very quickly and can be done manually or through a robotic system.

Traditional HTS focused on anti-AML drug discovery, measuring the ability of compounds to reduce the viability of leukemia cell lines, has identified many current day chemotherapeutic agents such as cytarabine.[15] However, these screens have mainly targeted AML blast cells, not LSCs. Ideally, screening LSCs as the therapeutic target would drive drug discovery towards what it is currently lacking – eradication of LSCs. Fortunately, due to recent development of LSC-like cell lines, this methodology can be implemented. Further, to design the most robust method for evaluating compounds for AML therapeutic potential, parallel screening using a normal cell line would be necessary to investigate cytotoxicity towards other cells.[15] As mentioned previously, the current chemotherapeutics are cytotoxic to normal haematopoietic cells, which is certainly unfavourable and difficult for elderly patients to withstand.

1.6.1. Advent of the LSC cell line

Assessing the toxicity of compounds against primary LSCs is difficult as they are exceedingly rare[4,40–42], very difficult to purify, and have high cytogenetic variation among patients.[15] Additionally, primary LSCs cannot be maintained in long term culture (only lasting up to 72 hours) without adopting very specialized, and expensive techniques.[15] To bypass the limitations of using common leukemia cell lines and primary

patient samples, the recent advent of new LSC-like cultures have made it possible to screen for LSC specific toxicity.

A novel cell line, TEX, was created by Warner, *et al.*[43] by transducing the leukemogenic fusion oncogene TLS-ERG, which is associated with many subtypes of AML, into normal HSC from lineage depleted cord blood.[43] TEX underwent step-wise transformation and immortalization through acquisition of additional genetic changes such as LSC markers CD34 (stem cell marker), CD44 (involved in cell-cell adhesion), and the stem cell gene CD123 (interleukin-3 receptor).[43]

This novel line is driven by LSC-like cells and is able to be cultured long-term, up to 24 months, differentiate *in vitro* upon removal of cytokines, and importantly, retain a hierarchical organization, unlike traditional leukemia cell lines.[15] *In vitro* and *in vivo* limiting dilution experiments revealed that TEX cells are functionally heterogeneous with a minority of cells possessing LSC function (1 in 120 cells *in vitro*; 1 in $3.8x10^5$ *in vivo*).[15] In addition this cell line, uniquely expresses many genes identified in LSCs, as well as within the shared LSC/normal HSC signature, indicating that these cells have some of the same stem cell regulatory networks and may reflect a more representative population for LSCs *in vivo.*[43] Finally, these cells are capable of bone marrow engraftment in mice following intrafemoral injection.[44] For the purpose of our projects, the TEX cell line is the surrogate cell line used as the target to evaluate compounds against LSCs.

1.7. Estrogen receptors

The estrogen receptor (ER) is a member of the nuclear family of receptor proteins that function as DNA transcription factors when bound to their corresponding hormone

ligand 17-β-estradiol (E2).[45] Once activated by E2, the ER is able to translocate to the nucleus and bind to DNA to regulate the activity of genes.[45] Elwood V. Jensen discovered the classical ER, ERα, at the University of Chicago in 1958.[46] It was not until 1996 that the gene for a second estrogen receptor, ERβ, was identified by Kuiper *et al*.[47] in rat prostate and ovary using degenerate ERα primers.

ERα and ERβ are encoded by separate genes and found on separate chromosomes (ESR1, locus 6q25.1 and ESR2 , 14q23-24 respectively).[48] Furthermore, they produce proteins of different sizes; 595 (ERα, 55 kDa) and 530 (ERβ, 59.2 kDa) amino acids.[48] The ERs are widely expressed in different tissue types; however, there are some notable differences in their expression patterns. ERα is expressed at high levels in several normal tissues classically associated with estrogenic activity including uterus, ovary (theca cells), bone and breast.[49] ERα is also expressed at high levels in prostate (stroma) and brain but to a lesser degree in bladder, liver and thymus.[50] ERβ is found at its highest levels in normal colon, prostate (epithelium), ovary (granulosa cells), bone marrow and brain, with smaller amounts reported in uterus, bladder, lung and testis.[49–52]

ERα and ERβ show significant overall sequence homology, and are both comprised of five domains (listed from N- to C- terminus; A-F; Figure 3).[53,54] The N-terminal A/B domain is the terminal region involved in activation of gene transcription. The C domain, known as the DNA binding domain (DBD), allows ER to dimerize and bind to the specific sequence of DNA.[55] The hinge region, the D domain, has a role in receptor dimerization and in binding to chaperone heat-shock proteins. At the C-terminus is the E/F domain, which functions as the ligand-binding domain (LBD), working with the N-terminal domain in the regulation of gene transcription.[48,56–59] The ER is composed

of two active transcriptional domains, activation factor 1 (AF-1) and activation factor 2 (AF-2). AF-1, located in the A/B domains, is hormonally independent and constitutively active when bound to DNA.[53] It is responsible for co-activator interaction and amplified receptor function.[58] AF-2 is a complex region located in the E/F domains and its structure and function are governed by ligand binding.[60] AF-2 is thought to interact indirectly with transcriptional machinery when ligands are bound to the receptor and is involved in chaperone protein binding when the receptor is unliganded.[58,59] Conformational changes occur at AF-2 domain when estrogen is bound, resulting in DNA binding and thus the initiation of transcription.[61]

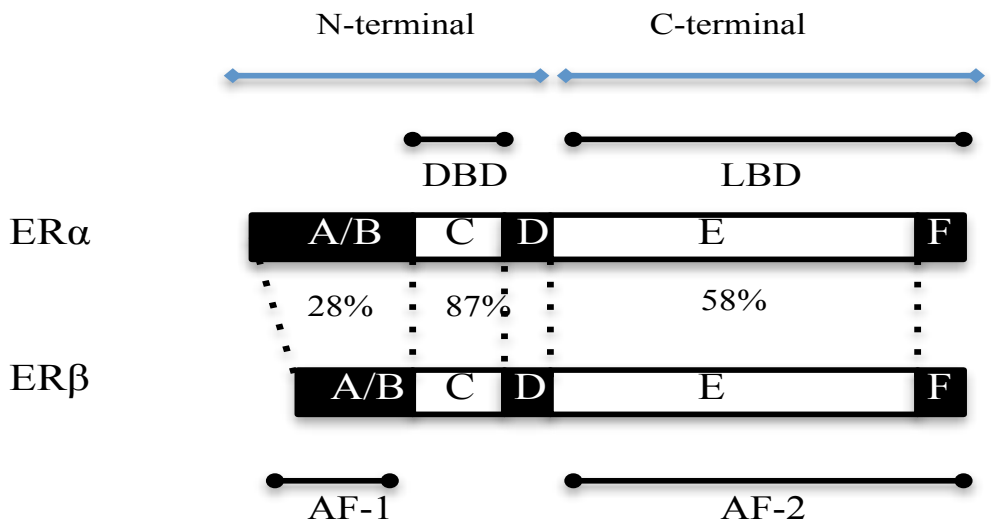


Figure 3. Domain organization and homology of ERα and ERβ. ERs consist of 5 domains, N- to -C terminus; labeled A-F. The A/B domain includes the AF-1 and is involved in transactivation. The C domain facilitates DNA binding and the D domain is the hinge region involved in receptor dimerization. The E/F domain includes the AF-2, and contains the ligand binding domain. The percentage indicates the homology retained in the corresponding domains between ERα and ERβ. Modified from Marino et al. *Estrogen Signaling Multiple Pathways to Impact Gene Transcription.* Curr. Genomics. 2006.[48]

1.7.1. Estrogen receptor signaling

Once activated, ERα and ERβ either form homo- or hetero-dimers[61], and exert their effects through a diverse array of pathways. These pathways mediate genomic and non-genomic events, which lead to either direct or indirect tissue specific transcriptional outcomes (Figure 4).

Estrogens classically regulate human physiology via diffusion through the plasma membrane of target cells and signalling through the intracellular ER. This classical genomic signalling, is referred to as *genomic signalling*, and occurs by direct binding of ER dimers to estrogen responsive elements (ERE), which are specific, inverted palindromic sequences located on the promoters of target genes (Figure 4A).[58] Because there are multiple EREs, activated ERs induce many effects. ERs also modulate gene expression by a second mechanism that involves protein-protein interaction of ER with other transcription factors (stimulating protein 1; Sp-1) and activating protein 1; Ap-1) through a process known as transcription factor cross talk (Figure 4B). Through this latter transcriptional activation or repression, ERs can therefore influence the expression of genes lacking EREs.[22-25] In both cases, the recruitment of co-regulators and transcription machinery to the transcription start site is initiated, which subsequently activates transcription of downstream genes.

More recently it has been elucidated that ER signalling can occur rapidly through *non-genomic mechanisms*, which involve membrane bound receptors (Figure 4C). Membrane-localized ER has elicited activation of downstream pathways such as MAPK, PKA and PKC.[45] This type of signalling does not require direct binding of DNA to the ER, but are mainly mediated by ER-dependent activation or repression of intracellular

second messengers.[62–64] The mechanistic details of non-genomic signalling have yet to be clearly characterized, especially for the E2-ERβ relationship.[48] ER can also be activated in the absence of a ligand through phosphorylation by the activation of kinases, for example, through growth factor receptor activation (Figure 4D).[45,65] This action thereby activates ERs or associated co-regulators. As an example, the HER2 downstream signalling molecules ERK1 and ERK2 can phosphorylate ER, leading to ligand-independent receptor activation.[48] The biological significance of this ER signalling remains unclear, but may contribute to hormone-independence of certain tumours.[48]

1.7.2. Estrogen receptor activity

Although ERα and ERβ have similar structure, they produce different biological effects.[45,66] The ERα and ERβ knock-out mice have different phenotypes demonstrating the different physiological roles of the two ERs.[67–69] The classical ER ligand, E2, binds to both ERα and ERβ, however, the two receptors elicit different, and opposing biological activity.[45] Hence, this suggests that in any given tissue the ERβ/ERα ratio is an important determinant to the response to estrogens.[70] Estrogens via ERα increases proliferation of the breast, uterus and developing prostate[71–73] , while estrogens via ERβ inhibits proliferation and promotes differentiation in response to ligands in the prostate, mammary gland, colon, lung, and immune cells.[74–77]

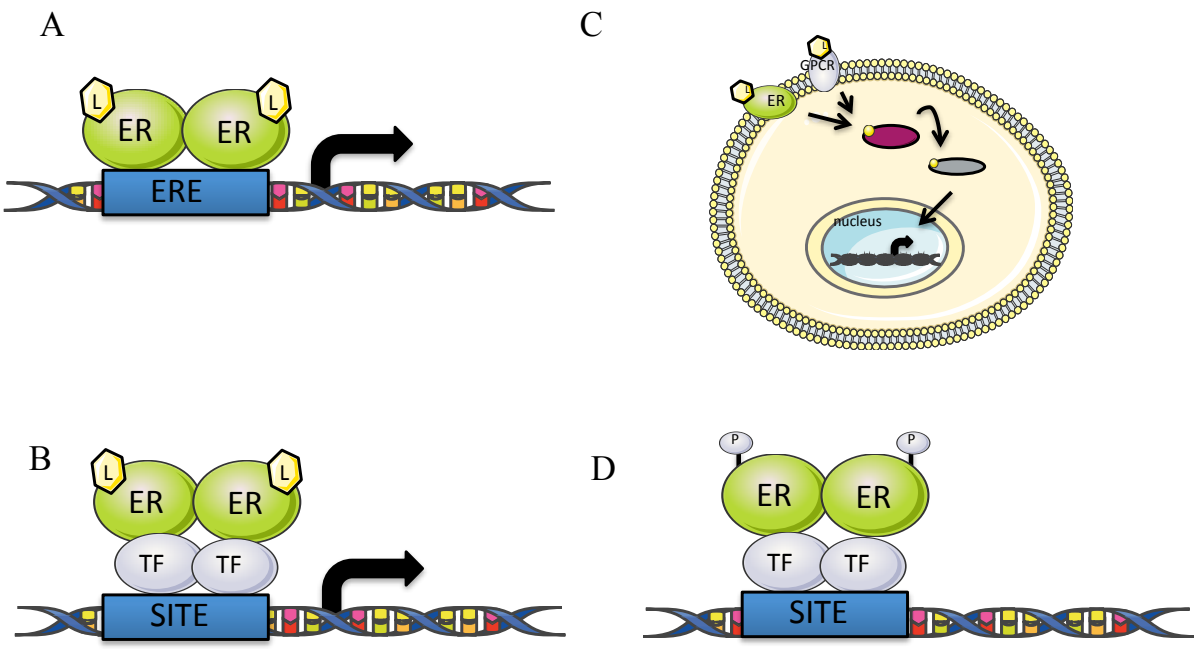


Figure 4. Estrogen receptor signalling mechanisms. Representation of the mechanistically distinct molecular pathways used by the estrogen receptors to regulate activity. (A) The classical pathway involves ligand activation and direct DNA binding to ERE to initiate transcription. (B) A second pathway that involves indirect DNA binding that occurs through protein-protein interactions of other transcription factors following ligand binding. (C) Non-genomic signalling occurs rapidly through membrane bound ERs which initiate signalling cascades through second messengers after activation by a ligand. (D) Ligand-independent signalling can occur through phosphorylation of the ERs by activation of kinases, and initiating DNA binding. Modified from Heldring *et al. Estrogen Receptors: How do They Signal and What Are Their Targets?* Physiol. Rev. 2007 [45]. Drawing made using Servier Medical Art Bank.

1.7.3. Estrogen receptors and cancer

ERs have been implicated in the onset, treatment and prevention of cancer. ERα is an important target in breast cancer, as it is increased in malignant breast cells, and it interacts with estrogen to result in increased proliferation of these cells.[78] Selective

estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, have been developed to antagonize this action as competitive inhibitors of the ER. Tamoxifen is widely used in the treatment of ER positive breast cancers and also as a preventative measure for women who have achieved remission.[78] While the role of ERβ is not well elucidated in breast cancer, it has been associated with favourable prognosis, and disease free survival rates, suggesting a protective nature for ERβ.[79] Alternatively, studies have suggested that ERβ can play an anti-proliferative or pro-apoptotic role in cancer. This effect has been observed in prostate and breast cancer[80,81], as well as lymphoma[70] through ERβ agonism. Many ERβ selective agonists are up for development in clinical trials, however, to date none are currently approved for the treatment of cancers.[57]

1.8. Nutraceuticals and AML

Nutraceuticals are food-derived bioactive compounds and part of a larger group of compounds known as natural health products (NHPs).[82] From the earliest days of drug discovery NHPs were often the original source of medicinal treatments, typically being secondary metabolites containing drug-like activities.[83] More recently, NHPs have been able to provide not only a drug source, but also templates for synthetic modification.[84] Between 1940 and 2010, a total of 27 anticancer drugs were obtained from natural sources.[85] Examples include commonly used chemotherapy agents in cancer treatment such as vincristine (Madagascar periwinkle), actinomycin D (soil bacteria), and paclitaxel (pacific yew tree).[85] Additionally, topotecan HCl, dexamethasone, etoposide, cytarabine and tamoxifen are mimics of natural products.[85]

Benefits of NHPs include: general low toxicity levels and thus harm to the body, or normal, healthy cells is minimized.[84,86] This can have positive implications for the

population of AML patients who are over the age of 65. Additionally, many NHPs have already been assessed for cytotoxicity[87], and are structurally defined. With only a very small portion of known nutraceutical compounds having been tested for novel, anti-cancer properties and even fewer for anti-leukemic activity, there remains much to be explored. Perhaps the most inspiring work while reviewing the clinical utility of nutraceuticals as treatment for haematological malignancies is the use of all-trans-retinoic acid (ATRA), a key active metabolite of vitamin A. ATRA is best known for the treatment of acute promyelocytic leukemia (APML), a subset of AML. After observation of ATRA's *in vitro* efficacy in the early 1980's[88] for inducing APML cell differentiation, ATRA was used successfully to achieve complete remission in APML patients.[89] Since ATRA was adopted as the main therapy for APML treatments, 5-year survival rates have improved to over 90 percent.[90,91]

1.9. Diosmetin

Flavonoids are polyphenolic compounds present in plants known for their protective function. More than 4,000 flavonoids mainly classified into flavonols, flavones, flavanols, flavanonols, flavanones, and isoflavones have been identified in the edible plants, fruit, vegetables, tea, wine, seeds, herbs, spices, whole grains and in the regular human diet.[92] In the normal US diet, estimated total consumption of flavonoids were found to be 1 g/day, including 160mg/day through vegetables and herbs, and an additional 290 mg/day through juices and fruits.[92] Numerous biological activities of flavonoids such as anti-allergic, antibacterial, oestrogenic, anti-tumoural, hepatoprotective, antithrombotic and antiviral have been reported in literature.[93–95]

To identify novel anti-leukemia agents, our lab screened close to 300 compounds from a unique in-house nutraceutical library. This screen identified diosmetin as the most potent compound reducing leukemia cell viability. Diosmetin (Figure 5), a compound belonging to the flavone class of flavonoids, is widely present in citrus fruits[96], plants belonging to the genus Teucrium (*Lamiacecea*)[97] and in Portuguese olive leaves[98]; and up to 30 other sources[92] found to date. Pharmacologically, diosmetin is reported to exhibit anticancer and antimicrobial activities. *In vitro* anticancer activity of diosmetin was evaluated against various tumour cell lines and found that it had significant anticancer activity against the brain carcinoma U251 cell lines and moderate activity against breast carcinoma MCF7 cell lines.[92] In MDA-MB 468 and MCF7 breast cancer cell line, diosmetin has been shown to inhibit proliferation, as a result of cytochrome P450 CYP1 bioactivation.[99] In addition, diosmetin has been shown to have growth inhibitory effects on P-388 lymphocytic leukemia cells[100] as well as pathogens such as *Bacillus subtilis* and *Trichophyton rubrum.*[101] Although these reports suggest diosmetin indeed has beneficial biological activity, the body of data evaluating its potential is still relatively small. We report in this thesis a novel function of diosmetin as a potential ERβ agonist.

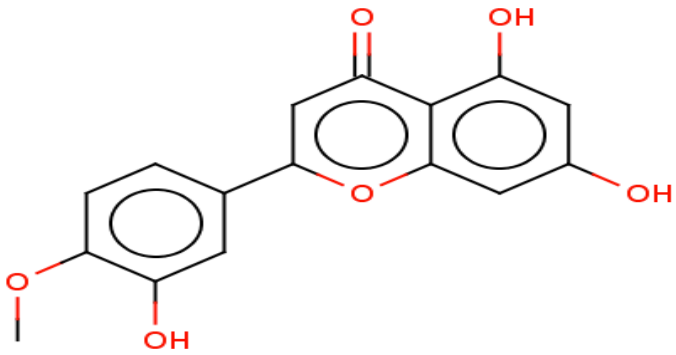


Figure 5. Structure of diosmetin. Diosmetin is a member of the flavone class of flavonoids, found in various sources such as citrus peel and olive tree leaf. Chemical name: 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-benzopyrone. Image taken from ChemBankID:2060301.

CHAPTER 2: OBJECTIVES AND HYPOTHESIS

The objectives of this project are:

1) Create and evaluate a nutraceutical specific library for anti-LSC activity;

2) characterize and evaluate lead compound diosmetin's mechanism of action and genetic target(s) in acute myeloid leukemia cells *in vitro* and; 3) determine diosmetin's *ex-vivo* efficacy in primary AML patient cells.

These objectives fulfilled the hypothesis that: Diosmetin imparts cell death in LSCs through estrogen receptor beta (ERβ) activation, initiating caspase-8 and TNFα mediated apoptosis.

Short-term goals:

The short term goals are to determine diosmetin's mechanism of action and activity in AML, which may further warrant investigation for it's clinical anti-AML application.

Long-term goals:

Upon successful completion of the aforementioned short term goals, the next steps would be to;

1) to investigate efficacy and safety in animal models, and

2) to further investigate the mechanism by which ERβ activation imparts LSC-specific death, including assessment of protein-gene interaction between ERβ and the promoter sequences and target genes.

CHAPTER 3: MATERIALS AND METHODS

3.1. Cell culture

Unless otherwise stated, all cells were cultured in media supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and antibiotics (100units/mL of streptomycin and 100μg/mL of penicillin; Sigma Chemical; St. Louis, MO). All cell lines were incubated in a humidified air atmosphere containing 5% CO_2 at 37°C.

KG1a AML, OCI-AML2 AML, K562 chronic myeloid leukemia, HL-60 promyelocytic leukemia and LP1 human myeloma cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies; Grand Island, NY). TEX leukemia cells were cultured in IMDM supplemented with 15% FCS, antibiotics, 20ng/mL stem cell factor, 2ng/mL IL3 (Peprotech; Hamburg, Germany), and 2mM L-glutamine (Sigma Chemical). DU145 prostate cancer, U937 human lymphoma, HeLa adenocarcinoma cell lines were cultured in RPMI (Life Technologies).

Primary human AML samples were obtained from Dr. Mark Minden (Princess Margaret Cancer Center), and were cultured at in IMDM, supplemented with 20% FCS and antibiotics. These cells contained at least 80% malignant cells among the mononuclear cells from consenting AML patients. $CD34^+$ hematopoietic cells from normal bone were purchased from Stem Cell Technologies (Vancouver, BC). The institutional ethics review boards (University Health Network, Toronto, ON, Canada and University of Waterloo, Waterloo, Ontario) approved the use of human tissue for this study.

3.2. Flow cytometry

All flow cytometry assays were performed on a Guava easyCyte 8HT Bench Top Flow Cytometer (Millipore, Billerica, MA) using GuavaSoft 2.2.3 (Millipore) flow cytometry software.

3.3. Cell growth and viability

Cell growth and viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay (Promega, Madison, WI)[87,102], Annexin V and PI (ANN/PI) staining (Biovision, Mountainview, CA) via flow cytometry[102], and colony formation cell assays (CFC)[44] , according to the manufacturer's protocol and as previously described. These assays will be described more thoroughly within this section.

3.4. MTS cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay is a common colorimetric method for determining the number of viable cells in proliferation. Typically, 5µL of drug or vehicle control was added to 95µL cells seeded in a 96-well plate at a density allowing 90% confluency at the end of the desired incubation time (24-72 hours). 20µL of MTS reduction assay (Promega) was added directly to each well and incubated for 2-4 hours at 37°C. This assay works on the principle that the mitochondrial dehydrogenase enzyme reduces the MTS salt to a coloured formazan and the absorbance is read directly by a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) at 492 nm.[32] Visibly, wells with proliferating cells results in a dark brown colour, whereas in wells with dead or non-proliferating cells a yellow colour will be present.

3.5. Nutraceutical screen

A unique nutraceutical library was created and screened similar to previously described methods.[44] Nutraceuticals were obtained from Chengdu Biopurify Phytochemicals Ltd. (n = 288, Sichuan, China), and were reconstituted in dimethyl sulfoxide (DMSO; Sigma Chemical) to make 20mM stock solutions. The compounds were then diluted further to 200μM stock solutions in phosphate buffered saline (PBS; Life Technologies). 100μL of TEX ($1.5x10^5$/well) or K562 ($1x10^5$/well) cells were seeded in 96-well polystyrene tissue culture plates (CELLSTAR, VWR, Mississauga, ON). After seeding, cells were treated with 5μL aliquots (1μM and 10μM final concentration) of library compound with a final DMSO concentration of less than 0.05% v/v. Kinetin riboside or tigecycline (Sigma Chemical), compounds known to induce TEX cell death, were used as positive controls at 10μM final concentration. Cell growth and viability were measured after 72 hours by the MTS assay. Twelve of the most promising compounds from the screen that induced cell death in TEX, but not K562 cells, were chosen for further validation in an additional MTS screen (5μM and 10μM final concentration). Lead compound, diosmetin (Sigma Chemical, Toronto Research Chemicals; Toronto, ON) was selected as the compound of interest in this project, and reconstituted in DMSO. The stock solution (5mM) was diluted in PBS, aliquoted and stored at -20°C to prevent excessive freeze thaw cycles.

3.6. Cell surface marker analysis

Eppendorf tubes containing $1x10^5$ cells from each cell line being tested were prepared. 2μL of a fluorescently conjugated cell surface receptor antibody was added to each tube, and incubated for 30 minutes at 2-8°C in the dark. Cells were then washed

twice in staining buffer (PBS containing 5% FCS) and analyzed using flow cytometry. The antibodies assessed are listed in Table 1. BD CompBeads anti- mouse Ig, k particles (BD Biosciences) were used as a positive control. In addition, a colony formation assay was used to assess progenitor capabilities of both TEX and K562. Refer to section 3.7 for full methodology.

Table 1. Cell Surface Marker Antibodies

Surface Marker	Isotype	Fluorescent tag	Manufacturer
CD33	Mouse IgG1	PE	BD Biosciences
CD19	Mouse IgG1	APC	BD Biosciences
CD34	Mouse IgG1	APC	BD Biosciences
CD38	Mouse IgG1	FITC	BD Biosciences

3.7. Colony formation cell assays

Colony formation cell (CFC) assays with primary AML and normal hematopoietic stem cells were performed, as previously described.[102] CD34+ bone marrow-derived normal stem cells (StemCell Technologies) or AML mononuclear cells ($1x10^5$cells/mL) were suspended in IMDM media containing 2% FCS. 300μL of cell suspension was added to 3mL of MethoCult GF H4434 medium (StemCell Technologies) containing 1% methylcellulose in IMDM, 30% FCS, 1% bovine serum albumin (BSA; Sigma Chemical), 3U/mL recombinant human erythropoietin, 10-4M 2-mercaptoethanol (2ME), 2mM L-glutamine, 50ng/ml recombinant human stem cell factor, 10ng/mL recombinant human granulocyte macrophage-colony stimulating factor and 10ng/mL recombinant human IL-3, in a 15 mL polystyrene tube. Treatment included diosmetin (10μM and 20μM concentrations) or a vehicle control added directly to the tubes, vortexing at medium speed for no longer than 7 seconds. 1.1mL of media was plated in each 35 mm dish (10^4 cells/dish) (Nunclon; Rochester, NY) using a 5mL syringe with a blunt tip

needle (Covodein, Minnapolis, MN). Duplicate dishes per treatment were stored in a 100mm Petri dish with an additional uncapped 35mm dish containing distilled water to control humidity. The plates were incubated for 7-14 days at 37°C with 5% CO_2 and 95% humidity. The colonies were counted on an inverted microscope with a cluster of 10 or more cells counted as one colony.

3.8. Annexin V/propidium iodide staining

Assessments of early apoptosis and cell death were achieved using Annexin V/Propidium Iodide (ANN/PI) staining and flow cytometry. When cell death is occurring, phosphotidylserine (PS) is translocated to the outer layer of the membrane, the external surface of the cell.[103] This occurs in the early phases of apoptotic cell death during which the cell membrane itself remains intact. Annexin V-FITC (ANN) is a calcium dependent phospholipid-binding protein with high affinity for PS.[103] Hence, this protein can be used as a sensitive indicator for PS exposure upon the cell membrane. Propidium iodide (PI) use is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content.[104] Use of a membrane impermeable fluorochrome, such as PI, that is capable of binding and labeling DNA can confirm that the cell is undergoing apoptosis and not necrosis.[104]

Early apoptosis was identified as ANN+/PI-, dead cells as ANN+/PI+, and viable cells ANN-/PI-. Cells were seeded in a 96-well plate, using the same method described in performing MTS assays. After treatment, cells were centrifuged and resuspended in 250μL Annexin buffer containing 1μL ANN (150μg/mL, Biovision) and 2.5μL of PI (250μg/mL, Biovision) per well. Each experiment included unstained and single stained

controls to set for compensation. The cells were incubated at room temperature in the dark for 15 minutes before collection and analysis using the flow cytometer.

3.9. SubG1 peak analysis

A cell cycle analysis was performed as previously described[102] to assess the presence of a sub G1 peak, an apoptosis indicator. During normal cell cycle, there are various phases of DNA within the cell. Cells will have an amount of DNA in the G0/G1 phase and double that amount of DNA when in the G2/M phase.[35] When cells are synthesizing their DNA during the S phase, the amount of DNA present is anywhere between these "1X" and "2X" amounts.[35] Anything less than the G0/G1 phase or "sub-G1" indicates that cells are undergoing DNA fragmentation, characteristic of apoptosis.[35] Briefly, TEX cells treated for 48 hours with 10μM diosmetin, were harvested, washed with cold PBS and re-suspended in PBS and cold absolute ethanol. Cells were incubated at 37°C for 30 minutes following treatment of 100ng/mL of DNase-free RNase A (Invitrogen; Carlsbad, CA). The cells were then washed with cold PBS, resuspended in PBS and incubated with 50μg/mL of PI for 15 minutes at room temperature in the dark. DNA content was measured by flow cytometry and analyzed with the Guava Cell Cycle software (Millipore).

3.10. Caspase activation

Caspase-3/7 activation, and thus apoptotic activity induced by diosmetin, was measured using the Apo-ONE Homogeneous Caspase-3/7 (Promega) kit and performed as previously described.[105] This assay involves the non-fluorescent caspase substrate Z-DEVD-R110, which upon cleavage by caspase 3/7 creates the fluorescent rhodamine 110. TEX cells ($1.5x10^5$) were seeded in a 96-well plate and treated with 10μM

diosmetin over a 72-hour time course, as well as 50μM Z-VAD-FMK (Z-VAD; R&D Systems, Minneapolis, MN) and 1μM doxorubicin (Sigma Chemical) as controls. After treatment, 50μL of APO-ONE homogeneous caspase-3/7 reagent was added to each well of a 96-well white walled plate containing 50μL of blank, control or assay treatment. The plate was gently centrifuged at 300 RPM for 30 seconds, and then incubated for 30 minutes at room temperature. Fluorescence was measured using a spectrophotometer at an excitation wavelength of 485nm and an emission wavelength of 530nm.

Caspase-8 substrate (Ac-LETD-AFC; Enzo Life Sciences, Farmingdale, NY) and caspase-9 substrate (Ac-LEHD-AMC; Enzo Life Sciences) were used according to the manufacturer protocol to measure caspase activation. Briefly, TEX cells were treated with diosmetin over a 48-hour time course and lysed. A reaction buffer (substrate stock solution, 1M DTT, 100mM EDTA and 20mM pH 7.4 Tris buffer) was prepared with a final substrate concentration of 50μM. Equal volumes of cell lysate and reaction buffer were mixed in a white walled 96 well plate and incubated for 60 minutes at 37°C. Fluorescent measurements were taken from the wells using the appropriate emission and excitation wavelengths (caspase 8; 404nm and 505nm respectively, caspase 9; 340nm and 440 nm respectively).

An additional study was performed to investigate caspase involvement. TEX cells were co-incubated with increasing concentrations of diosmetin (0-20μM final concentrations) and Z-VAD (50μM final concentration) and cytotoxicity was assessed using a 72-hour MTS assay. We would expect TEX cell death induced by diosmetin to be abrogated by Z-VAD if the mechanism of cell death is indeed apoptosis.

3.11. Reactive oxygen species detection

In response to treatment, intracellular reactive oxygen species (ROS) were detected by staining cells with the fluorescent dye 2',7'-dicholorofluorescein diacetate (DCFH/DA; Sigma Chemical) as previously described.[44] DCFH/DA is able to bypass the phospholipid bilayer, and gain access to the interior of the cell where esterases will hydrolyze its acetate group.[106] The molecule remains inactive until the interaction with peroxides, peroxide radicals and hydroxyl radicals, at which point it fluoresces at 529nm.[106] TEX cells ($5x10^5$) were treated with 10μM or 20μM diosmetin over a 48-hour time course prior to analysis. The treated cells were harvested and resuspended in 5μM DCFH/DA in PBS, incubated at 37°C for 30 minutes, then read and analyzed by flow cytometry.

With the use of antioxidants, we assessed if ROS served an important role in diosmetin induced apoptosis. Using an MTS assay, TEX cells ($1.5x10^5$) were incubated with 10μM diosmetin alone, or co-incubated with either 1mM N-acetylcysteine (NAC) or 1mM α-tocopherol (α-toc) (Sigma Chemical) antioxidants to assess if ROS played a role in diosmetin-mediated cell death. This study was then repeated using an ANN/PI assay. NAC is an artificial antioxidant known to inhibit ROS-induced apoptosis[107]. NAC's anti-ROS activity occurs through scavenging of free radicals via redox potential of thiols or increasing glutathione levels in the cell.[108] α-toc acts as a hydrophobic chain breaking antioxidant that exerts a protective role against free radical damage to unsaturated lipids, and hence membranes and tissues.[109] We would expect that diosmetin's activity would be abrogated by co-incubation with antioxidants, if ROS were functionally important in the cell death mechanism elicited by diosmetin.

3.12. Intracellular cytokine detection (TNFα)

TNFα detection was achieved using a flow cytometry assay, as previously described.[110] TEX and K562 ($5x10^5$) cells were seeded in 96-well plates and treated with 10μM diosmetin or 5mg/mL lipopolysaccarides (LPS) (Sigma Chemical) at time points over 48 hours. The cells were then harvested and fixed using 3% paraformaldehyde (Sigma Chemical) in PBS for 20 minutes at room temperature. The cells were then permeabilized using BD Perm/Wash buffer (BD Biosciences; San Jose, CA), and stained with APC mouse anti-human TNF antibody (BD Biosciences) according to the manufacturer protocols. Briefly, the fixed cells were incubated in 1X BD Perm/Wash buffer for 15 minutes at room temperature. The cells were collected and resuspended in 50μL of BD Perm/Wash buffer containing 2μL of TNF antibody and incubated for 30 minutes in the dark at 4°C. The cells were then washed in PBS, and resuspended in staining buffer (PBS containing 2% FCS and 0.09% sodium azide) to be analyzed via flow cytometry.

3.13. Predicted target using bioinformatics (DAVID)

We collaborated with Dr. Andrew Doxey's lab that specializes in bioinformatics at the University of Waterloo, to gain insight on possible predicted molecular targets of diosmetin's selective cytotoxic actions. Given the structure of diosmetin, the Doxey lab utilized the Protein Data Bank (PDB; a structural database) for protein structures physically bound to a compound similar to that of diosmetin. This search produced a small set of structures to analyze. This list of proteins was expanded, using the Possum Search K software, to related proteins within a similar binding site composition to obtain a list of additional structures, yielding approximately 30 structures in total. To summarize

the functions common to and statistically overrepresented within this group, the list of proteins was analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. DAVID organizes these lists by their functional annotations and assists in recognizing patterns within a set of similar structures.[111] A summary of clusters involving transcription and gene expression was produced to predict structures that are involved in the activity of diosmetin.

3.14. Evaluation of multiple cell lines

Multiple cancer cell lines were evaluated for sensitivity to diosmetin treatment using an MTS assay. Dose-response curves evaluating diosmetin treatment were generated for the following cell lines: TEX, K562, LP1, DU145, HL-60, HeLa, KG1a, U937, AML2 (see cell culture methods for cell line details). The two cell lines displaying the greatest sensitivity to diosmetin treatment, TEX and LP1, and the two cell lines that were most insensitive to diosmetin treatment, DU145 and K562, were further analyzed for ER protein expression via Western blotting.

3.15. Western blotting

3.15.1. Whole Cell Lysate Preparation

Cold RIPA buffer (Sigma Chemical) supplemented with protease inhibitors (Sigma Chemical) was added in a 2:1 ratio to cells collected from each cell line. The cells were lysed by vortexing continually for 20 minutes on ice. The protein was collected by centrifuging the tubes at 13,200 rpm for 20 minutes at 4°C and collecting the supernatant.

3.15.2. Protein Quantification and Sample Preparation

Total protein content was quantified using the BCA protein assay. For each assay, a standard curve was generated using bovine serum albumin (BSA) stock solution (10mg BSA, 10mL distilled water) diluted in distilled water to make increasing concentrations from 0mg/mL-10mg/mL. Each protein sample was diluted 10 fold with distilled water (eg. 10μL protein in 90μL distilled water). On a 96-well plate, 10μL of standards and diluted samples were loaded in triplicate. To each well, 200μL of bicinchoninic acid (BCA) working reagent (50 parts BCA to 1 part copper II sulphate; Sigma Chemical) was added. The plate was incubated at 37°C for 30-60 minutes and the optical density (OD) was read on the SpectraMax M5 spectrophotometer at 527 nm. Samples were then prepared as follows: 30-50μg total protein (calculated from standard curve) with 5μL sample buffer (240mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v sucrose, 0.02% w/v bromophenol blue, and 50mM DTT) and RIPA buffer to a total volume of 30μL. Samples were either stored at -80°C for later used or boiled at 95°C for 5 minutes, then loaded into 12 % polyacrylamide gel wells (25μL per sample per well).

3.15.3. SDS-PAGE and Immunodetection

Proteins were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels containing loaded protein samples were run in a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) with electrophoresis buffer (25mM Tris base, 190mM glycine, 3.5mM sodium dodecyl sulfate) at 150 V for 1 hour. Trans-Blot semi-dry transfer cell was used to transfer the proteins to PVDF membrane (Bio-Rad) in transfer buffer (25mM Tris base, 190mM glycine, 20% v/v methanol). The cell was run at 20 V for 45minutes.

Membranes were then blocked using 5% BSA in Tris-buffered saline (20mM Tris base, 150mM NaCl, pH 7.6) with 0.1% Tween (TBS-T) for 1 hour at 4°C. After discarding the blocking solution, the membranes were incubated with primary antibodies in blocking solution (Table 2) overnight at 4°C. Membranes were washed 4 times in TBS-T, and then incubated with the appropriate secondary antibody in blocking solution for 1 hour at room temperature. Membranes were washed an additional 5 times in TBS-T. Protein detection was achieved by incubating the membranes in Enhanced Chemiluminescence (ECL; GE Healthcare, Baie d'Urfe, QC) for 5 minutes at room temperature, according to the manufacturer's instructions. Luminescence was captured after 5-10 min using the Kodak Image Station 4000MM Pro and analyzed with a Kodak Molecular Imaging Software Version 5.0.1.27. To ensure equal loading, membranes were stained with Ponseau S, as previously described.[112]

Table 2. Antibodies

Antibody	Isotype	Concentration	Secondary Concentration	Molecular Weight	Manufacturer
ERβ	Rabbit	1:1000	1:8000	55 kDa	Santa Cruz
ERα	Rabbit	1:1000	1:8000	60 kDa	Santa Cruz
GAPDH	Rabbit	1:1000	N/A	37 kDa	Thermo Scientific
α-tubulin	Rabbit	1:5000	1:10000	50 kDa	Santa Cruz

3.16. mRNA quantification

3.16.1. RNA Isolation

RNA was isolated from untreated cells (TEX, LP1, DU145, K562 and primary AML patient samples) using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Chemical) according to the manufacturer's protocol. No more than 1 x 10^7 cells were pelleted, resuspended in 250-500μL of lysis buffer, and briefly vortexed. The lysate was

quickly added to a filtration column and centrifuged for 2 minutes at maximum speed (14,000 x g). Equal volume of 70% ethanol was added to the lysate and transferred to a binding column. The column was centrifuged at maximum speed for 15 seconds and the flow through liquid was discarded. The column was washed with Washing Buffer 1, followed by two washes with Washing Buffer 2. The column was centrifuged at maximum speed for 15 seconds each time, decanting the flow through liquid between washes. The binding column was centrifuged an additional time at maximum speed for 2 minutes to ensure filter drying. 50µL of elution buffer was added to the column and centrifuged for 1 minute to elute the RNA. The total RNA was quantified and checked for purity using the NanoDrop 2000c spectrophotometer (Thermo Scientific). The RNA was then either used immediately for cDNA synthesis or store temporarily at -20°C for later use.

3.16.2. cDNA synthesis

cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham MA) and performed according to the manufacturer's protocol. The following reagents were added to a sterile, nuclease-free tube on ice (1 tube per RNA sample): 1µL of oligo dT primer (0.5µg/µL), RNA extract amounting to 2.5µg, and nuclease free water up to 12µL. The tubes were then incubated at 65° C for 5 minutes in the Genius PCR thermal cycler (Techne, Staffordshire, UK). The tubes were retrieved from the thermal cycler and chilled on ice for 15 seconds before the following components were added to each sample: 4µL of 5X Reaction Buffer, 1µL of RiboLock RNase inhibitor (20U/µL), 2µL of dNTP Mix (10mM) and 1µL of RevertAid M-MuLV RT (200U/µL). The tubes were returned to the thermal cycler and incubated for 60

minutes at 42°C, then 70°C for 5 minutes. The cDNA was either used immediately or stored at -20°C short term.

3.17. Real Time Quantitative PCR

Real-time quantitative polymerase chain reaction (qPCR) was performed as previously described[87] in triplicate using an ABI 7900 Sequence Detection System (Applied Biosystems). For each reaction, 25ng RNA equivalent cDNA, 12.5μL of SYBR Green PCR Master mix (Thermo Scientific), 0.75μL (300nM) of ERα- and β-specific primers (Table 3), and nuclease free water up to 25μL were added to the wells of a PCR microplate (Thermo Scientific). GAPDH primers, supplied by the Thermo Scientific cDNA Synthesis Kit were used as a loading control. Relative mRNA expression was determined by using the ΔΔCT method as previously described[102], normalizing to the GAPDH controls, and efficiency was calculated using LinRegPCR analysis software.[113,114]

Table 3. PCR Primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ERα (ESR1)	TGCTCAATTCCAGTATGTACC-	ATGAGGTGAGTGTTTGAGAG
ERβ (ESR2)	CATTATGGAGTCTGGTCCTG	TTCGTATCCCACCTTTCATC

3.18. Estrogen receptor reporter assay

A Human Estrogen Receptors Reporter Assay Panel (Indigo Biosciences, State College, PA) was performed according to manufacturers protocol. The Panel provided the necessary materials to investigate ERβ and ERα activation and inhibition by a compound of interest. Reporter cells were provided, which are non-human mammalian cells engineered to express ERβ or ERα separately. The cells also include the luciferase reporter gene functionally linked to a responsive promoter. Thus, quantifying changes in

luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in ER activities. Luciferase gene expression occurs after ligand-bound ER undergoes nuclear translocation, DNA binding, recruitment and assembly of the co-activators, and ultimately expression of the target gene.

The ER reporter cells were thawed and resuspended in Cell Recovery Medium (CRM). Diosmetin was diluted to 2x the desired final concentrations in Compound Screening Media (CSM) supplied in the kit. Briefly, to evaluate diosmetin's capacity for ER activation (agonist assay), 100µL of ER reporter cell suspension was seeded in white walled 96-well plates, and immediately dosed with 100µL of increasing concentrations of diosmetin. Known ER agonist, 17-β-estradiol, was used as a positive control at the submaximal concentration, provided by the manufacturer, specific to the reporter cells (ERβ; EC_{75}=150pM, ERα; EC_{80}=1500pM). To investigate diosmetin's ER inhibition potential (antagonist assay), the same protocol as previously described was applied, however, 17-β-estradiol was added directly to the cell suspension before being plated at the $EC_{75\text{-}80}$ final concentrations. Following a 22-24 hour incubation period, the media was discarded from the plates and 100µL of luciferase detection reagent was added to the wells. The plates were incubated at room temperature in the dark for 20 minutes. Light emission was quantified by the SpectraMax M5 plate-reading luminometer every 7 minutes for a total of 35 minutes. The luminescence was then normalized to the control (untreated TEX cells).

3.19. Statistical analysis

Unless otherwise stated, the results are presented as mean ± SD. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, USA). $p \leq 0.05$ was accepted as being statistically significant.

3.20. Additional studies

3.20.1. Flavonoid Screen

From the 288 compound nutraceutical library, 54 flavonoids (Chendu Biosciences) were selected for additional assessment. We were interested in evaluating flavonoids, as a group, for selective toxicity in LSC. Using an MTS viability screen, these flavonoids were re-screened for cytotoxicity in TEX and K562 cell lines, similar to the original screen. Six flavonoids were identified that selectively induce cell death in TEX cells. The six "hits" were further validated using the ANN/PI assay via flow cytometry, and found that four flavonoids; genistein, luteloin, apigenin, and kaempferol, elicit dose-dependent TEX cytotoxicity, with little effect on K562 (control) cells.

3.20.2. Xenograft mouse models

TEX leukemia cells (2.5×10^6) were injected subcutaneously into the left flank of NOD/SCID (n = 7, Jackson Laboratory, Bar Harbor, ME). The mice were selected at random to receive either diosmetin (n = 4) (30 mg/kg/every other day; in 0.9% NaCl and 0.01% tween-80) or vehicle control (n = 3) (0.9% NaCl and 0.01% tween-80) intraperitoneally after palpable tumours were formed. Measurements of tumor volumes (tumor length × width^2 × 0.5236) were taken every other day using a caliper. Mice were sacrificed by asphyxiation by carbon dioxide at day 30, when tumour burden was apparent. At this time, tumours were excised, weighed and recorded. All animal studies

were carried out according to the regulations of the Canadian Council on Animal Care and with the approval of the University of Waterloo, Animal Care Committee.

3.20.3. Combination studies

Drug interactions between diosmetin and the common chemotherapies, cytarabine (Ara-C), daunarubicin (Da) (Sigma Chemical) were assessed using an MTS viability assay and analyzed using the Calcusyn (software) median effect model to determine combination index (CI) values. CI values of <1 indicate synergism, >1 indicate antagonism, and CI=1 indicates no interaction.[115,116] The MTS assay included a dose-response of diosmetin and each chemotherapy separately and together, at 0, 0.5x, 0.75x, 1x and 2x the IC_{50} for each drug.

3.20.4. Effect of tamoxifen on TEX cell viability

Tamoxifen is a SERM widely known for it's ER antagonist activity through ERα binding activity in breast tissue.[117] Some breast cancers use estrogen as a means of cell proliferation, by binding to the ER and eliciting this response.[78] Tamoxifen is used as a treatment option for ER positive breast cancers in pre-menopausal women.[78] In breast tissue, tamoxifen is metabolized into compounds that bind the ERα, but does not activate it, therefore blocking the proliferative action of estrogen.[117] However, tamoxifen has been shown to have agonist activities as well, in tissues such as the endometrium and bone[118], thus eliciting cell- and tissue-specific responses. To evaluate tamoxifen's potential activity in LSC, we treated TEX cells with increasing concentrations of tamoxifen (0-100μM, Sigma Chemical) over 72-hours and assessed cell viability using the MTS assay. In addition, we co-incubated these increasing concentrations of tamoxifen with 10μM diosmetin in a 72-hour MTS assay.

CHAPTER 4: RESULTS

4.1. A nutraceutical screen for novel anti-AML compounds identifies diosmetin

To identify nutraceuticals with anti-AML activity we compiled a unique library consisting of 288 nutraceuticals from commercially available sources. The library was screened against the surrogate LSC cell line TEX and a control cell line, K562; which is a CML cell line that does not possess LSC properties, using the MTS assay following a 72-hour incubation period (Figure 6A&B). Twelve lead compounds from this screen, which imparted the greatest reduction in TEX cell viability without affecting K562 cell viability, were validated in secondary screens (Figure 6C&D). It was found that diosmetin, a flavonoid largely found in citrus fruits[96] (Figure 6E), was the most potent compound, inducing cytotoxicity at relatively low doses. Diosmetin's activity was further validated by generating a dose response curve using the Annexin/PI assay following 72 hours of incubation (IC50: 6.1 ± 0.5μM) and by time course analysis (Figure 6F&G).

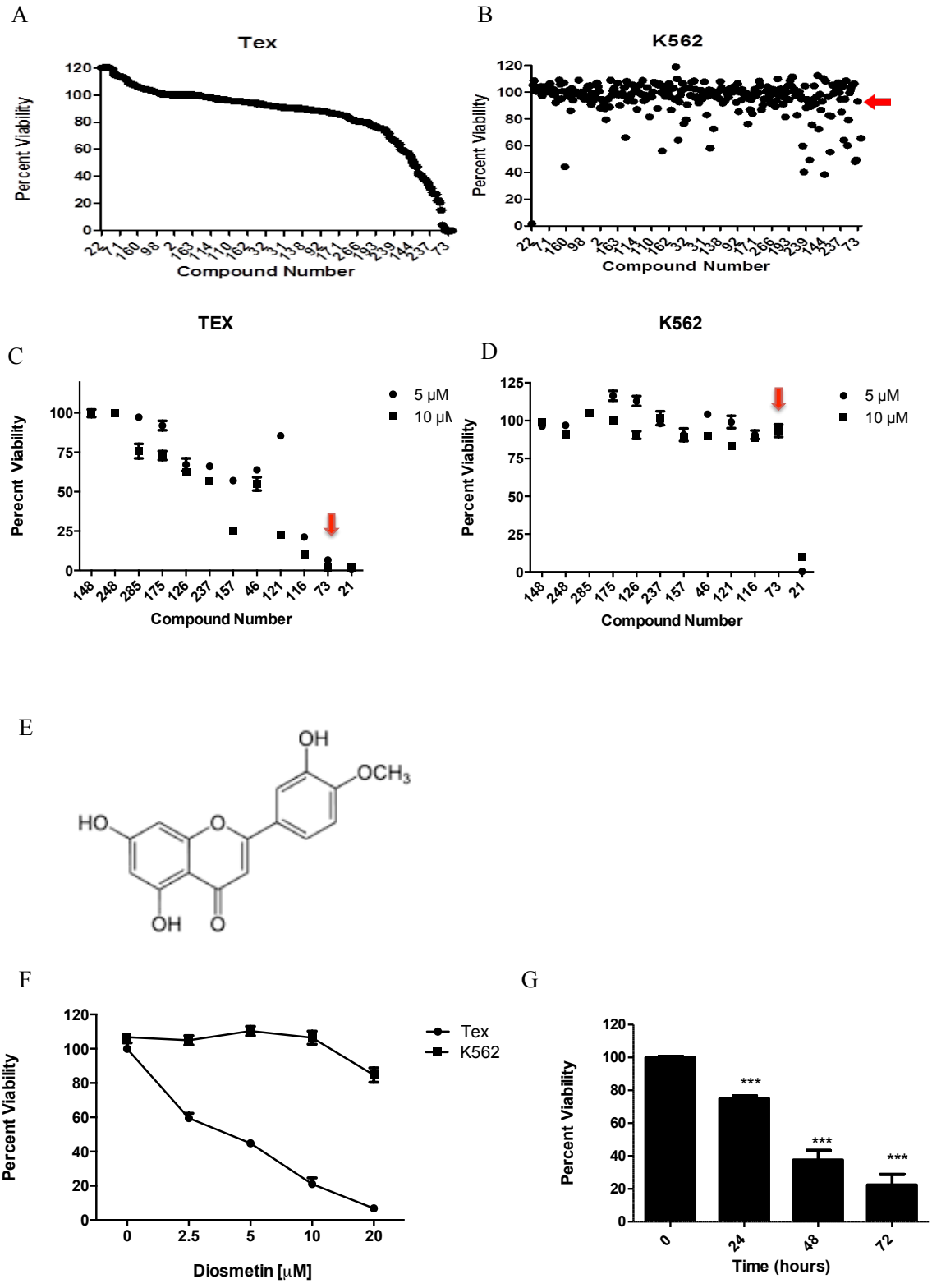

A
Tex
Percent Viability
Compound Number
B
K562
Percent Viability
Compound Number
TEX
C
Perecnt Viability
5 μM
10 μM
Compound Number
K562
D
Percent Viability
5 μM
10 μM
Compound Number
E
OH
OCH3
HO
O
OH
O
F
Percent Viability
Tex
K562
Diosmetin [μM]
G
Percent Viability
***
***
***
Time (hours)

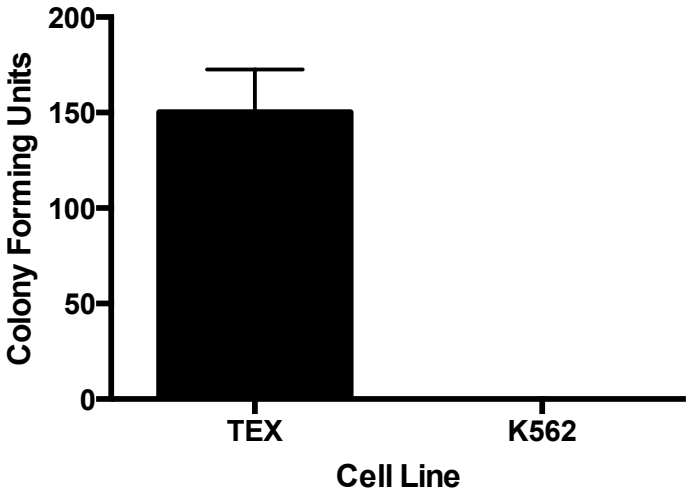
Figure 6. Diosmetin (Dios) was identified as a novel anti-LSC agent. (A) A MTS screen of 288 nutraceutical compounds, determined that dios was the lead "hit", reducing the viability of a surrogate LSC cell line, TEX with the highest potency, (B) but does not reduce the viability of the control line, K562, a chronic myeloid leukemia cell line. (C) Displayed are the top 12 cytotoxic compounds validated from the initial screen in Tex and (D) K562. (E) The structure of diosmetin, a flavonoid found in citrus fruits, among many other sources. (F) Dios reduces TEX cell viability in a dose and (G) time dependent manner. ***$p<0.001$. All experiments, with exception of the initial screen, were performed three times in triplicate, representative figures are shown.

4.2. Cell surface marker analysis

To ensure that our cell line TEX is representative of an LSC population, and that the control line is not, our lab performed cell surface marker analysis. Stem cell markers CD34+/38- and myeloid markers CD33+/19- were screened using fluorescently tagged antibodies. Flow cytometry was used to quantify the proportion of these stained stem cell and myeloid markers. We determined that our Tex cell line contains a high proportion of both myeloid and leukemia stem cells (77 and 60% respectively), and K562 has little to none (Table 4). These markers were tested periodically in TEX cells to assure standardization across experiments. We also assessed our cell lines for potential progenitor properties using colony formation assays. While TEX cells produced colonies in these assays, no colony growth was observed from K562 cells, further indicating TEX cells contain characteristics of LSCs while K562 cells do not.

Table 4. Assessment of cell surface receptors validate the use of TEX as a surrogate stem cell population. Myeloid surface markers CD33+/CD19- and stem cell surface markers CD34+/CD38- were assessed in the TEX and K562 cell lines using fluorescent antibodies via flow cytometry. It was revealed that our chosen cell line of study, TEX, does in fact contain a high proportion of myeloid and stem cells. Colony formation assays assessing progenitor content in TEX and K562 cells revealed that TEX cells produce colonies, while K562 do not have this capability. This indicates that K562 cells are not representative of an LSC cell line, while TEX cells display characteristics of LSCs such as colony formation.

Cell line	% Cells	
	CD33+/CD19- (myeloid)	CD34+/CD38- (LSC)
TEX	77.36±0.18	59.77±0.038
K562	0.46±0.12	0.16±0.038



4.3. Diosmetin reduces clonogenic formation in primary AML patient samples

Given the cytotoxicity of diosmetin in our surrogate LSC cell line, we tested its activity in a functionally defined subset of primitive leukemia cells using the colony formation assay. Here, diosmetin (10μM-20μM final concentration) was added to semi-solid culture media of primary AML patient samples or normal hematopoietic stem cells and colonies were counted after 7-14 days of incubation. Diosmetin reduced clonogenic growth of primary AML patient samples (n = 3; Figure 7A). However, when incubated with normal, CD34+ bone marrow cells (n = 3; Figure 7B), diosmetin had no effect on colony formation. Together, these results demonstrate diosmetin's selective toxicity toward AML progenitor cells.

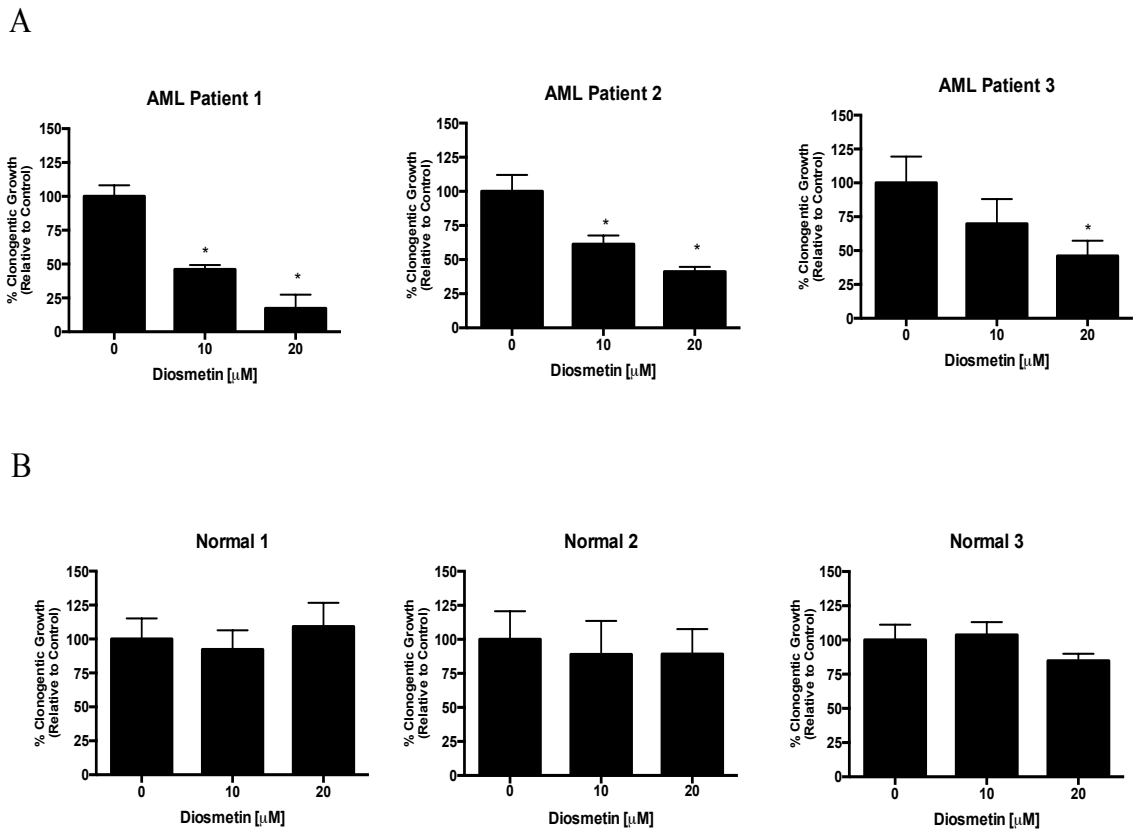


Figure 7. Diosmetin reduces colony formation in primary AML patient samples. Primary AML patient samples and CD34+ normal hematopoietic stem cells were incubated with 10 and 20μM dios in semi-solid media and assessed for colony formation. (A) Dios reduces colony formation in primary patient samples (n=3). (B) but had no effect in CD34+ normal hematopoietic cells (n=4). *p<0.05. All experiments were performed in duplicate. Due to limited number of cells available in each patient sample, graphs shown represent a single experiment performed in duplicate.

4.4. Diosmetin induced death is independent of reactive oxygen species

Diosmetin is a member of the flavonoid family, which can induce death through the generation of reactive oxygen species (ROS).[119–121] Initially, we assessed if diosmetin treatment in TEX cells would lead to an increase in ROS. TEX cells were treated with 10μM diosmetin over 48 hours, stained with DCFH/DA and analyzed via flow cytometry to assess diosmetin's ability to activate ROS. A significant increase is observed starting at the 12-hour time point, with the highest increase observed at 24 hours (Figure 8A). Thus,

we assessed whether death was ROS-dependent by co-incubating diosmetin with the anti-oxidants N-acetylcysteine (NAC) or α-tocopherol (αtoc). Cell death was not affected by co-incubations with NAC or α-toc, indicating that unlike the typical activity of flavonoids, diosmetin-induced death is independent of ROS (Figure 8B).

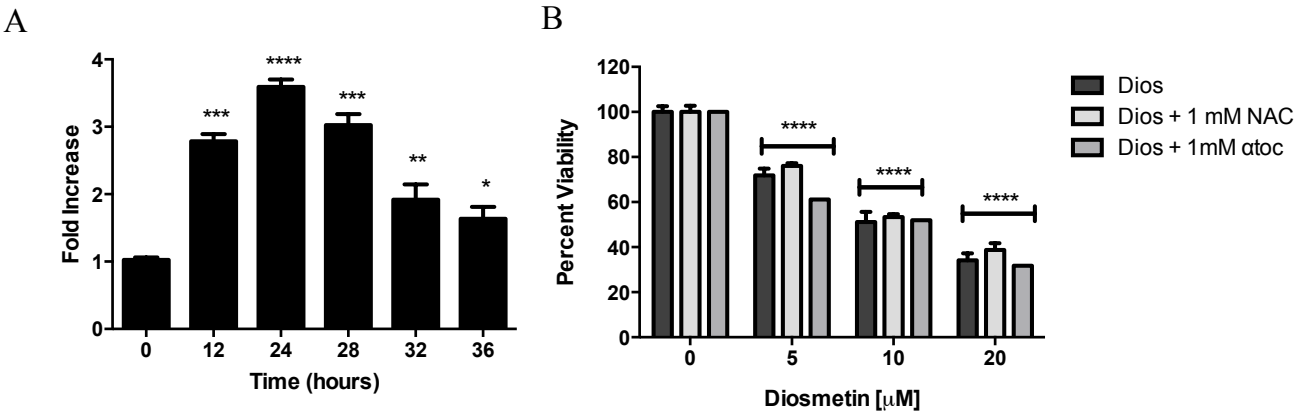


Figure 8. Diosmetin treatment increases ROS levels in TEX cells. (A) ROS were measured in TEX cells treated with 10μM diosmetin for increasing time by DCFH-DA by flow cytometry. A 2-3.5 fold increase was observed between 12-14 hours of treatment. (B) TEX cells were treated with 10μM diosmetin in the presence or absence of the anti-oxidants, N-acetyl cysteine (NAC) or α-tocopherol (αtoc). Viability was measured by the ANN/PI assay. *p<0.05, **p<0.01, ***p<0.001. Statistics were calculated relative to the untreated controls. All experiments were performed three times in triplicate, and representative figures are shown.

4.5. Diosmetin induces extrinsic apoptosis mediated by TNFα

We next assessed the mode of diosmetin-induced leukemia cell death. Early apoptosis (i.e., ANN^+/PI^-) was detected by flow cytometry in diosmetin treated TEX cell (Figure 9A). Apoptosis was confirmed as diosmetin treated TEX cells demonstrated a subG1 peak increase, which is an indirect measure of DNA fragmentation - a hallmark of apoptosis (Figure 9B). To determine if caspase enzymes were involved in diosmetin-induced cell death, we performed a caspase 3/7 assay, as previously described.[105] Significant caspase-3/7 activation in TEX cells was observed 24-48 hours after diosmetin

treatment (Figure 9C). In addition, we blocked caspase activity using the pan-caspase inhibitor Z-VAD (50μM final concentration), that was co-incubated with increasing concentrations of diosmetin in TEX cells. Z-VAD partially attenuated diosmetin's activity, further confirming caspase-3/7 enzyme involvement in diosmetin mediated apoptosis (Figure 9D). To further elucidate on the mechanism of cell death, we measured caspase 8 and 9 activity, which are upstream enzymes that activate caspases 3 and 7. Interestingly, at a time that precedes caspase 3/7 activation, caspase 8 but not caspase 9 was increased following diosmetin treatment (Figure 9E&F). Induction of caspase-8 is consistent with the extrinsic (death receptor) pathway of apoptosis.[122]

The death receptor pathway is activated following ligation of cell surface receptors. The tumour necrosis factor (TNF) receptor superfamily are an example of a death receptor and ligation of these receptors begins a multi-protein cascade starting with caspase-8 activation.[123] For this reason, we next investigated TNFα levels in diosmetin treated TEX and K562 cells by flow cytometry. Here, we observed a 10-fold increase in TNFα production following 12 hours of 10μM diosmetin treatment in TEX cells (Figure 4G). In contrast, there was no change in fluorescence following diosmetin treatment in K562 cells (Figure 9H) Lipopolysaccharide (LPS) was used as a positive control, as it known to potently activate TNF-α (Figure 9I).[124,125] Together, these results demonstrate that diosmetin increases levels of TNF-α and induces caspase-8 and -3 mediated apoptosis.

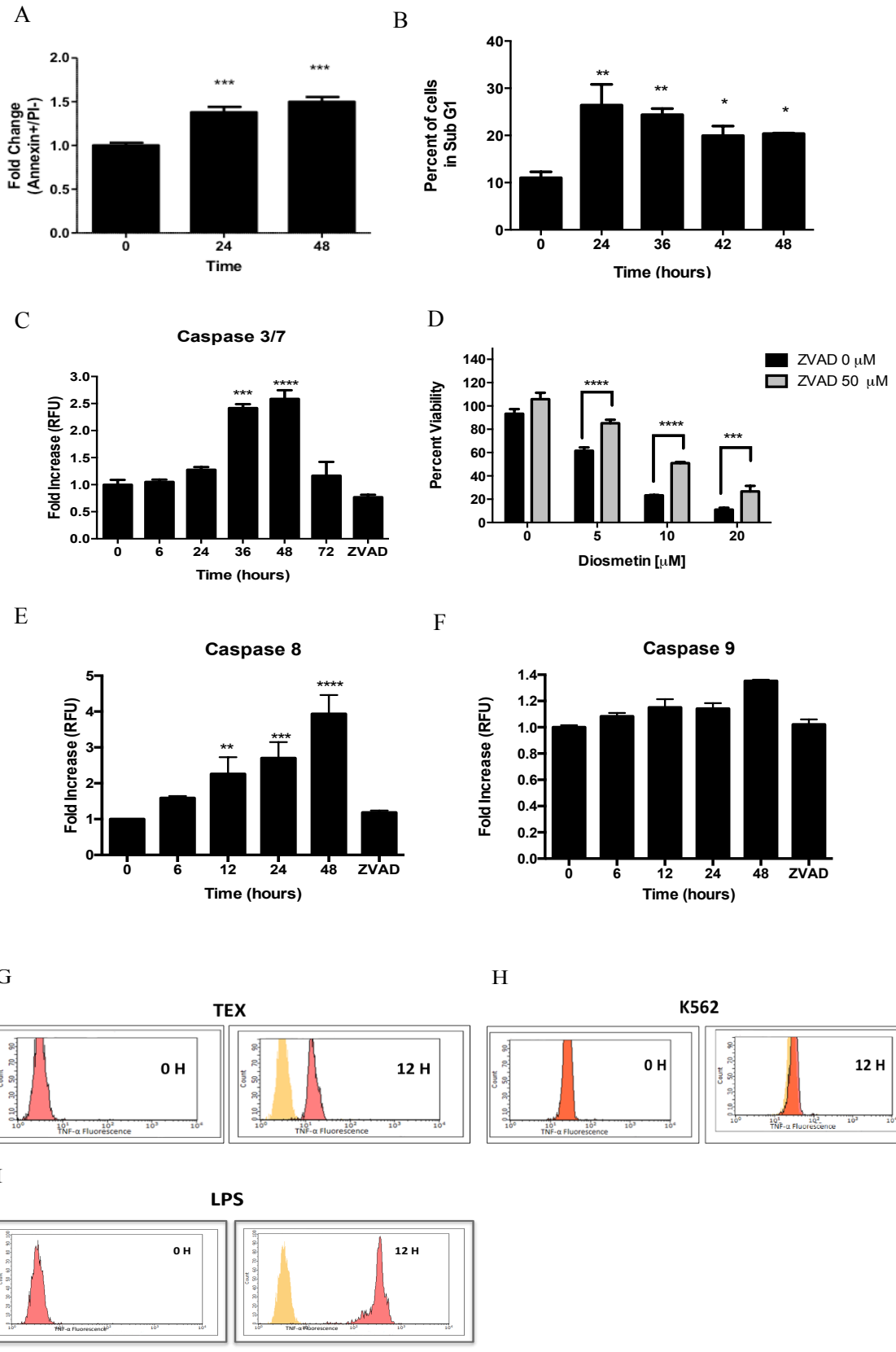

A
Fold Change (Annexin+/PI-)
2.0
1.5
1.0
0.5
0.0
***
***
0
24
48
Time
B
Percent of cells in Sub G1
40
30
20
10
0
**
**
*
*
0
24
36
42
48
Time (hours)
C
Caspase 3/7
Fold Increase (RFU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
****
0
6
24
36
48
72
ZVAD
Time (hours)
D
Percent Viability
140
120
100
80
60
40
20
0
ZVAD 0 μM
ZVAD 50 μM
****
****
***
0
5
10
20
Diosmetin [μM]
E
Caspase 8
Fold Increase (RFU)
5
4
3
2
1
0
**
***
****
0
6
12
24
48
ZVAD
Time (hours)
F
Caspase 9
Fold Increase (RFU)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
24
48
ZVAD
Time (hours)
G
TEX
0 H
12 H
TNF-α Fluorescence
H
K562
0 H
12 H
TNF-α Fluorescence
I
LPS
0 H
12 H

Figure 9. Diosmetin activates the extrinsic pathway of apoptosis and is mediated by TNFα. (A) Diosmetin induces apoptosis in TEX cells (% Annexin+/PI-) as measured by flow cytometry using the Annexin/PI assay. (B) In addition, the presence of a subG1 peak following diosmetin treatment is observed, characteristic of apoptosis. (C) Diosmetin significantly activated caspase 3/7, (D) which was blocked when co-treated with pan-caspase inhibitor, Z-VAD, indicating caspase activity is important in diosmetin mediated cell death. Doxorubicin produced an approximately 2.5 fold change after 12 hours. To elucidate the apoptotic pathway it was observed that (E) diosmetin increases activation of caspase 8, (F) but not caspase 9. Intracellular cytokine staining measured by flow cytometry demonstrated that (G) TEX cells treated for 12 hours with 10μM diosmetin increased TNFα production. (H) No increase was shown in K562 cells. (I) LPS was used as a positive control for TNFα production. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. All experiments were performed three times in triplicate and representative figures are shown.

4.6. Estrogen receptors are predicted molecular targets

Out of 12 hits in our primary and secondary screen, 7 were compounds similar to diosmetin's structure. Thus, to determine the mechanism of diosmetin's selective toxicity, we utilized the DAVID tool, which provides a comprehensive set of annotation tools for investigators to use to understand biological meaning behind lists of genes. The Protein Data Bank (PDB) was searched for protein structures that were bound to diosmetin. To expand this list, the PDB was searched for proteins that appear to have similar binding sites using the Possum software. Table 5A displays known protein types that diosmetin, and similar structures bind to. Then, using DAVID, these proteins were analyzed to find functional clusters involving gene transcription and expression that are common among the proteins, which are displayed in Table 5B. From these tables it was predicted that nuclear hormone receptors (ERα and ERβ) are involved in diosmetin activity.

Table 5. Estrogen receptors are a potential target. (A) Using the protein structural database, diosmetin was predicted to bind to estrogen receptors alpha and beta (ERα, ERβ). (B) Using the DAVID tool, functional clusters common among diosmetin binding was defined.

A

Species	Gene Name
Homo sapiens	**Estrogen receptor 1 (ER Alpha)**
Homo sapiens	**Estrogen receptor 2 (ER beta)**
Homo sapiens	Peroxisome proliferator-activated receptor gamma
Homo sapiens	TRF1-interacting ankyrin-related ADP-ribose polymerase 2
Homo sapiens	Transthyretin

B

Annotation Cluster	Count	P Value
Nuclear hormone receptor, ligand-binding, core	12	1.4E-18
Nuclear hormone receptor, ligand-binding	12	1.4E-18
Steroid hormone receptor activity	12	2.2E-17
Ligand-dependent nuclear receptor activity	12	1.7E-16
HOLI	12	3.0E-3
Transcription factor activity	12	1.0E-3

4.7. Cell line sensitivity is linked to ERβ

A panel of cell lines was assessed for their sensitivity to diosmetin treatment using the MTS assay following 72-hour incubation (Figure 10A). The cell lines TEX and LP1 displayed the highest level of sensitivity, while the cell lines K562 and DU145 were insensitive to diosmetin (Figure 10A; Left). We next questioned whether expression of ERβ and ERα would reveal a pattern consistent with diosmetin sensitivity observed in these cell lines. Western blotting of ERβ and ERα protein levels in untreated TEX and LP1 showed increased ERβ protein expression compared with untreated K562 and DU145 (Figure 10B). No such pattern was observed for ERα protein expression in the cell lines (Figure 10C). Similar patterns were observed for mRNA expression.

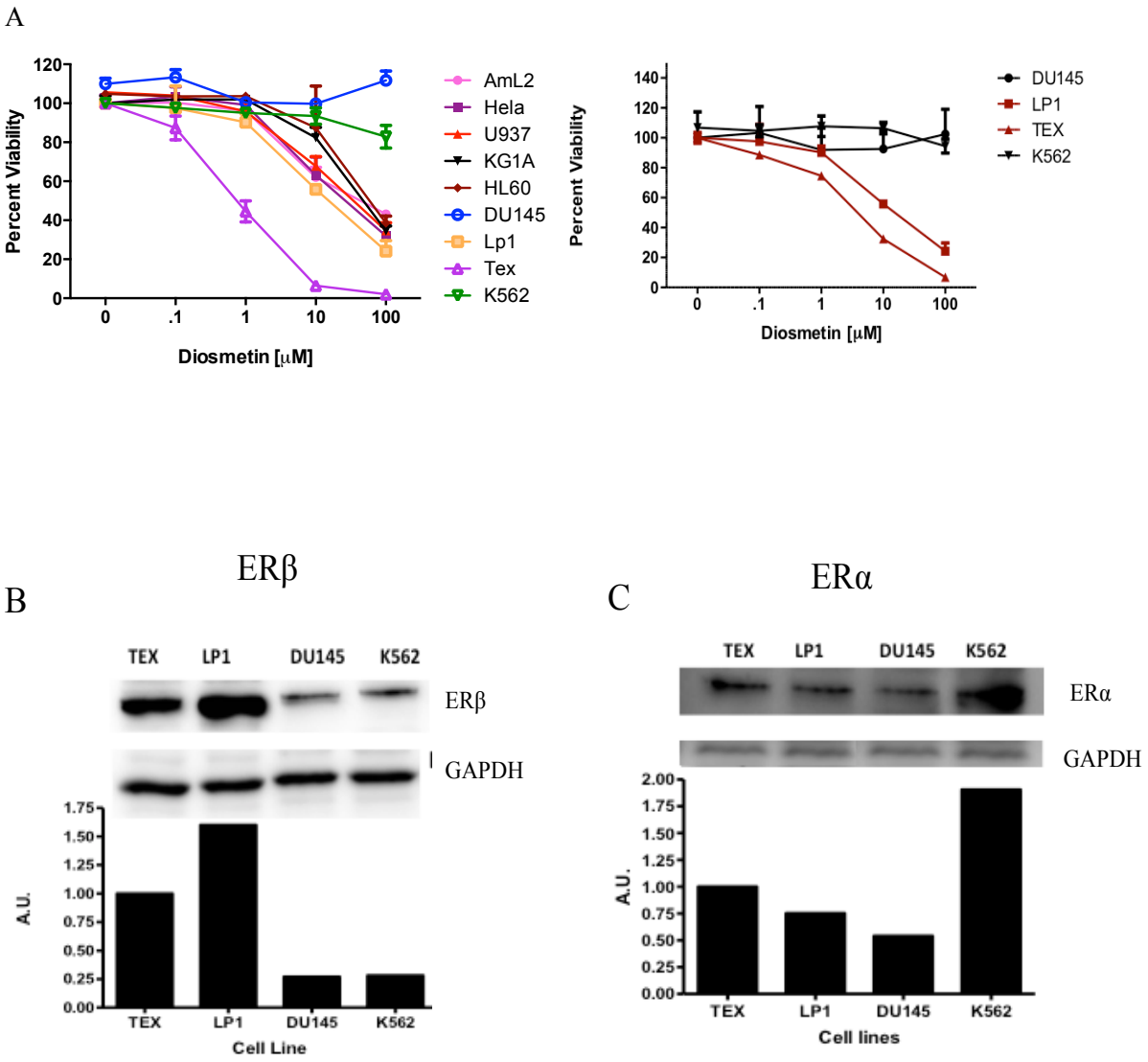


Figure 10. ERβ as the receptor of interest. (A) Several cell lines were tested for sensitivity to diosmetin using the MTS viability assay. Shown more clearly in (A; right) TEX and LP1 displayed the greatest sensitivity to diosmetin, while K562 and DU145 were insensitive. (B) Western blotting showed that diosmetin sensitive cell lines have elevated ERβ compared diosmetin insensitive cell lines. (C) This pattern was not observed for ERα protein expression in these cell lines. All experiments were performed three times in triplicate.

4.8. Increased ERβ mRNA expression in diosmetin sensitive cells

TEX and K562 cell lines were assessed for their relative *ESR1* and *ESR2* gene expression through the use of qRT-PCR. TEX cells expressed levels of *ESR2*

(ERβ) mRNA, with undetectable amounts of *ESR1* (ERα) mRNA expression (Figure 11A). Conversely, K562 cells expressed small amounts of *ESR1* (ERα) mRNA, and undetectable amounts of *ESR2* (ERβ) mRNA (Figure 11 A). The patterns observed through ERβ and ERα mRNA expression of TEX and K562 cells are consistent with the patterns observed in ERβ and ERα protein expression of these cells lines.

The primary AML patient samples (n = 3) that had previously demonstrated reduced clonogenic formation following diosmetin treatment were also assessed for their relative *ESR1* and *ESR2* mRNA levels using qRT-PCR. All three primary samples showed expression of *ESR2* mRNA levels and undetectable amounts *ESR1* mRNA (Figure 11B). Together, with the observed protein expression patterns, this suggests that diosmetin targets ERβ.

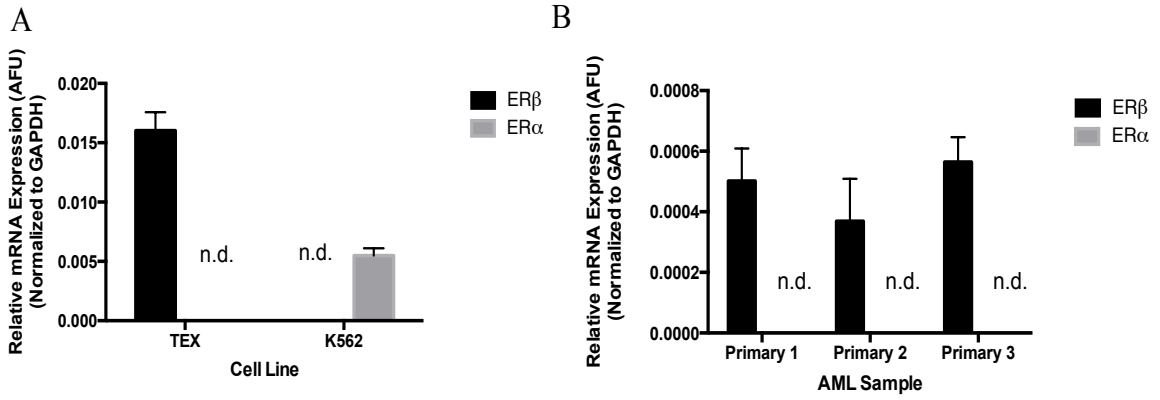


Figure 11. TEX cells express ERβ mRNA. Expression of *ESR1* (ERα) and *ESR2* (ERβ) mRNA levels in TEX and K562 cells, and Primary AML patient samples that with previously observed diosmetin sensitivity was assessed using quantitative PCR. *ESR1* and *ESR2* mRNA expression was normalized and reported relative to the GAPDH control. (A) TEX cells displayed expression of *ESR2* (ERβ) mRNA levels, but undetectable levels of *ESR1* (ERα) mRNA. Opposite results were observed in K562 cells, as small amounts of *ESR1* mRNA were present, with undeletable expression of *ESR2*. (B) In all three primary cells assessed, *ESR2* mRNA expression was present, with undetectable amount of *ESR1* observed. Cell line experiments were performed three times, in triplicate.

4.9. Diosmetin acts as an ERβ agonist

To determine if diosmetin targets and binds to ERβ we performed ER agonist and antagonist luciferase reporter assays. In the antagonist assay, reporter cells were co-incubated with the classical ligand, 17β-Estradiol (E2; optimized EC75 concentration) and increasing concentrations of diosmetin. At concentrations, which impart toxicity (8-10μM), diosmetin had no effect as an antagonist, as luminescence was unaffected (Figure 12A). In the agonist assay, where reporter cells were treated solely with diosmetin; agonist activity was observed at concentrations at or below the EC_{50}, as luminescence increased with each dose of diosmetin (Figure 12A). Antagonist activity was also observed in ERα cells at diosmetin concentrations above 25μM, and some agonist activity was observed for lower concentrations (3-6μM). It was observed that the agonist luciferase activity induced by diosmetin in ERα reporter cells were much lower than that induced by E2. Furthermore, diosmetin also induced greater luciferase activity in ERβ reporter cells than in ERα reporter cells, suggesting that diosmetin has a greater affinity for ERβ over ERα, and E2 binds with a higher affinity for ERα than diosmetin (Figure 12B). Together, this demonstrates that diosmetin acts best as an ERβ agonist.

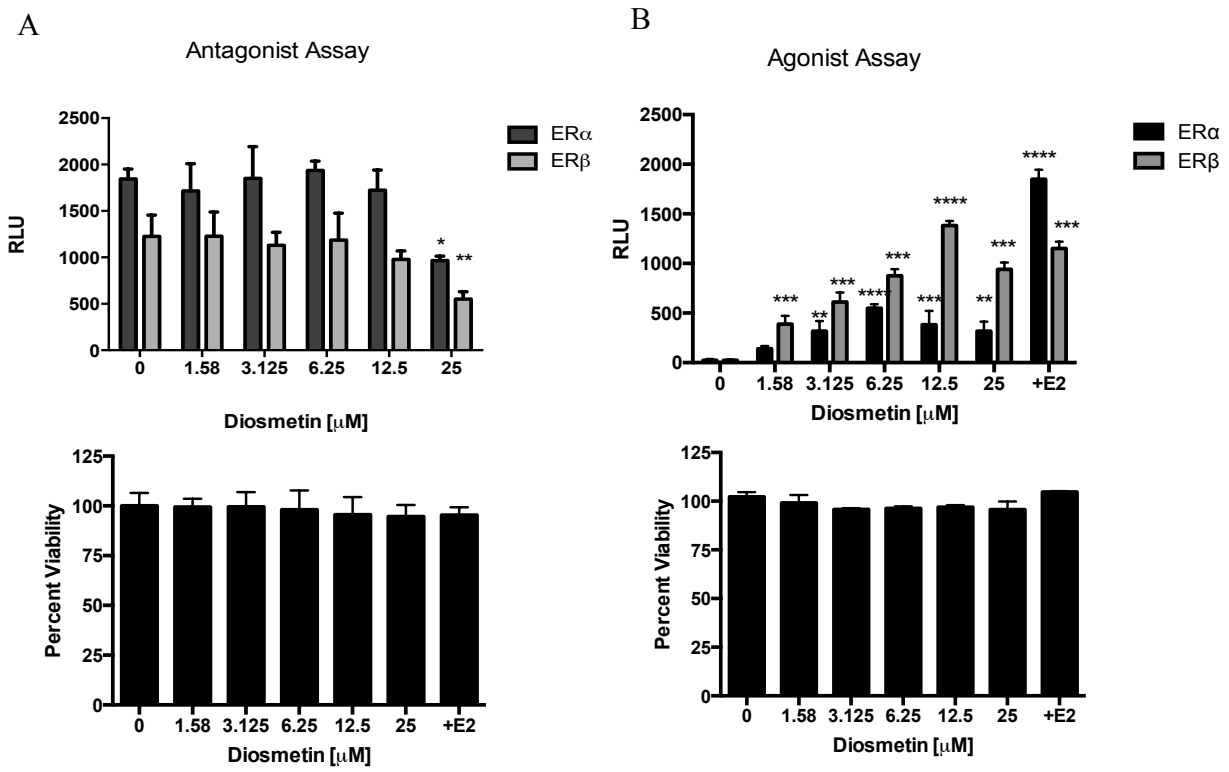


Figure 12. Diosmetin acts as a ERβ agonist. (A) Diosmetin acts as an antagonist in ERβ and ERα reporter cells at concentrations >25 μM as measured in the ER antagonist luciferase reporter assay. (B) Diosmetin acts as an agonist in both ERβ reporter cells at concentrations <25 μM, as measured by the ER agonist luciferase reporter assay, However the luciferase activity produced in ERα reporter cells was much lower than that produced in ERβ reporter cells. Below each graph shows the corresponding MTS viability results, performed parallel to the reporter assays, showing that reporter cells were viable after 24 hours of treatment. Statistics were calculated in comparison to respective untreated controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All experiments were performed three times in triplicate and representative graphs are shown.

4.10. Additional studies

4.10.1. Xenograph animal model

We also performed small scale mouse xenographs. Given diosmetin's cytotoxic activity *in vitro*, we decided to assess diosmetin's capcity to reduce tumour weight *in vivo*. A small number of NOD/SCID mice were injected with TEX cells subcutaneously in the left flank, then treated intraperitoneally with either diosmetin (n = 4) or vehicle

control (n = 3) every other day for 30 days. Following treatment, the mice were sacrificed, and tumours were excised and weighed. The data showed a reduction in tumour weight in diosmetin treated mice compared to the controls (Figure 13A). Although these results seem promising, tumour weight reduction was insignificant. Additional animal studies, which include a greater number of mice, are needed to further assess diosmetin's *in vivo* efficacy. In addition, mouse body weight was measured continuously throughout the experiment and remained unchanged for both diosmetin and vehicle treated mice (Figure 13B); however a more robust measure of toxicity, such as blood bioanalysis is required to strengthen *in vivo* safety claims. This lends evidence that diosmetin may not impart toxicity while inducing anti-cancer activity.

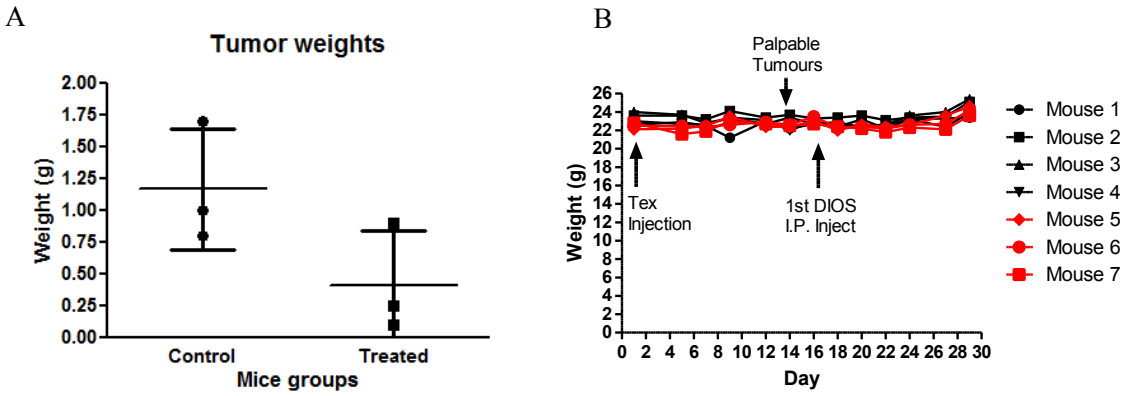


Figure 13. Diosmetin shows some efficacy in reducing tumor weight in LSC mouse xenografts. NOD/SCID mice were subcutaneously injected with TEX cells (n= 4 dios treated, n = 3 control) and following implantation were treated intraperitoneally with 30 mg/kg dios or a PBS control every other day for 18 days. Tumor volume and body weights were measured every other day. At the termination of the experiment, mice were sacrificed, blood was collected and tumors were excised and weighed. (A) Dios showed a reduction in tumor weights, however not significant. (B) Dios treated mice had no changes in body weight compared to control treated animals, suggesting little toxicity was imparted**.**

4.10.2. Effects of tamoxifen in TEX cells

Tamoxifen is a well-known SERM that is used most commonly for the treatment of ER positive breast cancers.[126] Although it is best characterized by its antagonist activity in breast tissue mediated through ERα, tamoxifen has agonist activity in other tissues such as the endometrium and bone, and has shown some affinity for ERβ.[127] With this knowledge, we were interested to know if tamoxifen would induce a response in TEX cells. When TEX cells were co-incubated with increasing concentrations of tamoxifen, no change in viability was observed using the MTS assay (Figure 13). This was perhaps an expected outcome, as tamoxifen acts primarily as an ERα antagonist with known affinity for ERα. Interestingly however, when increasing concentrations of tamoxifen were co-incubated with diosmetin at 10μM, tamoxifen potentiated a further decrease in cell viability than with diosmetin alone (Figure 13). Thus, tamoxifen sensitized TEX cells to diosmetin-induced cytotoxicity.

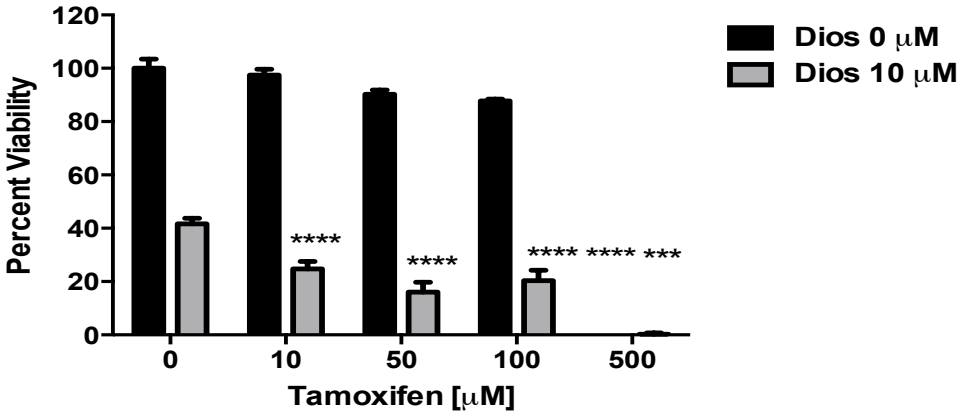


Figure 14. TEX cells respond to ER ligands. (A) When TEX cells are incubated with well-known ERα, tamoxifen, at increasing concentrations no decrease in viability is observed. Interestingly, when 10μM diosmetin is co-incubated with increasing concentrations of tamoxifen, a reduction in viability is seen in comparison to diosmetin treatment alone. ***p<0.001, ****p<0.0001. All experiments were performed three times in triplicate and representative graphs are shown.

4.10.3. Additional flavonoid screen

To assess flavonoids, as a group, for their selective cytotoxicity towards LSC, 54 flavonoids from our nutraceutical library were selected for an additional MTS viability screen. Similar to our initial screen, the flavonoids were assessed for their cytotoxicity in both TEX (surrogate LSC) and K562 (control) cells (results not shown. Six compounds were identified that selectively killed TEX cells. These six "hits" were further validated using the ANN/PI flow cytometry assay, and found flavonoids genistein, luteloin, apigenin, and kaempferol elicit dose-dependent TEX cell cytotoxicity, with little effect on K562 cells. The EC_{50} concentrations in TEX cells of genistein, luteolin, and apigenin are approximately 7μM, and kaempferol is approximately 10μM (Figure 15A&B). Interestingly, there have been numerous reports showing that both genistein and apigenin to have strong affinity for ERβ and agonist activity through this receptor.

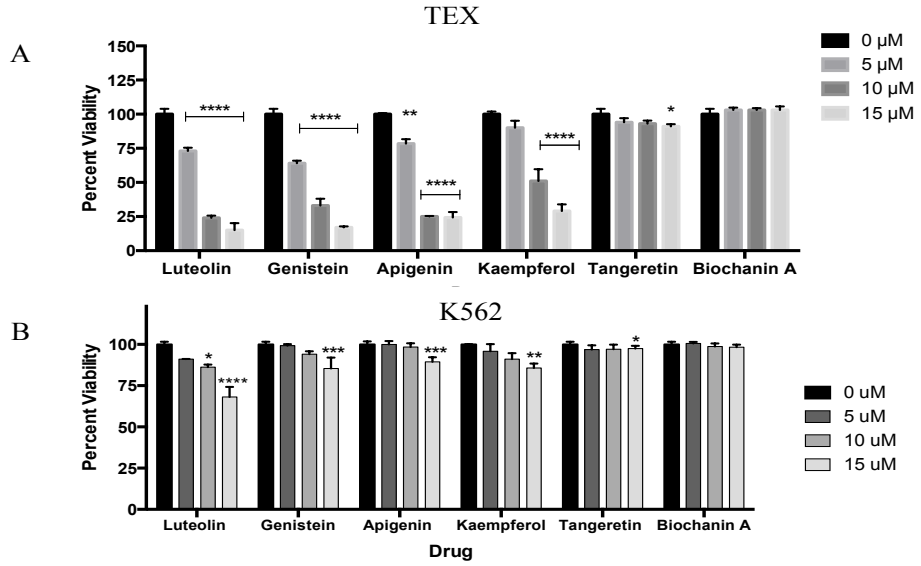
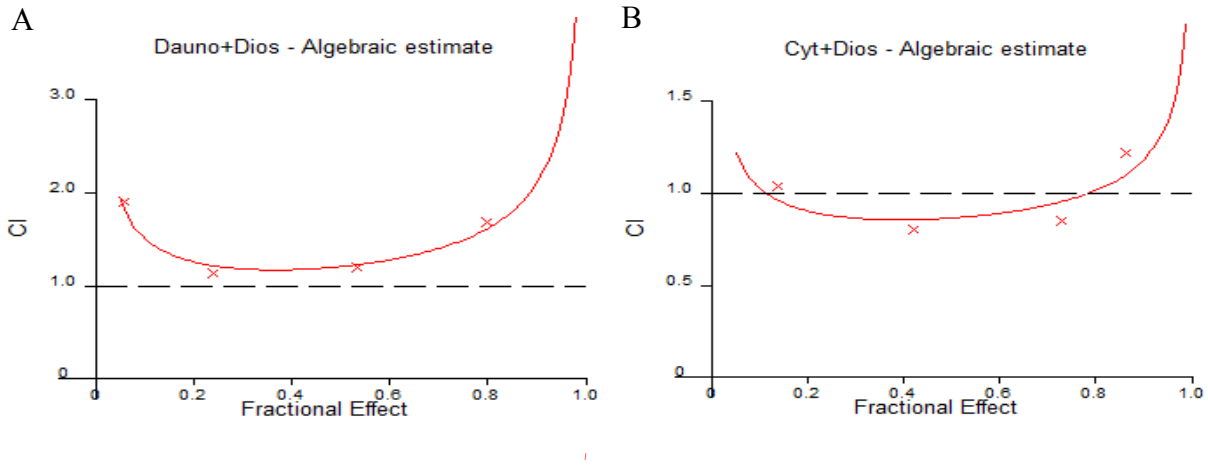


Figure 15. Flavonoids induce selective toxicity in LSC cell line, TEX. 54 flavonoids were screened for selective toxicity in LSC, using the surrogate LSC cell line, TEX, and control line, K562. Of these compounds, apiginin, luteolin, genistein and kaempferol showed reduced viability in (A) TEX cells, with little reduction in (B) K562 cells, validated through the ANN/PI flow cytometry assay. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All experiments were performed three times in triplicate.

4.10.4. Combination studies

To determine if diosmetin treatment sensitizes cells to chemotherapy, diosmetin was combined with cytarabine (Ara-C) and daunorubicin, two common AML chemotherapeutics, and assessed via the MTS assay. The drugs were co-incubated in TEX cells at 0.5x, 1x, 1.5x, and 2x of each compounds specific EC_{50} concentration. Synergistic treatments have a greater effect in combination than they do individually, whereas antagonistic treatments are weaker in combination. A CI value <1 indicates synergism, CI>1 indicates antagonism, and CI=1 indicates that there is no interaction (i.e. additive effect). CI values for the drug combination are shown in Figure 16A&B. We found that when co-incubated with daunorubicin, diosmetin produced an antagonistic effect. It was also observed that when co-incubated with cytarabine, there was a small

window of concentrations that produced a small synergistic effect (between approximately 1x and 1.5x) (Figure 16B). On either side of these concentrations, the combination produced an antagonistic effect. In a recent combination screen in our lab, evaluating the CI of other flavonoids with cytarabine and daunorubicin (Figure 16C), we have shown flavonoids to have antagonistic effects when co-incubated with these chemotherapeutics. Therefore, perhaps flavonoids should be used with caution if considered for clinical development or even used as over the counter supplements.



C

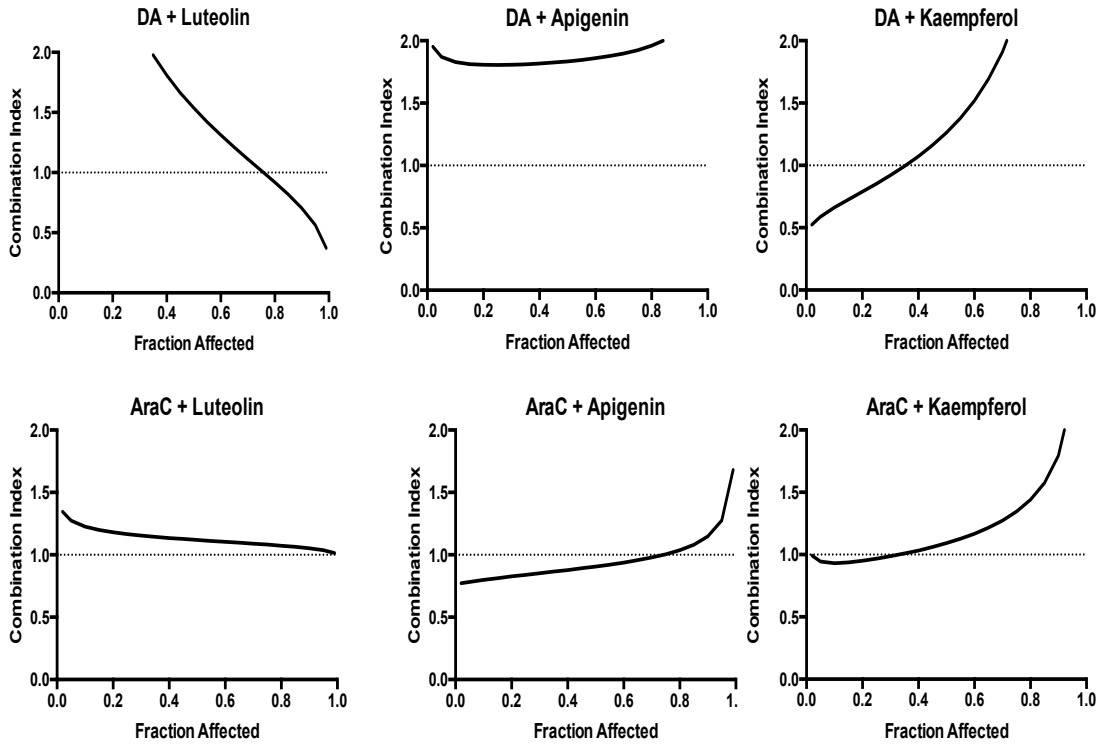


Figure 16. Combination indices (CIs) for diosmetin and chemotherapy treatments in TEX cells. Tex cells were treated with diosmetin and common chemotherapy treatments alone, and in combination with each other to assess for possible potentiating effects by calculating a CI. CI < 1 indicates synergism, CI > 1 indicates antagonism and CI = 1 indicates there is no drug interaction. Diosmetin was co-incubated with (A) daunorubicin and (B) cytarabine, common AML therapeutics. Co-incubations with cytarabine resulted in what seems to be a dual effect, with a slight synergism within a small window of concentrations, and otherwise antagonism. All experiments were performed three times in triplicate. (C) Combination indices of other flavonoids with common chemotherapeutics, cytarabine (AraC) and daunorubicin (DA), show both antagonistic and synergistic effects.

CHAPTER 5: DISCUSSION

5.1. Summary of findings

A screen of a unique nutraceutical library demonstrated that diosmetin is a novel anti-AML agent. *In vitro* and *ex vivo* data has demonstrated that diosmetin has selective toxicity in AML and AML progenitors with no effect in normal cells. Mechanistically, we highlight ERβ activation inducing extrinsic apoptosis as a novel approach to selectively eliminating LSCs.

We have shown that diosmetin, a flavonoid found in citrus fruits and other various plant sources, induces selective cytotoxicity in AML cells and AML progenitors and that death was related to ERβ expression. Protein and mRNA levels in cell lines and primary AML patient samples sensitive to diosmetin possessed elevated ERβ levels whereas cell lines insensitive to diosmetin had low levels. Moreover, reporter assays demonstrated that diosmetin acted as a partial ERβ agonist (Figure 18A).

5.2. The nature of ERβ in cancer

Activation of ERβ has demonstrated anti-proliferative and pro-apoptotic properties, which have been observed in both normal tissue types and pre-clinical studies involving prostate, breast, and colon cancer cell lines.[80] More recently, literature regarding chronic lymphocytic leukemia (CLL) and lymphoma, there have been an observed role for ERβ in prognosis and potential treatment.[77,70] For example, it has been shown in CLL that patients expressing higher ERβ correlated with shorter therapy requirement.[128] In addition, it has been observed that ERβ selective agonists induced an anti-proliferative effect in lymphoma cell (EG7) xenograft mice models.[70]

An anti-proliferative effect of ERβ is suggested from studies involving ERβ knockout mice, which display prostate hyperplasia and a myeloproliferative disease resembling CML.[77,74] Similarly, prostate cancer progression is associated with a reduction in ERβ expression suggesting possibility that this receptor exerts a restraining effect on proliferation.[129,130] Finally, ERβ ligand activation or transfection of ER negative cell lines with ERβ results in reduced proliferation, induction of apoptosis, and inhibition of tumour formation in prostate[80,131], colon[132], lymphoma[70], or breast cancer[81,133] cell lines. While positive effects have been observed in the aforementioned tumour types, the role of ERβ has not widely been addressed for AML, or LSC specifically. Collectively, ERβ activation is a potential anti-cancer target and the studies presented here extend these findings to demonstrate the pro-apoptotic/anti-proliferative role for ERβ in AML.

5.3. LSC selectivity of diosmetin

We propose this selectivity is due to diosmetin's ability to activate ERβ, as several of our observations show a relationship between ERβ and diosmetin sensitivity. For example, using structural analysis and the DAVID tool, it was predicted that ERs could be potential molecular targets. We also show a link between diosmetin sensitivity and ERβ expression through Western blotting and qPCR analysis in both cell lines and primary AML patient samples. TEX cells, sensitive to diosmetin treatment, showed an expression profile that included increased ERβ protein expression, as well as presence of ERβ mRNA and an absence of ERα mRNA. Similarly, primary AML patient samples that were diosmetin sensitive also revealed a presence of ERβ mRNA and an absence of ERα mRNA. In contrast, K562 cells, insensitive to diosmetin treatment, had a differential ER protein expression pattern, where ERα mRNA was observed with a lack of ERβ

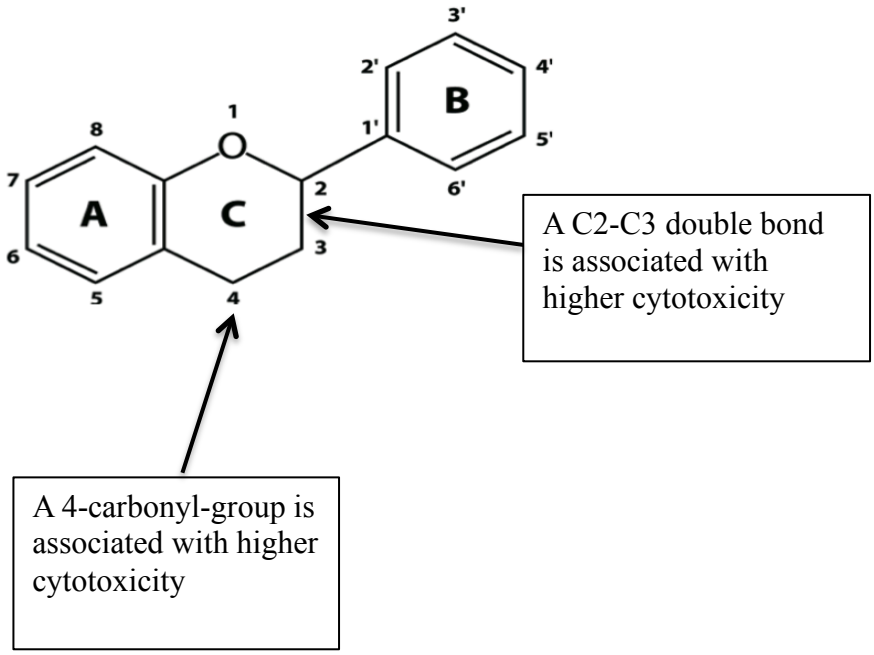
mRNA, as well as larger proportions of ERα protein expression with little ERβ protein expression. These results give preliminary evidence for the prediction that diosmetin sensitivity is directly associated with a specific expression profile of the ERs.

Future studies will be aimed at assessing additional AML patient samples for diosmetin sensitivity and ERβ expression, through Western blotting and qPCR analysis to further confirm this link. We would expect that the primary AML patient samples that displayed sensitivity to diosmetin treatment would also show similar ERβ expression profiles of the primary patient samples assessed and described previously. In addition, ERβ expression will be measured in normal CD34+ bone marrow derived cells, which were previously insensitive to diosmetin treatment, in order to strengthen the hypothesis that ERβ expression is linked to diosmetin sensitivity. We would expect the normal CD34+ to have differential ERβ expression than the diosmetin sensitive primary AML samples.

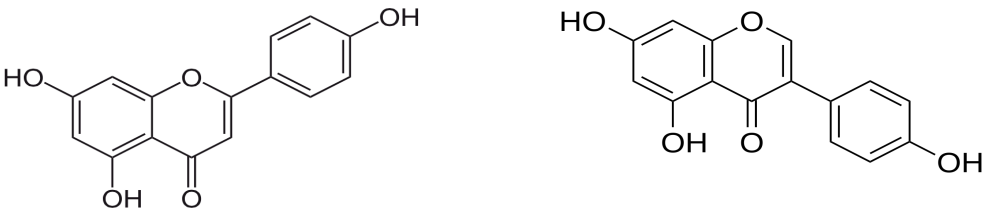
Given that diosmetin is a flavonoid, it is not surprising that ERs were predicted to be involved in diosmetin's activity, as many flavonoids have been shown to possess ER binding. Diosmetin is an o-methylated flavone of the flavonoid family which has not been previously studied for its cytotoxicity in leukemia cells. Structure-activity relationship studies confirm that flavonoids with a C2-C3 double bond and a 4-carbonyl-group are typically associated with higher cytotoxicity.[134] Both of these features are present in the structure of diosmetin (Figure 17, left panel). Flavonoids are known for their ability to induce ROS-mediated apoptosis[119–121]; however, diosmetin's anti-AML activity was shown to be independent of ROS. Instead, we show diosmetin's activity was related to its ability to bind to ERβ. Other flavonoids have also shown a distinct affinity

for ERβ binding. For example, genistein (Figure 17B) has shown high binding affinity for ERβ and a suppressive effect on CLL and lymphoma cells *in vitro* and in combination with other chemotherapy treatments.[135,136] Apigenin (Figure 17B, right panel) has also been found to have anti-proliferative activities, but in breast and prostate cancer cell lines, through ERβ mediated apoptosis.[137] Together, this information points to flavonoids as potential anti-cancer agents, specifically through targeting ERβ. Interestingly, in an additional screen focused on evaluating flavonoids for their anti-LSC activity, genistein and apigenin (Figure 17B) were among the four flavonoids that imparted cytotoxicity in TEX cells, while having no effect on the control cell line K562. Perhaps future studies should be aimed at attempting to characterize the relationship between flavonoids and ERβ and identifying the potential alternate mechanisms by which this relationship can induce apoptosis.

A.



B.



Apigenin Genistein

Figure 17. Basic structure of flavonoids. (A) Basic structure of flavonoids and structure-related activity. (B) Chemical structures of apigenin and genistein, two flavonoids with known affinity for ERβ, and have been observed to produce pro-apoptotic responses. These structures are very similar to that of diosmetin (Figure 5, Chapter 1). Figure modified from Chan et al*, Structurally related cytotoxic effects of flavonoids on human cancer cells in vitro.*Arch Pharm Res. 2008. [138]

5.4. Diosmetin's mechanism of action

Diosmetin induced caspase-mediated extrinsic apoptosis through the activation of caspase-8 and increases in TNFα (Figure 18B). These findings are consistent with data supporting a similar mechanisms involving ERβ activation in prostate cancer and in human hepatocellular carcinoma cell (HCC) lines [80,139] TNFα, a biologically active cytokine, can elicit a wide range of biological responses such as inflammation, apoptosis,

cell proliferation, and immunity.[140] TNFα signals through two receptors, TNFR1 and TNFR2, which are expressed in most cells. The binding of TNFα to TNFR1 promotes the recruitment of several adaptor molecules, including TNFR-associated death domain (DD), Fas-associated DD (FADD), and pro-caspase 8.[32]

It was found that ERβ-mediated apoptotic response following siRNA knockdown of TNFα in a human prostate cancer cell line.[80] In the same study, the authors quantified prostatic apoptosis in TNFα knock-out (TNFαKO) mice following ERβ agonist treatment. While the TNFαKO mice failed to show any increase in apoptosis following ERβ agonist treatment, an increase was observed in the wild type mice.[80] In another study, HCC cell line HATT2 were transfected to over-express ERβ (HA22T-hER-β) and assessed for ERβ mediated apoptosis through TNFα production.[139] Increased DNA fragmentation, as well as caspase-3 and -8 (but not caspase-9 activity) were found in hERβ-overexpressed HA22T cells treated with estrogen. These effects were not observed in hERα-overexpressed cells. An increase in TNFα RNA and protein levels were strongly correlated with overexpressed hERβ and E2 treatment in HA22T cells. In addition, the HA22T-hERβ was transfected with the hTNFα promoter-luciferase reporter gene and assessed for luciferase activity upon treatment with E2. It was observed that hER-β induced luciferase activity in a ligand dependent manner. Future studies will be aimed at elucidating on the mechanism by which diosmetin activates TNF-α in AML.

While additional studies are required to display show that ERβ activates the transcription of TNFα genes, searching the Champion ChiP Transcription Factor Portal (SAbioscience database) revealed that there is an ERα binding domain on the TNFα promoter region. Since ERα and ERβ have a high level of homology for the DNA

binding domain it could be possible that there is an ERβ binding site that may not have yet been validated. A potential experiment that could elucidate this question would be the chromatin immunoprecipiation (ChIP) assay, which is used to determine whether specific proteins are associated with specific genomic sequences, such as DNA binding sites. Through a ChIP experiment, it would be possible to further investigate the interaction between ERβ and the TNFα promoter.

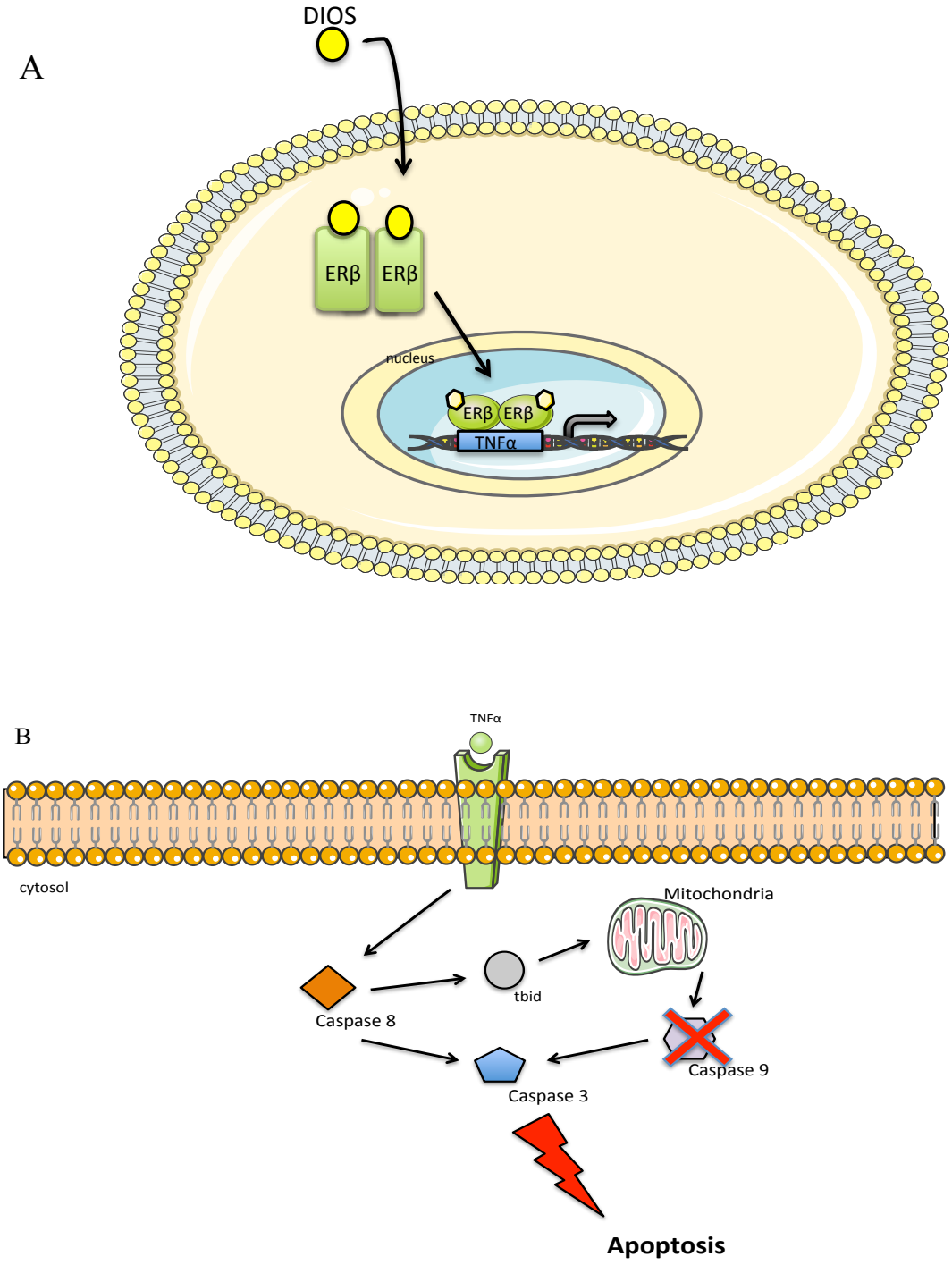

A
DIOS
ERβ
ERβ
nucleus
ERβ
ERβ
TNFα
B
TNFα
cytosol
Mitochondria
tbid
Caspase 8
Caspase 9
Caspase 3
Apoptosis

Figure 18. Diosmetin's mechanism of action. (A) Diosmetin was identified as the most potent compound to selectively reduce cell viability, by apoptosis, of the surrogate LSC cell line, TEX. It is proposed that diosmetin induces apoptosis through binding to the ERβ, a nuclear receptor that acts as a transcription factor, and instigating agonist activities. Once bound by dios, the ERβ receptors form a dimer and translocates to the nucleus to initiate gene transcription. Evidence presented in this thesis demonstrate that it is likely that the ERβ dimer binds to the TNFα promoter and induces TNFα gene transcription. (B) TNFα, an intracellular cytokine, is a ligand to the TNFR1 death receptor, which initiates the extrinsic pathway of apoptosis. The cascade begins through the activation of caspase 8, followed by the activation of caspase 3/7, which then initiates apoptosis (refer to Chapter 1, Figure 2). It was observed that dios treatment increased caspase 8 activation, but not caspase 9 activation, consistent with the proposed mechanism of action. Figure modified from *Huang et al. Opposing action of estrogen receptors alpha and beta on tumor necrosis factor-alpha gene expression and caspase-8-mediated apoptotic effects in HA22T cells.* Mol. Cell. Biochem. 2006.[139]Drawing made using Servier Medical Art Bank.

5.5. Effects of tamoxifen in TEX cells

Tamoxifen is shown to have no effect on TEX cells when incubated at increasing concentrations, however, when co-incubated with diosmetin, it increases diosmetin-mediated cytotoxicity. When investigating the CI values of diosmetin and tamoxifen (results not shown), it appeared there was a synergistic effect, however since tamoxifen had no effect on TEX cells, this relationship cannot be accurately calculated. The rationale for an increased sensitivity to diosmetin treatment in TEX cells may be that tamoxifen is a well-known ERα antagonist, blocking the proliferative response following of ERα ligation.[141] When treated in TEX cells, we would expect to see a lack of response as TEX cells have been shown to possess smaller amounts of ERα protein and mRNA levels. However, when co-incubated with diosmetin, it is possible that tamoxifen is binding to ERα, which has a small presence in TEX cells. It was observed that diosmetin had some affinity for ERα in an agonist reporter assay, in which case it may have induced a small amount of proliferation, but not enough to overcome the anti-

proliferative effects through ERβ. Tamoxifen treatment may have blocked ERα from diosmetin binding, through competition with tamoxifen binding. With the antagonistic effects of tamoxifen on ERα, this may have reduced the opposing agonist action of diosmetin bound to ERα, therefore increasing diosmetin's efficacy through ERβ.

5.6. Clinical relevance

Although a wide spectrum of ERβ specific agonists are under clinical development (i.e. ERB-041, WAY-202196, diarylpropionitrile, etc.), none have yet been FDA approved[57] and have not been widely tested as therapeutic potential in hematological malignancies.[70] This study supports the rationale for preclinical testing and evaluation of the potential for clinical applications of ER-based therapies for AML, specifically focusing on ERβ agonists, either alone or in combination with existing chemotherapy regimens. For example, as previously described, CLL patient treatment duration is positively correlated with ERβ expression further supporting the need for additional investigation regarding the use of ERβ in deciding on treatment options. Moreover, some studies have shown a suppressive effect of genistein in vitro, alone or in combination with fludarabine.[135,136] While studies such as this observe positive effects of combination therapy, our studies investigating diosmetin co-incubation with common chemotherapies showed antagonistic effects, stressing the importance of evaluating the effect of potential therapeutic strategies on current ones. An interesting detail to consider is ERβ methylation, resulting in decreased ERβ activity. Increased ERβ methylation has been observed in colon or breast tumors[142,143], especially those that are at a more advanced stage, further highlighting the need for more specific interrogation into the role of ERβ in

AML, including expression patterns in normal and malignant cells. Nonetheless, our research provides seminal evidence into the role or ERβ in AML.

5.6.1. ERβ tissue expression patterns

While considering ERβ as a potential treatment option for AML and other cancers, monitoring for side effects, given the differential expression of ER in many different tissues, is necessary. For example, concerns regarding immunosuppression, as a high expression of ER splice variants in normal immune cells have been observed.[70,128] However, as previously mentioned, the net effect of a ligand is determined by the balance between ERα and ERβ within different cells and tissues, as the two receptors induce opposing biological activity.[77] Therefore, side effects will largely depend on the expression profiles of the cell and tissue type, and may not be easily predicted.

ERα and ERβ regulate different genes in response to E2 and SERMs.[52,144] For example, one study found that only 40% of the genes regulated by E2 in U2OS cells that express ERα are also regulated in U20S cells that express ERβ.[52] Furthermore, different ligands may differ in their affinity for ERα and ERβ isoforms. Although the DNA-binding domains of ERα and ERβ share a high level of homology, their ligand-binding domains (LBDs) have approximately 58% homology.[48] Because of this discrepancy in their LBDs, the two ER subtypes can bind ligands, both agonists and antagonists, with different affinities.[71,145] Moreover, the different ER dimer combinations may respond differently to various ligands, which may translate into tissue- and cell-specific agonistic and antagonistic effects.[146] Finally, studies have suggested that differences in co-regulator levels and recruitment to a promoter can determine the functionality of the ER in different tissue types.[147] Needless to say, ER action can be rather complicated and future

preclinical *in vivo* studies using ER agonists, such as diosmetin, are warranted to determine the full potential and safety of this class of agonist for treatment against LSC.

5.7. Limitations

While this project provides good support for the use of nutraceuticals in discovering new therapeutics or molecular targets for future development, there are some limitations. Although our focus was specifically to investigate nutraceuticals for anti-cancer activity in LSCs, our initial screen involved the use of our surrogate LSC cell line TEX, and a control line lacking LSC characteristics, K562. It is more common for a screen to involve a number of cell lines across a list of cancers for a more robust report. For example, the US National Cancer institute conducts large scale screens to identify novel anticancer drugs. A typically screen would involve upwards of 60 different cell lines from 9 different tumour types.[148] Unfortunately our lab does not have access to such a wealth of cell lines; however, we were able to screen diosmetin in 9 cell lines representing a range of cancers. In addition, having only one cell line acting as the target cell line might limit the validity of the results, however, the LSC line, TEX, used was designed to be highly representative of both LSC and myeloid blasts. Furthermore, to ensure TEX cells maintained the LSC phenotype (high CD34+/CD38-), cell surface markers were analyzed regularly and cells were only used up to 15 passages. Although another surrogate LSC line exists, it was unavailable for use in our lab.

We were fortunate enough to obtain primary AML patient samples through collaboration with Princess Margaret Cancer Center to assess for reduction of progenitor cells through diosmetin treatment, as well as assess ER mRNA expression. Colony formation cell assays have been widely accepted as a robust measure to evaluate

cytotoxicity of a compound against progenitor populations in samples.[3] These assays provided further validation to effects that were observed *in vitro.* Although the use of these samples was extremely valuable to this project, the number of samples we were provided with were still relatively few, with a small number of isolated cells per sample. For example, similar drug discovery studies report the use of 20 or more primary patient samples.[149] In addition, studies using primary patient samples to evaluate protein expression would have been beneficial, however these studies require a large number of cells. Unfortunately, our lab does not have direct access to primary patient samples, and thus we rely on our collaborator's availability to issue samples to us.

Another limitation to these studies is the lack of data regarding diosmetin's efficacy and safety *in vivo*. While we showed a reduction in tumour volume following diosmetin treatment in xenograft mice models, these results were insignificant, likely due to the low number of mice used in the study (n = 3). While we were fortunate enough to have tested diosmetin's capacity *in vivo* in some capacity, more mice are needed to investigate the true efficacy and safety of both diosmetin as a compound, and targeting ERβ *in vivo.* A more robust measure of toxicity is needed, more than just preliminary measurement for weight changes during treatment, including a blood bioanalysis. Animal models can be time intensive and relatively expensive, so for the purposes of this project, were not extensively used. In addition, designing an *in vivo* study using an engraftment mouse model to ensure better investigation of treatment effects on LSCs. Given the differential expression patterns ERs found in the human body, and the natural progression of pre-clinical studies, *in vivo* models would be a clear future consideration for this project.

In addition, it is important to consider potential post-translational modifications of compounds that can occur. It is possible that the active compound may be a modification of diosmetin's structure. For example, *Vasilis et al*. found that there were two metabolites of diosmetin, luteolin, and an un-identifiable metabolite, which were responsible for the cytotoxic activity in breast cancer cells.[99] It would be beneficial to assess if diosmetin is metabolized in TEX cells to a more active form with a slightly altered structure. It would also be of value to assess if luteloin possesses ERβ affinity and activating properties.

Finally, to have strengthened the validity of proposing that ERβ mediates apoptosis induced by diosmetin, obtaining an ERβ knock out cell line, or alternatively using siRNA transfection to knock out ERβ in TEX cells would have been valuable. More definitive evidence of the role of ERβ in diosmetin induced, LSC specific cell through observations of abrogation of cell death following diosmetin treatment in either ERβ knock out cells, or ERβ- TEX cells. Unfortunately, despite efforts in contacting researchers who have previously utilized ERβ knockout cells to potentially obtain these cells, we were unable to make a connection. Additionally, due to time restraints, generating ERβ knock out TEX cells was not possible, but would be a future consideration of this project to further validate the proposed mechanism of action.

5.8. Conclusions

Given the devastatingly poor prognosis for the majority of AML patients, the need to increase the efficacy of treatment is clear. It has been widely observed that LSCs are responsible for initiation, maintenance and relapse of the disease, and also express biological properties that allow them to evade current chemotherapy treatment. Thus, goals for improving patient outcome should be focused on developing therapeutic

strategies to eliminate LSCs. Nutraceuticals provide a virtually untapped resource for the discovery of new anti-cancer agents. In this thesis, we discuss the efficacy of the flavonoid compound diosmetin in imparting selective cell death in LSCs. Through the project presented here, apoptotic cell death in our surrogate LSC line, TEX, was proposed to be mediated by ERβ, which is linked to an increase in TNFα. The presented studies highlight a promising novel target, ERβ, for AML treatment, as well as nutraceutical as a vast resource for novel anti-cancer drug discovery.

Appendix A: Research activity resulting from this program

PUBLICATIONS AND MANUSCRIPTS

Rota SG, Doxey A, Dude I, and Spagnuolo PA. Estrogen receptor is a novel target in acute myeloid leukemia. *In preparation*.

Lee EA, Angka L, **Rota SG**, Hurren R, Wang XM, Gronda M, Bernard D, Minden M, Mitchell A, Joseph JW, Datti A, Wrana J, Quadrilatero J, Schimmer AD, Spagnuolo PA. Targeting mitochondria with avocatin B induces selective leukemia cell death. Cancer Research. April 24, 2015.

Angka L, Lee EA, **Rota SG**, et al. Glucopsychosine increases cytosolic calcium to induce calpain- mediated apoptosis of acute myeloid leukemia cells. Cancer letters. Mar 12 2014.

PRESENTATIONS

Oral presentation. High throughput screening as a method to identify novel anti-cancer nutraceuticals. Natural Health Product Research Society of Canada. Windsor, ON. May 2013

Poster. Estrogen receptor is a novel target in acute myeloid leukemia. Till and McCulloch Meetings 2014. Ottawa, ON. Oct 2014

Poster. Estrogen receptor is a novel target in acute myeloid leukemia. AFPC Rx&D Poster Competition, University of Waterloo School of Pharmacy. Kitchener, ON. May 2014. *Awarded second place.*

BIBLIOGRAPHY

1. Jones, R. J. & Matsui, W. Cancer stem cells: From bench to bedside. *Biol. Blood Marrow Transplant.* **13,** 47–52 (2007).

2. Estey, E. & Dohner, H. Acute myeloid leukaemia. *Lancet* **368,** 1894–1907 (2006).

3. Dick, J. E. Stem cell concepts renew cancer research. *Blood* **112,** 4793–4807 (2008).

4. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3,** 730–737 (1997).

5. Lowenberg, B., Griffin, J. D. & Tallman, M. S. Acute myeloid leukemia and acute promyelocytic leukemia. *Hematology* **2003,** 82–101 (2003).

6. Rowe, J. M. & Tallman, M. S. How I treat acute myeloid leukemia. *Blood* **116,** 3147–3156 (2010).

7. Craig, C. M. & Schiller, G. J. Acute myeloid leukemia in the elderly: conventional and novel treatment approaches. *Blood Rev* **22,** 221–234 (2008).

8. Roboz, G. J. Novel approaches to the treatment of acute myeloid leukemia. *Hematology Am. Soc. Hematol. Educ. Program* **2011,** 43–50 (2011).

9. Robak, T. *et al.* Novel and emerging drugs for acute myeloid leukemia: pharmacology and therapeutic activity. *Curr. Med. Chem.* **18,** 638–666 (2011).

10. Lowenberg, B., Downing, J. R. & Burnett, A. Acute Myeloid Leukemia. *New England Journal of Medicine* **341,** 1051–1062 (1999).

11. Ferrara, F. & Schiffer, C. A. Acute myeloid leukaemia in adults. *Lancet* **381,** 484–495 (2013).

12. Estey, E. What is the optimal induction strategy for older patients? *Best Pract. Res. Clin. Haematol.* **24,** 515–522 (2011).

13. Jordan, C. T. *et al.* The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **14,** 1777–1784 (2000).

14. Huntly, B. J. P. *et al.* MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* **6,** 587–596 (2004).

15. McDermott, S. P. *et al.* A small molecule screening strategy with validation on human leukemia stem cells uncovers the therapeutic efficacy of kinetin riboside. *Blood* **119,** 1200–1207 (2012).

16. Snauwaert, S., Vandekerckhove, B. & Kerre, T. Can immunotherapy specifically target acute myeloid leukemic stem cells? *Oncoimmunology* **2,** e22943 (2013).

17. Lane, S. W. & Gilliland, D. G. Leukemia stem cells. *Seminars in Cancer Biology* **20,** 71–76 (2010).

18. Blair, a, Hogge, D. E., Ailles, L. E., Lansdorp, P. M. & Sutherland, H. J. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood* **89,** 3104–3112 (1997).

19. Hosen, N. *et al.* CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 11008–11013 (2007).

20. Jan, M. *et al.* Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. *Proc Natl Acad Sci U S A* **108,** 5009–5014 (2011).

21. Saito, Y. *et al.* Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci. Transl. Med.* **2,** 17ra9 (2010).

22. Jin, L. *et al.* Monoclonal Antibody-Mediated Targeting of CD123, IL-3 Receptor ?? Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells. *Cell Stem Cell* **5,** 31–42 (2009).

23. Zhao, X. *et al.* Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia. *Haematologica* **95,** 71–78 (2010).

24. Van Rhenen, A. *et al.* High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clin. Cancer Res.* **11,** 6520–6527 (2005).

25. Huff, C. A., Matsui, W. H., Smith, B. D. & Jones, R. J. Strategies to eliminate cancer stem cells: clinical implications. *Eur J Cancer* **42,** 1293–1297 (2006).

26. Guzman, M. L. & Jordan, C. T. Considerations for targeting malignant stem cells in leukemia. *Cancer Control* **11,** 97–104 (2004).

27. Jordan, C. T. The leukemic stem cell. *Best Pr. Res Clin Haematol* **20,** 13–18 (2007).

28. Sun, Y. & Peng, Z.-L. Programmed cell death and cancer. *Postgrad. Med. J.* **85,** 134–140 (2009).

29. Clarke, P. G. H. Apoptosis: from morphological types of cell death to interacting pathways - Comment from Clarke. *Trends Pharmacol. Sci.* **23,** 308–309 (2002).

30. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–674 (2011).

31. Codogno, P. & Meijer, A. J. Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ.* **12 Suppl 2,** 1509–1518 (2005).

32. Hotchkiss, R., Strasser, A., McDunn, J. & Swanson, P. Cell Death. *N. Engl. J. Med.* **361,** 1570–83 (2009).

33. O'Donovan, N. *et al.* Caspase 3 in breast cancer. *Clin. Cancer Res.* **9,** 738–742 (2003).

34. Stennicke, H. R. & Salvesen, G. S. Properties of the caspases. *Biochim. Biophys. Acta-Protein Struct. Mol. Enzymol.* **1387,** 17–31 (1998).

35. Shi, Y. G. Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell* **9,** 459–470 (2002).

36. Igney, F. H. & Krammer, P. H. Death and anti-death: Tumour resistance to apoptosis. *Nat. Rev. Cancer* **2,** 277–288 (2002).

37. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35,** 495–516 (2007).

38. Van Loo, G. *et al.* The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Differ* **9,** 1031–1042 (2002).

39. Couty, S. *et al.* The discovery of potent ribosomal S6 kinase inhibitors by high-throughput screening and structure-guided drug design. *Oncotarget* **4,** 1647–61 (2013).

40. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367,** 645–648 (1994).

41. Hope, K. J., Jin, L. & Dick, J. E. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat. Immunol.* **5,** 738–743 (2004).

42. Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat. Med.* **17,** 1086–1093 (2011).

43. Warner, J. K. *et al.* Direct evidence for cooperating genetic events in the leukemic transformation of normal human hematopoietic cells. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **19,** 1794–1805 (2005).

44. Skrtić, M. *et al.* Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* **20,** 674–88 (2011).

45. Heldring, N. *et al.* Estrogen receptors: how do they signal and what are their targets. *Physiol. Rev.* **87,** 905–931 (2007).

46. Jensen, E. V. On the Mechanism of Estrogen Action. *Perspect. Biol. Med.* **6,** 47–60 (1962).

47. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* **93,** 5925–5930 (1996).

48. Marino, M., Galluzzo, P. & Ascenzi, P. Estrogen signaling multiple pathways to impact gene transcription. *Curr. Genomics* **7,** 497–508 (2006).

49. Shughrue, P. J., Lane, M. V, Scrimo, P. J. & Merchenthaler, I. Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63,** 498–504 (1998).

50. Kuiper, G. G. J. M. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138,** 863–870 (1997).

51. Veeneman, G. H. Non-steroidal subtype selective estrogens. *Curr. Med. Chem.* **12,** 1077–1136 (2005).

52. Tee, M. K. *et al.* Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. *Mol. Biol. Cell* **15,** 1262–1272 (2004).

53. Vivar, O. I. *et al.* Estrogen receptor beta binds to and regulates three distinct classes of target genes. *J. Biol. Chem.* **285,** 22059–22066 (2010).

54. Nilsson, S. *et al.* Mechanisms of estrogen action. *Physiol. Rev.* **81,** 1535–1565 (2001).

55. Black, B. E., Holaska, J. M., Rastinejad, F. & Paschal, B. M. DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr. Biol.* **11,** 1749–1758 (2001).

56. Braich, P. S., Almeida, D. R., Hollands, S. & Coleman, M. T. Effects of pictograms in educating 3 distinct low-literacy populations on the use of postoperative cataract medication. *Can. J. Ophthalmol.* **46,** 276–281 (2011).

57. Mohler, M. L. *et al.* Estrogen receptor beta selective nonsteroidal estrogens: seeking clinical indications. *Expert Opin. Ther. Pat.* **20,** 507–534 (2010).

58. Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* **29,** 2905–2919 (2001).

59. Ascenzi, P., Bocedi, A. & Marino, M. Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol. Aspects Med.* **27,** 299–402 (2006).

60. Tora, L. *et al.* The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59,** 477–487 (1989).

61. Locker, G. Y. Hormonal therapy of breast cancer. (1998).

62. Ordóñez-Morán, P. & Muñoz, A. Nuclear receptors: Genomic and non-genomic effects converge. *Cell Cycle* **8,** 1675–1680 (2009).

63. Singh, M., Sétáló, G., Guan, X., Warren, M. & Toran-Allerand, C. D. Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J. Neurosci.* **19,** 1179–1188 (1999).

64. Kato, S. *et al.* Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270,** 1491–1494 (1995).

65. Driggers, P. H. & Segars, J. H. Estrogen action and cytoplasmic signaling pathways. Part II: The role of growth factors and phosphorylation in estrogen signaling. *Trends in Endocrinology and Metabolism* **13,** 422–428 (2002).

66. Katzenellenbogen, B. S. *et al.* Estrogen receptors: selective ligands, partners, and distinctive pharmacology. *Recent Prog. Horm. Res.* **55,** 163–193; discussion 194–195 (2000).

67. Hewitt, S. C., Harrell, J. C. & Korach, K. S. Lessons in estrogen biology from knockout and transgenic animals. *Annu. Rev. Physiol.* **67,** 285–308 (2005).

68. Krege, J. H. *et al.* Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15677–15682 (1998).

69. Lubahn, D. B. *et al.* Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11162–11166 (1993).

70. Yakimchuk, K. *et al.* Effect of ligand-activated estrogen receptor β on lymphoma growth in vitro and in vivo. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **25,** 1103–1110 (2011).

71. Warner, M. & Gustafsson, J.-A. The role of estrogen receptor beta (ERbeta) in malignant diseases--a new potential target for antiproliferative drugs in prevention and treatment of cancer. *Biochem. Biophys. Res. Commun.* **396,** 63–66 (2010).

72. Cheng, G., Weihua, Z., Warner, M. & Gustafsson, J.-A. Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 3739–3746 (2004).

73. Omoto, Y., Imamov, O., Warner, M. & Gustafsson, J.-A. Estrogen receptor alpha and imprinting of the neonatal mouse ventral prostate by estrogen. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1484–1489 (2005).

74. Imamov, O. *et al.* Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9375–9380 (2004).

75. Wada-Hiraike, O. *et al.* Role of estrogen receptor beta in uterine stroma and epithelium: Insights from estrogen receptor beta-/- mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 18350–18355 (2006).

76. Morani, A. *et al.* Lung dysfunction causes systemic hypoxia in estrogen receptor beta knockout (ERbeta-/-) mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7165–7169 (2006).

77. Shim, G.-J. *et al.* Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 6694–6699 (2003).

78. Pearce, S. T. & Jordan, V. C. The biological role of estrogen receptors alpha and beta in cancer. *Crit. Rev. Oncol. Hematol.* **50,** 3–22 (2004).

79. Mann, S. *et al.* Estrogen receptor beta expression in invasive breast cancer. *Hum Pathol* **32,** 113–118 (2001).

80. McPherson, S. J. *et al.* Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFalpha mediated. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3123–3128 (2010).

81. Ström, A. *et al.* Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1566–1571 (2004).

82. Milner, J. A. Nutrition and cancer: Essential elements for a roadmap. *Cancer Letters* **269,** 189–198 (2008).

83. Ovadje, P. *et al.* Advances in the Research and Development of Natural Health Products as Main Stream Cancer Therapeutics. **2015,** (2015).

84. Patwardhan, B. & Vaidya, A. D. B. Natural products drug discovery: Accelerating the clinical candidate development using reverse pharmacology approaches. *Indian J. Exp. Biol.* **48,** 220–227 (2010).

85. Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products* **75,** 311–335 (2012).

86. Patel, K., Gadewar, M., Tripathi, R., Prasad, S. K. & Patel, D. K. A review on medicinal importance, pharmacological activity and bioanalytical aspects of beta-carboline alkaloid " Harmine." *Asian Pac. J. Trop. Biomed.* **2,** 660–664 (2012).

87. Spagnuolo, P. A. *et al.* The antihelmintic flubendazole inhibits microtubule function through a mechanism distinct from Vinca alkaloids and displays preclinical activity in leukemia and myeloma. *Blood* **115,** 4824–4833 (2010).

88. Breitman, T. R., Selonick, S. E. & Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2936–2940 (1980).

89. Schenk, T., Stengel, S. & Zelent, a. Unlocking the potential of retinoic acid in anticancer therapy. *Br. J. Cancer* **111,** 2039–2045 (2014).

90. Hasserjian, R. P. Acute myeloid leukemia: Advances in diagnosis and classification. *International Journal of Laboratory Hematology* **35,** 358–366 (2013).

91. O'Donnell, M. R. *et al.* Acute myeloid leukemia, version 2.2013. *J. Natl. Compr. Canc. Netw.* **11,** 1047–55 (2013).

92. Patel, K., GadewPatel, K., Gadewar, M., Tahilyani, V., & Patel, D. K. (2013). A review on pharmacological and analytical aspects of diosmetin: a concise report. Chin J Integr Med, 19(10), 792–800. doi:10.1007/s11655-013-1595-3ar, M., Tahilyani, V. & Patel, D. K. A review on pharmacological and analytical aspects of diosmetin: a concise report. *Chin J Integr Med* **19,** 792–800 (2013).

93. Lin, J.-K. & Weng, M.-S. in *The science of flavonoids* 213–238 (2006). doi:10.1007/978-0-387-28822-2

94. Ciolino, H. P., Wang, T. T. Y. & Yeh, G. C. Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. *Cancer Res* **58,** 2754–2760 (1998).

95. Lee, S. *et al.* Naringenin derivatives as anti-atherogenic agents. *Bioorganic Med. Chem. Lett.* **13,** 3901–3903 (2003).

96. Spanakis, M., Kasmas, S. & Niopas, I. Simultaneous determination of the flavonoid aglycones diosmetin and hesperetin in human plasma and urine by a validated GC/MS method: in vivo metabolic reduction of diosmetin to hesperetin. *Biomed Chromatogr* **23,** 124–131 (2009).

97. Kadifkova Panovska, T., Kulevanova, S. & Stefova, M. In vitro antioxidant activity of some Teucrium species (Lamiaceae). *Acta Pharm.* **55,** 207–214 (2005).

98. Meirinhos, J. *et al.* Analysis and quantification of flavonoidic compounds from Portuguese olive (Olea europaea L.) leaf cultivars. *Nat. Prod. Res.* **19,** 189–195 (2005).

99. Androutsopoulos, V. P., Mahale, S., Arroo, R. R. J. & Potter, G. Anticancer effects of the flavonoid diosmetin on cell cycle progression and proliferation of MDA-MB 468 breast cancer cells due to CYP1 activation. *Oncol. Rep.* **21,** 1525–1528 (2009).

100. Bai, N. *et al.* Antioxidative flavonoids from the flower of Inula britannica. *Journal of Food Lipids* **12,** 141–149 (2005).

101. Meng, J. C., Zhu, Q. X. & Tan, R. X. New antimicrobial mono- and sesquiterpenes from Soroseris hookeriana subsp. erysimoides. *Planta. Med.* **66,** 541–544 (2000).

102. Spagnuolo, P. a *et al.* Inhibition of intracellular dipeptidyl peptidases 8 and 9 enhances parthenolide's anti-leukemic activity. *Leukemia* **27,** 1236–44 (2013).

103. Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **184,** 39–51 (1995).

104. Riccardi, C. & Nicoletti, I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat. Protoc.* **1,** 1458–1461 (2006).

105. Angka, L. *et al.* Glucopsychosine increases cytosolic calcium to induce calpain-mediated apoptosis of acute myeloid leukemia cells. *Cancer Lett.* **348,** 29–37 (2014).

106. Carmody, R. J., Costa-Pereira, a P., McKenna, S. L. & Cotter, T. G. Detection of molecular events during apoptosis by flow cytometry. *Methods Mol. Med.* **38,** 71–83 (2000).

107. Curtin, J. F., Donovan, M. & Cotter, T. G. Regulation and measurement of oxidative stress in apoptosis. *J. Immunol. Methods* **265,** 49–72 (2002).

108. Sun, S.-Y. N-acetylcysteine, reactive oxygen species and beyond. *Cancer Biol. Ther.* **9,** 109–110 (2010).

109. Pédeboscq, S. *et al.* Non-antioxidant properties of α-tocopherol reduce the anticancer activity of several protein Kinase inhibitors in vitro. *PLoS One* **7,** (2012).

110. Gagro, A. *et al.* Detection of intracellular cytokines by flow cytometry. *Central-European Journal of Immunology* **24,** 125–131 (1999).

111. Huang, D. W. *et al.* The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol.* **8,** R183 (2007).

112. Dam, A. D., Mitchell, A. S. & Quadrilatero, J. Induction of mitochondrial biogenesis protects against caspase-dependent and caspase-independent apoptosis in L6 myoblasts. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833,** 3426–3435 (2013).

113. Ramakers, C., Ruijter, J. M., Deprez, R. H. L. & Moorman, A. F. . Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339,** 62–66 (2003).

114. Ruijter, J. M. *et al.* Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* **37,** (2009).

115. Chou, T.-C. Preclinical versus clinical drug combination studies. *Leuk. Lymphoma* **49,** 2059–2080 (2008).

116. Chou, T. C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22,** 27–55 (1984).

117. Shiau, A. K. *et al.* The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95,** 927–937 (1998).

118. Domont, J., Namer, M., Khayat, D. & Spano, J. P. [Neoadjuvant endocrine therapy for breast cancer: an overview]. *Bull Cancer* **91,** 55–62 (2004).

119. Birt, D. F., Hendrich, S. & Wang, W. Dietary agents in cancer prevention: Flavonoids and isoflavonoids. *Pharmacology and Therapeutics* **90,** 157–177 (2001).

120. Galati, G., Sabzevari, O., Wilson, J. X. & O'Brien, P. J. Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* **177,** 91–104 (2002).

121. Ju, W. *et al.* A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor-kappaB pathway and sensitization of apoptosis in lung cancer cells. *Mol. Pharmacol.* **71,** 1381–1388 (2007).

122. Hotchkiss, R. S., Strasser, A., McDunn, J. E. & Swanson, P. E. Mechanisms of Disease Cell Death. *N. Engl. J. Med.* **361,** 1570–1583 (2009).

123. Chen, G. & Goeddel, D. V. TNF-R1 signaling: a beautiful pathway. *Science* **296,** 1634–1635 (2002).

124. Xaus, J. *et al.* LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. *Blood* **95,** 3823–3831 (2000).

125. Van Der Bruggen, T., Nijenhuis, S., Van Raaij, E., Verhoef, J. & Van Asbeck, B. S. Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the Raf-1/MEK1-MEK2/ERK1-ERK2 pathway. *Infect. Immun.* **67,** 3824–3829 (1999).

126. Jensen, E. V & Jordan, V. C. The estrogen receptor: a model for molecular medicine. *Clin. Cancer Res.* **9,** 1980–1989 (2003).

127. Pearce, S. T., Liu, H. & Jordan, V. C. Modulation of estrogen receptor alpha function and stability by tamoxifen and a critical amino acid (Asp-538) in helix 12. *J Biol Chem* **278,** 7630–7638 (2003).

128. Yakimchuk, K. *et al.* Up-regulated estrogen receptor β2 in chronic lymphocytic leukemia. *Leukemia & Lymphoma* **53,** 139–144 (2012).

129. Linja, M. J., Savinainen, K. J., Tammela, T. L. J., Isola, J. J. & Visakorpi, T. Expression of ERalpha and ERbeta in prostate cancer. *Prostate* **55,** 180–186 (2003).

130. Zhu, X. *et al.* Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. *Am. J. Pathol.* **164,** 2003–2012 (2004).

131. Cheng, J., Lee, E. J., Madison, L. D. & Lazennec, G. Expression of estrogen receptor beta in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis. *FEBS Lett* **566,** 169–172 (2004).

132. Hartman, J. *et al.* Tumor repressive functions of estrogen receptor β in SW480 colon cancer cells. *Cancer Res.* **69,** 6100–6106 (2009).

133. Hartman, J. *et al.* Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res.* **66,** 11207–11213 (2006).

134. Plochmann, K. *et al.* Structure-activity relationships of flavonoid-induced cytotoxicity on human leukemia cells. *Arch. Biochem. Biophys.* **460,** 1–9 (2007).

135. Mansour, A., Chang, V. T., Srinivas, S., Harrison, J. & Raveche, E. Correlation of ZAP-70 expression in B cell leukemias to the ex vivo response to a combination of fludarabine/genistein. *Cancer Immunol. Immunother.* **56,** 501–514 (2007).

136. McCall, J. L., Burich, R. A. & Mack, P. C. GCP, a genistein-rich compound, inhibits proliferation and induces apoptosis in lymphoma cell lines. *Leuk. Res.* **34,** 69–76 (2010).

137. Mak, P., Leung, Y.-K., Tang, W.-Y., Harwood, C. & Ho, S.-M. Apigenin suppresses cancer cell growth through ERbeta. *Neoplasia* **8,** 896–904 (2006).

138. Chang, H. *et al.* Structurally related cytotoxic effects of flavonoids on human cancer cells in vitro. *Arch. Pharm. Res.* **31,** 1137–1144 (2008).

139. Huang, E.-J. *et al.* Opposing action of estrogen receptors alpha and beta on tumor necrosis factor-alpha gene expression and caspase-8-mediated apoptotic effects in HA22T cells. *Mol. Cell. Biochem.* **287,** 137–145 (2006).

140. Liu, Z. G. Molecular mechanism of TNF signaling and beyond. *Cell Res.* **15,** 24–27 (2005).

141. Cheung, K. L. Endocrine therapy for breast cancer: an overview. *Breast* **16,** 327–343 (2007).

142. Zhao, C. *et al.* Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* **22,** 7600–7606 (2003).

143. Rody, A. *et al.* Methylation of estrogen receptor β promoter correlates with loss of ER-β expression in mammary carcinoma and is an early indication marker in premalignant lesions. *Endocr. Relat. Cancer* **12,** 903–916 (2005).

144. Monroe, D. G. *et al.* Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERalpha or ERbeta. *J. Cell. Biochem.* **90,** 315–326 (2003).

145. Gustafsson, J. A. Novel aspects of estrogen action. *J. Soc. Gynecol. Investig.* **7,** S8–9 (2000).

146. Monroe, D. G. *et al.* Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. *Mol. Endocrinol.* **19,** 1555–1568 (2005).

147. Shang, Y. & Brown, M. Molecular determinants for the tissue specificity of SERMs. *Science* **295,** 2465–2468 (2002).

148. Cardarelli, R. *et al.* Improving accuracy of medication identification in an older population using a medication bottle color symbol label system. *Bmc Fam. Pract.* **12,** 142 (2011).

149. Skrtic, M. *et al.* Inhibition of Mitochondrial Translation as a Therapeutic Strategy for Human Acute Myeloid Leukemia. *Cancer Cell* **20,** 674–688 (2011).